

Special Issue Reprint

Advanced Preparation and Application of Cellulose

Edited by
Marta Fernandes and Jorge Padrão

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Guest Editors

Marta Fernandes

Jorge Padrão



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Guest Editors

Marta Fernandes

Centre for Textile Science and

Technology (2C2T)

University of Minho

Guimarães

Portugal

Jorge Padrão

Centre for Textile Science and

Technology (2C2T)

University of Minho

Guimarães

Portugal

Editorial Office

MDPI AG

Grosspeteranlage 5

4052 Basel, Switzerland

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About the Editors

Marta Fernandes

Marta Fernandes works at the Centre for Textile Science and Technology (2C2T), University of Minho, Portugal. She received her master's degree in Textile Engineering in 2008 and her PhD in Textile Engineering in 2021, both from the University of Minho. Her research interests focus on bacterial nanocellulose, fibrous materials, advanced textiles, medical textiles, sustainable processes, and plasma technology.

Jorge Padrão

Jorge Padrão is a Junior Researcher at the Centre for Textile Science and Technology (2C2T), University of Minho, Portugal. He received his degree in Applied Biology in 2006 and his PhD in Chemical and Biological Engineering in 2014, both from the University of Minho. His research is focused on advanced multifunctional textile-based materials and the development of novel protocols for their generation and characterization. His work involves the development and characterization of functional textile materials with applications in health, automotive, aerospace, living textiles, and sustainable technologies.

Preface

Growing environmental awareness has brought about a widespread urgency to address global warming, energy crises, and increasing waste generation, which has catalyzed a global shift toward renewable, low-impact products. Front and center of the sustainable materials revolution is cellulose—a biobased polymer that has emerged as one of the most promising candidates for future applications due to its abundant availability and remarkable versatility.

This collection of works explores the cutting-edge developments in the preparation, modification, and application of cellulose and nanocellulose. While traditional wood pulp remains an industrial flagship raw material, the slow growth of trees has driven innovative research into fast-growing annual plants. For instance, Maguey fiber has proven to be a highly effective and sustainable raw material, yielding high-crystallinity nanocellulose with strong thermal stability. Furthermore, advancements in the packaging industry demonstrate that rigorous recycling processes, such as deinking flotation, can successfully remove heavy metals from pharmaceutical packaging, ensuring the safe and circular reuse of cardboard.

A significant portion of this collection delves into the chemical and physical modification of cellulose. Through its highly reactive hydroxyl groups, cellulose can be tailored for highly specific functionalities. Researchers are continually overcoming the polymer's natural insolubility (mainly due to its strong hydrogen bonding) by developing novel homogeneous modification processes, such as the use of aromatic oxy-ionic liquids for rapid, uniform esterification. These structural modifications open the door to advanced materials, ranging from rigid rod-like cellulose ethers in aqueous solutions to cholesteric liquid crystals with tunable, temperature-responsive optical properties for sustainable photonics. Non-chemical approaches, such as low-frequency ultrasound treatments, are also highlighted for their ability to reduce particle size and enhance the rheology of cellulose nanocrystal suspensions without altering their fundamental chemical structure or hydrophilicity.

Beyond fundamental materials science, the research presented herein showcases the critical role of cellulose in solving modern, high-tech challenges across three major frontiers:

- **Energy Storage and Electronics:** Cellulose is rapidly replacing non-renewable materials in the fast-growing energy sector due to its high specific surface area and thermal stability. Innovations in this collection include porous activated carbons derived from bacterial nanocellulose for high-performance supercapacitor electrodes, the use of nanocellulose as a carbon source in lithium battery cathodes, and the application of ethyl cellulose in advanced electronic and dielectric devices.
- **Environmental Remediation:** To address critical water pollution, modified cellulose aerogels—rendered superhydrophobic and magnetic—are being deployed for the efficient, reusable cleanup of oil spills. Similarly, functionalized cellulose acetate capsules offer a powerful solution for extracting toxic cadmium ions from contaminated water and battery leachates.
- **Biomedical Engineering:** Capitalizing on its exceptional biocompatibility and tunable properties, cellulose is transforming medicine. Highlights include lyophilized nanofibrillated hydrogels that support viable 3D cell cultures for advanced drug screening, and ethyl cellulose–ethanol gels engineered for controlled, minimally invasive tumor ablation therapies.

Ultimately, the undeniable versatility of cellulose positions it as a cornerstone of sustainable innovation. The research presented here not only underscores the material's immense scientific potential but also injects a vital sustainability factor into some of the most critical and advanced applications of our time.

Marta Fernandes and Jorge Padrão

Guest Editors

Advanced Preparation and Application of Cellulose

Marta Fernandes * and Jorge Padrão

Centre for Textile Science and Technology (2C2T), University of Minho, Campus de Azurém, 4800-058 Guimarães, Portugal; padraoj@2c2t.uminho.pt

* Correspondence: marta.fernandes@det.uminho.pt

Growing environmental awareness of global warming, energy crises, and waste generation is driving demand for renewable low-impact products. The scientific community is increasingly focused on developing sustainable and renewable materials for future applications, with particular interest in nanocellulose. Cellulose is a biobased polymer that stands out as one of the most prominent candidates in this area, due to its abundant availability from various sources.

Wood pulp is the main source for industrial cellulose production; however, slow tree growth drives interest in annual fast-growing plants. Sumarago et al. studied the effects of acid concentration and temperature on the yield and properties of nanocellulose extracted from Maguey fiber. A nanocellulose yield of 81.58% (50 °C and 50 wt.% H₂SO₄) with high crystallinity, good thermal stability, and strong colloidal stability corroborates this fiber as a sustainable and effective raw material for nanocellulose production [1]. The packaging industry uses a considerable amount of recycled cellulose pulp. Klemenčič et al. investigated the removal of heavy metals during paper and cardboard recycling from pharmaceutical packaging via deinking flotation. The results gathered indicate that the presence of adhesives and metal electronegativity affect separation. Nevertheless, the recycling process effectively removes certain heavy metals, indicating strong potential for safe cardboard recycling [2].

Cellulose can be chemically modified through its abundant and highly reactive hydroxyl groups, enabling tailored functionalities. However, strong hydrogen bonding makes it insoluble in common organic solvents and water. Thus, homogeneous modification processes are increasingly explored to improve cellulose modification efficiency and homogeneous application. Yoshizawa et al. developed aromatic oxy-ionic liquids (ILs) for the modification of cellulose, achieving fast (30 min) and efficient esterification with a high degree of substitution and uniform structures, overcoming the side effects of acetyl groups common in carboxylate ILs [3]. Unlike cellulose, industrially synthesized cellulose ethers are often soluble in water, where they form rod-like conformations in solution. Yoshida et al. investigated the conformation of hydroxyethyl cellulose ether in aqueous solutions, concluding that the molecules behave like rigid rod-like particles across a wide molar-mass range, regardless of molar mass [4]. Ogiwara et al. investigated the cholesteric liquid crystals derived from hydroxypropyl cellulose esterified with two alkanol side-chain groups to control the helical structure and optical properties. These materials have tunable color responses to temperature, highlighting their potential for sustainable photonic and optical applications [5]. In the work carried out by Perrin et al., using a non-chemical approach, it was shown that low-frequency ultrasound treatment affects cellulose nanocrystals by breaking aggregates and reducing particle size. The treatment improves the rheology of suspensions and promotes liquid-crystal formation, while leaving chemical structure, crys-

tallinity, surface charge, and hydrophilicity unchanged, enhancing their use in stabilizing Pickering emulsions [6].

In energy applications, cellulose is an alternative material for replacing non-renewable sources, satisfying the currently high demand for sustainability in this fast-growing sector. It has attractive properties such as high carbon content, ease of surface modification and doping, three-dimensional structure, high specific surface area, and high thermal and structural stability. Villarreal-Rueda et al. investigated the production of activated carbon from bacterial nanocellulose grown in fiqué juice for use in supercapacitor electrodes. These materials, chemically activated with KOH at 800 °C, created porous carbons, achieving a high surface area (up to 893 m²/g) and exhibiting excellent electrochemical performance for sustainable energy storage [7]. Kroff et al. evaluated the use of nanocellulose as a reducing agent and carbon source in the microwave-assisted hydrothermal synthesis of lithium iron phosphate/carbon cathodes [8]. Puente-Córdova et al. highlighted the potential of ethyl cellulose for electronic and dielectric applications by analyzing the electrical conduction mechanisms under alternating and direct current electric fields, where they concluded that electric conduction is governed by the mobility of the chain segments [9].

Addressing water pollution is a critical environmental challenge. The intrinsic hydrophilicity and recycling challenges of cellulose-based aerogels have hindered their practical application as sorbents for oil and organic pollutant cleaning. To overcome this, Zhai and Yuan fabricated a cellulose-based aerogel incorporating Fe₃O₄ nanoparticles via a simple freeze-drying method, followed by chemical vapor deposition, resulting in superhydrophobic, magnetically recoverable and reusable composites for treating oil pollution in water [10]. In the work of Pérez-Silva et al., cellulose acetate capsules functionalized with Cyanex 923 for removing cadmium ions from aqueous solutions and battery leachates were explored [11].

Cellulose is widely utilized in biomedical engineering due to its excellent biocompatibility, tunable properties, and renewable nature. For example, Merivaara et al. developed lyophilized nanofibrillated cellulose hydrogels with tunable stiffness for three-dimensional cell cultures. The hydrogels have demonstrated success in forming viable 3D spheroids of mesenchymal stem cells and cancer cells, offering an efficient alternative for drug screening [12]. In another work, Chelades et al. investigated ethyl cellulose-ethanol gel as a controlled alternative to liquid ethanol for percutaneous ablation. Experiments in ex vivo swine liver established relationships between infusion volume, concentration, and needle gauge to predict the size of the ablation zone, aiming to optimize injection protocols for controlled, minimally invasive tumor ablation therapies [13].

The versatility of cellulose is unquestionable, and further promotes its relevance in adding an important sustainability factor to advanced critical applications.

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Article

Production and Characterization of Nanocellulose from Maguey (*Agave cantala*) Fiber

Erwin C. Sumarago ¹, Mary Frahnchezka M. dela Cerna ¹, Andrea Kaylie B. Leyson ¹, Noel Peter B. Tan ^{2,*} and Kendra Felizimarie Magsico ²

¹ Department of Chemical Engineering, University of San Carlos, Cebu City 6000, Philippines; erwincsumarago@gmail.com (E.C.S.); mfrahnz@gmail.com (M.F.M.d.C.); andrealeyson@gmail.com (A.K.B.L.)

² Center for Advanced New Materials, Engineering, and Emerging Technologies (CANMEET), University of San Agustin, Iloilo City 5000, Philippines; kfmagsico@usa.edu.ph

* Correspondence: dtan@usa.edu.ph

Abstract: Plant fibers have been studied as sources of nanocellulose due to their sustainable features. This study investigated the effects of acid hydrolysis parameters, reaction temperature, and acid concentration on nanocellulose yield from maguey (*Agave cantala*) fiber. Nanocellulose was produced from the fibers via the removal of non-cellulosic components through alkali treatment and bleaching, followed by strong acid hydrolysis for 45 min using sulfuric acid (H₂SO₄). The temperature during acid hydrolysis was 30, 40, 50, and 60 °C, and the H₂SO₄ concentration was 40, 50, and 60 wt. % H₂SO₄. Results showed that 53.56% of raw maguey fibers were isolated as cellulose, that is, 89.45% was α-cellulose. The highest nanocellulose yield of 81.58 ± 0.36% was achieved from acid hydrolysis at 50 °C using 50 wt. % H₂SO₄, producing nanocellulose measuring 8–75 nm in diameter and 72–866 nm in length, as confirmed via field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) analysis. Fourier-transform infrared spectroscopy (FTIR) analysis indicated the chemical transformation of fibers throughout the nanocellulose production process. The zeta potential analysis showed that the nanocellulose had excellent colloidal stability with a highly negative surface charge of −37.3 mV. Meanwhile, X-ray diffraction (XRD) analysis validated the crystallinity of nanocellulose with a crystallinity index of 74.80%. Lastly, thermogravimetric analysis (TGA) demonstrated that the inflection point attributed to the cellulose degradation of the produced nanocellulose is 311.41 °C.

Keywords: *Agave cantala*; maguey fiber; alkali treatment; bleaching; acid hydrolysis; nanocellulose; nanocrystal

1. Introduction

Nanocellulose is one of today's most prominent sustainable materials due to its numerous appealing properties, such as biodegradability, high mechanical capabilities, excellent biocompatibility, and many more [1]. It refers to a natural cellulose nanomaterial extracted from plant cell walls with nano-scale dimensions of less than 100 nanometers in diameter. This nanomaterial offers the potential for surface modification due to its abundant and highly reactive hydroxyl groups, providing an opportunity to introduce specific chemical functionalities [2]. It can be classified into three main categories: nanocrystalline cellulose, nanofibrillated cellulose, and bacterial cellulose [3]. Nanocellulose properties include high surface area, excellent strength, good stiffness, and sustainable features such as eco-friendliness, biocompatibility, non-toxicity, and renewable characteristics [4]. With its sustainable nature and excellent mechanical properties, its application ranges from material science to biomedical engineering [5]. One primary application of nanocellulose is as a polymer matrix reinforcement in biocomposite processing due to its crystal size and versatility in chemical surface modifications [6]. Nanocellulose crystals are used as

reinforcing agents in various polymer matrices like polyurethane and starch, forming mechanically durable polymer nanocomposites [7].

The primary source for industrial cellulose production is wood pulp from hardwoods and softwoods [8]. However, since it takes several years for trees to become a source of cellulose, annual plants are used as an alternative source for cellulose instead of wood [9]. The search for alternative sources of cellulose fibers should be directed to plants with large biomass in which the production is low-cost, the usage time is shorter, and the source material is renewable and biodegradable [9]. Plant fibers qualify these characteristics, making them extensively studied as an alternative source for nanocellulose.

Cellulose, the essential structural component in plant fiber, is isolated through an alkali protocol, which involves alkali treatment followed by bleaching [10]. Alkali treatment is the process of cellulose isolation that utilizes sodium hydroxide solution to treat the plant fibers, removing the hemicellulose, lignin, extractive substances, and waxes and oil covering the cell walls of plant fibers [11]. This is followed by a bleaching process using oxidizing chemicals like hypochlorite and peroxide that removes the colored lignin and decomposed wax materials from the cellulosic residues of the plant fibers [12].

Nanocellulose can be produced from cellulose via various methods based on the material source and final intended application. These include chemical methods like acid hydrolysis, enzymatic hydrolysis, subcritical water hydrolysis, and 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) oxidation, and mechanical methods like grinding, cryo crushing, and steam explosion [1,13]. Acid hydrolysis, the most common and effective method of nanocellulose production, involves using diluted or concentrated acids to hydrolyze the amorphous regions of the cellulose [14]. Hydrolysis techniques tend to remove the amorphous regions in the fiber, producing nanocellulose with higher crystallinity [15]. Studies have reported that nanocellulose produced via acid hydrolysis has a higher crystallinity index and is of smaller sizes compared with nanocellulose produced using other methods [16].

Acid hydrolysis is carried out primarily by using concentrated acids to avoid the need for high pressure and temperature to hydrolyze the amorphous regions of cellulose effectively. Employing concentrated acids like sulfuric, sulfurous, hydrochloric, hydrofluoric, phosphoric, nitric, and formic acid facilitates the generation of high yields of nanocrystalline cellulose with enhanced adaptability to different raw materials, as opposed to the utilization of diluted acids [17]. The most preferred acid is sulfuric acid (H_2SO_4) because the esterification of the hydroxyl group by sulfate ions causes the cellulose nanocrystals to be strongly isolated. However, the production method using concentrated acids presents significant drawbacks owing to the corrosive and toxic nature of acids involved together with the generation of acid wastewater, which necessitates the implementation of acid waste recycling to mitigate the cost associated with acid waste disposal [1].

Numerous studies have reported the pivotal role of hydrolysis parameters, specifically acid concentration, temperature, and hydrolysis duration, in influencing the yield of nanocellulose [14]. According to the study by Bacha (2022) on the effects of acid hydrolysis process parameters for extracting nanocellulose from teff straw, the reported findings reveal that under constant reaction times and temperature conditions, the yield of nanocellulose exhibits a decreasing trend with increasing acid concentration [18]. This phenomenon implies an over-degradation of the cellulose due to heightened acid concentrations leading to the decomposition of the crystalline regions within the cellulose. Furthermore, the study also reported the effects of increasing temperature in terms of this facilitating acid–fiber interaction, where higher temperature aids deeper acid penetration into the fiber structure and subsequently degrades its components. Moreover, it was reported that, under constant temperature and acid concentrations, a prolonged hydrolysis duration results in a marginal decrease in the yield. This can be attributed to the extended contact time between the cellulose and acid, which facilitates deeper acid diffusion into the cellulose components, subsequently degrading the crystalline regions, reducing the yield [18].

The effects of varying the hydrolysis parameters as independent variables on the nanocellulose yield and the corresponding optimized conditions depend on factors such

as the type, composition, and purity of raw material used, the optimization method implemented, and the range of the conditions being investigated [14]. Consequently, it is imperative to determine the best conditions resulting in high nanocellulose yields for the acid hydrolysis of a new and different raw material, which is, in this case, the maguey (*Agave cantala*) fiber.

Agave cantala, locally known as, “maguey”, is a plant abundant in fiber belonging to the *Agavaceae* family, most likely native to Mexico. *Agave cantala* is an *Agave* species that has been cultivated in the Philippines. Maguey plant fibers have exemplary properties such as length, strength, and luster and are characterized by their low density, high tenacity, and high moisture absorbency compared with other leaf fibers [19,20]. In the Philippines, these fibers were used to manufacture twines and ropes for fishing and agriculture. Unfortunately, these products are not widely sold anymore [21]. There are minimal studies conducted on the usage of *Agave* species as a raw material for nanocellulose production, such as *Agave tequilana* weber bagasse by Palacios Hinestroza, *Agave americana* leaves by Ortega et al., and *Agave angustifolia* leaves by Rosli et al. [7,22,23]. However, as of the time of writing, there has yet to be an existing study on the potential of using the maguey plant as a source of nanocellulose. Moreover, there is no study on the effects of hydrolysis parameters, sulfuric acid concentration, and temperature, on the yield of nanocellulose derived from this *Agave* species. Thus, this research aims to fill the knowledge gap on the production of nanocellulose from the maguey plant, which is essential to future research on exploiting local non-woody renewable sources of nanocellulose like the maguey fiber.

This study investigated the potential of using maguey fibers as an alternative source of nanocellulose through a series of processes, starting with the preparation of a raw maguey fiber sample, removal of non-cellulosic components of the fiber, and strong acid hydrolysis of cellulose from the fibers. The objective of this research is to investigate the effects of acid hydrolysis parameters, sulfuric acid concentration (40, 50, 60 wt. % H_2SO_4), and reaction temperature (30, 40, 50, and 60 °C) on the nanocellulose yield at a constant reaction time of 45 min. Further investigations were carried out on the chemical and physical properties of the nanocellulose obtained from the hydrolysis conditions that gave the highest yield.

2. Materials and Methods

Maguey fiber samples (MFS) were obtained via natural retting from the *Agave cantala* plant species, locally known as “maguey” in Tabogon, Cebu, Philippines. The collection and utilization of such plant species adhered to the local, national, and international guidelines and regulations. The chemical reagents used included sodium hydroxide pellets (98% NaOH, HIMEDIA GRM 467, Nashik, India), sodium hypochlorite (7% NaClO stock solution, Parañaque City, Philippines), sulfuric acid (98% H_2SO_4 , AJAX 534, Scoresby, Australia), methanol (100% CH_3OH , Scharlau ExpertQ®, Quezon City, Philippines), and acetic acid (CH_3COOH , glacial, AJAX 01, Scoresby, Australia). These chemicals were used without further purification.

2.1. Drying and Size Reduction for Maguey Fibers

The researchers manually harvested the maguey leaves. The leaves were then retted via natural water retting to obtain their fibers. The obtained raw maguey fibers were washed three times using distilled water to remove remaining impurities before actual experimental execution and then dried in direct sunlight for 8 h. The dried maguey fibers were cut into sections measuring 3–5 cm long and then oven-dried at 80 °C or until a moisture content of <10% was achieved. A moisture content of 5.34% was achieved, as shown in Section S.I.1 in the Supplementary Material. The maguey fibers were then cut into 2–2.5 mm length pieces as part of the preparation before being pulverized in Thomas Model 4 Wiley® Mill, Thomas Scientific, Swedesboro, NJ, USA, with a 1 mm screen plate. Maguey fibers were then sieved for 5 min using an ISO 3310-1 Laboratory Test Sieve, Endecotts Ltd., London, UK, sieve shaker with sieve sizes of 850, 450, 250, and 180 µm.

The obtained mean particle size diameter was 582.6820 μm , as shown in Table S.I.2 in the Supplementary Material.

2.2. Removal of Non-Cellulosic Components of Maguey Fibers

2.2.1. Alkali Treatment

A series of alkali treatments with sodium hydroxide (4% *w/v* NaOH) were performed on MFS. In total, 100 g of MFS was treated with 2500 mL of NaOH solution (1:25 g/mL ratio) at 75 °C for 2 h in a laboratory water bath (Model No. WNB 7, Memmert GmbH, Schwabach, Germany) with intermittent stirring every 15 min [7]. After heating, the mixture was quenched in iced water until 30 °C. The cooled samples were then filtered using cheesecloth and washed with distilled water until a filtrate of pH 7 was achieved. The wet alkali-treated MFS was retreated under the same process and conditions for the second alkali treatment. With pH 7, the alkali-treated MFS was oven-dried at 105 °C for 3 h and then further dried at 80 °C for another 3 h.

2.2.2. Bleaching

The dried alkali-treated MFS with an approximate mass of 78.37 g was treated with 783.7 mL of 5% aqueous NaClO solution at 70 °C to room temperature for 1 h (1:10 g/mL ratio) with intermittent stirring every 15 min. The process was carried out at room temperature under a fume hood. After an hour of bleaching, the bleached MFS was filtered using cheesecloth and thoroughly washed with distilled water until a filtrate of pH 7 was reached. The filtered bleached MFS was oven-dried at 80 °C for 3 h. The mass of dried bleached MFS (m_C) is the amount of cellulose obtained. From the obtained cellulose, the MFS cellulose yield (Y_C) was calculated using Equation (1):

$$\% \text{ Cellulose yield } (Y_C) = \frac{m_C}{m_{MFS}} \times 100\% \quad (1)$$

where Y_C is the cellulose yield from MFS, m_C is the mass of cellulose (g), and m_{MFS} is the mass of MFS (g).

2.3. Determination of the Chemical Composition of Cellulose

2.3.1. Determination of Holocellulose Content

Holocellulose refers to the total alpha and hemicelluloses in the extracted cellulose [24]. The holocellulose of the cellulose from MFS was extracted after 3 h of water bath shaking [25]. The process was explicitly carried out by weighing ~1 g of the cellulose sample and mixing with 40 mL of distilled water. To the mixture, 5 g of 0.1269 mM NaClO and 0.25 mL of glacial acetic acid were added, and placed it in a water bath shaker (Model No. SHZ-88 Laboratory Thermostatic Shaking Water Bath, Ningbo, China) for 1 h at 70 °C. The process of adding NaClO and CH₃COOH was repeated every hour for 3 h, resulting in a total of 15 g of 0.1269 mM NaClO and 0.75 mL of glacial acetic acid in a 40 mL mixture of distilled water and a gram of cellulose [25].

The mixture was then cooled in the chiller to nearly 5 °C for at least 1 h. After cooling, the mixture was then brought to room temperature and then washed with a water-methanol mixture (1:1 *v/v* ratio) over Whatman No. 42, Merck KGaA, Darmstadt, Germany filter paper until a filtrate of pH 7 was obtained. The residue in the pre-weighed filter paper was then oven-dried at 40 °C until a constant weight was achieved. The mass obtained from the residue is the amount of holocellulose from the cellulose. Holocellulose content was calculated using Equation (2):

$$\% \text{ Holocellulose content } (\% \text{ HC}) = \frac{m_{HC}}{m_{\text{cellulose}}} \times 100\% \quad (2)$$

where % HC is the holocellulose content of cellulose from MFS, m_{HC} is the mass of oven-dried holocellulose (g), and $m_{\text{cellulose}}$ is the mass of cellulose sample (g).

2.3.2. Determination of α -Cellulose Content

α -cellulose content of the produced cellulose was determined by placing the previously dried holocellulose in a mortar and adding 7 mL of 17.5 wt. % NaOH solution [25]. The contents in the mortar were then mashed slowly for 8 min in a circular motion. Afterward, another 7 mL of 17.5 wt. % NaOH solution was added to the mixture and mashed for another 8 min. The mashed sample in the mortar was transferred to a 100 mL beaker. A total of 40 mL of distilled water was added to the sample in the beaker and mixed thoroughly for 5 min. The mixture was then filtered over Whatman No. 42 filter paper and washed with a mixture of distilled water and 10% acetic acid until the residue became off-white. The residue was oven-dried at 40 °C until a constant weight was achieved. The mass obtained after drying is the mass of the dried α -cellulose (m_C). The α -cellulose content was calculated using Equation (3):

$$\% \alpha - \text{cellulose content (\% C)} = \frac{m_C}{m_{\text{cellulose}}} \times 100 \% \quad (3)$$

where % C is the α -cellulose content of cellulose from MFS, m_C is mass of the dried α -cellulose (g), and $m_{\text{cellulose}}$ is the mass of the cellulose sample (g).

2.3.3. Determination of Hemicellulose Content

The amount of hemicellulose is the difference between holocellulose and α -cellulose amounts. Using Equation (4), the hemicellulose content (% H) was determined:

$$\% \text{ Hemicellulose (\% H)} = \% \text{ HC} - \% \text{ C} \quad (4)$$

2.3.4. Determination of Lignin and Other Extractive Contents

Having determined the hemicellulose and α -cellulose content, by difference, the amount of lignin together with the other extractives was also determined. The content lignin and other extractives (% L and O) was calculated using Equation (5):

$$\% \text{ Lignin \& Other Extractives (\% L \& O)} = 100\% - (\% \text{ H} + \% \text{ C}) \quad (5)$$

2.4. Strong Acid Hydrolysis of Cellulose

Cellulose from MFS was hydrolyzed using sulfuric acid at varying concentrations (40, 50, and 60 wt. %) and temperatures (30, 40, 50, and 60 °C) for 45 min. The hydrolysis process was carried out using 50 mL glass lab vials with screw caps in a water bath shaker at 250 rpm. The process was carried out explicitly by weighing ~0.5 g of extracted cellulose and then deliberately adding it to the preheated solutions of sulfuric acid (10 mL) under the desired conditions. The mixture was then transferred to a 250 mL Erlenmeyer flask with 80 mL of cold distilled water under a fume hood to quench the solution. Another 20 mL of cold distilled water was used to wash off the remaining residue of the hydrolysis mixture in the glass lab vials. The mixture was then homogenized using a vortex mixer for 10 s and then centrifuged (HSIANGTAI Centrifuge, New Taipei, Taiwan) at 3500 rpm for 30 min. After centrifugation, a layer of supernatant and precipitate was observed in the vials. The supernatants were discarded while the precipitates were then dialyzed using cellulose dialysis tubing (MW14000 Regenerated Cellulose RC Dialysis Bag, Hefei, China) with a 14,000 Da cut-off until a solvent (water) of pH 7 was achieved (7 days). The dialyzed samples were freeze-dried (ilShinbiobase Co., Ltd., Dongducheon, Republic of Korea) at −40 °C for 54 h and weighed [4,7,26].

Calculation of Nanocellulose Yield from Maguey Cellulose

The post-hydrolyzed cellulose was designated as the nanocellulose product after the presence of nanoparticles was verified via morphological analysis. The nanocellulose yield (Y_N) was calculated using Equation (6):

$$\% \text{ Nanocellulose yield } (Y_N) = \frac{m_N}{m_{CCS}} \times 100 \% \quad (6)$$

where m_N is the mass of nanocellulose (g), and m_{CCS} is the mass of cellulose sample (g).

Response surface methodology (RSM) through DX-Expert Version 22.0.3 software was administered to analyze the results of the experiment and ensured that the accuracy and sensitivity of the results were carried over. Furthermore, a two-way analysis of variance (ANOVA) without replication was also used to analyze nanocellulose yields and to statistically evaluate the impact of temperature and acid concentration as independent variables on nanocellulose yield. This evaluation assesses the statistical significance of these effects, ruling out chance occurrences.

2.5. Characterization

2.5.1. Morphological Analysis via Scanning Electron Microscopy and Transmission Electron Microscopy

Field emission scanning electron microscope (FE-SEM) analyses were conducted on raw maguey fibers, cellulose, and post-hydrolyzed cellulose. FE-SEM was carried out for both maguey raw fibers and post-hydrolyzed cellulose and analyzed under a Zeiss microscope, Jena, Germany at 5.0 kV; the FE-SEM analysis of the extracted cellulose was analyzed uncoated with benchtop SEM model JCM-7000, JEOL Ltd., Tokyo, Japan at 10.0 kV, and another FE-SEM for post-hydrolyzed cellulose was analyzed using JEOL JSM-IT800, JEOL Ltd., Tokyo, Japan at 15.0 kV. For the raw MFS, the samples were coated with gold, with a 3 nm thickness, using a sputtering machine. The dimensions of the nanoparticles were manually measured using AutoCAD Version S.51.0.0 software.

Moreover, transmission electron microscopy (TEM) analysis was carried on the post-hydrolyzed cellulose using JEOL JEM-2100F, JEOL Ltd., Tokyo, Japan with 200 accelerating voltages in a 2 μ m and 50 nm micron marker. Information on the inner structure of the post-hydrolyzed cellulose sample was analyzed. The dimensions of the nanoparticles were also manually measured using AutoCAD Version S.51.0.0 software.

2.5.2. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analyses for the raw MFS, cellulose, and post-hydrolyzed cellulose were performed using IRAffinity-1s, SHIMADZU, Kyoto, Japan, through zinc selenide attenuated total reflectance (ZnSe, ATR, SHIMADZU, Kyoto, Japan) in the range of 600 to 4000 cm^{-1} . The samples were finely powdered using an electric rotary-type mill before the analysis.

2.5.3. Zeta Potential Analysis

The zeta potential of the nanocellulose was measured using the SZ-100, HORIBA, Kyoto, Japan instrument. The measurement was performed by suspending approximately 0.05 g of the nanocellulose sample in 50 mL of water, followed by sonication for 3 h to ensure uniform dispersion. The instrument was set to an electrode voltage of 3.3 V and a conductivity of 0.285 mS/cm. The measurement was performed at constant temperature of 25 $^{\circ}\text{C}$ and dispersion medium viscosity of 0.895 mPa.s with a range from -200 mV to 200 mV.

2.5.4. X-ray Diffraction (XRD)

The XRD analysis was performed using a LabX-6000, SHIMADZU, Kyoto, Japan X-ray diffractometer with an aluminum plate sample holder (25 mm diameter and 1 mm depth) at 40.0 kV and 30.0 mA with Cu $K\alpha$ radiation (1.5148 $\text{\AA}$). The post-hydrolyzed cellulose samples were finely powdered for 1 min using an 800Y Kankeirr, Guangzhou,

China electric rotary-type mill with sharp steel blades and sent for analysis. The post-hydrolyzed cellulose sample was analyzed without any further processing of the raw data. The data were acquired in a 2θ range from 2° to 70° . The detailed description of the XRD analysis profile data can be accessed in Section S.IV.4. in the Supplementary Material. The crystallinity index (I_C) of the nanocellulose from maguey fibers obtained via a series of chemical treatments was calculated using the XRD peak height method by Segal et al. (1959), as shown in Equation (7) [27]:

$$I_C = \frac{I_{200} - I_{am}}{I_{200}} \times 100 \% \quad (7)$$

where I_{200} is the maximum intensity of diffraction of the (200) lattice peak around 22° to 23° and I_{am} is the minimum intensity between 18° and 19° , representing the amorphous material in this empirical equation.

2.5.5. Thermogravimetric Analysis (TGA)

The TGA of the post-hydrolyzed cellulose was performed using a Simultaneous Thermal Analyzer (STA) 6000, PerkinElmer, Waltham, MA, USA measuring cell to characterize the thermal stability of the nanocellulose obtained from the maguey fibers. Approximately 13.549 mg of sample was held in a ceramic crucible and heated from 30°C to 600°C at a heating rate of $10^\circ\text{C}/\text{min}$. The measurement was performed under a nitrogen atmosphere at 20 mL/min from 30°C to 600°C .

3. Results and Discussion

3.1. Removal of Non-Cellulosic Components of Maguey Fiber by Alkali Treatment and Bleaching

3.1.1. Cellulose Yield from Maguey Fiber

MFS presents a cellulose yield of 53.56% after alkali treatment and bleaching processes. This corresponds well with the cellulose yield of other *Agave* plant species and competes with other natural fibers that underwent similar processes, as presented in Table 1. MFS has a relatively higher cellulose yield than other natural fibers with similar isolation processes. This implies the significance of MFS as a better alternative cellulose source that is locally available.

Table 1. Cellulose yield and composition of various *Agave* plant species and other natural fibers.

Fiber Sources	Process Parameters		Lignocellulose Yield, %	Components, %			Reference
	Alkali Treatment	Bleaching		α -Cellulose	Hemicellulose	Lignin and Other Extractives	
<i>Agave cantala</i>	4 wt. % NaOH, 75°C , 2 h *	5 v/v % NaClO, 70°C to room temp., 2 h	53.56	89.45 ± 2.64	2.96 ± 0.13	7.59 ± 2.77	Present study
<i>Agave americana</i>	2–4 w/v % NaOH, 80°C , 2 h	2 w/v % NaClO ₂ , 80°C , 4 h	65.00 ± 2.00	90.00 ± 5.00	9.00 ± 5.00	0.04 ± 0.10	[28]
<i>Agave angustifolia</i>	4% NaOH, 70 – 80°C , 2 h *	1.7 w/v % NaClO ₂ , 70 – 80°C , 4 h	67.01 ± 0.15	97.31 ± 0.02	3.14 ± 0.35	0.23 ± 0.04	[7]
<i>Agave gigantea</i>	5 w/v % NaOH, 80°C , 2 h	1.7 wt. % NaClO ₂ , 80°C , 1 h *	n.a.	89.39	3.73	0.53	[29]
Apple pomace	6% NaOH, 60°C , 30 min	1% NaClO, 95°C , 1 h	33.17	84.72	n.a.	n.a.	[26]

Table 1. Cont.

Fiber Sources	Process Parameters		Lignocellulose Yield, %	Components, %			Reference
	Alkali Treatment	Bleaching		α -Cellulose	Hemicellulose	Lignin and Other Extractives	
Banana sheath	2% NaOH, room temp., 6 h	5% NaClO ₂ , 50 °C, 1 h	28.40	46.50	9.34	11.62	[30]
Pineapple leaf	2 w/v % NaOH	4 w/v % NaClO, 85 ± 5 °C, 4 h	70.90 ± 1.10	85.53 ± 2.30	0.30 ± 0.90	0.40 ± 0.30	[31]
Sugar palm	10 w/w % NaOH, 150 °C, 2 h	15% H ₂ O ₂ , 60 °C, 1.5 h	n.a.	86.99 ± 1.98	9.95 ± 0.87	2.90 ± 1.38	[32]

n.a.: not available; * repeated twice.

3.1.2. Holocellulose, α -Cellulose, Hemicellulose, and Lignin and Other Extractive Contents

After removing non-cellulosic components via alkali treatment and bleaching, the bleached MFS was composed of α -cellulose and traces of hemicellulose, lignin, and other extractives. These components were determined via percent composition following various determination procedures. The bleached product comprised holocellulose, which refers to α -cellulose and hemicellulose combined, and lignin and other extractives. The holocellulose content was determined to be 92.41%. The composition of the bleached MFS is presented in Table 1. Detailed calculations of the holocellulose, α -cellulose, hemicellulose, and lignin and other extractive contents can be seen in Section II of the Supplementary Material.

As a result of alkali treatment and bleaching carried out on the MFS, bleached MFS with α -cellulose content of 89.45% is presented in Table 1. This α -cellulose content is competitive with other reported α -cellulose contents of other *Agave* plant species and is relatively higher than that of other natural fibers. The bleached MFS has a hemicellulose content of 2.96%, which is lower than that of other reported hemicellulose and has a lignin content of 7.59%.

The hemicellulose content of the bleached MFS corresponds well with the alkali treatment process. The repetition of the alkali treatment process further removed more hemicellulose in the MFS, leading to lower hemicellulose content compared with those under processes without repeated alkali treatment. Moreover, the lignin content of the bleached MFS was relatively higher, corresponding with the bleaching process carried out on MFS. The bleaching process directly affected the lignin composition, which reacted as lignin chloride [7]. The alkali-treated maguey fiber was bleached from the preheated temperature to room temperature in a shorter period than other reported bleached fibers. The significant amount of α -cellulose produced indicates that maguey fiber is an excellent alternative cellulose source for nanocellulose production.

3.2. Strong Acid Hydrolysis of Cellulose

3.2.1. Nanocellulose Yield from Strong Acid Hydrolysis of Cellulose

The nanocellulose yield from strong acid hydrolysis of cellulose is analyzed with respect to the varying conditions of two independent variables, namely acid concentrations and reaction temperatures. The relationship between the two independent variables with the nanocellulose yield varies as presented in Figure 1.

The acid hydrolyses at all acid concentrations demonstrate a maximum nanocellulose yield along with a 50 wt. % acid concentration while the acid hydrolyses at all reaction temperatures depict a maximum nanocellulose yield at 50 °C, as shown in Figure 1a and Figure 1b, respectively.

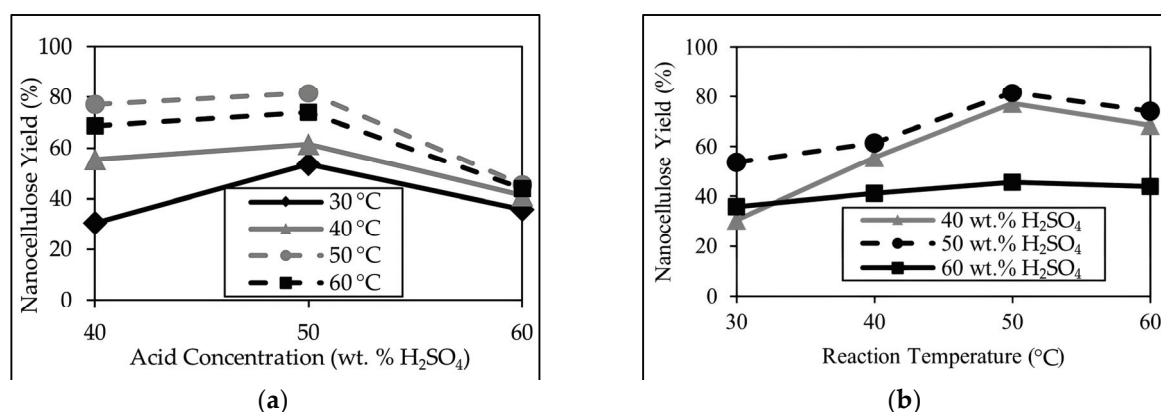


Figure 1. Graph of average nanocellulose yield with respect to (a) varying acid concentrations and (b) reaction temperatures.

Under ideal acid hydrolysis conditions, the amorphous regions of the cellulose fibrils are hydrolyzed and removed by the acid while maintaining the crystalline areas of the cellulose fibrils. However, at temperatures below 50 °C and at an acid concentration of 50 wt. % H₂SO₄, cellulose is under-degraded due to the inability of these conditions to solubilize and degrade the amorphous regions, preventing the formation of nanocrystalline cellulose. On the other hand, under hydrolysis conditions exceeding 50 °C and a 50 wt. % H₂SO₄ concentration, cellulose is over-degraded, causing the decomposition of both crystalline and amorphous regions, resulting in a lower yield.

The acid hydrolysis at 50 °C and a 50 wt. % H₂SO₄ concentration has the highest observed yield of $81.58 \pm 0.36\%$, while hydrolysis at the temperature of 30 °C and a 40 wt. % H₂SO₄ concentration has the lowest observed yield of $30.41 \pm 1.71\%$.

The observed variance in nanocellulose yields is analyzed via a two-way analysis of variance (ANOVA), and simple main effects analysis concludes, at a 95% level of confidence, that both temperature and acid concentration have statistically significant effects on nanocellulose yield.

Moreover, undergoing a multilevel categoric full factorial design type via the response surface methodology of DX-Design Version 22.0.3 software, the analysis obtained a final equation, Equation (8):

$$\begin{aligned} \text{Nanocellulose Yield} &= 57.07 - 1.56A + 12.20A^2 - 9.27B - 9.88B^2 + 13.36B^3 - 10.62AB \\ &+ 3.37A^2B - 3.73AB^2 - 0.9168A^2B^2 + 7.27AB^3 - 0.7924A^2B^3 \end{aligned} \quad (8)$$

where A refers to the acid concentration and B is the reaction temperature. The equation can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as −1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. A very detailed discussion on ANOVA and RSM results can be found in Section III of the Supplementary Material.

The nanocellulose produced from maguey fibers was compared to other nanocellulose obtained from other *Agave* species and other non-wood nanocellulose sources with similar process parameters together with the nanocellulose characteristics including morphological dimensions, crystallinity, and thermal stability, as shown in Table 2. The comparative analysis of nanocellulose yield and characteristics against other *Agave* and non-wood nanocellulose sources exhibits competitive results. Notably, the yield from the produced nanocellulose is within the range of that of *Agave tequilana* Weber var. Azul based on the cellulose sample demonstrating levels similar to the reported nanocellulose yield. Furthermore, the yield of the nanocellulose produced in this study appears relatively higher when compared with that of other reported nanocellulose based on the fiber sample

shown in Table 2. This observation implies that maguey fiber is a significant alternative source for nanocellulose.

Table 2. Nanocellulose yield and characteristics from various *Agave* species and other natural fibers.

Fiber Sources	Process Parameters	Nanocellulose Yield, %	Characteristics				Reference
	Acid Hydrolysis		Diameter (nm)	Length (nm)	Crystallinity Index (%)	Thermal Stability (°C)	
<i>Agave cantala</i>	50 wt. % H ₂ SO ₄ , 50 °C, 45 min	81.58 ± 0.36 (43.50–43.89 *)	8–75	72–866	74.80	311.41	Present study
<i>Agave americana</i>	70% HNO ₃ and 80% CH ₃ COOH, 100 °C, 30 min	27.0 *	n.a	18.2 ± 10.14	64.11	374.70	[28]
<i>Agave angustifolia</i>	60 wt. % H ₂ SO ₄ , 45 °C, 45 min	n.a	8–15	170–500	82.40	361	[7]
<i>Agave sisalana</i>	55 wt. % H ₂ SO ₄ , 45–60 °C, 20–30 min	n.a	5.9 ± 1.0–10.5 ± 2.9	177 ± 56–433 ± 132	n.a	n.a	[33]
<i>Agave tequilana</i>	65 wt. % H ₂ SO ₄ , 50 °C, 60 min	n.a	11 ± 4	323 ± 113	71	324	[34]
<i>Agave tequilana</i> Weber var. Azul	60–65 wt. % H ₂ SO ₄ , 40–60 °C, 40–70 min	4.2–96	8.6–9.1	216–829	88.4–90.1	n.a	[35]
Apple pomace	45% H ₂ SO ₄ , 50 °C, 45 min	n.a	7.9 ± 1.25	28 ± 2.03	78	187	[26]
<i>Acacia fornesiana</i>	60–65 wt. % H ₂ SO ₄ , 45–55 °C, 45–65 min	n.a	n.a	100–260	n.a	n.a	[36]
L. Willd	11 M H ₂ SO ₄ , 50 °C, 30–240 min	10 *	13 ± 4–19 ± 6	319 ± 68–466 ± 159	69–74	n.a	[37]
Banana pseudostem	65 wt. % H ₂ SO ₄ , 50 °C, 60 min	n.a	10 ± 4	329 ± 123	66	357	[34]
Barley	60 w/v % H ₂ SO ₄ , 50 °C, 5 h	n.a	35	170	n.a	345	[38]
Sugarcane bagasse							

n.a: not available; * based on fiber.

Moreover, the morphological dimensions of the produced nanocellulose coincide with those of other reported nanocellulose. This morphological similarity displays the consistency of maguey nanocellulose with the broader variety of non-wood cellulose sources. In addition, the crystallinity index and thermal stability of the produced nanocellulose demonstrate competitive qualities that further enhance its significance and potential applications.

3.2.2. Morphological Analysis of Maguey Fiber, Cellulose, and Post-Hydrolyzed Cellulose

The FE-SEM micrographs of the maguey fiber, cellulose, and post-hydrolyzed cellulose are presented in Figure 2. The following figures show a change in the maguey fiber's morphology after going through various chemical processes: alkali treatment, bleaching, and acid hydrolysis.

The FE-SEM micrograph of the raw maguey fiber shown in Figure 2 has a length range from 400 µm to 700 µm and a diameter range from 90 µm to 110 µm. The fiber surface is uneven and rough, and has an irregular shape, implying the presence of impurities that ought to be hemicelluloses, lignin, and other extractives.

Meanwhile, the FE-SEM micrograph of the extracted cellulose depicts an apparent change in the morphology of the maguey fibers. A smoother surface and narrower microscale cellulose strands can be observed as it underwent alkali treatment and bleaching processes. However, there are inconsistent structures aside from the fibrous structures along the cellulose strands, implying that there are still impurities, such as hemicellulose and lignin. The length and diameter of the cellulose strands range from 23 µm to 256 µm and 5 µm to 13 µm, respectively.

Moreover, the micrograph of the post-hydrolyzed cellulose produced under the best hydrolysis conditions based on the highest yield (at 50 °C using 50 wt. % H₂SO₄) shows an apparent change in morphology as the extracted cellulose underwent the acid hydrolysis process. Figure 2c presents the sonicated post-hydrolyzed cellulose sample prior to FE-SEM

analysis, displaying a dispersed nanocellulose in the micrograph, as indicated with the red arrows. Small rod-like structures at the nanoscale are scattered throughout the image, observed at an approximate magnification of 10,000 times. On the other hand, in Figure 2d, the FE-SEM image without the sonication of the post-hydrolyzed cellulose sample displays agglomerated nanocellulose in the micrograph with a magnification of exactly 30,000 times. The length and diameter of the nanoparticles range from 72 nm to 866 nm and 8 nm to 75 nm, respectively. With the length of the nanoparticles exceeding 100 nm and differing significantly from their diameter, the nanoparticles produced can also be considered nanofibrillar. However, nanofiber is mostly associated with a length of several micrometers; thus, the produced nanoparticles will be acknowledged as nanocellulose. A very detailed characterization of nanocellulose can be seen in Section IV of the Supplementary Material.

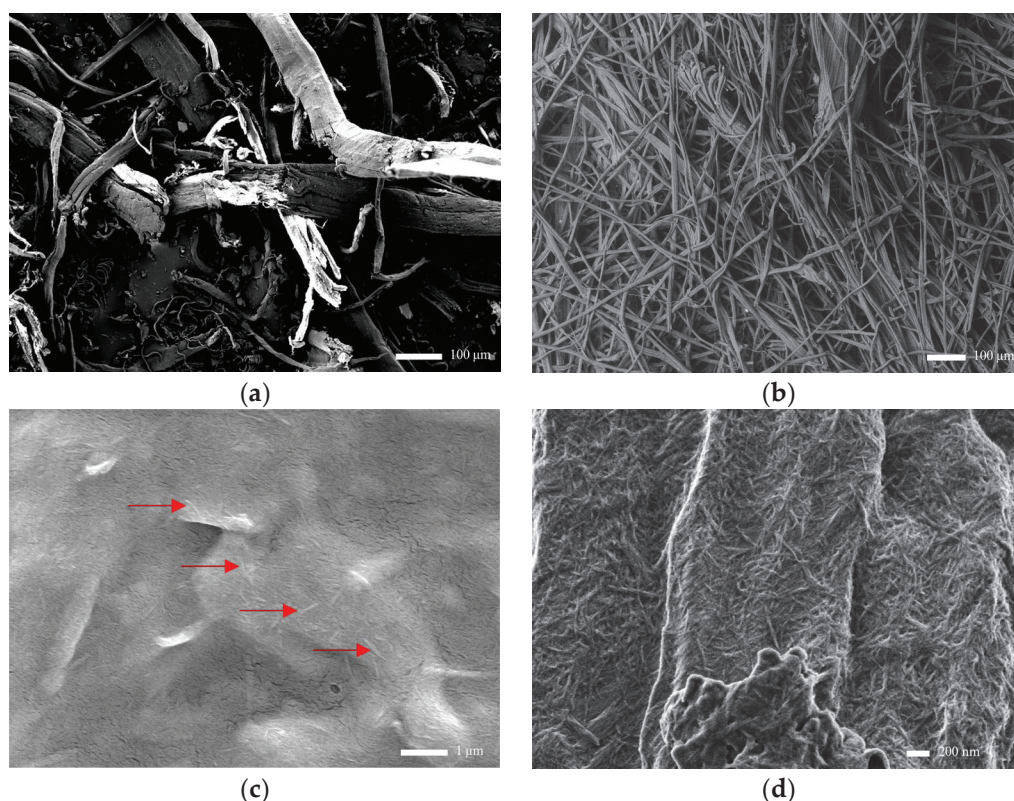


Figure 2. Field emission scanning electron microscopy (FE-SEM) micrographs of (a) raw maguey fiber sample (MFS), (b) extracted cellulose, (c) sonicated post-hydrolyzed cellulose (dispersed nanocellulose indicated with red arrows), and (d) non-sonicated post-hydrolyzed cellulose.

TEM analysis was also carried out to examine the morphology of the post-hydrolyzed cellulose, which provides an excellent resolution, enabling a closer look at the structure and size of the sonicated post-hydrolyzed cellulose sample, as shown in Figure 3. Figure 3a clearly shows rod-like structures similar to what is observed in Figure 2c. The length and diameter coincide with the manually measured structures in the SEM micrographs. The measured dimensions present an average length of 491.57 nm and an average diameter of 69.99 nm. In Figure 3b, the inner structure of the nanoparticles is solid, which corresponds to the formation of a crystalline rod-like structure, attributed to the cleavage of the amorphous region of the cellulose during acid hydrolysis [39].

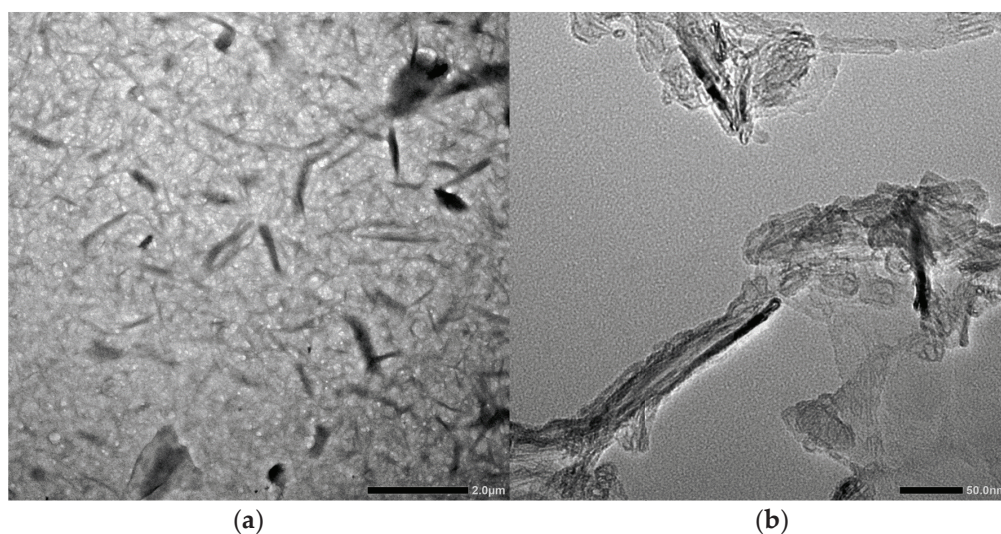


Figure 3. Transmission electron microscopy (TEM) micrograph of post-hydrolyzed cellulose at (a) 2.0 μm scale and (b) 50.0 nm scale.

3.2.3. Fourier Transform Infrared (FTIR) Spectroscopy Analysis of Maguey Fiber, Cellulose, Nanocellulose

The results of the FTIR analysis for the raw maguey fiber, cellulose, and nanocellulose are shown in Figure 4. The figure presents infrared transmittance, shown in peaks, for the raw maguey fiber, cellulose, and nanocellulose spectrum. The appearance and absence of certain peaks indicate changes in the chemical composition of the fibers as they underwent alkali treatment, bleaching, and acid hydrolysis processes. The actual spectrum of the samples can be seen in Section S.IV.3. in the Supplementary Material.

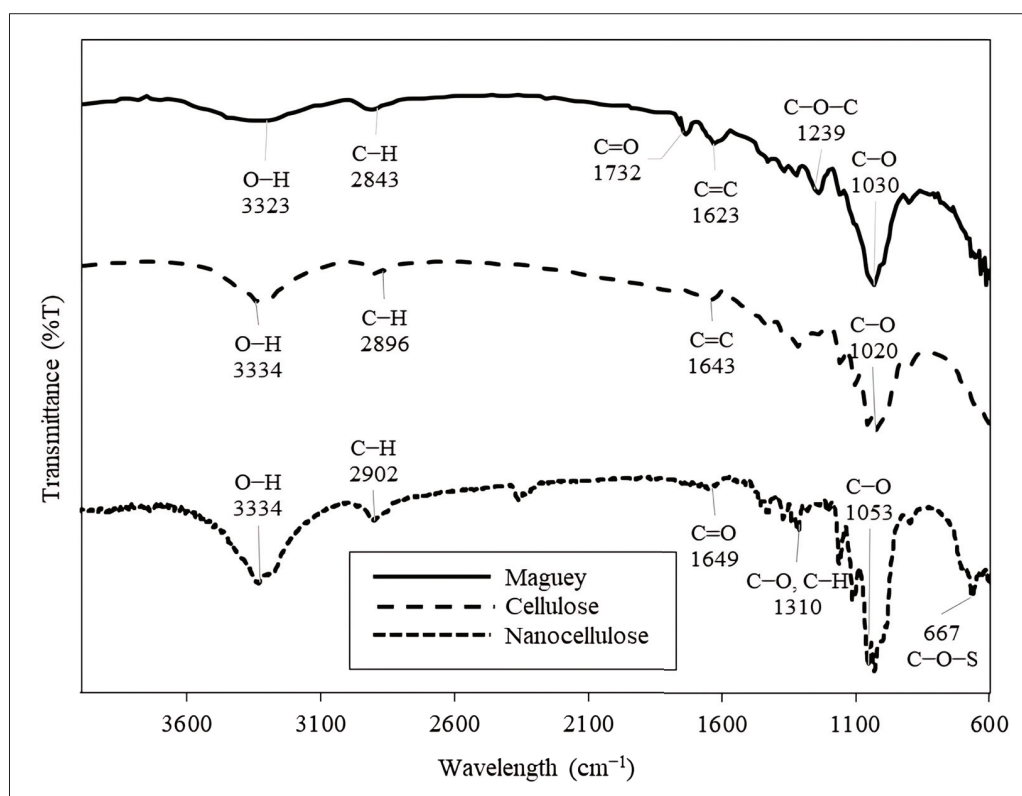


Figure 4. Fourier transform infrared spectroscopy (FTIR) spectrum of raw maguey fiber (data were generated using OriginPro).

The raw maguey fiber spectrum shows peaks at 1732.94 cm^{-1} and 1623.69 cm^{-1} , indicating the presence of C=O and C=C functional groups, respectively [40]. The C=O stretching likely refers to the carbonyl, carboxyl, and acetyl groups corresponding to the presence of cellulose and hemicellulose. Meanwhile, the C=C stretching corresponds to the presence of lignin. Other peaks occur at 1239.30 cm^{-1} , 1030.79 cm^{-1} , and 2843.29 cm^{-1} , and a broad-shallow peak occurs in the $3100\text{--}3600\text{ cm}^{-1}$ range, associated with the presence of cellulose and represent aryl-alkyl ether in lignin, primary alcohol in hemicellulose, hydrocarbons, and the hydroxyl stretching vibrations, respectively [41].

The peaks of the raw maguey fiber spectrum appear to be of a lower intensity than the peaks of the cellulose and nanocellulose spectra. The resulting peak of an untreated fiber is influenced by several factors, including different surface conditions due to the presence of impurities. The chemical bond under different surface conditions will absorb varying intensities and frequencies, which likely causes lower absorption of infrared radiation, resulting in a lower intensity of the spectrum peaks [42].

On the other hand, the cellulose spectrum shows the result of the alkali-treated and bleached maguey fibers. The broad peak at 3334.88 cm^{-1} and small peak at 2896.96 cm^{-1} are attributed to the O–H stretching vibration group of cellulose and C–H stretching, respectively. Moreover, the peak at 1643.80 cm^{-1} in this spectrum indicates the presence of a C=C functional group attributed to lignin [43]. The lack of C=O vibrations indicates the effectiveness of the alkali treatment and bleaching processes, as it implies the removal of most hemicellulose.

The spectrum of the nanocellulose produced from maguey fiber exhibits a broad peak at 3334.92 cm^{-1} , which is attributed to the O–H stretching vibration and intermolecular hydrogen bonding. The O–H vibration peaks exhibit a correlation with hydrolysis, as prolonged hydrolysis results in a deeper peak within this range [44]. Thus, the change in peak depth is observable from the raw maguey fiber to the nanocellulose sample. Additionally, a peak at 2902.87 cm^{-1} is associated with C–H bending, indicating the enhanced exposure and sharpening of the cellulose components [44–46].

Furthermore, an observable peak at 1649.14 cm^{-1} is attributed to the carboxyl groups corresponding to the presence of cellulose (higher intensity peaks), hemicellulose, and lignin (lower intensity peaks), while smaller vibrations ($<1315.45\text{ cm}^{-1}$) is attributed to the bending vibration of C–O of secondary alcohols and aromatic C–H bonds in a polysaccharide structure [47]. However, the absence of the C=O attributed to hemicellulose and C=C attributed to lignin, which are commonly found in most untreated fibers, implies the removal of most hemicellulose and lignin during the alkali treatment, bleaching, and the acid hydrolysis process. In the fingerprint region, the peak at 667.37 cm^{-1} is attributed to C–O–S bending, indicating a sulfation peak attributed to the hydrolysis process or the reaction of sulfuric acid [48].

3.2.4. Zeta Potential Analysis of Nanocellulose

The zeta potential of the nanocellulose was measured to evaluate its surface charge and the stability of the nanocellulose suspension. The nanocellulose displays a highly negative surface charge with a mean zeta potential value of -37.3 mV . This negative zeta potential was aided by the negative electrostatic layers formed on the nanocellulose due to the grafting of sulfate groups during sulfuric acid hydrolysis that link to the nanocellulose chains [49]. Nanocellulose with a negative zeta potential is highly preferred as it can resist aggregation in a colloidal environment, leading to a wider distribution [49]. The reported zeta potential value presents a more stable dispersed state compared with values between -15 and 15 mV where agglomeration forms due to the lack of necessary charges for a repelling effect [49]. This result implies that the produced nanocellulose would lead to a uniform nanocellulose suspension due to the strong electrostatic repulsive forces [49,50]. The measurement results can be obtained from Section S.IV.4 in the Supplementary Material.

3.2.5. X-ray Diffraction Analysis of Nanocellulose

Figure 5 shows the X-ray diffraction pattern of the nanocellulose from maguey fiber. The diffraction pattern showed peaks around $2\theta = 15.52^\circ$ and 22.64° , which indicate a typical cellulose I structure [7,15]. The high peak exhibited a higher crystallinity index, indicating the effective removal of amorphous regions during the chemical treatments. The crystallinity index refers to the order of crystallinity of the material rather than the crystallinity of crystalline regions. A higher crystallinity value is mainly attributed to removal of the amorphous constituents during acid hydrolysis.

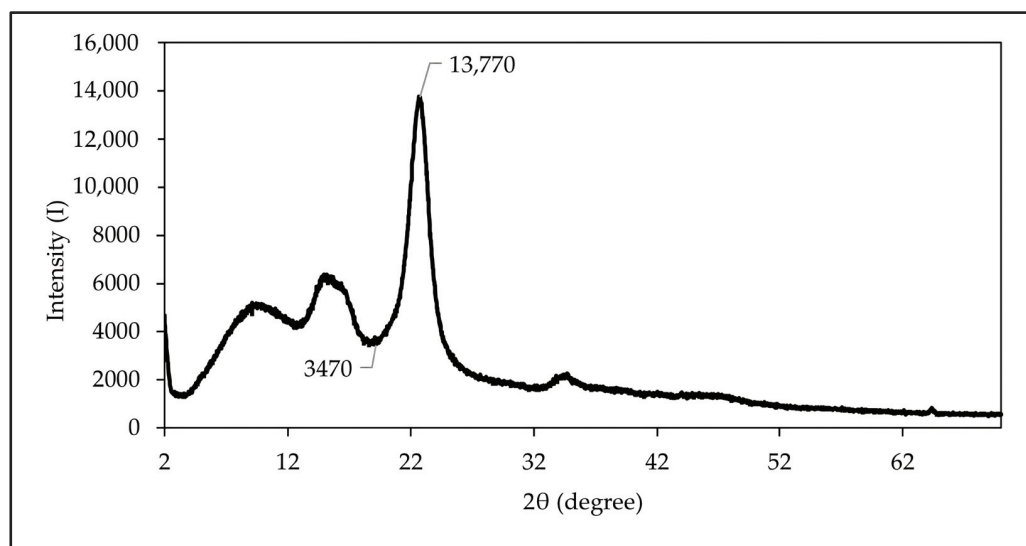


Figure 5. X-ray diffraction (XRD) patterns of nanocellulose from maguey fibers.

In Figure 5, the crystallinity of the sample implies the presence of a sharp peak. The height of the highest diffraction peak represents the amount of crystalline material, while the amorphous material is represented by the height of the minimum intensity between the major peaks. The crystallinity of the nanocellulose increases when the lignin, hemicellulose, and other amorphous components are almost completely removed during the series of chemical treatments [48].

The method of Segal et al. (1959) is used to determine the crystallinity index of maguey fibers, known as the XRD peak height method [27]. This method examined the changes in the XRD spectra during the decrystallization of nanocellulose from maguey fiber using a series of chemical treatments. The crystallinity index (I_C) was determined from the maximum (I_{200}) and minimum (I_{am}) intensity of the diffraction, which signify the crystallinity and the amorphous material peaks at 22° to 23° and 18° to 19° for a 2θ diffraction angle, respectively. The crystallinity index of the nanocellulose from maguey fiber was found to be 74.80%, while the crystallinity index of some of the nanocellulose from various natural fibers was ~64–82% [7,15,28,38]. In that case, the calculated crystallinity index was within the range of the crystallinity index of nanocellulose among the natural fibers. This high value of the crystallinity index is attributed to the elimination of amorphous constituents during the chemical treatments.

3.2.6. Thermogravimetric Analysis of Nanocellulose

The thermal decomposition parameters of the obtained nanocellulose sample from the maguey fibers were determined from the thermogravimetric–differential thermogravimetric (TG–DTG) of the nanocellulose sample in solid white powder form.

Evident weight loss and corresponding peak temperatures are observed in the resulting TG and DTG curves of the nanocellulose sample, presented in Figure 6. The TG curve illustrates how the weight of the post-hydrolyzed cellulose changes with temperature while the DTG curve indicates the point at which weight loss is most apparent.

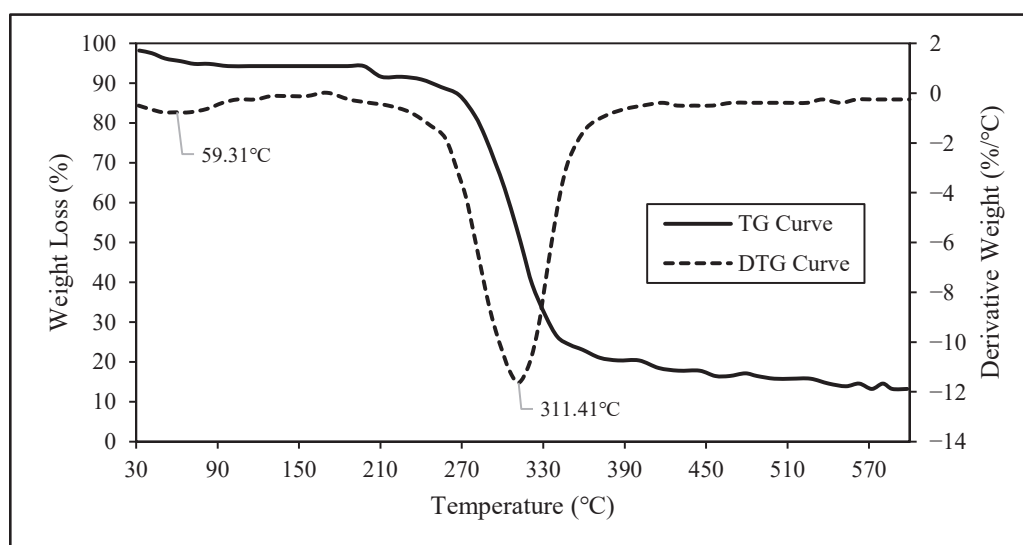


Figure 6. Thermogravimetric (TG) and differential thermogravimetric (DTG) curves of nanocellulose from maguey fibers.

Based on the DTG curve presented in Figure 6, it is evident that within the temperature range of 30.00 °C to 129.13 °C, the peak temperature of 59.31 °C corresponds to 4.72% weight loss, primarily attributed to the evaporation of moisture during the heating process. During the heating in the temperature range of 129.13 °C to 427.81 °C, the inflection point or the first derivative peak temperature, indicating that the point of the greatest rate of change on the weight loss curve was at 311.41 °C, where 81.22% weight loss occurred. This weight loss is attributed to the alpha-cellulose degradation at this heating temperature [7]. The residual weight at the final temperature of 600 °C is found to be 14.06% of the initial weight of the sample. A fraction of this residual weight of post-hydrolyzed cellulose is attributed to the remaining lignin. Lignin has a slow degradation rate, and, when it is present in the sample, a higher residual weight is expected [7]. The remaining fraction of the residual weight can be attributed to the remaining ash present, the thermal decomposition of which occurs above the final temperature. A detailed report of this analysis can be found in Section S.IV.6 in the Supplementary Material.

4. Conclusions

Cellulose was successfully obtained from maguey (*Agave cantala*) fibers via alkali treatment and bleaching processes with a yield of 53.56%. The high cellulose yield of maguey fiber demonstrates an efficient process and embodies promising qualities in terms of sustainability and cost-effectiveness. This high yield not only enhances productivity and resource efficiency but also contributes significantly to sustainable nanocellulose production. Maximizing output through an efficient process leads to reductions in cost and resource conservation. The availability of maguey fiber as a local source of cellulose also has economic advantages in terms of transportation, resource acquisition, and flexible production. Additionally, local availability can also contribute to lower carbon emissions and other environmental impacts, leading to more sustainable production.

Cellulose nanocrystals were also successfully produced from maguey cellulose via strong acid hydrolysis, as proven by the nanoscale dimensions measured in the morphological analysis and the crystallinity index measured via XRD analysis. Variations in the acid concentration and reaction temperatures of the acid hydrolysis of cellulose from maguey fibers show significant effects on the yield of nanocellulose produced, as supported by the two-way ANOVA.

This study found that the acid hydrolysis conditions that provided the highest nanocellulose yield are 50 °C and a 50 wt. % H_2SO_4 concentration. FE-SEM and TEM analyses showed that the maguey fibers were successfully disintegrated from 90–110 μm to 8–75 nm

in diameter and from 400–700 μm to 72–866 nm in length. FTIR analysis showed that the chemical composition of the maguey fibers changed from that of raw fibers to that of nanocellulose, indicating the removal of most impurities. The highly negative surface charge of the nanocellulose with a mean zeta potential value of -37.3 mV indicates a more stable dispersed state of the nanocellulose suspension due to the strong electrostatic repulsion. Through XRD analysis, the crystallinity index of the nanocellulose was found to be 74.80%. On the other hand, thermogravimetric analysis of the nanocellulose indicated that the point of the greatest rate of thermal degradation occurred at 311.41 $^{\circ}\text{C}$, which is attributed to cellulose degradation.

The nanocellulose produced from maguey fiber embodies desirable properties, thereby revealing the potential of maguey fiber as an alternative source of nanocellulose. This offers an efficient solution for diverse practical applications such as in polymers due to its competitive crystallinity index and stable dispersed state. Other essential qualities of nanocellulose include biocompatibility, sustainability, and cost-effectiveness, making it an ideal candidate for advanced material applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym16101312/s1>, contains data gathered from SEM micrographs analysis, raw data from FTIR analysis, Zeta Potential analysis, XRD analysis profile data, and TGA data. Supplementary I: Characterization of the maguey fiber sample; Supplementary II: Determination of cellulose, hemicellulose, and lignin and other extractive contents; Supplementary III: Raw data and analysis via Response Surface Methodology and two-factor ANOVA of nanocellulose yield from maguey fiber; Supplementary IV: Characterization of nanocellulose.

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Article

Determination of the Mass Fractions of the Heavy Metals in the Recycled Cellulose Pulp

Mia Klemenčić ^{1,*}, Ivana Bolanča Mirković ¹, Nenad Bolf ² and Marinko Markić ²

¹ Faculty of Graphic Arts, University of Zagreb, 10 000 Zagreb, Croatia; ivana.bolanca.mirkovic@grf.unizg.hr

² Faculty of Chemical Engineering and Technology, University of Zagreb, 10 000 Zagreb, Croatia; marinko.markic@fkit.unizg.hr (M.M.)

* Correspondence: klemencic.mia@gmail.com

Abstract: In the process of paper recycling, certain amounts of metals can be found in the cellulose suspension, the source of which is mainly printing inks. The paper industry often uses different technologies to reduce heavy metal emissions. The recycling of laminated packaging contributes to the formation of sticky particles, which affects the concentration of heavy metals. This study aimed to determine the mass fraction of metals in the different phases of the deinking process to optimize the cellulose pulp's quality and design healthy correct packaging products. In this research, the deinking flotation of laminated and non-laminated samples was carried out by the Ingede 11 method. As a result of the study, the mass fractions of metals in cellulose pulp were divided into four groups according to the mass fraction's increasing value and the metals' increasing electronegativity. The quantities of metals were analyzed using Inductively Coupled Mass Spectrometry (ICP-MS). The separation of metals from cellulose pulp is influenced by the presence of adhesives and the electronegativity of the metal. The results of the study show that the recycling process removes certain heavy metals very well, which indicates the good recycling potential of pharmaceutical cardboard samples.

Keywords: recycling paper; pharmaceutical packaging; flotation deinking; heavy metals; IPC-MS

1. Introduction

Cellulose is the most abundant natural polymer on Earth [1]. It is a flexible, renewable, and biodegradable raw material, making it widely used in the packaging industry [2–4]. All plant matter has, on average, a cellulose concentration of roughly 33% [5]. Cellulose is a complex carbohydrate found in plant cell walls, providing rigidity and strength to plant cells. It is made up of repeating units of β -D-glucose linked together by β -1,4-glycosidic bonds. It has a linear semicrystalline structure containing a long chain of repeated D-glucose units linked by a β -1,4 glycosidic bond between D-glucopyranosyl units. The key process of cellulose pulp production is the extraction of cellulose in its fibrous form [6,7]. Most of the paper today is prepared from the cellulose pulp of coniferous trees (spruce and pines), whose three main components are cellulose, hemicellulose, and lignin [8]. Cellulose has fiber-forming properties due to the presence of straight, long, and parallel chains. It provides strength and stability to the paper structure. Hemicelluloses are a group of polysaccharides that can influence characteristics such as paper porosity, absorbency, and printability. Lignin is a complex polymer that is a natural binding component of wood cells that helps hold cellulose chains together [9]. Paper is a flat material made of plant cellulosic fibers, usually mechanical and/or chemical wood pulp, but also recycled fibers, non-fibrous components (minerals and additives), and water [10]. Different paper products will have different compositions [11,12].

Packaging is known as a protective outside layer, which tends to protect its contents from any physical, chemical, or biological hazards. Food and drugs are subject to the same

rules and regulations [13]. Cellulosic paper and paper-based materials are one of the oldest and most widely used food contact materials (FCMs) [14,15]; however, they are porous and offer little resistance to the migration of chemical compounds [16,17]. To improve functional barriers against the transfer of various permeates such as moisture, gases, and lipids through the cellulose wall, they are often treated with additives or laminated [18,19]. Therefore, FCMs are made from raw materials and intentionally added substances (IASs) that extend the service life, but also improve production, stability, mechanical properties, and aesthetics [20,21]. Numerous toxic chemicals such as inks, phthalates, surfactants, bleaches, and hydrocarbons are introduced into paper during the production process [10,22]. Raw materials made from recycled paper and cardboard are more likely to contain some heavy metals as well as other chemical additives [23]. Muncke et al. reported that more than 10,000 chemicals are intentionally used in the production of food contact materials [24].

In paper production, pigments are added to improve the structural and surface properties of the paper. Zinc sulfate is used to increase the opacity of special papers, while zinc oxide is sometimes used to make photocopy paper [25]. Zinc and cadmium pigments are additives that give paper fluorescent properties and increase the cohesive strength of paper surfaces. Zinc is also commonly used in papermaking for fine art applications when white pigments are used [26]. Metals such as copper and aluminum are used for engraving on various packaging [27]. Additionally, the quantity of toxic metals can increase when treating corrugated board packaging using dyes that dissolve in water and an acidic platform without previous surface treatment [25]. The main sources of heavy metals are dyes, which mainly consist of conventional inks and pigments, as well as spot inks and Pantone Matching System (PMS) inks [28]. Inks are considered to increase the content of Cd, Pb, Zn, and Cu [28]. It was concluded that most pigments used in printing inks are based on metal compounds of Zn and Cu, including Pb and Cr, which is why they are already banned for food packaging in some countries. Green and yellow packaging contained compounds such as lead chromate, lead sulfate, and lead oxide [29]. Mohammadpour et al. detected harmful metals such as Pb in high concentrations in most pastry packaging made from recycled paper [30]. In another study, different types of packaging and levels of heavy metals such as Al, As, Ba, Cr, Co, Ni, Pb, and V were analyzed, which in some samples exceeded the permitted concentration [31]. Chang et al. developed a new Liquid Chromatography Mass Spectrometry (LC-MS/MS) analytical method. This method was found to be effective in the rapid analysis of photoinitiators with a high degree of reliability [32]. The ICP-MS method developed for determining the mass fractions of chemical elements (Al, Ba, Fe, Mg, Mn, Pb, Sr, Zn) in paper samples proved to be linear and precise. Values of relative measurement uncertainty ranged from 7.7% to 13.6%, and the used approach allows for improving the quality of data and decision making [33].

However, FCMs may also contain unintentionally added substances (NIASs). The recycled paper contains more total NIAS than virgin paper [34,35]. Identification of these substances is often difficult, sometimes impossible [36,37]. For NIAS screening, substances can be divided into three main groups: volatile, semi-volatile, and non-volatile [38,39]. High-resolution precision mass spectrometry (MS) is a valuable tool for the analysis of non-target substances, including screening and chemical identification, and LC-Orbitrap MS is used to screen suspected migrating compounds in paper food packaging [40,41]. Universal detectors that ensure the detection of the largest possible amount of substances are preferred [39,42]. However, there is no single technique for the assessment of trace metals in materials or their migration, and usually several of them must be combined [16]. In packaging that comes into direct contact with food, contaminants can migrate, that is, chemical compounds from the structure of paper and cardboard packaging can move into the food [28]. This requires a comprehensive analysis of all ingredients that can reach toxicological concentrations in food [16].

In recent years, there have been several research efforts to provide recommendations for the safety of chemicals used in specific packaging and to provide a science-based basis for the development of risk management strategies [40,43–47]. For cardboard and

paper packaging products, some standards and regulations limit the content of heavy metals and other harmful substances to ensure safety and environmental protection. In the European Union, the Packaging and Packaging Waste Directive (94/62/EC) provides the framework for limiting the presence of heavy metals in packaging [48]. The directive limits the concentrations of lead, cadmium, mercury, and hexavalent chromium in packaging materials to a maximum value of 100 ppm (mg/kg) for the total content of all four metals combined. This standard applies to all packaging within the EU and to imported packaging material. In the United States, packaging regulations are often state-specific, such as California's Proposition 65, which requires labeling of products containing chemicals known to cause cancer, birth defects, or other reproductive harm [49]. Although Proposition 65 does not specifically target packaging, its provisions affect packaging materials used for products sold in California. The circular economy is the primary goal of socially responsible and sustainable businesses, but the aforementioned standards emphasize the importance of knowing the composition of recycled raw materials and their suitability for health.

In this study, the separation of metals from the different stages of deinking flotation, which is carried out using the INGEDE 11 method, from cellulose pulp was investigated. The deinking flotation method is based on the separation of impurities based on their hydrophilicity or hydrophobicity. Cellulose fibers remain in suspension, and hydrophobic impurities and cellulose chains that are shorter in length or are aggregated with adhesives into sticky particles are largely separated in the flotation foam. The study aims to determine the concentration of some heavy metals in a sheet of paper obtained from cellulose pulp and made from cardboard packaging before and after the deinking flotation process. The content of heavy metals in the packaging was analyzed using ICP-MS. The results can be used to determine the quality of the cellulose pulp, i.e., its chemical suitability for use in the production of recycled cellulose. The research results will also show which metals have a lower affinity for separation from the cellulose suspension. If possible, it would be good to omit such metals in the design and production of paper and cardboard products, which can lead to a higher quality of cellulose pulp.

2. Materials and Methods

In this study, printing substrate, printed quire, and printed packaging were analyzed. The printing substrate was GC2 cardboard, to which an acrylic polymer-based dispersion was applied. The cellulose and other components of the cardboard printing substrate meet the conditions for food and pharmaceutical products, which means that it does not contain increased concentrations of metals and other harmful components. This is crucial for analyzing the concentration of metals, which is the subject of this research. The printing substrate is also a sustainable product, as it consists of 60% virgin fibers and 30% high-quality recycled fibers. The front side of the printing substrate is coated with a three-layer pigment varnish, while the back side is only coated once.

The laminated printing surface is produced with a dispersion based on acrylic polymers (biaxially oriented polyethylene terephthalate), also known as metalized BoPET film (Ultralen Film GmbH, Rhein, Germany) on the same GC2 cardboard. These self-crosslinking acrylics are free of plasticizers and alkylphenol ethoxylates (APEOs). The APEO compounds can affect the environment, aquatic organisms, and humans. The data show that short-chain compounds have a much lower impact than long-chain compounds [50]. The function of the dispersion is to apply a biaxially oriented polyethylene terephthalate film (BOPET metalized) to obtain a laminated packaging material. The film complies with the requirements of the repealed Directive 20/590/EEC.

The samples used are laminated and non-laminated substrate, quire, and packaging. The prints were produced on a five-color offset machine with standard UV offset colors from a European manufacturer, white cover offset printing ink, CMYK (cyan, magenta, yellow, black) inks, and dark purple-blue Pantone Matching System (PMS) inks (Zagreb, Croatia). The printing samples were produced using a standard printing form on a Roland 705 five-color offset press machine. The prints were prepared with UV offset inks from

recognized European ink factories. The printing form consists of CMYK (cyan, magenta, yellow, and black) and RGB (red, green, and blue) profiles with a raster tonal value of 10 to 100%, full tones of CMYK (cyan, magenta, yellow, and black) and RGB (red, green, and blue colors), and packaging for medicines. The accuracy of the printed form is 83%. The printing process started with white offset printing ink, continued with CMYK separation, and at the end of the printing process, a dark purple-blue Pantone ink was used for the text on the packaging. For rub resistance, the prints were varnished with a UV-curable varnish, a highly reactive photopolymerizable acrylate system that is free of volatile organic compounds (VOCs) and has a low odor and optimal wetting properties. The UV-cured varnish mentioned is VP 1038 high gloss (marked L2). For the assembly of the packaging, an adhesive was applied to the edges that comply with the European Framework Directive 89/109/EEC, the specific regulations for adhesives in the food industry, and the Regulation (EC) of the European Parliament and the Council on materials and articles intended to come into contact with foodstuffs, as well as repealed Directives 80/590/EEC and Commission Regulation (EU) No. 10/2011 on plastic materials and articles intended to come into contact with foodstuffs [51,52].

The samples were disintegrated into cellulose pulp according to ISO 5263-2:2004 [53]. The standard flotation deinking method INGEDE 11 [54] was used to separate the ink particles from the cellulose pulp. The handsheets were produced according to the INGEDE 1 procedure [55] and the ISO 5269-2:2004 standard [56]. The standard handsheets for this study were produced with a device for the automatic production of paper sheets, the Rapid-Köthen-Blattformer sheet former from Frank—PTI, according to the ISO 5269 standard method.

Figure 1 shows Flowchart of the study, exhibiting all steps of the research. In all procedures, sodium hydroxide was added to the cellulose pulp, giving it an alkaline character ($\text{pH} = 9.0\text{--}11.0$). This pH value favors the reactions of saponification and/or hydrolysis of resins from printing inks as well as the swelling of cellulose fibers, which makes them more flexible (better separation of the ink particles). H_2O_2 is used to lighten and prevent darkening of the pulp. Therefore, in the procedures with H_2O_2 and Na_2SiO_3 , a silicate is included to prevent the decomposition of H_2O_2 . The positive properties of Na_2SiO_3 are also evident in the reduction in surface tension, the effect on particle dispersion, and the prevention of the binding of impurities.

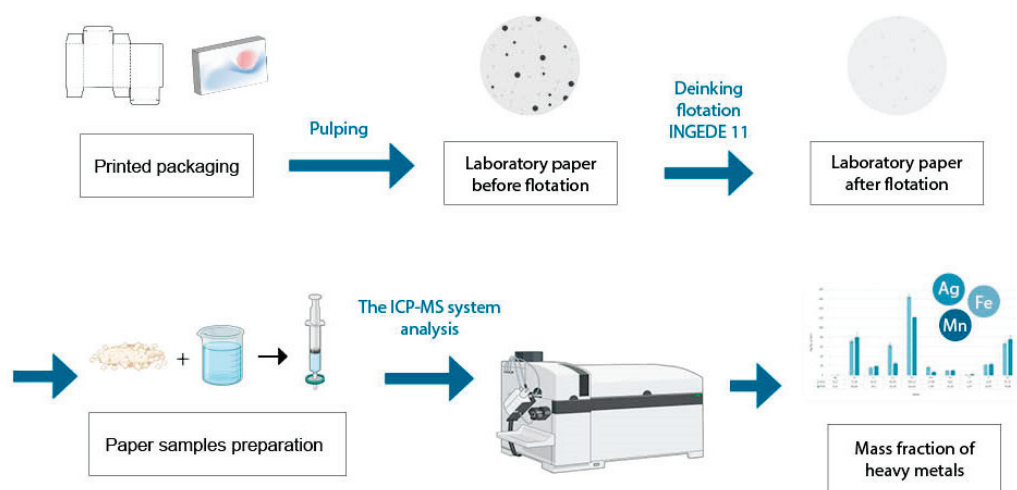


Figure 1. Flowchart of the study.

Table 1 shows samples of laboratory papers made from non-laminated and laminated prints on a cardboard printing base intended for pharmaceutical packaging, before and after the process of separating the ink particles from the cellulose fibers. The quality of the cellulose suspension is also affected by adhesive particles that have formed in the laminated sheet samples and the packaging sample.

Table 1. Samples of laboratory papers made from non-laminated and laminated prints.

Symbol	Sample
P_BF	printed packaging before flotation
P_AF	printed packaging after flotation
Q_BF	printed quire before flotation
Q_AF	printed quire after flotation
S_BF	printing substrate before flotation
S_AF	printing substrate after flotation

The ICP-MS Analysis

The next step of the study was to determine the metal amounts in the laboratory paper and in the cellulose pulp. The method used for the analysis is inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS system measures the concentration of elements quantitatively and gives the total amount of each element of interest. The process can be divided into four stages: sample feed, ICP torch, interface, and MS.

The recycled paper sample was cut into small pieces and then weighed to approximately 100 mg. To extract the metals from the paper, 5 mL of hydrochloric acid and nitric acid (1:3 ratio) were added to the sample (J.T. Baker, p.a. purity). After dilution, the analysis was carried out; the sample was filtered with a syringe filter and then diluted ten times. Elemental analysis (Ag, Co, Cr, Cu, Fe, Mn, Ni, Ti, V, Zn analysis) was performed by inductively coupled plasma mass spectrophotometry (ICP-MS PerkinElmer SCIEX™ ELAN® DRC-e, Concord, ON, Canada), which applies continuous scattering (Table 2). ICP-MS is a tool for analyzing trace metals in environmental samples. In this method, the sample is atomized to generate atomic and small polyatomic ions, which are then detected. The working conditions of the device are listed in Table 2. It is possible to detect metals and various non-metals in liquid samples at very low concentrations.

Table 2. The working conditions of ICP-MS PerkinElmer.

Parameters	Working Conditions
Spray gas flow rate	0.85 L/min
Auxiliary gas flow rate	1.2 L/min
Plasma flow rate	14 L/min;
Lens Voltage	8.5 V; ICP RF
Power supply	1100 W; CeO/Ce = 0.016; Ba ⁺⁺ /Ba ⁺ = 0.015

The ICP-MS calibration was carried out using certified standards. Internal standards are used to compensate for possible measurement deviations. A large number of elements can be detected with an ICP-MS. More than 70 elements can be measured simultaneously in a single analysis. It can measure virtually every naturally occurring element plus many non-natural “radiogenic” isotopes such as technetium, neptunium, plutonium, and americium. The only elements that ICP-MS cannot measure are H and He (which are below the mass range of the mass spectrometer), Ar, N, and O (which are present at high levels from the plasma and air), and F and Ne (which cannot be ionized in an argon plasma) [57]. The advantages of using plasma over other ionization methods, such as flame ionization, are that the ionization takes place in a chemically inert environment, which prevents the formation of oxides, and the ionization is more complete. In addition, the temperature profile of the torch is relatively uniform, which reduces self-absorption effects. Linear calibration curves over several orders of magnitude are observed for ionization processes.

For mass spectrometry, the generation of particles in the submicron range with efficient particle transport to the ICP plays a decisive role [58]. The development and use of the

plasma torch as an excitation and ionization source in spectrometry has brought about an important development in analytical elemental analysis. Nowadays, ICP-MS is an essential analytical technique in various fields. This technique requires simple spectra, adequate spectral resolution, and low detection limits for nearly all the elements it can measure. It can detect many elements at levels below 0.1 parts per trillion (ppt). It can also measure elements at concentrations up to 100 s or even 1000 s parts per million (ppm). All metals whose presence was analyzed in this work have a detection limit of up to 10 ppt [57]. For this reason, a mass spectrometer was used as a detector and a high-pressure plasma as an ion source [59]. This method is suitable for the comparison of laboratory sheets, as it is fast, accurate, and precise and allows the analysis of trace elements at low concentrations. The ICP-MS method was used for multi-element analysis for the quantitative determination of silver (Ag), cobalt (Co), copper (Cu), chromium (Cr), nickel (Ni), iron (Fe), manganese (Mn), titanium (Ti), vanadium (V), and zinc (Zn). The mass fraction of all metals is determined according to Formulas (1) and (2).

$$\text{Species mass fraction in the mixture} = W_i = \frac{\text{Mass of specie 'i' in mixture (mg)}}{\text{Total mass of mixture (kg)}} \quad (1)$$

$$\sum_{i=1}^{I=n} W_i = 1 \quad (2)$$

3. Results and Discussion

We have divided the measured mass fractions of metals in laminated and non-laminated samples into four groups so that the graphical representation of the results is as easy to read as possible (mass fractions up to 0.025 mg/kg, 0.04 mg/kg, 0.4 mg/kg, and 7 mg/kg). The mass fractions of silver and cobalt in the unlaminated and laminated samples before and after the deinking flotation process are shown in Figure 2. The mass fractions of silver in the laminated samples are higher than those of the cobalt samples, which is due to the lamination of the sheets. Examination of the mass fractions of the metals in the laboratory paper samples from laminated boxes and sheets before and after deinking flotation leads to the conclusion that cobalt is better separated in the flotation foam. The separation of impurity particles by the deinking flotation method depends on many factors. The quality of the paper collected for recycling, the age of the printed product, and the climatic conditions during its life cycle as well as the coatings, varnishes, laminates, and adhesives can affect their deinkability [12,60–65]. Some of these are the small or large size of the ink particles (particles from 10 to 100 μm are best separated), the weak hydrophobicity of the ink particles, the difficulty of separating the particles from the cellulose, the presence of adhesives in the cellulose pulp (formation of sticky particles), and others. The INGEDE 11 method defines the added surfactants sodium hydroxide and sodium silicate, which combine the various necessary functional properties of surfactants and non-surfactants. At the same time, the oleic acid influences the value of the hydrophilic–lipophilic balance (HLB) [66,67]. All this had an impact on the extraction of metal particles from the cellulose pulp, i.e., on the separation from the cellulose fibers. It can be said that the polarity of an atom depends on its electronegativity. In the research, we used the Allred–Rochow electronegativity, which determines the values of the electrostatic force with which the effective nuclear charge acts on the valence electrons. According to the given scale, the electronegativity of the elements is as follows: Ag (1.42), Co (1.70), Cu (1.75), Cr (1.56), Ni (1.75), Fe (1.64), Mn (1.60), Ti (1.32), V (1.45), and Zn (1.66). The value of the effective nuclear charges can be described by the following rule; the higher the charge, the more likely it is to attract electrons. This value is estimated using Slater's rules.

$$Z_{\text{eff}} = Z - S \quad (3)$$

Effective nuclear charge (Z_{eff})

Actual nuclear charge (Z)

Shielding constant (S)

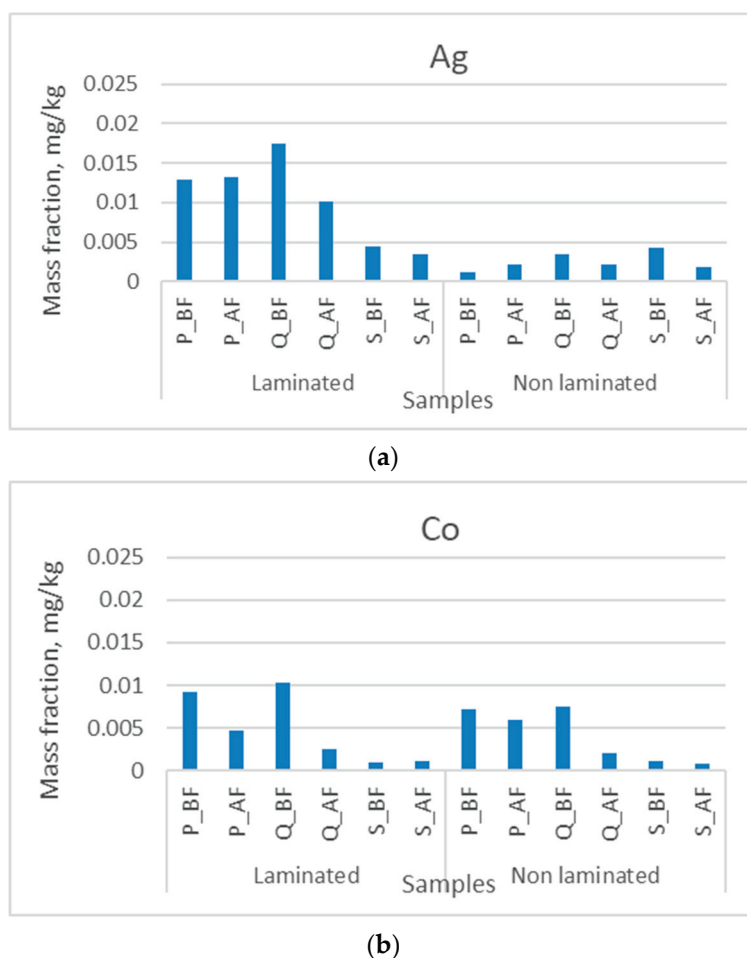


Figure 2. Mass fractions in unlaminated and laminated samples before and after the deinking flotation process for (a) silver and (b) cobalt.

Cobalt has a higher electronegativity (1.70) than silver (1.42), which could be associated with better extraction methods for cobalt from cellulose suspensions. Figure 2 shows that the mass fraction of cobalt decreases more than that of silver after the deinking flotation process.

When examining the mass fractions of the metals in Figure 3, it is noticeable that the smallest quantities of nickel were detected. It can also be seen that the concentrations in laminated prints are much higher than in non-laminated packaging. It can be assumed that adhesives significantly influence the presence of the mentioned metals, but nickel can be successfully separated by the deinking flotation process. For the samples of manganese and chromium, there is no significant difference between the mass fraction of the metal in laminated and non-laminated samples, but it must be emphasized that slightly higher mass fractions were detected in non-laminated samples. It can be seen that manganese is better separated during deinking flotation, which could also be attributed to the higher electronegativity (1.60). In this series of metals, the least electronegative metal is titanium (1.32), which was found to have significantly higher mass fractions in laminated samples. Increased concentrations of titanium in laminated samples may be associated with the use of white opaque paints applied to the plastic foil for lamination [68,69]. Sticky particles are formed in the cellulose pulp of the laminated samples, to which titanium can adhere and which also contribute to the increase in the mass concentration values. Confirmation of the above can be seen in the low values of the mass fraction of the non-laminated samples.

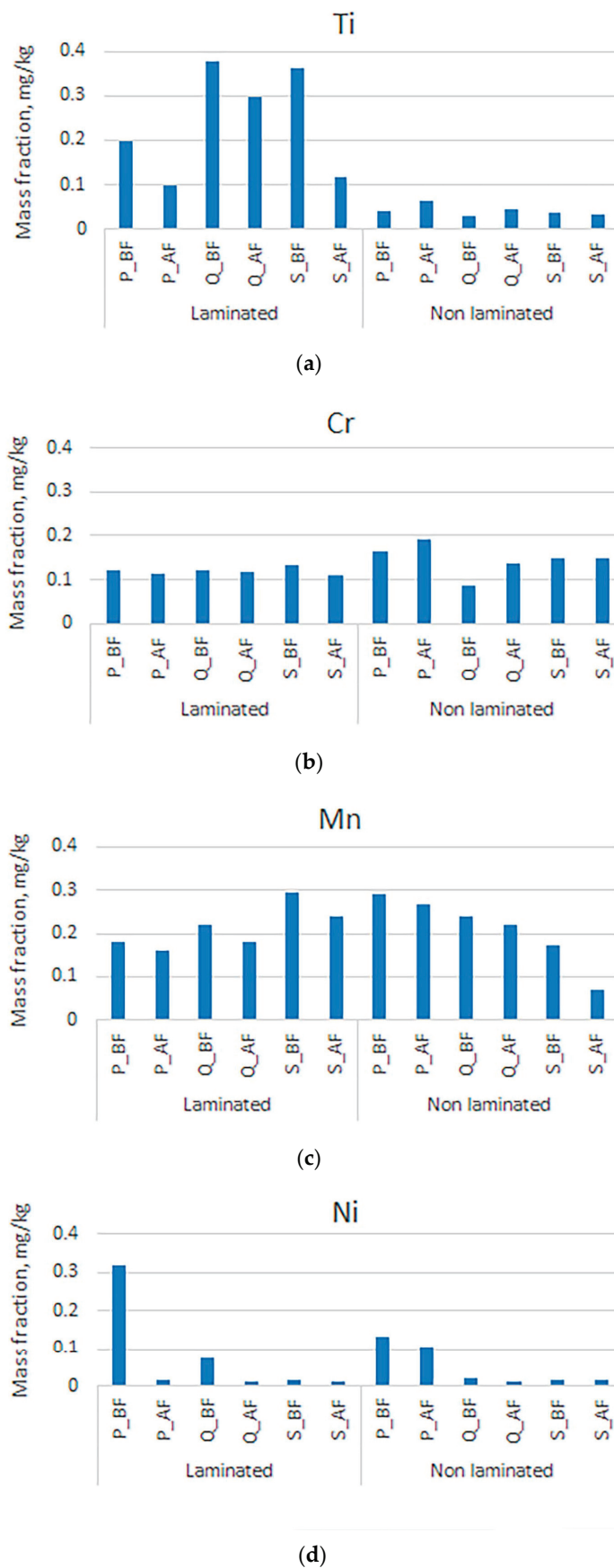


Figure 3. Mass fractions in unlaminated and laminated samples before and after the deinking flotation process for (a) titanium, (b) chromium, (c) manganese, and (d) nickel.

Figure 4 shows the mass fractions of vanadium and copper. Slightly higher concentrations of copper were detected in samples of laboratory paper sheets. When examining the mass fractions of copper before and after the deinking flotation process, it can be seen that copper separation is most successful in the sample made from non-laminated boxes. From this, it could be concluded that the adhesive for lamination, which is applied to the entire surface of the printing substrate with a laminated box and quire samples, is more effective than the adhesive for gluing the edges of the box. When examining the influence of the type of adhesive mentioned on the extraction of ink particles from cellulose pulp in the deinking flotation process, the trend is the opposite [70]. When examining the mass fractions before and after the deinking flotation process, it can also be seen that the laminating adhesive has no significant influence on the results. It is likely that the lower electronegativity (1.45) and the adhesive attached to the edges of the box contribute to the formation of larger particles, which contribute to a slight increase in the mass fraction of vanadium after the deinking process.

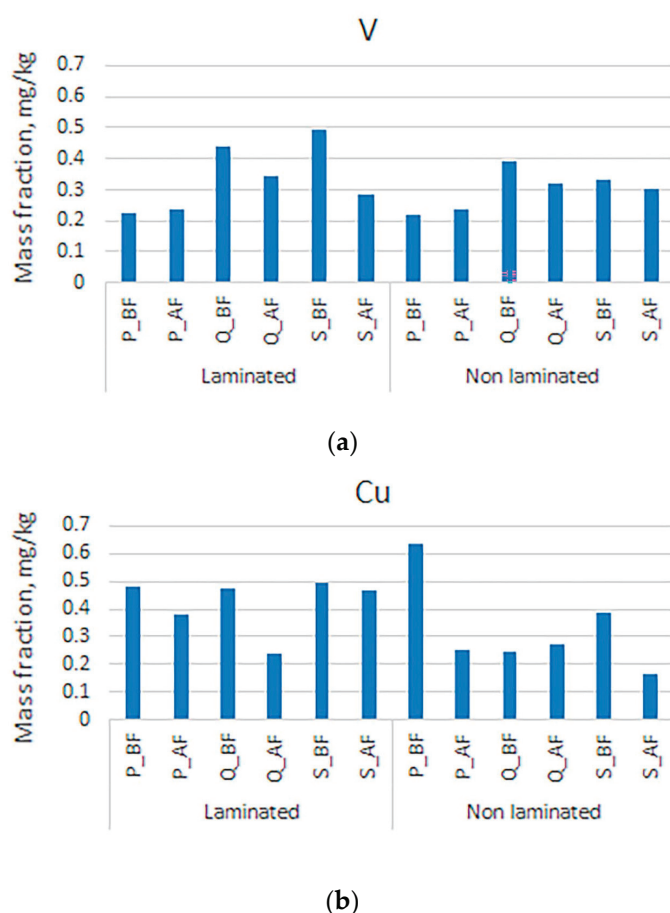


Figure 4. Mass fractions in unlaminated and laminated samples before and after the deinking flotation process for (a) vanadium and (b) copper.

Figure 5 shows that mass fractions of zinc in the samples are slightly higher than those of iron. It can be noticed that the fat content of zinc increases in non-laminated boxes and quires increase after the deinking flotation process. Since no adhesive is present in the mentioned samples, this behavior of zinc could be explained by the lower electronegativity of zinc (1.66), which may negatively affect the extraction process and contribute to its concentration in the cellulose pulp. The literature does not describe frequent occurrences of negative consequences of elevated zinc concentrations for humans, which can lead to gastrointestinal symptoms, reduced absorption of other minerals (copper and iron), immune disorders, neurological symptoms, liver and kidney damage, etc. The diseases

mentioned are more likely to be related to occupational exposure than to contamination of food or packaging [71,72]. The results obtained in this research also confirm the facts from the literature about unlikely effects on human health (the mass fraction is below 3.5 mg/kg). The extraction of iron in the process is not among the most efficient in this study, but it has a constant trend of reducing the mass fraction in the cellulose pulp. It should be noted that in the case of iron, the negative influence of the lamination adhesive on the reduction in the mass fraction could be threatened. In support of this, the results confirm the increase in the mass concentration of iron in the samples after the printing substrate deinking flotation process.

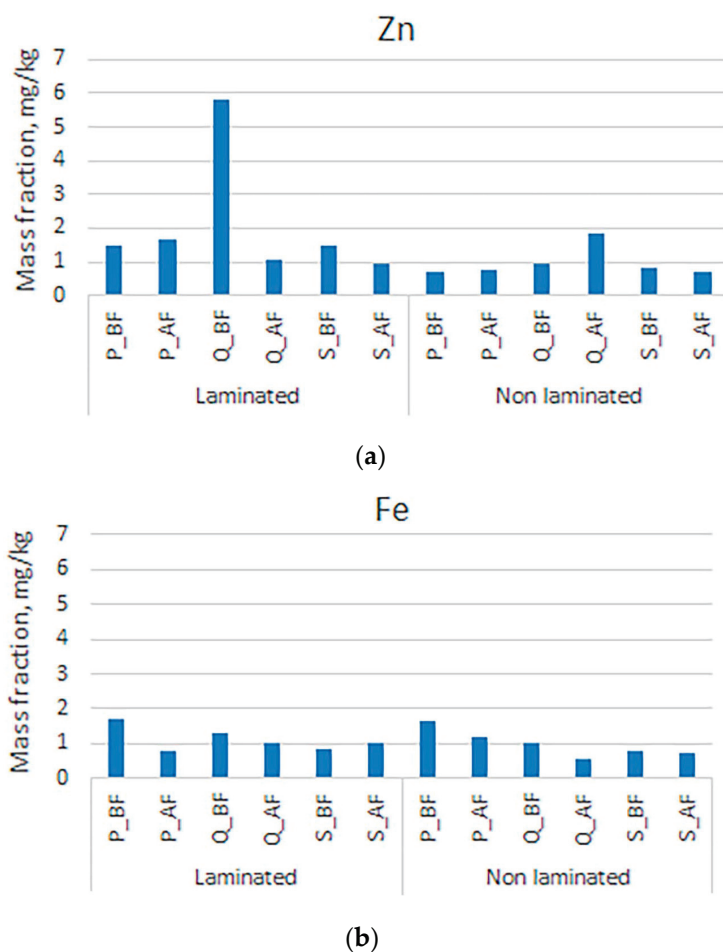


Figure 5. Mass fractions in unlaminated and laminated samples before and after the deinking flotation process for (a) zinc and (b) iron.

From repeated measurements, silver (Ag), cobalt (Co), copper (Cu), chromium (Cr), nickel (Ni), iron (Fe), manganese (Mn), and titanium (Ti) were calculated for all metals. Vanadium (V) and zinc (Zn) values are presented in terms of standard deviation (SD) and variance (σ^2). Table 3 shows the values for laminated samples, and Table 4 shows the values for non-laminated samples.

Variance is a measure of the dispersion of measured variables, the average sum of the squares of the deviations of the quantity value from the arithmetic mean, while the standard deviation is the positive square root of the variance. Standard deviation is a measure of deviation. It is evident from Tables 3 and 4 that there are no major deviations obtained in the process of determining the mass fractions of metals, which points to the advantage of the chosen method.

Table 3. Standard deviation and variance of the mass fractions of the metals for the laminated samples.

		Ag	Co	Cu	Cr	Ni	Fe	Mn	Ti	V	Zn
Printed packaging before flotation	SD	0.031	0.125	0.025	0.125	0.025	0.045	0.035	0.025	0.055	0.125
	σ^2	0.025	0.101	0.001	0.081	0.001	0.031	0.021	0.001	0.041	0.078
Printed packaging after flotation	SD	0.030	0.106	0.006	0.096	0.016	0.036	0.026	0.016	0.032	0.099
	σ^2	0.021	0.101	0.001	0.071	0.011	0.021	0.021	0.011	0.029	0.077
Printed quire before flotation	SD	0.020	0.085	0.015	0.055	0.025	0.025	0.025	0.025	0.025	0.085
	σ^2	0.015	0.081	0.001	0.041	0.011	0.011	0.011	0.011	0.016	0.074
Printed quire after flotation	SD	0.020	0.076	0.011	0.056	0.016	0.016	0.016	0.016	0.032	0.075
	σ^2	0.015	0.061	0.001	0.041	0.011	0.011	0.011	0.011	0.016	0.068
Printed substrate before flotation	SD	0.025	0.055	0.015	0.035	0.025	0.025	0.025	0.025	0.025	0.042
	σ^2	0.015	0.031	0.001	0.021	0.009	0.011	0.011	0.011	0.011	0.031
Printed substrate after flotation	SD	0.020	0.036	0.006	0.026	0.016	0.016	0.016	0.016	0.022	0.032
	σ^2	0.011	0.021	0.001	0.015	0.011	0.011	0.011	0.011	0.011	0.016

Table 4. Standard deviation and variance of the mass fractions of the metals for the non-laminated samples.

		Ag	Co	Cu	Cr	Ni	Fe	Mn	Ti	V	Zn
Printed packaging before flotation	SD	0.025	0.089	0.025	0.095	0.025	0.035	0.025	0.025	0.045	0.093
	σ^2	0.020	0.077	0.001	0.075	0.001	0.022	0.011	0.001	0.031	0.067
Printed packaging after flotation	SD	0.025	0.086	0.006	0.066	0.016	0.026	0.016	0.006	0.022	0.073
	σ^2	0.011	0.071	0.001	0.041	0.011	0.011	0.011	0.011	0.013	0.056
Printed quire before flotation	SD	0.017	0.065	0.015	0.035	0.025	0.020	0.025	0.020	0.020	0.070
	σ^2	0.011	0.041	0.001	0.021	0.010	0.005	0.001	0.001	0.011	0.055
Printed quire after flotation	SD	0.016	0.056	0.010	0.045	0.015	0.010	0.010	0.010	0.021	0.062
	σ^2	0.010	0.040	0.001	0.032	0.011	0.001	0.005	0.004	0.015	0.044
Printed substrate before flotation	SD	0.020	0.050	0.015	0.030	0.021	0.015	0.020	0.020	0.015	0.035
	σ^2	0.011	0.028	0.001	0.015	0.007	0.007	0.001	0.010	0.005	0.025
Printed substrate after flotation	SD	0.020	0.030	0.004	0.020	0.010	0.011	0.010	0.010	0.015	0.025
	σ^2	0.009	0.017	0.001	0.011	0.007	0.008	0.007	0.008	0.007	0.014

4. Conclusions

This research aimed to investigate the composition of pulp and paper concerning the mass fraction of metals, which can help to assess the correctness of the use of recycled cellulose in the production of cardboard packaging in which food or medicines are packaged. By creating databases, the data obtained from the research as well as those that are planned to be obtained in subsequent studies can contribute to the selection of materials that do not contribute to the increase in metals in the cellulose pulp. The recycling of printed materials such as packaging, newspapers, magazines, or other printed products may contain metals in the composition of the paper. The source of the heavy metals is usually in inks and dyes that are applied to the surface of the printing substrate during the printing process. The possible increase in the concentration of certain metals during multiple cycles of recycling would contribute to the health problems of cellulose pulp for the production of packaging products for food and pharmaceutical purposes.

In the study, it has been found that the extraction of metals from cellulose pulp is influenced by the factor of using or not using adhesives and the electronegativity of the metal. We believe that the electronegativity factor is related to the process properties of deinking flotation, which depends on the hydrophilicity and hydrophobicity of the substance. The study shows that the metals Ag, Ti, Cr, V, and Zn, which have a lower electronegativity, have a smaller increase in mass fraction in some phases after deinking flotation. The influence of adhesives and the formation of sticky particles and the influence on the deinking process were also investigated, as were processes related to the separation of ink particles. The results of this study show that the processes for extracting metals from cellulose pulp are significantly influenced by the composition of the adhesive, which should be taken into account in the design of cardboard packaging. Adhesives for lamination have a greater effect on the separation of the mat from the cellulose pulp than the adhesive applied to the edges of the packaging during its assembly. It must be emphasized that the surface on which the lamination adhesive is applied is much larger, so perhaps the reason for this phenomenon is hidden there. In most cases, the deinking flotation method has proven to be a suitable process for the extraction of metals, and the mass fractions of metals measured in the samples do not belong to the categories that would be of concern for human health.

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Article

Aryloxy Ionic Liquid-Catalyzed Homogenous Esterification of Cellulose with Low-Reactive Acyl Donors

Akina Yoshizawa¹, Chie Maruyama¹, Samuel Budi Wardhana Kusuma^{1,2}, Naoki Wada¹, Kosuke Kuroda¹, Daisuke Hirose^{1,*} and Kenji Takahashi^{1,*}

¹ Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

² Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Negeri Semarang, Semarang City 50229, Indonesia

* Correspondence: dh Hirose@se.kanazawa-u.ac.jp (D.H.); ktkenji@staff.kanazawa-u.ac.jp (K.T.)

Abstract: Ionic liquids (ILs) are recyclable, non-volatile, and can dissolve cellulose, a natural polymer that is insoluble in versatile solvents. Therefore, ILs have been used to modify cellulose. However, 1-ethyl-3-methylimidazolium acetate (EmimOAc), a commercially available IL often used to dissolve and modify cellulose to prepare cellulose-based materials, causes the undesired introduction of an acetyl group derived from the acetate anion of EmimOAc onto the hydroxy group of cellulose during esterification. In this study, for cellulose esterification, we prepared aryloxy ILs as non-carboxylate-type and basic ILs, which can theoretically prevent the undesired introduction of an acyl group from the IL onto the hydroxy group of cellulose. The optimized 1-ethyl-3-methylimidazolium 2-pyridinolate (Emim2OPy) and mixed solvent system achieved rapid cellulose esterification (within 30 min) with an excellent degree of substitution (DS) value (up to >2.9) derived from the employed low-reactive vinyl esters and bio-based unsaturated aldehydes, without any undesired substituent introduction from side reactions.

Keywords: ionic liquid; aryloxy anion; pyridinolate; biobased polymer; cellulose; esterification

1. Introduction

Currently, the growing awareness about the environmental impacts of petroleum-based materials is necessitating the development of their replacements, such as bio-based polymers, to reduce reliance on petroleum resources in the polymer industry [1]. Cellulose is a polysaccharide with a linear chain of β -1,4 linked D-glucose units and is the most abundant renewable bio-based polymer in the world [2,3]. Cellulose can be chemically modified into a range of polymeric derivatives with remarkable properties that are suitable for various applications [4]. However, cellulose is insoluble in common organic solvents and water because of the inter- and intramolecular hydrogen bonds between the hydroxyl groups in the glucose unit [3]. Therefore, many cellulose modifications, such as esterification [5], etherification [6,7], and silylation [8,9], are commonly achieved through heterogeneous routes, and the heterogeneous reaction process in cellulose modification requires overstoichiometric amounts of reactants and acids or bases for activating the hydroxyl groups in cellulose. Furthermore, controlling the degree of substitution (DS) of the resulting modified cellulose derivatives using a heterogeneous modification process is difficult. Therefore, cellulose modification under homogeneous reaction conditions is an attractive research area in cellulose chemistry. In 2002, Rogers et al. first demonstrated that molten organic salts with relatively low melting points (<100 °C), called ionic liquids (ILs) [10,11], can dissolve cellulose under mild reaction conditions [12].

Since their report, chemical modifications of cellulose, such as esterification under homogeneous reaction conditions utilizing ILs, have been reported. The first-generation esterification of cellulose in the presence of ILs is usually performed with highly reactive

and corrosive carboxylic acid chlorides or anhydrides [13–16]. The utilization of acyl chlorides or anhydrides for cellulose esterification leads to high conversion rates but generates environmentally unfriendly halides or useless carboxylic acids as side products. A promising alternative to acyl chloride or anhydrides for the esterification of cellulose is the use of vinyl esters (VEs). In 2015, our group reported the acetylation of cellulose with low-reactive isopropenyl acetate (IPA) in the presence of 1-ethyl-3-methylimidazolium acetate (EmimOAc 1) as a catalyst and solvent (Figure 1) [17].

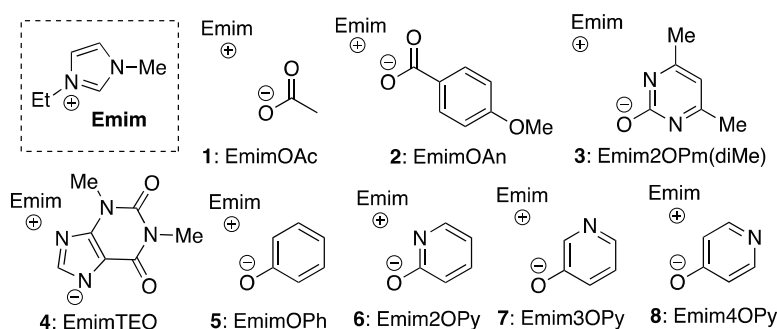
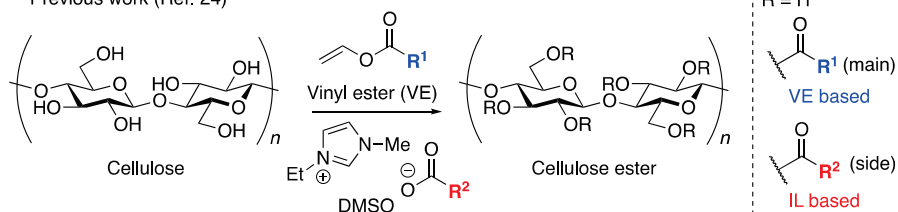


Figure 1. Chemical structures of ILs.

This cellulose esterification is a green and efficient reaction that does not require any additional catalyst, such as a strong acid or base, and an environmentally unfriendly acyl donor. However, for vinyl esters, except IPA, this process causes the undesired introduction of an acetyl group derived from the acetate anion of EmimOAc 1 (Scheme 1a) [18]. The introduction of undesired substituents onto cellulose might alter the thermal [19] and chemical [20,21] properties of the resulting cellulose derivatives. Therefore, undesired anion contamination as ester substituents from ILs must be avoided during cellulose esterification.

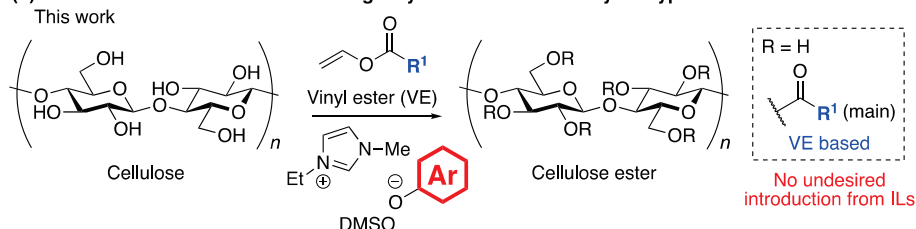
(a) Transesterification of cellulose using vinyl ester in carboxylate type ILs

Previous work (Ref. 24)



(b) Transesterification of cellulose using vinyl ester in non-carboxylate type ILs

This work



Scheme 1. Schematic illustration of the transesterification of cellulose using vinyl esters in ILs. (a,b) Transesterification of cellulose using vinyl ester in carboxylate type ILs (a) and non-carboxylate type aryloxy ILs (b).

Previous studies have predicted that the mixed acid anhydride is formed from the IL anion and acyl donor. Then, the hydroxy groups of cellulose nucleophilically attack the carbonyl carbon of the mixed acid anhydride, leading to side reactions [22,23]. Based on this finding, the design of ILs with different carboxylate anions was investigated in our previous reports to prevent the generation of mixed acid anhydrides and proceed with

the desired acylation with high selectivity [24,25]. ILs with optimized anion substituents and bulkiness, for example, 1-ethyl-3-methylimidazolium *p*-anisate (EmimOAn **2**), were able to suppress the formation of mixed acid anhydrides while maintaining high cellulose solvation and transesterification reactivity [24]. However, in the transesterification reaction of cellulose using carboxylate-type ILs, it is theoretically undeniable that mixed acid anhydrides are formed from the anions of ILs and VE, and it is difficult to completely prevent the introduction of undesired acyl groups from the anions of the ILs.

Therefore, in this study, we investigated a non-carboxylate-type basic IL that mainly includes an aryloxy anion that cannot form mixed acid anhydrides in the esterification reaction between cellulose and VEs (Scheme 1b). The cellulose solvation and catalytic activity of the prepared ILs were evaluated for trans- and oxidative esterification with low-reactivity acyl donors.

2. Materials and Methods

2.1. Materials

1-Ethyl-3-methylimidazolium chloride (EmimCl), DMSO anhydrous, and cellulose (Avicel®PH-101, particle size < 50 µm) were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). EmimCl was used after recrystallization twice from acetonitrile. The number-average degree of polymerization of Avicel was calculated to be 105 [22]. EmimOAc **1** was obtained from Nippon Nyukazai Co., Ltd. (Tokyo, Japan). 4-Methoxybenzoic acid, 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine, theophylline, 4,6-dimethyl-2-hydroxypyrimidine, vinyl pivalate, vinyl propionate, vinyl laurate, and cinnamaldehyde were purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan). Reichardt's Dye, *N,N*-diethyl-4-nitroaniline and 4-nitroaniline were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Other chemicals were obtained from commercial sources and were used without further purification.

2.2. Instruments

¹H and ¹³C NMR spectra were recorded at 600 MHz on a JNM-ECA 600 spectrometer (JEOL Ltd., Tokyo, Japan) in deuterated solvents. Chemical shifts (δ, ppm) are reported in ppm relative to tetramethylsilane (δ 0.00) for the ¹H NMR and to chloroform (δ 77.0) for the ¹³C NMR measurements. Fourier transform-infrared (FT-IR) spectra were recorded using a Thermo Fisher Scientific Nicolet IS10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) spectrometer equipped with an attenuated total reflection (ATR) unit at room temperature. Mass spectra were recorded in fast atom bombardment (FAB) mode using a JMS-700 (JEOL Ltd., Tokyo, Japan). The UV–vis spectrum was measured at room temperature on a UV-Cary 300 Spectrometer (Agilent, Santa Clara, CA, USA).

2.3. Preparation of ILs from EmimCl [24,26,27]

EmimOAn **2** [24], 1-ethyl-3-methylimidazolium phenoxide (EmimOPh **5**) [26] and 1-ethyl-3-methylimidazolium 2-pyridinolate (Emim2OPy **6**) [26] were prepared using the reported method from our group. 1-Ethyl-3-methylimidazolium 3-pyridinolate (Emim3OPy **7**), 1-ethyl-3-methylimidazolium 4-pyridinolate (Emim4OPy **8**), 1-Ethyl-3-methylimidazolium theophyllinate (EmimTEO **4**) and 1-ethyl-3-methylimidazolium 4,6-dimethyl-2-hydroxypyrimidinolate (Emim2OPm(diMe) **3**) were synthesized according to conventional protocols, as shown below [26].

Potassium hydroxide (85%, 4.94 g, 68.2 mmol)/2-propanol (120 mL) and equal amount of EmimCl (10 g, 68.2 mmol) as 2-propanol (20 mL) solutions were prepared. Both solutions were cooled to −60 °C. The cooled solutions were mixed and filtered twice to remove precipitated potassium chloride. The filtrate contained 1-ethyl-3-methylimidazolium hydroxide (EmimOH) dissolved in 2-propanol. EmimOH in the solution was neutralized with an equal amount of corresponding hydroxy aryls or theophylline in 2-propanol (10 mL) solution. The reaction mixture was filtered using a glass fiber filter (Advantec, GA-55, 21 mm), 2-propanol was evaporated, and the filtrate was dried in vacuo at room tempera-

ture. The resultant ILs were quantitatively obtained as yellow, brown, sticky oils, or white solids.

2.4. Solubility of Cellulose in ILs

The solubility of cellulose in a mixed solution of ILs and DMSO (1/20, molar ratio) was tested using the following method: The cellulose was pre-dried for four hours in vacuo at 60 °C. At 80 °C, the pre-dried cellulose (15 mg) was added to a solution (1 mL) containing DMSO and the respective IL, and kept for 15 min. After 15 min, the dissolution of cellulose in the reaction solution was visually confirmed. If complete dissolution of the cellulose was confirmed, an additional 15 mg of cellulose was added. The final process was continued until the saturation of cellulose in the DMSO/IL mixture.

2.5. General Procedure for the Transesterification of Cellulose

As a typical case, cellulose (240 mg, [AGU (anhydrous glucose unit)] = 1.48 mmol, [OH] = 4.44 mmol) was mixed with the Emim2OPy **6** (470 mg, 2.29 mmol) and dried for three hours in vacuo at 80 °C. After complete drying, anhydride DMSO (5.2 mL) was added, and the mixture was stirred for one hour at 80 °C under an argon atmosphere. Vinyl pivalate (10.5 mL, 71.6 mmol) was added, and the reaction mixture was stirred for 24 h at 80 °C. The resulting solution was poured into a large amount of 50% methanol solution, and precipitated cellulose ester was collected by filtration. The polymer was purified by precipitation (from acetone solution into water) to obtain cellulose pivalate as a white powder (DS = 2.99, 283 mg, 48%).

2.6. General Procedure for the Oxidative Esterification of Cellulose

The synthesis of cellulose esters via oxidative esterification was conducted using a previously reported method [26,28]. As a typical example, cellulose (120 mg, [AGU] = 0.740 mmol, and [OH] = 2.22 mmol) was mixed with the Emim2OPy **6** (455 mg, 2.22 mmol) and dried for three hours in vacuo at 80 °C. After complete drying, anhydride DMSO (3.15 mL) was added, and the mixture was stirred for one hour at 60 °C under an argon atmosphere. In the next step, after adding cinnamaldehyde (0.6 mL, 2.22 mmol), the reaction mixture was stirred at 60 °C for 24 h. After the reaction, the resulting solution was poured into methanol. The polymer was purified by reprecipitation (from acetone solution into methanol) to obtain cellulose phenylpropionate as a pale yellowish powder (DS = 2.78, 312 mg, 79%).

2.7. Evaluation of the DS in Cellulose Esters

The DS in the cellulose esters was determined by ^1H NMR spectroscopy conducted in DMSO- d_6 , acetone- d_6 , or CDCl_3 depending on the solubility of the product in deuterated solvents and the overlap between the target and solvent peaks. To determine the DS values of the low-modified cellulose derivatives, the DS values were calculated after perbenzoylation or peracetylation. The DS values determined from the desired substituents (DS_{main}) of the modified cellulose derivatives were calculated using the following Equation (1):

$$\text{DS}_{\text{main}} = (\text{I}_{\text{main}}/\text{X})/(\text{I}_{\text{AGU}}/7) \quad (1)$$

The DS_{main} values for cellulose pivalate, propionate, laurate, and phenylpropionate were calculated from the integrals of the corresponding peaks (I_{main}) at approximately 1.3–0.9 ppm ($\text{X} = 9$), 1.3–1.0 ppm ($\text{X} = 3$), 1.0–0.7 ppm ($\text{X} = 3$) and 7.8–6.5 ppm ($\text{X} = 5$), respectively, and the AGU peaks (I_{AGU}) at approximately 5.5–3.0 ppm. The DS values determined from the undesired substituents (DS_{side}) were calculated using Equation (2):

$$\text{DS}_{\text{side}} = (\text{I}_{\text{side}}/\text{X})/(\text{I}_{\text{AGU}}/7) \quad (2)$$

DS_{side} values of cellulose esters derived from EmimOAc **1** and EmimOAn **2** were calculated from the integrals of the peaks (I_{side}) at approximately 2.2–1.7 ppm ($\text{X} = 3$) and

8.3–6.8 ppm ($X = 4$), corresponding to a methyl group and an aromatic ring, respectively, and the areas of AGU signals (I_{AGU}) at 3.0–5.5 ppm.

2.8. General Procedure for Per-Benzoylation of the Synthesized Cellulose Ester

The DS_{side} values for cellulose esters synthesized by transesterification were determined after perbenzoylation to produce accurate DS_{side} values. The synthesized cellulose ester (50 mg, $[AGU] = 0.308$ mmol, calculated as cellulose) was dissolved in *N,N*-dimethylacetamide (DMAc, 3 mL). Benzoic acid (1.85 mmol, 226 mg), 4-dimethylaminopyridine (DMAP, 1.85 mmol, 226 mg), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC HCl, 1.85 mmol, 355 mg) were added, and the reaction mixture was stirred at room temperature for 24 h. After the reaction, the perbenzoylated cellulose ester was precipitated from methanol (30 mL), collected, and washed with methanol three times. The collected product was dried overnight in vacuo in the oven at 70 °C.

2.9. General Procedure for Per-Acetylation of the Synthesized Cellulose Esters

The synthesized cellulose ester (50 mg, $[AGU] = 0.308$ mmol, calculated as cellulose) was dissolved in DMAc (3 mL). DMAP (1.85 mmol, 226 mg), acetic acid (1.85 mmol, 0.106 mL), and EDC HCl (1.85 mmol, 355 mg) were added, and the reaction mixture was stirred at room temperature for 24 h. After the reaction, the peracetylated cellulose ester was precipitated from methanol and collected. The collected product was dried overnight in vacuo in the oven at 70 °C.

2.10. Kamlet–Taft Parameters

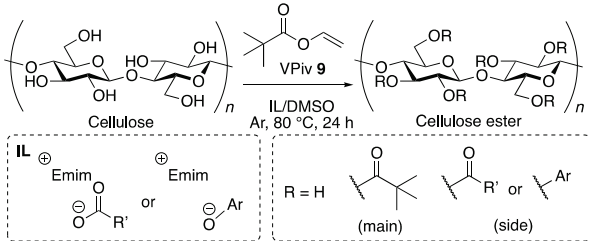
From the spectroscopic data, the acidity (α) and basicity (β) were calculated using equations previously published by Kamlet et al. [29,30].

3. Results and Discussion

3.1. ILs Synthesis and Cellulose Dissolution in ILs

Non-carboxylate-type ILs that do not form mixed acid anhydride intermediates were designed to synthesize ILs with high cellulose solvation and without the undesired introduction of anions into the hydroxy groups on cellulose (Figure 1). ILs containing various non-carboxylate anions were prepared to investigate the influence of IL on transesterification of cellulose with VE. Next, the solubility of cellulose in the prepared ILs was tested. A mixed-solvent system of DMSO and IL has been reported to enhance the performance of ILs in the dissolving cellulose [31,32] and accelerate some cellulose-modification reactions [22]. Thus, the solubility of cellulose in mixed-solvent systems containing non-carboxylate-type ILs and DMSO was tested and compared with those reported for mixed-solvent systems containing carboxylate-type ILs and DMSO [24]. ILs 2–8, with melting points below 100 °C, were synthesized via the anion-exchange method using potassium hydroxide [24,27]. ILs 3 and 5–8, with aryloxy ions, were prepared from EmimCl and its corresponding aromatic hydroxyl compounds. EmimTEO 4 was synthesized from EmimCl and theophylline, a bio-based alkaloid. IL 4 does not contain an aryloxy structure, but it exhibits basicity similar to that exhibited by other aryloxy ILs. Therefore, the cellulose solubility and catalytic ability of 4 were also investigated, as with the other non-carboxylate-type ILs in this study.

The solubility of cellulose, in the range of 15–75 mg in a mixed-solvent system of synthesized ILs and DMSO (1/20 molar ratio), was examined at 80 °C (Table 1) [31]. In our previous report [24], the solubility of cellulose in a mixed-solvent system of IL/DMSO was determined to depend on the basicity of the anion of the IL. Bulky anions effectively prevented undesired anion introduction onto cellulose, but the steric hindrance of the IL anions decreased its efficiency in dissolving cellulose.

Table 1. Effect of ILs in transesterification of cellulose ^a.


Entry	IL	pK _a ^b	Solubility ^c	DS _{main} ^d	DS _{side} ^d	Selectivity (%)
1	1	4.79	<75	2.43	0.54	82
2	2	4.47	<45	2.78	0.03	99
3	3	10.3	<15	2.85	n.d.	>99
4	4	8.60	<15	2.73	n.d.	>99
5	6	12.0	<75	2.99	n.d.	>99
6	7	9.15	<60	2.85	n.d.	>99
7	8	5.19	<30	2.74	n.d.	>99

^a Reaction conditions: [OH]/[IL]/[DMSO]/[VPiv 9] = 1/0.5/17/16 (molar ratio); for 24 h, 80 °C under Ar atmosphere. ^b The pK_a value of the conjugated acid. ^c Solubility test (mg mL⁻¹) in IL/DMSO = 1/20 (molar ratio) solvent system at 80 °C. ^d Determined by ¹H NMR spectroscopy. n.d.: not detected (estimated DS_{side} << 0.03).

Based on the pK_a value of the conjugated acid [33], which is an indicator of anion basicity (Table 1), the basicity of the anions in the synthesized ILs was in the order **6** (12.0) > **3** (10.3) > **7** (9.15) > **4** (8.60) > **8** (5.19) > **1** (4.79) > **2** (4.47). The pK_a values of the conjugated acid of anions are shown in parentheses. The trend in the solubility of cellulose in the carboxylate ILs was **1** > **2**, similar to the order of the basicity of the anions of the ILs (Table 1, entries 1 and 2). Although ILs **3** and **4** had sufficient basicity for cellulose dissolution (Table 1, entries 3 and 4), these two ILs showed low cellulose solubilities, probably due to their large anion size. To improve cellulose solvation, EmimOPh **5** (the pK_a value of the conjugated acid is 9.86), which has a smaller size, higher basicity anion, and is a better acceptor of hydrogen bonds, was synthesized. However, the synthesized **5** was not sufficiently stable for cellulose modification [26,34,35]. To stabilize the negative charge on the anions of ILs, we tested EmimOPys **6–8** which have a nitrogen atom at the 2-4 position of the aromatic ring. These **6–8** showed sufficient cellulose solvation and stability for cellulose modification. In the case of imidazolium-type ILs with 2OPy-4OPy anions, the cellulose solvation in the mixed solvent system of ILs and DMSO was also in the order **6** (12.0) > **7** (9.15) > **8** (5.19), corresponding to the order of basicity of the anion.

To further discuss the solubility of cellulose in the synthesized ILs, the polarity of the ILs was evaluated through the Kamlet-Taft (KT) polarity evaluation method based on the solvatochromism of dyes, using parameters such as hydrogen bond basicity and acidity (β and α value, respectively) [36]. The KT parameters of Emim2OPy **6**, which has the highest cellulose solvation, and Emim4OPy **8**, which has the lowest cellulose solvation among EmimOPys **6–8**, were calculated from the solvatochromic behavior of three dyes: 4-nitroaniline, *N,N*-diethyl-4-nitroaniline, and Reichardt's Dye 30 (Table 2) [29,37]. To discuss the KT parameters considering the cellulose solvation conditions, this measurement was performed in DMSO. Due to the breaking of hydrogen bonds between cellulose molecules, imidazolium-type ILs with β > 0.8 show the ability to dissolve cellulose [38]. Mixed-solvent systems containing DMSO with Emim2OPy **6** and Emim4OPy **8** (IL/DMSO = 1/20 (molar ratio)) showed relatively high β values of 1.04 and 0.93, respectively, similar to that of pure EmimOAc **1** (0.95–1.09) [38,39]. This observation supports the high solubility of cellulose in both ILs. However, the difference between the β values was slightly smaller than that between the solubility values, indicating that the KT parameters may not be the main factors responsible for solubility. It was difficult to obtain reproducible KT parameters for Emim2OPy **6** and Emim4OPy **8** under neat conditions without dilution with DMSO, probably due to their high viscosities.

Table 2. Kamlet–Taft parameters α and β of solvent.

Entry	Solvents	α	β
1	Emim2OPy/DMSO (1/20)	0.20	1.04
2	Emim4OPy/DMSO (1/20)	0.26	0.93
3	EmimOAc	0.40	0.95–1.09 [38,39]
4	DMSO	−0.11	0.72 [40]

3.2. Catalytic Activity of Synthesized ILs in Transesterification of Cellulose

The reactivity of the prepared ILs in the transesterification reaction was confirmed using vinyl pivalate (VPiv, **9**), an acyl donor that is difficult to introduce and causes easy introduction of undesired acyl groups derived from the ILs anions (Table 1). At first, the transesterification of cellulose was carried out using a mixed solvent system of DMSO and Emim2OPy **6**, which had the highest solubility for cellulose among the prepared ILs at 80 °C (Table 1, entry 5). The reaction solution was homogeneous throughout the process, indicating that the ILs effectively solvated cellulose in this system. After the reaction, the resulting cellulose derivative was recovered from the reaction mixture by precipitation in the 50% methanol solution (*v/v*). The structure of the resulting cellulose derivative was confirmed using infrared (IR) and ^1H NMR spectroscopy. The IR absorption spectrum of the obtained cellulose derivative showed an absorption band at 1737 cm^{-1} derived from the C=O stretching vibrations of the carbonyl group (Figure 2a), supporting the progress of cellulose esterification. The ^1H NMR spectrum revealed proton peaks derived from a pivaloyl group at 0.9–1.3 ppm and a cellulose backbone at 3.0–5.5 ppm (Figure 2b), the resulting cellulose derivative was confirmed to be cellulose pivalate. Interestingly, there were no proton peaks at 6.5–8.0 ppm derived from an aromatic ring in the ^1H NMR spectrum, indicating that the undesired substituents derived from 2OPy anion were not introduced into the hydroxyl group of the cellulose. Even an aryl ester formed by the nucleophilic attack of the aryloxy anion of Emim2OPy **6** on VPiv **9** may introduce an aromatic ring onto the hydroxy group of cellulose by aromatic nucleophilic substitution reactions ($\text{S}_{\text{N}}\text{Ar}$). However, this transesterification of cellulose with VPiv **9** in the presence of Emim2OPy **6** proceeded with only the desired transesterification (Figure 3) [22,41].

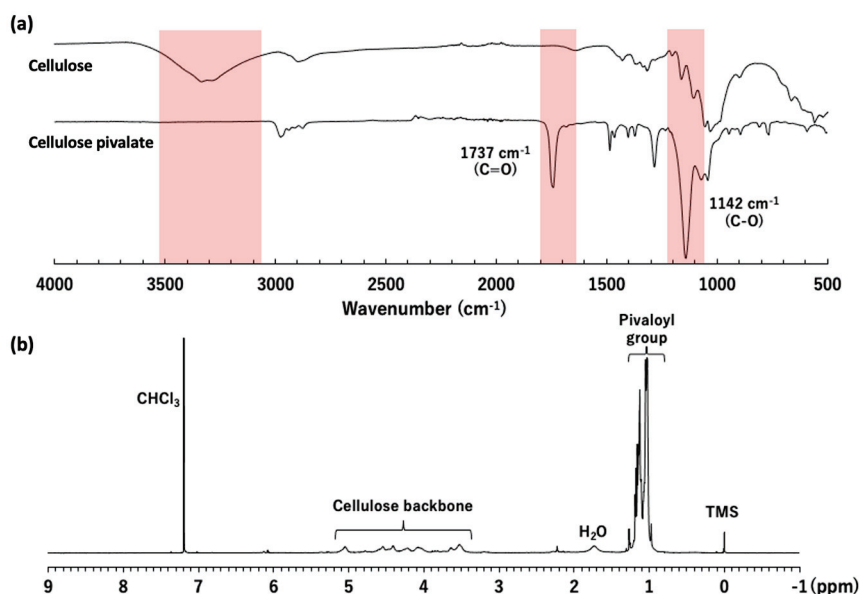


Figure 2. (a) ATR-mode FT-IR spectra of the original cellulose (Avicel® PH-101) and the cellulose pivalate prepared in the presence of Emim2OPy **6** (Table 1, entry 5). (b) ^1H NMR spectra of the resulting cellulose pivalate in CDCl_3 measured at room temperature.

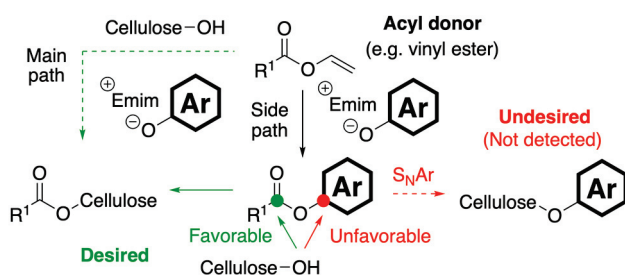


Figure 3. Predicted mechanism of the side pathways in transesterification of cellulose in the presence of aryloxy ILs.

To determine accurate DS values, the residual hydroxyl groups of the resulting cellulose pivalate were perbenzoylated and peracetylated to determine the DS value of the desired pivaloyl group (DS_{main}) and undesired group (DS_{side}), followed by ^1H NMR measurements. In the ^1H NMR spectrum of the perbenzoylated sample of the obtained cellulose pivalate, a comparison of the integrated values of the proton peaks of the pivaloyl group and the cellulose backbone showed that the DS_{main} value of the cellulose pivalate synthesized using Emim2OPy **6** reached $DS_{\text{main}} = 2.99$. This result demonstrated that pure cellulose pivalate with an almost complete substitution ($DS_{\text{main}} > 2.9$) was successfully synthesized with a mild acyl donor.

Subsequently, we tested the transesterification of cellulose with VPiv **9** in the presence of other ILs **1–8** (except **5**) prepared in this study (Table 1). In all experiments, DS_{main} and DS_{side} values were obtained using the same method as transesterification with Emim2OPy **6**. However, the DS_{side} value of the transesterification in EmimOAc **1** was calculated from ^1H NMR spectra measured after per-benzoylation. From all the experiments, it was clear that the transesterification proceeded smoothly in all the mixed solvent systems of DMSO and the prepared imidazolium-type ILs **1–8** (except **5**), yielding cellulose pivalates with high DS_{main} values of 2.43 to 2.99. As previously reported, the transesterification reaction of cellulose with VPiv **9** in carboxylate-type ILs **1** and **2** (Table 1, entries 1 and 2) resulted in anion contamination from ILs with ester substituents ($DS_{\text{side}} = 0.54$ and 0.03) [24]. The introduction of undesired anions from ILs **1** and **2** was confirmed by the ^1H NMR of the obtained cellulose pivalate. Furthermore, EmimOAc **1** limited the DS_{main} value of cellulose pivalate, and it never even reached 2.5 due to the side reaction derived from its acetate anion. On the other hand, the transesterification reactions in non-carboxylate-type ILs **3–8** (except **5**) showed much higher DS_{main} values of 2.73–2.99 than those in EmimOAc **1** (Table 1, entries 3–7). Notably, the results show that the selectivity of transesterification in non-carboxylate type ILs was almost 100%, indicating that these ILs prevented the undesired introduction of the aryl oxide group from anions of ILs.

3.3. Optimization of Reaction Conditions and Kinetics of Cellulose Esterification

Next, the kinetics of transesterification using VPiv **9** was investigated. Figure 4a shows the time-dependent change in DS_{main} values of cellulose pivalate prepared using one equivalent of VPiv **9** per hydroxy group of cellulose in a mixed solvent system of DMSO with EmimOAc **1**, EmimOAn **2**, and Emim2OPy **6** at 30 and 80 °C. The transesterification of cellulose and VPiv **9** in a mixed solvent of DMSO and EmimOAn **2** at 30 °C resulted in a DS_{main} of only 0.17 for the cellulose pivalate obtained after 6 h (Figure 4a, green). Even after extending the reaction time to 24 h, DS_{main} was 0.31 and little progress was made, meaning that the catalytic activity of EmimOAn **2** was very low at room temperature due to its steric hindrance. EmimOAc **1**, which has a smaller carboxylate, exhibited relatively higher catalytic activity at 30 °C than EmimOAn **2**. Cellulose pivalate obtained from the reaction mixture in the mixed solvent system of DMSO and EmimOAc **1** after 24 h showed a better DS_{main} value (1.76) (Figure 4a, orange).

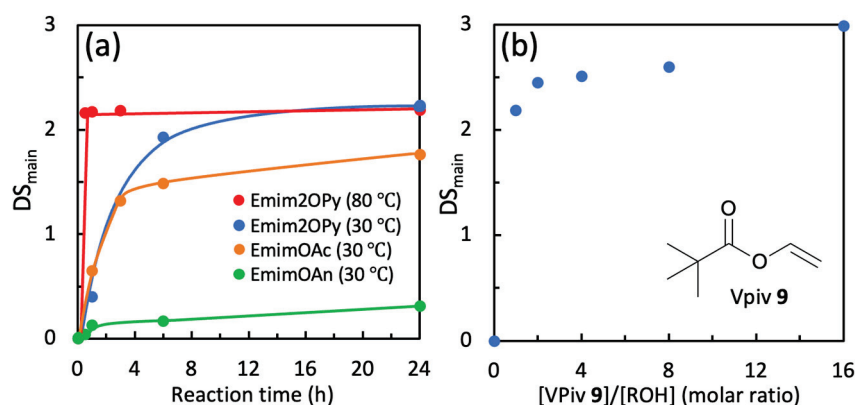


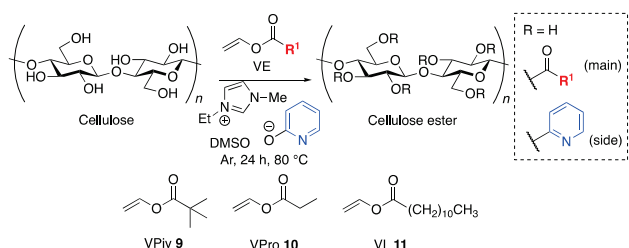
Figure 4. (a) Time-dependent change in DS_{main} value in the synthesis of cellulose pivalate. Reaction conditions: $[OH]/[IL]/[DMSO]/[VPiv\ 9] = 1/0.5/17/1$ (molar ratio), Ar atmosphere. (b) Effect of the amount of VPiv 9 on cellulose OH groups. Reaction conditions: $[OH]/[Emim2OPy\ 6]/[DMSO] = 1/0.5/17$ (molar ratio), 24 h, 80 °C, Ar atmosphere.

In contrast, transesterification in the presence of Emim2OPy 6 for 24 h afforded the desired cellulose ester with a higher DS_{main} value (2.23) than the reactions in EmimOAc 1 and EmimOAn 2 without any anion introduction (Figure 4a, blue). This result indicates that the catalytic activity of Emim2OPy 6 in transesterification is high, even at a low temperature of 30 °C. Furthermore, when cellulose was esterified at 80 °C in a mixed solvent system of Emim2OPy 6 and DMSO, it required less than 30 min to reach a saturated DS_{main} value (2.16), suggesting satisfactory fast kinetics of cellulose esterification (Figure 4a, red). These results show that Emim2OPy 6 has higher catalytic activity in the transesterification of cellulose with VPiv 9 than that of previously reported carboxylate ILs.

Next, the amount of VPiv 9 was varied to maximize the DS_{main} value of the main ester group in the obtained cellulose ester (Figure 4b). Although VPiv 9 is a bulky acyl donor, this transesterification in the presence of Emim2OPy 6 using one equivalent of VPiv 9 per hydroxyl group of cellulose provided the synthesized cellulose pivalate with a high DS_{main} value (2.19). The synthesized cellulose pivalate ($DS_{main} = 2.19$) exhibited sufficient solubility in various lipophilic solvents and molding processability. The DS_{main} values of the obtained cellulose pivalate were improved from 2.19 to 2.60 by increasing the amount of VPiv 9 from one equivalent to eight equivalents per hydroxy group of cellulose, and a fully substituted cellulose pivalate ($DS_{main} = >2.9$) was obtained by using 16 equivalents of vinyl pivalate.

3.4. Scope of Vinyl Esters

Emim2OPy 6 which had the highest cellulose solvation in the above study (Table 1) and the optimized reaction conditions were applied to the transesterification of cellulose with different vinyl esters such as vinyl propionate (VPro 10) and vinyl laurate (VL 11) (Table 3). After perbenzoylation of the synthesized cellulose esters, the DS_{main} values of the cellulose esters were determined by 1H NMR spectroscopy using the same method as in Table 1. In these three experiments, the DS_{main} values of the cellulose esters showed almost full substitution on the side chain (pivalate: $DS_{main} = 2.96$ (entry 1), propionate: $DS_{main} = 2.85$ (entry 3), and laurate: $DS_{main} = 2.96$ (entry 4)) without any anion contamination of Emim2OPy 6 as ether substituents based on 1H NMR spectra (Figures S9 and S10). Even in transesterification with bulky vinyl pivalate, the introduction of the desired ester group with complete selectivity was successful, although a side reaction was observed when EmimOAc 1 was used ($DS_{side} = 0.54$) (Table 3, entry 2).

Table 3. Scope of vinyl ester in the transesterification in the Emim2OPy/DMSO mixed solvent system ^a.


Entry	VE	DS _{main} ^b	DS _{side} ^b	Selectivity (%)
1	9	2.96	n.d.	>99
2	9	2.43 ^c	0.54 ^c	82 ^c
3	10	2.85	n.d.	>99
4	11	2.96	n.d.	>99

^a Reaction conditions: [OH]/[Emim2OPy 6]/[DMSO]/[VE] = 1/0.5/17/16 (molar ratio); for 24 h, 80 °C under Ar atmosphere. ^b Determined by ¹H NMR spectroscopy. ^c The esterification was performed in the presence of EmimOAc instead of Emim2OPy.

The solubilities of the synthesized cellulose esters using VPiv, VPro, and VL were tested in various solvents, and these three cellulose esters with high DS_{main} values showed high solubility in various organic solvents such as chloroform, tetrahydrofuran, and acetone (Table S1). The high solubilities of the synthesized cellulose derivatives in various organic solvents indicated easy processing of the obtained cellulose derivatives [42,43].

3.5. Effect of the IL Species on Oxidative Esterification of Cellulose

Recently, we reported a synthetic method for fully bio-based cellulose esters using bio-based unsaturated aldehydes such as cinnamonaldehyde **12** as acyl donors based on different mechanisms of transesterification [26,28]. This reaction proceeds via an oxidative esterification mechanism in which the carbon–carbon double bond (C=C) works as an intramolecular oxidant and is an environmentally benign method because there is no leaving group, unlike conventional esterification methods, including transesterification, that use acid chloride, anhydride, and condensation reagents. Under mild reaction conditions, oxidative esterification hardly proceeds without *N*-heterocyclic carbene (NHC)-based catalytic ability of the ionic liquid [26,28,44]. Therefore, aldehydes are regarded as inactive acyl donors in conventional cellulose esterification without an ionic liquid. However, the effect of the aryloxy IL structure on this new green cellulose esterification method is still not clear. Synthesized aryloxy ILs EmimOPys **6–8** showing high cellulose solvation were applied to oxidative cellulose esterification for discussion of cellulose solvation and reactivity.

To investigate the kinetics of the oxidative esterification of cellulose with cinnamonaldehyde **12**, time-dependent DS_{main} value changes in the resulting cellulose phenylpropionate were evaluated [26,28]. After the dissolution of cellulose in the mixed solvent of synthesized EmimOPys **6–8** and DMSO, cinnamonaldehyde **12** was added to the reaction mixture and stirred at 60 °C (Figure 5). After the reaction, the resulting cellulose esters were recovered and washed with methanol. The DS_{main} values were determined from ¹H NMR spectra of the synthesized cellulose phenylpropionate after per-acetylation. The DS_{main} value of cellulose phenylpropionate was calculated from the integrated peak ratio between the cellulose backbone and aromatic ring in ¹H NMR spectra. In the oxidative esterification of cellulose in EmimOPys **6–8**, the undesired introduction of ether groups from the anions of the ionic liquid due to side reactions was not observed (Figures S11–S13). In the case of the oxidative esterification of cellulose in the mixed solvent of Emim2OPy **6** and DMSO, the DS_{main} value of the synthesized cellulose phenylpropionate was quite high (2.55) during the first hour. Subsequently, extending the reaction time to 24 h only increased the DS_{main} value of cellulose phenylpropionate to a maximum of 2.78, and extending the reaction time

did not significantly contribute to improving the DS_{main} value. The oxidative esterification of cellulose in the presence of Emim3OPy 7 showed almost the same behavior as that in the presence of Emim2OPy 6. On the other hand, in the oxidative esterification of cellulose using Emim4OPy, the DS_{main} value was lower than that when using Emim2OPy 6 or Emim3OPy 7. Emim4OPy 8 catalyzed oxidative esterification of cellulose also afforded the desired cellulose phenylpropionate after one hour, but its DS_{main} value was relatively small (2.27) and the reaction rate was slower than that of Emim2OPy 6 or Emim3OPy 7. These results indicate that aryloxy ILs with higher cellulose solvation (Emim2OPy 6 or Emim3OPy 7) are excellent catalysts and solvents for cellulose esterification with low-reactive acyl donors, including oxidative esterification and transesterification.

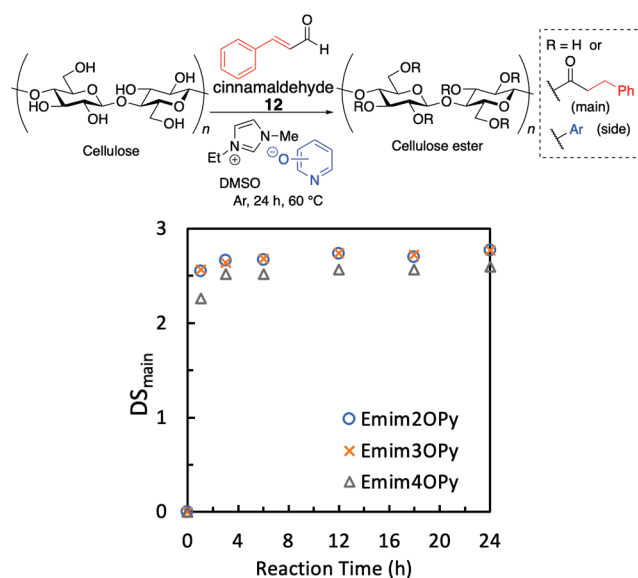


Figure 5. Time-dependent change in DS_{main} value in the synthesis of cellulose phenylpropionate. Reaction conditions: $[OH]/[cinnamaldehyde\ 12]/[IL]/[DMSO] = 1/1/1/20$ (molar ratio), 24 h, 60 °C, Ar atmosphere.

4. Conclusions

For the precise synthesis of cellulose esters using mild low-reactive acyl donors in ILs, we prepared non-carboxylate-type ILs that theoretically negate the formation of mixed acid anhydrides that cause the undesired introduction of anions as acyl groups onto hydroxy groups. Furthermore, we evaluated the effects of the basicity and bulkiness of the anions of ILs on cellulose solvation and catalytic activity for cellulose esterification. Aryloxy ILs, which have a relatively small anion with high basicity, show high cellulose solvation and effectively catalyze cellulose esterification. In a mixed solvent system of DMSO and aryloxy-type ILs, especially 1-ethyl-3-methylimidazolium 2-pyridinolates (Emim2OPy), cellulose esters could be produced with a high DS_{main} value of up to >2.9 without any undesired introduction of an acyl group onto the hydroxy group of cellulose by transesterification of cellulose with low-reactive vinyl esters. Furthermore, a series of ILs also promoted the oxidative esterification of cellulose and afforded the target product with a high DS_{main} value (up to 2.78) without introducing an IL anion-based structure due to side reactions. The results of this study show that the synthesis of cellulose esters with high substitutions and structural uniformity in ILs can be achieved by two different esterification reactions using aryloxy-type ILs that can completely suppress the formation of side reactions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym15020419/s1>, Figure S1: 1H NMR spectrum of 1-ethyl-3-methylimidazolium 4,6-dimethyl-2-hydroxypyridinate (Emim2OPm(diMe)) (3) in DMSO- d_6 at rt.; Figure S2: ^{13}C NMR spectrum of 1-ethyl-3-methylimidazolium 4,6-dimethyl-2-hydroxypyrimidinolate

(Emim2OPm(diMe)) (3) in DMSO- d_6 at rt.; Figure S3: ^1H NMR spectrum of 1-ethyl-3-methylimidazolium theophyllinate (EmimTEO) (4) in DMSO- d_6 at rt.; Figure S4: ^{13}C NMR spectrum of 1-ethyl-3-methylimidazolium theophyllinate (EmimTEO) (4) in DMSO- d_6 at rt.; Figure S5: ^1H NMR spectrum of 1-ethyl-3-methylimidazolium 3-pyridinolates (Emim3OPy) (7) in DMSO- d_6 at rt.; Figure S6: ^{13}C NMR spectrum of 1-ethyl-3-methylimidazolium 3-pyridinolates (Emim3OPy) (7) in DMSO- d_6 at rt.; Figure S7: ^1H NMR spectrum of 1-ethyl-3-methylimidazolium 4-pyridinolates (Emim4OPy) (8) in DMSO- d_6 at rt.; Figure S8: ^{13}C NMR spectrum of 1-ethyl-3-methylimidazolium 4-pyridinolates (Emim4OPy) (8) in DMSO- d_6 at rt.; Figure S9: ^1H NMR spectrum of cellulose propionate (DS = 2.85) in CDCl_3 at rt (Table 3, entry 3); Figure S10: ^1H NMR spectrum of cellulose laurate (DS = 2.96) in CDCl_3 at rt (Table 3, entry 4); Figure S11: ^1H NMR spectrum of cellulose phenylpropionate (DS = 2.78) synthesized from Emim2OPy 6 in CDCl_3 at rt (Figure 5, blue, 24 h); Figure S12: ^1H NMR spectrum of cellulose phenylpropionate (DS = 2.78) synthesized from Emim3OPy 7 in CDCl_3 at rt (Figure 5, orange, 24 h); Figure S13: ^1H NMR spectrum of cellulose phenylpropionate (DS = 2.60) synthesized from Emim4OPy 8 in CDCl_3 at rt (Figure 5, gray, 24 h).

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Article

Conformation and Structure of Hydroxyethyl Cellulose Ether with a Wide Range of Average Molar Masses in Aqueous Solutions

Misato Yoshida ^{1,2}, Hiroki Iwase ³ and Toshiyuki Shikata ^{1,2,*}

¹ Division of Natural Resources and Eco-Materials, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

² Cellulose Research Unit, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

³ Neutron Science and Technology Center, Comprehensive Research Organization for Science and Society (CROSS), 162-1 Shirakata, Tokai, Naka 319-1106, Ibaraki, Japan

* Correspondence: shikata@cc.tuat.ac.jp

Abstract: The solution properties of a water-soluble chemically modified cellulose ether, hydroxyethyl cellulose (HeC), were examined using static light scattering (SLS), dynamic light scattering (DLS), small-to-wide-angle neutron scattering (S-WANS), small-to-wide-angle X-ray scattering (S-WAXS) and viscometric techniques at 25 °C. The examined HeC samples had average molar substitution numbers ranging from 2.36 to 2.41 and weight average molar masses (M_w) that fell within a wide range from 87 to 1500 kg mol^{−1}. Although the relationship between the determined radius of gyration (R_g) and M_w was described as $R_g \propto M_w^{-0.6}$, as is observed usually in flexible polymer solutions in good solvents, the observed scattering vector (q) dependencies of excess Rayleigh ratios were well interpreted using a rigid rod particle model, even in high- M_w samples. Moreover, the ratios of the formed particle length (L) evaluated assuming the model for rigid rods to the determined R_g showed the relationship $LR_g^{-1} \sim 3.5$ irrespective of M_w and were close to those theoretically predicted for rigid rod particle systems, i.e., $LR_g^{-1} = \sqrt{12}$. The observed SLS behavior suggested that HeC molecules behave just like rigid rods in aqueous solution. As the L values were not simply proportional to the average molecular contour length calculated from the M_w , the chain conformation or structure of the formed particles by HeC molecules in aqueous solution changed with increasing M_w . The q dependencies of excess scattering intensities observed using the S-WANS and S-WAXS experiments demonstrated that HeC molecules with M_w less than 200 kg mol^{−1} have a diameter of ~1.4 nm and possess an extended rigid rod-like local structure, the size of which increases gradually with increasing M_w . The observed M_w dependencies of the translational and rotational diffusion coefficients and the intrinsic viscosity of the particle suspensions strongly support the idea that the HeC molecules behave as rigid rod particles irrespective of their M_w .

Keywords: hydroxyethyl cellulose; small-to-wide-angle neutron scattering; small-to-wide-angle X-ray scattering; static light scattering; dynamic light scattering; weight average molar mass; radius of gyration; translational diffusion coefficient; rotational diffusion coefficient

1. Introduction

Hydroxyethyl cellulose (HeC) is a water-soluble chemically modified cellulose ether that is derived from cellulose by additional reaction with ethylene oxide by many manufacturers at present [1–3]. As HeC is obtained from an abundant and eco-friendly natural product, cellulose, it has been produced by many chemical manufacturers and is used in many practical applications in daily life; one such use is as an effective rheology modifying agent [4–8]. The fascinating rheological behavior of aqueous HeC solutions, including their shear and extensional behavior, has attracted much attention from rheologists in recent

years [9,10]. The water solubility of HeC samples is controllable by the molar substitution number (MS) of the hydroxyethyl groups of each constituent glucose ring; most commercial water-soluble HeC samples have MS numbers between 1.3 and 3.4, and HeC samples maintain rather high water solubility over a wide temperature range irrespective of the weight average molar mass (M_w) [11]. Other water-soluble chemically modified cellulose ethers such as methyl cellulose (MC) and hydroxypropylmethyl cellulose (HpMC) show a characteristic temperature dependence of water solubility; MC and HpMC are soluble in water only at temperatures lower than, e.g., 40 °C and show rather sharp clouding behavior and sometimes gelation above this temperature, depending on the MS numbers of the hydroxypropyl groups and the degree of substitution (DS) by methyl groups in each glucose unit [12]. The mechanisms through which the clouding and gelation phenomena observed in aqueous MC solutions occur have been investigated in detail [13,14]. The characteristic clouding behavior is understood to be a steep dehydration phenomenon that occurs with increasing temperature and in which the molecules fail to achieve the critical hydration state necessary to maintain water solubility. In the case of water-soluble HeC samples, the temperature dependence of the hydration number (n_H) per glucose ring is much weaker than that of HpMC and MC samples, and n_H does not fall to the critical value at which the HeC clouds or gels in water, even at temperatures higher than 70 °C [15]. Thus, HeC samples possess stable fluidity even at higher temperatures without showing clouding and gelation phenomena; thus, they are useful in applications that require stable solubility and fluidity and provide the desired rheological behaviors in aqueous solutions over wide temperature ranges from 0 to 90 °C.

We recently reported that MC samples with a wide range of M_w possess a highly elongated rigid rod-like conformation and structure in aqueous solution at room temperature (25 °C) and that these MC samples maintain high solubility [16,17]. As many macromolecular scientists believe that water-soluble cellulose derivatives of sufficiently high M_w behave as semiflexible polymer chains with persistence lengths of, e.g., ~5 nm and act as flexible chains in aqueous solutions [18,19], the rigid rod-like structure found in MC samples over a wide M_w range is rather curious. Many investigators might ask the simple question “Do HeC samples that exhibit stable water solubility without showing clouding behavior in aqueous solutions also have conformations and structures identical to the rigid rod-like particles found in MC samples?” To obtain a clear answer to this question, we decided to investigate the conformation and structure in aqueous solution at room temperature (25 °C) of HeC samples with an MS value of ~2.4 and a wide range of M_w values ranging from less than 10² kg mol^{−1} to greater than 10³ kg mol^{−1} using scattering techniques such as static light scattering (SLS), dynamic light scattering (DLS), small-to-wide-angle neutron scattering (S-WANS) and small-to-wide-angle X-ray scattering (S-WAXS) techniques and viscometric measurements. As the chemical modifications, MS values and M_w values of the HeC samples examined in this study do not differ from those of many commercially available HeC samples, the information obtained on the conformation and structure of the HeC samples in this work provides basic fundamental knowledge related to many types of applications of HeC in aqueous solution.

2. Experimental

2.1. Materials

The five HeC samples investigated in this study were supplied by Daicel Corporation (Tokyo; Japan), and were used without any further purification. The molar substitution numbers, MS , for each HeC sample were determined by use of the so-called hydrogen iodide decomposition reaction method, and the weight average molar masses were determined using SLS techniques. The determined MS values were somewhat dependent on the sample species and ranged from 2.36 to 2.41, as summarized in Table 1. The samples were coded using numbers related to their M_w (in kg mol^{−1}); for example, the HeC sample with $M_w = 87$ kg mol^{−1} was coded “HeC87”. For each HeC sample, the polydispersity index, $M_w M_n^{-1}$, in which M_n indicates the number average molar mass, was roughly determined

using size-exclusion chromatography. The determined $M_w M_n^{-1}$ values of all samples were greater than 2, as summarized in Table 1.

Table 1. Characteristics of the examined HeC samples: molar substitution number, MS (by hydroxyethyl groups), weight average molar mass, M_w , radius of gyration, R_g , second virial coefficient, A_2 , polydispersity index, $M_w M_n^{-1}$, average molecular contour length, l , particle length, L , particle diameter, d , and ratios, LR_g^{-1} and lL^{-1} .

Code	MS	$M_w/\text{kg mol}^{-1}$	R_g/nm	$A_2/\text{mL mol g}^{-2}$ *	$M_w M_n^{-1}$	l/nm	L/nm **	d/nm **	LR_g^{-1}	lL^{-1}
HeC87	2.38	87.0	27	1.0×10^{-3}	2.0	161	93.0	1.4	3.4	1.7
HeC170	2.41	170	43	1.0×10^{-3}	2.7	315	150	1.6	3.5	2.1
HeC520	2.36	520	80	4.9×10^{-4}	4.0	972	290	2.5	3.6	3.4
HeC1000	2.39	1000	120	1.1×10^{-3}	2.4	1860	420	3.0	3.5	4.4
HeC1500	2.36	1500	140	1.5×10^{-3}	2.2	2800	500	5.0	3.6	5.6

* With uncertainty of $\pm 2.0 \times 10^{-4}$. ** Evaluated assuming rigid rod particles (see text).

Deuterium oxide (D_2O , deuterium content > 99.8%) was purchased from Eurisotope (Saint-Aubin; France) and used as a solvent in the S-WANS experiments. Highly deionized water with a specific electrical resistance greater than $18 \text{ M}\Omega \text{ cm}$ obtained using a Direct-Q UV 3, Millipore (Darmstadt; Germany) was used as a solvent in the preparation of aqueous solutions of HeC used in experiments other than the S-WANS experiments.

HeC samples at several concentrations (c), each of which was lower than the reciprocal of the intrinsic viscosity ($[\eta]^{-1}$) resulting from viscometric measurements, were used in the SLS and DLS experiments. The c values of the HeC87 samples in D_2O used in the S-WANS measurements were $c = 5.0 \times 10^{-3}$ and $1.0 \times 10^{-2} \text{ g mL}^{-1}$. The c values in prepared solutions of the samples used in the S-WAXS experiments were $c = 2.5 \times 10^{-3}$, 5.0×10^{-3} and 1.0×10^{-2} for HeC87, $c = 1.0 \times 10^{-3}$ for HeC1000 and $c = 8.0 \times 10^{-4} \text{ g mL}^{-1}$ for HeC1500.

2.2. Methods

SLS and DLS measurements were conducted using an extensively modified DLS7000 originally manufactured by Otsuka Electronics Co., Ltd. (Osaka, Japan). A single-frequency Sapphire laser, SF488-100, Coherent, Inc. (Santa Clara, CA, USA), at a wavelength of $\lambda = 488.0 \text{ nm}$, was equipped with the modified DLS7000 as a light source. As a light detector, a photomultiplier, R9880U-01, Hamamatsu Photonics K. K. (Hamamatsu, Japan), was installed. To record intensity fluctuations in scattered light as a function of time, an LSI correlator, LS Instrument AG (Fribourg, Swiss), was used. An autocorrelation function of the scattered light intensity was precisely calculated by the correlator. A Pyrex glass tube which possesses an inner diameter of 19.0 mm and a thickness of 0.5 mm was used as a measuring cell. The measuring temperature for both SLS and DLS measurements was 25.0°C . The scattering angle (θ) was varied from 30° to 150° in 10° increments. The magnitudes of the scattering vectors covered from 9.78×10^{-3} to $3.65 \times 10^{-2} \text{ nm}^{-1}$. Toluene was employed as the standard material for scattered light intensity in the SLS experiments. The refractive index increment ($\partial n/\partial c$) of the HeC in aqueous solution was determined to be 0.139 mL g^{-1} by use of an Abbemat MW multiwavelength refractometer, Anton Paar (Graz, Austria), at a wavelength of $\lambda = 486 \text{ nm}$. This value was used to determine the M_w values for each HeC sample from the SLS data. $\partial n/\partial c = 0.138 \text{ mL g}^{-1}$ was also determined at a different wavelength of $\lambda = 589 \text{ nm}^{-1}$, and the λ dependence of $\partial n/\partial c$ of the HeC samples was weak.

S-WANS experiments were conducted at the Materials and Life Science Experimental Facility (MLF) at the Japan Proton Accelerator Research Complex, J-PARC (Tokai, Japan), using a small-angle neutron scattering facility (TAIKAN) [20], which is installed on a

beamline BL15. The range of the scattering vector, q , used in the S-WANS experiments covered from 9.0×10^{-2} to $1.0 \times 10^2 \text{ nm}^{-1}$. As sample cells for S-WANS experiments, square-type quartz cuvettes with a neutron beam path length of 4.0 mm were used. The standard exposure time was 2 h for each sample solution. S-WANS measurements were conducted at 25 °C. The obtained scattering data were converted to absolute values by use of a standard “glassy carbon” data. Absolute scattering value for the glassy carbon had been precisely determined.

S-WAXS measurements were conducted using a small-to-wide-angle X-ray scattering analyzer, SAXSpace, Anton Paar (Graz, Austria), at 25 °C. The scattering vector, q , in the S-WAXS measurements ranged from 1.3×10^{-1} to $1.9 \times 10^1 \text{ nm}^{-1}$. Quartz capillaries with outer diameter and thickness of 1.0 mm and 10 μm , respectively, were used as sample cells. The X-ray exposure time was 8 h for aqueous HeC solutions at $c \leq 2.5 \times 10^{-3} \text{ g mL}^{-1}$ and 30 min for aqueous HeC87 solutions at $c = 5.0 \times 10^{-3}$ and $1.0 \times 10^{-2} \text{ g mL}^{-1}$.

The intrinsic viscosities, $[\eta]$, of HeC samples in aqueous solutions were determined at 25.0 °C with a Ubbelohde-type capillary viscometer.

3. Results and Discussion

3.1. SLS Behaviors

Using values of $\lambda = 488.0 \text{ nm}$ for light course and $\partial n/\partial c = 0.139 \text{ mL g}^{-1}$ for the examined HeC in aqueous solution, the apparatus constant (K) of the system was determined to be $K = 3.89 \times 10^{-7} \text{ cm}^2 \text{ mol g}^{-2}$. The excess Rayleigh ratios, R_θ , compared to the standard liquid, toluene, were determined at each scattering angle for all the aqueous HeC solutions examined. As one of the typical SLS experimental results, the square of the scattering vector, q^2 , and the dependencies of the $\left(\sqrt{Kc(R_\theta)^{-1}}\right)_{c=0} (= \lim_{c \rightarrow 0} \sqrt{Kc(R_\theta)^{-1}})$ data, so-called Berry plots [21], for the HeC170 sample are shown in Figure 1a. As the HeC samples possess rather broad molar mass distributions, Berry plots rather than Zimm plots [22] were used to analyze the data in this study. The intercept determined from the solid line in Figure 1a indicates $M_w = 170 \text{ kg mol}^{-1}$, and the initial slope of the line indicates a radius of gyration $R_g = 43.0 \text{ nm}$ for HeC170. The c dependencies of the $\left(\sqrt{Kc(R_\theta)^{-1}}\right)_{q=0} (= \lim_{q \rightarrow 0} \sqrt{Kc(R_\theta)^{-1}})$ data for the same HeC170 sample are shown in Figure 1b. The intercept determined from the solid line in Figure 1b yields the same M_w value as that obtained using the solid line in Figure 1a, and the initial slope of the line indicates a second virial coefficient of $A_2 = 1.0 \times 10^{-3} \text{ mL mol g}^{-2}$ for HeC170. Using essentially the same procedures as described above, the values of M_w , R_g and A_2 were determined for each HeC sample examined in this study. Table 1 summarizes these values.

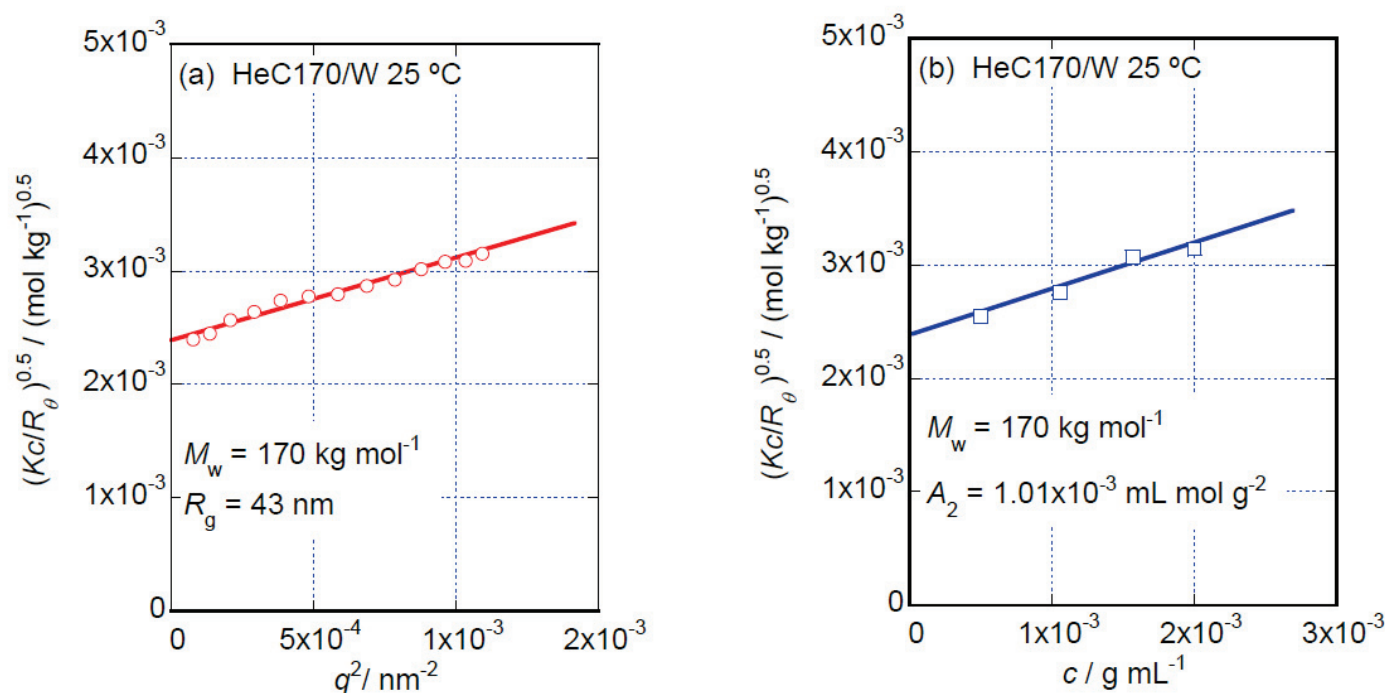


Figure 1. q^2 dependencies of the $\left(\sqrt{Kc(R_\theta)^{-1}}\right)_{c=0}$ data (a) and c dependencies of the $\left(\sqrt{Kc(R_\theta)^{-1}}\right)_{q=0}$ data (b) for aqueous solutions of the HeC170 sample at 25 °C.

The dependencies of R_g and A_2 on the M_w values of the HeC samples are shown in Figure 2a,b, respectively. Based on the slope of the solid line in Figure 2a, which is ~ 0.6 , the relationship $R_g \propto M_w^{0.6}$ is approximately obtained. This M_w exponent is close to the values that are usually reported for flexible polymer chains dissolved in moderately good solvents [23]. However, we cannot simply conclude that the HeC samples behave as flexible polymer chains in aqueous solution, because the q dependence of the $(R_\theta (Kc)^{-1})_{c=0}$ obtained in the SLS experiments and that of the S-WANS and S-WAXS data, which are discussed in detail in later sections, do not show flexible polymer chain behavior. The value of A_2 indicates the strength of the interparticle interaction between two solute molecules. The decrease in the A_2 value observed in Figure 2b up to the M_w value of ~ 500 kg mol $^{-1}$ indicates that there is a decrease in the repulsive interaction between two HeC molecules with increasing M_w that leads to an increase in attractive interactions between HeC molecules with increasing M_w . After reaching its minimum, A_2 displays a steep increase in its M_w dependence, as is clearly seen in Figure 2b; this is related to an increase in repulsive interactions between HeC molecules with increasing M_w . The observed complicated M_w dependence of A_2 in the HeC samples appears to be caused by conformational and structural changes induced by alterations in the intramolecular interactions between constituent segments of HeC molecules with increasing M_w .

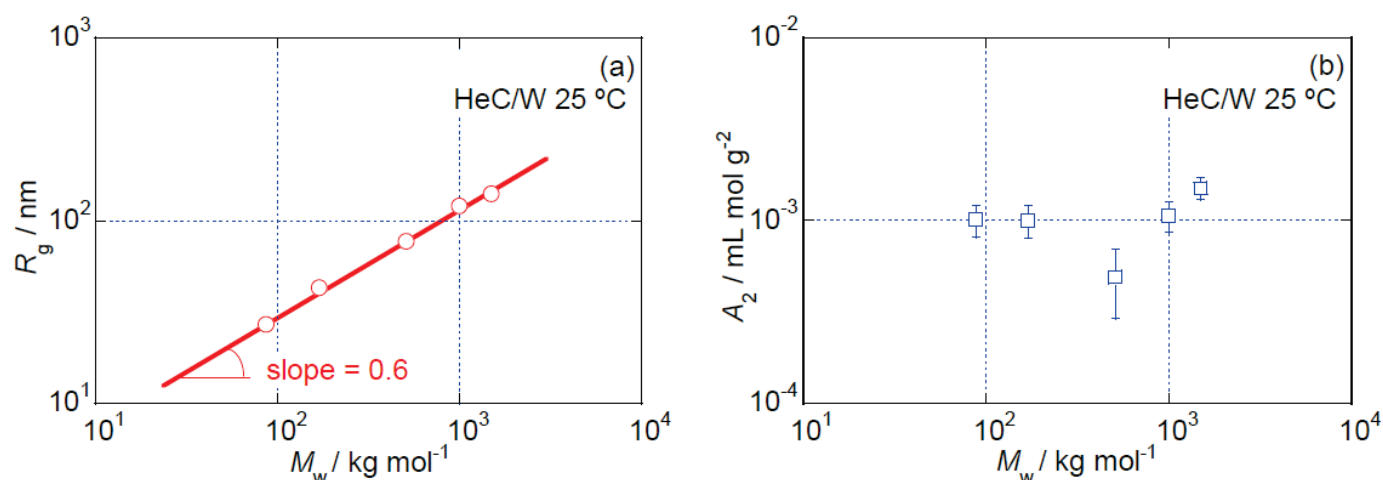


Figure 2. M_w dependencies of R_g (a) and A_2 (b) for HeC samples in aqueous solution at 25 °C.

As the A_2 values of most flexible and semiflexible polymer chains that maintain constant persistence lengths irrespective of M_w when dissolved in solutions show monotonous weak decreasing behavior with increasing M_w [23,24], the M_w dependence of A_2 observed in Figure 2b suggests a clear difference between the HeC molecules analyzed in this study and the usual flexible and semiflexible polymer chains from the viewpoint of intermolecular interactions. In the case of aqueous solutions of another water-soluble chemically modified cellulose ether, methyl cellulose, MC, the observed A_2 decreases rather drastically with increasing M_w and does not show a subsequent steep increase up to an M_w value of $\sim 1.0 \times 10^3$ kg mol^{-1} , as observed in Figure 2b [17]. Thus, it is likely that the structural change that occurs in the aqueous HeC samples is not identical to that in the aqueous MC samples [17].

The q dependencies of $R_\theta(Kc)^{-1}_{c=0}$ data for all HeC samples are shown in Figure 3 on a double-logarithmic scale. For HeC samples with M_w values lower than 200 kg mol^{-1} , the $R_\theta(Kc)^{-1}_{c=0}$ data show weak q dependencies, for which suitable form factors, $P(q)$, cannot be obtained precisely from the SLS data alone. However, the HeC samples with lower M_w values demonstrated obvious proportionality to q^{-1} in their excess scattering intensities, $\Delta I(q)c^{-1}$, in the data obtained in the S-WANS and S-WAXS experiments covering a q range higher than that of the SLS experiments, as discussed in detail in a later section. In a high q range of the SLS data, the HeC samples with M_w values higher than 500 kg mol^{-1} clearly show the relationship $R_\theta(Kc)^{-1}_{c=0} \propto q^{-1}$, as seen in Figure 3. As the relationship $R_\theta(Kc)^{-1}_{c=0}$ (or $\Delta I(q)c^{-1}$) $\propto q^{-1}$ indicates characteristic behavior of the form factors, $P(q)$, of rigid rod particles in the q range higher than the reciprocal of rod particle length (L) [25,26] and $R_\theta(Kc)^{-1}_{c=0}$ theoretically corresponds to $M_w P(q)$, it is possible that the $R_\theta(Kc)^{-1}_{c=0}$ data for all the HeC samples in this study are describable based on the form factors of rigid rod particles that possess different L values that depend on the M_w of the sample.

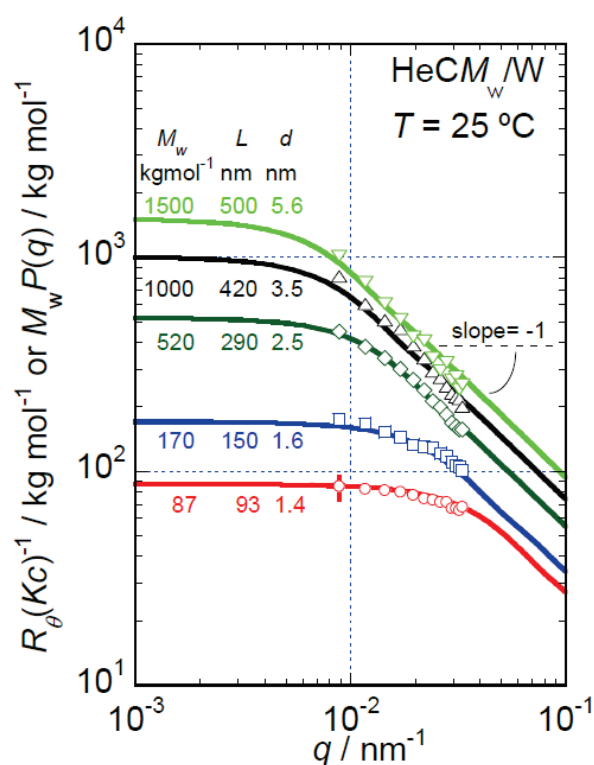


Figure 3. q dependencies of the $R_\theta(Kc)^{-1}_{c=0}$ data and fitted $M_w P(q)$ curves for HeC samples in aqueous solution at 25 °C.

The solid lines shown in Figure 3 represent $M_w P(q)$ curves resulting from curve fitting to the $R_\theta(Kc)^{-1}_{c=0}$ data for each HeC sample assuming rigid rod particle form factors. In the curve fitting procedure, the open source software SasView [27] was employed to calculate rigid rod form factors [26], $P(q)$. Varying L values, $M_w P(q)$ curves were fitted to the $R_\theta(Kc)^{-1}_{c=0}$ data to permit identification of the most adequate L values for each HeC sample. The q dependence of the $P(q)$ curves in the q range covered by the SLS experiments ($q < 4 \times 10^{-2} \text{ nm}^{-1}$) was not sensitive to the diameter (d) of the assumed rigid rod particles when d was set at values smaller than, e.g., 10 nm. The values of L (and d) determined as the most adequate average values based on fitting of the data are shown in Figure 3. As the observed agreement between the $M_w P(q)$ curves and the $R_\theta(Kc)^{-1}_{c=0}$ data is reasonable for HeC samples with M_w greater than 500 kg mol $^{-1}$, the rigid rod particle form factors appear to be suitable for describing the SLS behavior of HeC samples in aqueous solutions. The $R_\theta(Kc)^{-1}_{c=0}$ data for the HeC samples with M_w values lower than 100 kg mol $^{-1}$ display quite weak q dependence in the q range covered by the SLS experiments for the determination of $M_w P(q)$ curves precisely. Then, the combination of the q dependencies of the $R_\theta(Kc)^{-1}_{c=0}$ data and the excess scattering intensity data, $\Delta I(q)c^{-1}$, resulting from the S-WANS and S-WAXS experiments will be quite useful to determine $M_w P(q)$ curves for the low- M_w HeC samples as discussed in detail in the next section.

Figure 4 shows the M_w dependencies of L and LR_g^{-1} determined for each HeC sample. Although the value of L increases monotonically with increasing M_w , the L value is not proportional to the M_w value, and the ratio LR_g^{-1} appears to remain at a constant value close to ~ 3.5 irrespective of M_w . As the relationship found in this study is close to the simple relationship $L^2 = 12R_g^2$, i.e., $L(R_g)^{-1} = \sqrt{12} \sim 3.46$, which holds in suspensions of rigid rod particles, the results might be interpreted as strongly supporting a rigid rod structure of the particles formed by HeC molecules in aqueous solution. However, the HeC molecules cannot have simply elongated straight conformations or systematic helical conformations because the L of the formed particles is not simply proportional to M_w , as described above. The average contour lengths (l) of the HeC molecules in each HeC sample

can be calculated assuming the repeating length of the glucose unit to be 0.5 nm [28], and in this way a holding number defined as lL^{-1} , which indicates the average molecular chain number per cross section of the rod particles formed by the HeC molecules, can be calculated, considering that an essential characteristic of cellulose molecules that form the framework of HeC is that they prefer to assume a straight elongated conformation. The M_w dependence of the lL^{-1} value for HeC samples is also shown in Figure 4. For HeC samples of M_w less than 20 kg mol^{-1} , the lL^{-1} value is not far from two. This observation suggests that short HeC molecules have hairpin-like particle structures with $lL^{-1} = 2$ on average. The presence of a similar hairpin-like structure has already been demonstrated in aqueous solutions of MC and HpMC [16,17]. The observed change in the lL^{-1} value with increasing M_w in Figure 4 suggests alterations in the size and shape of the cross sections of HeC molecules in aqueous solution. However, the SLS data cannot provide useful information in the length scale shorter than 10 nm^{-1} , which is related to the cross section of HeC molecules.

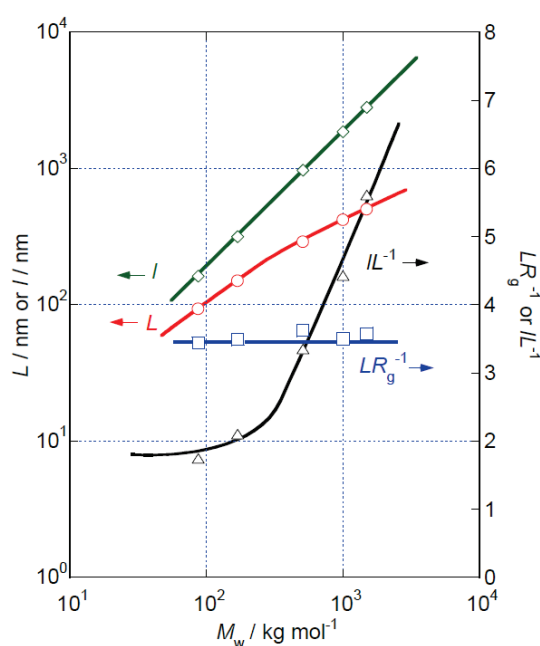


Figure 4. M_w dependencies of L , LR_g^{-1} and lL^{-1} for HeC samples in aqueous solution at 25°C . Lines are drawn in this figure as a guide for the eye.

3.2. S-WANS and S-WAXS Behaviors

The q dependencies of concentration-reduced excess scattering intensities, $\Delta I_N(q)c^{-1}$, determined via the S-WANS measurements for HeC87 at $c = 0.005$ and 0.010 g mL^{-1} , are shown in Figure 5a. As the $[\eta]$ value for HeC87 was determined to be 180 mL g^{-1} , the $\Delta I_N(q)c^{-1}$ data obtained at $c = 0.005 \text{ g mL}^{-1}$ should be more reliable than those obtained under the condition of the so-called isolated state for HeC molecules given by $c < [\eta]^{-1}$. The difference between the $\Delta I_N(q)c^{-1}$ data obtained at these concentrations is small, as seen in Figure 5a. Thus, the condition at $c = 0.010 \text{ g mL}^{-1}$ is not far from the isolated state. One can easily recognize the relationship $\Delta I(q)c^{-1} \propto q^{-1}$ in the q range $0.1 \text{ nm}^{-1} < q < 1.0 \text{ nm}^{-1}$; such a relationship is characteristic of rigid rod or long columnar particles with $L > 10 \text{ nm}$. A steeper decrease in the $\Delta I(q)c^{-1}$ data is observed over the q range greater than 2.0 nm^{-1} ; there, two broad interference-type peaks are clearly recognized at $q \sim 6.5$ and 14 nm^{-1} , as seen in Figure 5a. As the presence of two similar interference-type peaks has also been observed in aqueous solutions of MC and HpMC, it is speculated that the local structures assumed by several water-soluble chemically modified cellulose ethers have common characteristics showing these peaks [16,17]. The observed peak at $\sim 6.5 \text{ nm}^{-1}$ corresponds to the periodic distance of $\sim (2\pi/6.5) 1.0 \text{ nm}$, close to the molecular size of cellobiose, the

structural repeating unit of cellulose. Another periodic distance of 0.4 nm corresponding to the other peak at 14 nm^{-1} , $2\pi/14 \sim 4.5 \text{ nm}$, is not far from the separation between facing cellulose molecules in the crystalline structure of cellulose II [16,17].

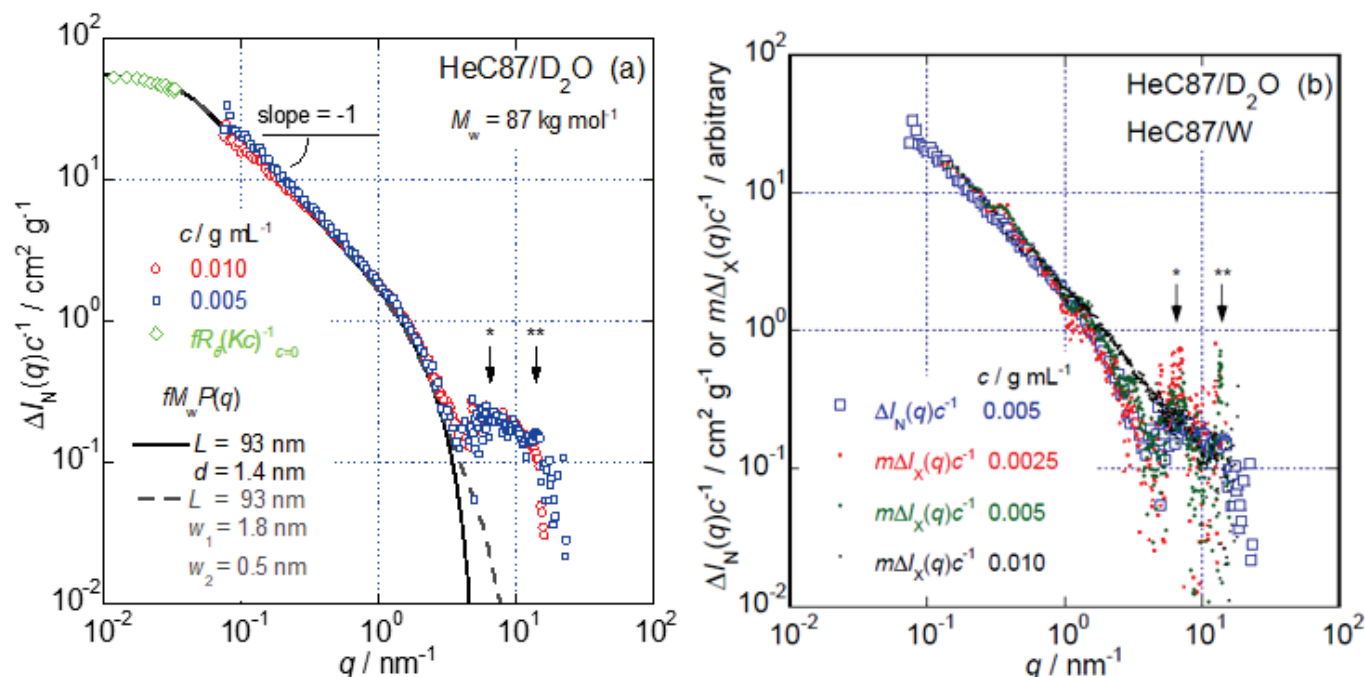


Figure 5. (a) q dependencies of the $\Delta I_N(q)c^{-1}$ (S-WANS) data for D_2O solutions of HeC87 at $c = 0.005$ and 0.010 g mL^{-1} at 25°C ; (b) q dependencies of the $m\Delta I_X(q)c^{-1}$ (S-WAXS) data for aqueous HeC87 solutions at $c = 0.0025, 0.0050$ and 0.010 g mL^{-1} at 25°C and that of $\Delta I_N(q)c^{-1}$ data for the same HeC87 sample shown in (a). The SLS data obtained for the same system, $fR_\theta(Kc)^{-1}$, and the two fit curves, $fM_wP(q)$, obtained assuming $f = 6.5 \times 10^{-4} \text{ cm}^2 \text{ g}^{-2} \text{ mol}$, are also shown in (a). The numerical constant of $m = 0.17$ is used for all the $m\Delta I_X(q)c^{-1}$ data in (b). The places of * and ** in the figures mean the positions of the broad interference-type peaks.

The solid line in Figure 5a shows the curve fit to the $\Delta I(q)c^{-1}$ data by SasView [27] assuming the rigid rod particle form factor, $P(q)$, with $L = 93$ and $d = 1.4 \text{ nm}$. Although the L value cannot be determined uniquely from the S-WANS data alone, the SLS data providing the value of $R_g = 27 \text{ nm}$ and the weak q dependence of $R_\theta(Kc)^{-1}_{c=0}$ data in the q range lower than 0.04 nm^{-1} were useful in determining the L and d values. The SLS data multiplied by the constant $f = 6.5 \times 10^{-4} \text{ cm}^2 \text{ g}^{-2} \text{ mol}$, i.e., $fR_\theta(Kc)^{-1}_{c=0}$, are also plotted in Figure 5a. The value $f = (6.5 \pm 0.3) \times 10^{-4} \text{ cm}^2 \text{ g}^{-2} \text{ mol}$ connects the S-WANS and SLS data quite smoothly using the rigid rod form factor, maintaining the characteristics of almost flat $P(q)$ in the range of $q < 0.04 \text{ nm}^{-1}$ and $P(q) \propto q^{-1}$ in the range of $0.1 \text{ nm}^{-1} \leq q < 2.0 \text{ nm}^{-1}$. The agreement between the solid fit curve, $fM_wP(q)$, with the identical f value to the $fR_\theta(Kc)^{-1}_{c=0}$ data and the experimental $\Delta I_N(q)c^{-1}$ data appears reasonable except for the presence of the two interference-type peaks. The broken line in the same figure represents the fit curve calculated assuming a rectangular columnar particle with $L = 93 \text{ nm}$, width $w_1 = 1.8 \text{ nm}$ and thickness $w_2 = 0.5 \text{ nm}$. As the difference between the fit curves indicated by the solid and broken lines is inconspicuous, especially in the q range below 2.5 nm^{-1} , we might conclude that these structural factors describe the local structure of HeC87 molecules rather reasonably. An elliptical rod particle [29] with structural parameters similar to those of the rectangular columnar particle would have a form factor, $P(q)$, suitable for the $\Delta I_N(q)c^{-1}$ data seen in Figure 5a.

The S-WAXS measurements covered a similar q range to the S-WANS measurements in this study. To confirm the validity of the data obtained using these two techniques, we compared the q dependencies of the $\Delta I_X(q)c^{-1}$ data determined in S-WAXS measure-

ments with those obtained from S-WANS measurements. As the $\Delta I_X(q)c^{-1}$ data were not converted into absolute values in this study, the $m\Delta I_X(q)c^{-1}$ data multiplied by a certain numerical constant, m , can be used for comparison. Figure 5b shows the q dependence of $\Delta I_N(q)c^{-1}$ for the HeC87 sample obtained at $c = 0.005 \text{ g mL}^{-1}$ (the same data as seen in Figure 5a) and that of $m\Delta I_X(q)c^{-1}$ at $c = 0.0025, 0.0050$ and 0.010 g mL^{-1} using the numerical constant $m = 0.17$. Although all the c values are rather low and the obtained $\Delta I_N(q)c^{-1}$ and $m\Delta I_X(q)c^{-1}$ data are poorly dispersed, the agreement between the two sets of data, with the exception of the $m\Delta I_X(q)c^{-1}$ data at $c = 0.010 \text{ g mL}^{-1}$, is fairly good, especially in the q range 0.1 to 4.0 nm^{-1} ; within that range, the scattering data are proportional to q^{-1} up to $q = 1.0 \text{ nm}^{-1}$ and decrease more steeply above that q value. The data obtained at $c = 0.010 \text{ g mL}^{-1}$ clearly show substantially greater values than other data in the q range from 1.5 to 5.0 nm^{-1} and show the interference-type peaks less significantly than $m\Delta I_X(q)c^{-1}$ data at other lower c . It appears that the $m\Delta I_X(q)c^{-1}$ data obtained from the S-WAXS measurements demonstrate the presence of the two interference peaks at $q = 6.5$ and 14 nm^{-1} slightly more clearly than do the $\Delta I_N(q)c^{-1}$ (S-WANS) data. Based on the observed fair agreement between the $\Delta I_N(q)c^{-1}$ data and the $m\Delta I_X(q)c^{-1}$ data in the q range 0.1 to 4.0 nm^{-1} , we might conclude that both the S-WANS measurements and the S-WAXS measurements show the precise q dependence of scattering data for isolated HeC molecules under the condition of $c \leq [\eta]^{-1}$, as we expected.

The $m\Delta I_X(q)c^{-1}$ data for HeC1000 at $c = 1.0 \times 10^{-3} \text{ g mL}^{-1}$ and those for HeC1500 at $c = 0.8 \times 10^{-3} \text{ g mL}^{-1}$ are shown in Figure 6a,b, respectively. The $fR_\theta(Kc)^{-1}_{c=0}$ data for the HeC1000 and HeC1500 samples obtained from the SLS measurements and the $fM_wP(q)$ curves resulting from the rigid rod particle model are also included in Figure 6a,b. As the f and m values used in Figure 5b are also employed in Figure 6a,b, the q dependencies of the $m\Delta I_X(q)c^{-1}$ data in these figures are directly comparable with the $fR_\theta(Kc)^{-1}_{c=0}$ data and the $fM_wP(q)$ curves quantitatively with respect to their magnitude. It is likely that the q range over which the relationship $m\Delta I_X(q)c^{-1} \propto q^{-1}$ is observed becomes narrower with increasing M_w . In the case of HeC1000, the $m\Delta I_X(q)c^{-1}$ data show a steeper decrease in magnitude with a negative exponent of q , obviously greater than unity, in the q range higher than $\sim 0.8 \text{ nm}^{-1}$, as seen in Figure 6a. A similar change in the q dependence of $m\Delta I_X(q)c^{-1}$ data can also be recognized for HeC1500 in the q range higher than $\sim 0.3 \text{ nm}^{-1}$; this range is clearly lower than the q range observed for the solution of HeC1000(850), as seen in Figure 6b. This suggests that there is a change in the local particle structure formed by HeC molecules that is a function of M_w . An increase in the diameter, d , of the rigid rod decreases the q range, showing the relationship $m\Delta I_X(q)c^{-1} \propto q^{-1}$. The solid line indicating $fM_wP(q)$, shown in Figure 6a, is the fit curve for the $m\Delta I_X(q)c^{-1}$ data calculated using SasView [27] assuming the form factor of a rigid rod, $P(q)$, with $L = 420 \text{ nm}$ and $d = 3.0 \text{ nm}$. The agreement between the calculated curve and the data is reasonable in the q range below 1.0 nm^{-1} . It is likely that the $m\Delta I_X(q)c^{-1}$ data have a certain characteristic q dependence that is related to the local structure of the formed particles in the q range higher than 2.0 nm^{-1} . However, the poor quality of the obtained data due to the low concentrations of HeC used in the experiments did not allow us to distinguish between a more local particle structure formed by HeC1000 molecules and other sophisticated particle models such as rectangular columnar and/or elliptical rod models.

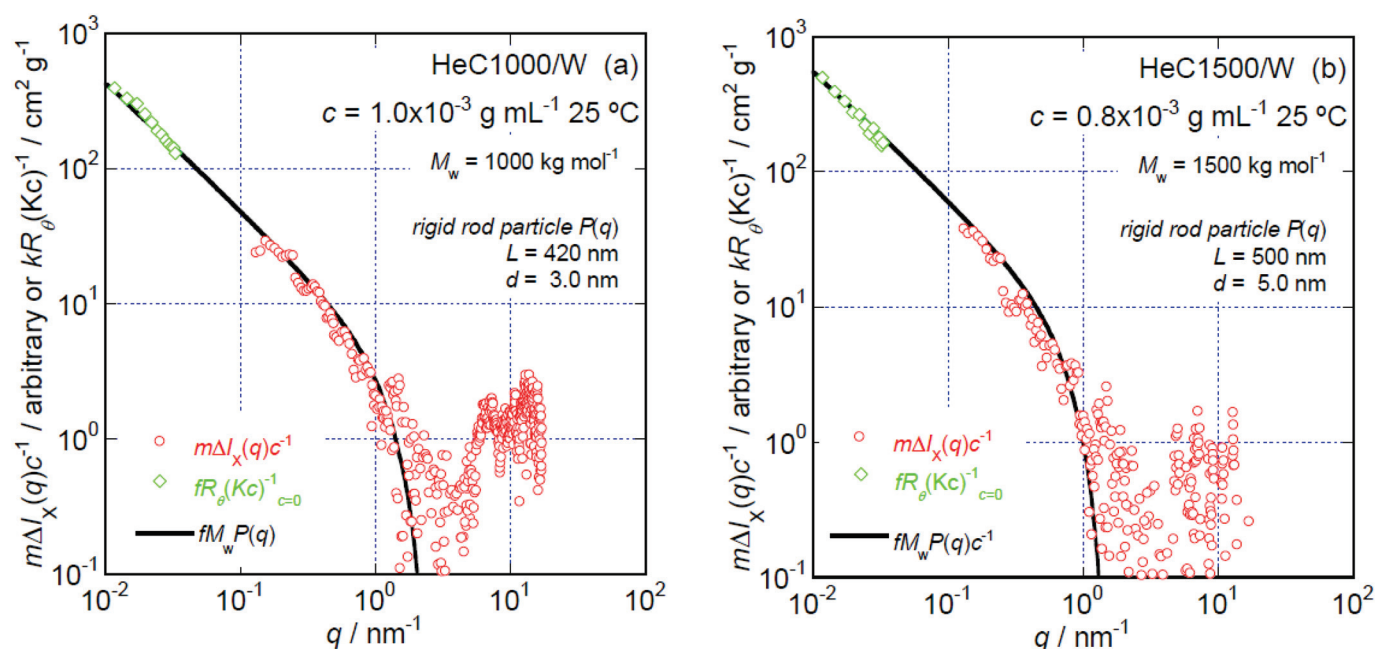


Figure 6. q dependencies of the $m\Delta I_X(q)c^{-1}$ (S-WAXS) data obtained for HeC1000 at $c = 1.0 \times 10^{-3} \text{ g mL}^{-1}$ (a) and those obtained for HeC1500 at $c = 0.8 \times 10^{-3} \text{ g mL}^{-1}$ at 25°C (b). The same numerical constants as those used to generate the fitted curves shown in Figure 5b, $f = 6.5 \times 10^{-4} \text{ cm}^2 \text{ g}^{-2} \text{ mol}$ and $m = 0.17$, were employed.

HeC1500 molecules also appear to have rigid rod-like local structures, as clearly suggested by the dependence of the $m\Delta I_X(q)c^{-1}$ data on q seen in Figure 6b. The solid line representing $fM_w P(q)$ in Figure 6b indicates the form factor, $P(q)$, of a rigid rod with $L = 500 \text{ nm}$ and $d = 5.0 \text{ nm}$ as a function of q . The agreement between the fitted curve obtained using the rigid rod particle model and the $m\Delta I_X(q)c^{-1}$ data is fair, especially in the q range lower than 1.0 nm^{-1} . We did not consider more local structures of particles formed by HeC1500(900) in aqueous solution due to the poor quality of the data in the q range higher than 1 nm^{-1} , as also seen in Figure 6b. However, despite the low quality of the data, two interference peaks at approximately 6.5 and 14 nm^{-1} can be discerned, as seen in Figure 6a,b.

The fair agreement between the fitted curves obtained assuming the form factors of rigid rods with diameter, d , varying from 1.4 to 5.0 nm with increasing M_w and the $m\Delta I_X(q)c^{-1}$ data obtained for aqueous solutions of HeC samples, as seen in Figures 5 and 6, strongly suggests that the particles formed by the HeC molecules can be described as having local structures and conformations that correspond to rigid rods with diameters that increase gradually with increasing M_w .

3.3. Viscometric Behaviors

The M_w dependence of the intrinsic viscosity, $[\eta]$, of the HeC samples in aqueous solution is shown in Figure 7. Although the relationship $[\eta] \propto M_w^{-0.67}$, which is usually observed for flexible polymer chain samples dissolved in good solvents [23], appears at a glance to hold, we would like to consider this relationship based on the idea of formation of rigid rod particles by the HeC samples. This consideration is based on the fact that all the scattering data obtained in the SLS and S-WANS and S-WAXS experiments can be fairly well explained using a form factor corresponding to that of rigid rod particles. Based on this consideration, we do not think that the lengths, L , of the formed particles are simply proportional to M_w and that the radii, d , of the formed rigid rod particles maintain a constant value irrespective of M_w , despite the fact that this is a usually accepted idea.

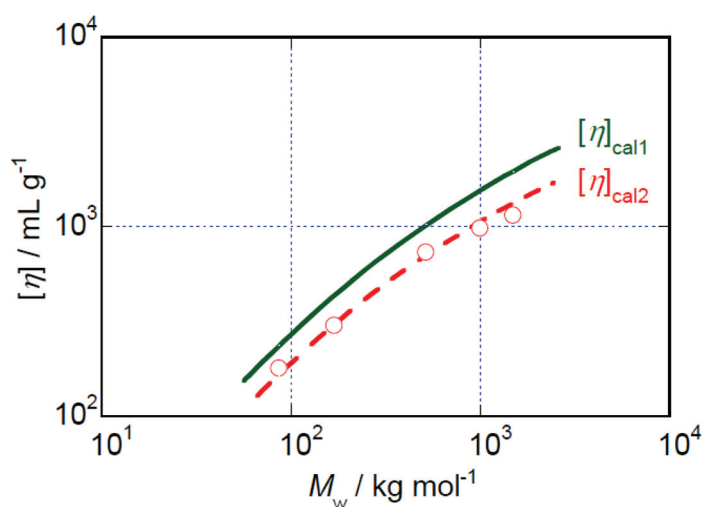


Figure 7. M_w dependence of $[\eta]$ data for HeC samples in aqueous solution at 25 °C. The solid line labeled $[\eta]_{cal1}$ in this figure shows the M_w dependence of $[\eta]_{cal}$ assuming the first simple relationship, $L_\eta = L$ and $d_\eta = d$, and the broken line labeled $[\eta]_{cal2}$ represents the M_w dependence obtained assuming the second relationship, $L_\eta = 0.87L$ and $d_\eta = d$.

According to theoretical calculations [30,31], the intrinsic viscosity of a suspension of rigid rod particles ($[\eta]_{cal}$) with viscometric particle length (L_η) and d_η is given as Equation (1).

$$[\eta]_{cal} = \frac{2\pi N_A L_\eta^3}{45M_w \{ \ln(L_\eta/d_\eta) + C_\eta \}} \quad (1)$$

In Equation (1), N_A and C_η represent, respectively, Avogadro's number and a numerical constant that shows the contribution of hydrodynamic interaction formulated using polynomials of the ratio L_η/d_η [30]. As the first simple choice, we can select the relationship $L_\eta = L$ and $d_\eta = d$ to calculate $[\eta]_{cal}$. It is known that C_η approaches a constant value of -0.93 when L_η is much longer than d_η . We used this value in our calculations of $[\eta]_{cal}$. The solid line representing $[\eta]_{cal1}$ in Figure 7 shows the M_w dependence of $[\eta]_{cal}$ resulting from the first simple choice. Although the agreement between the calculated $[\eta]_{cal1}$ curve and the experimental data is not perfect, the M_w dependence of the $[\eta]$ data is reproduced semiquantitatively. We then tested a second assumption, $L_\eta = 0.87L$ and $d_\eta = d$; the curve obtained in that way is shown in Figure 7 as the broken curve labeled $[\eta]_{cal2}$. The agreement between the $[\eta]_{cal2}$ curve and the $[\eta]$ data is much better than that between $[\eta]_{cal1}$ and the $[\eta]$ data. Changing the d_η value, e.g., using $d_\eta = 0.87d$, hardly affected the value of $[\eta]_{cal}$. Consequently, we might conclude that the viscometric behavior of HeC samples in aqueous solution is reasonably described as corresponding to that of rigid rod particles with viscometric lengths slightly shorter than the structural lengths determined using scattering methods such as SLS, S-WANS and S-WAXS. Viscometric lengths that are shorter than structural lengths have also been reported for aqueous solutions of MC samples [17] and for *N*-methylpyrrolidone solutions of poly(vinylidene difluoride) [32].

3.4. Diffusional Behaviors

The first cumulants, Γ_1 , calculated from the initial slopes of the obtained autocorrelation functions of the scattered light electric field provide translational diffusion coefficients, D_t , of particles formed by solute molecules dissolved in sample solutions. As typical experimental results for HeC samples in aqueous solutions, Figure 8a,b show the q^2 dependencies of Γ_1 data for the shortest (HeC87) and the longest (HeC900) samples, respectively. The Γ_1 data for the HeC87 sample seen in Figure 8a can be described as following a straight line with a constant slope over the q^2 range covered in this study. In this case, the value of D_t can be simply evaluated as the constant slope of $2.3 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$. However, the Γ_1 data for the HeC1500 sample shown in Figure 8b are not simply proportional to q^2 ; instead,

they show a sigmoidal shape dependence. For Γ_1 data without linear q^2 dependence, the D_t value can be obtained from the initial slope of the line conforming to the Γ_1 data using the equation $D_t = \lim_{q^2 \rightarrow 0} \Gamma_1 q^{-2}$. In the case of large particles such as HeC1500, the Γ_1 data reflect the contribution of rotational diffusion, especially in the high- q^2 region. When the Γ_1 data possess another $\Gamma_1 \propto q^2$ relationship for which the proportional coefficient is identical to D_t for the high- q^2 region, the q^2 dependence of the Γ_1 data can be approximately described as $\Gamma_1 = 6D_r + D_t q^2$ [17,33,34]. The rotational diffusion coefficient, D_r , can then be evaluated from the intercepts of the straight lines, which follow the Γ_1 data in the high- q^2 range and are extrapolated to $q^2 = 0$. This is shown in Figure 8b for the HeC1500 sample. However, in this study, the values of D_r were obtained only for the three high- M_w HeC samples. Although depolarized dynamic light scattering (DDLS) techniques [33] are better methods for determining D_r values for solute particles and we, of course, performed some DDLS measurements, scattering intensity data sufficient to determine D_r values precisely were not obtained under depolarized conditions for all the samples examined in this study.

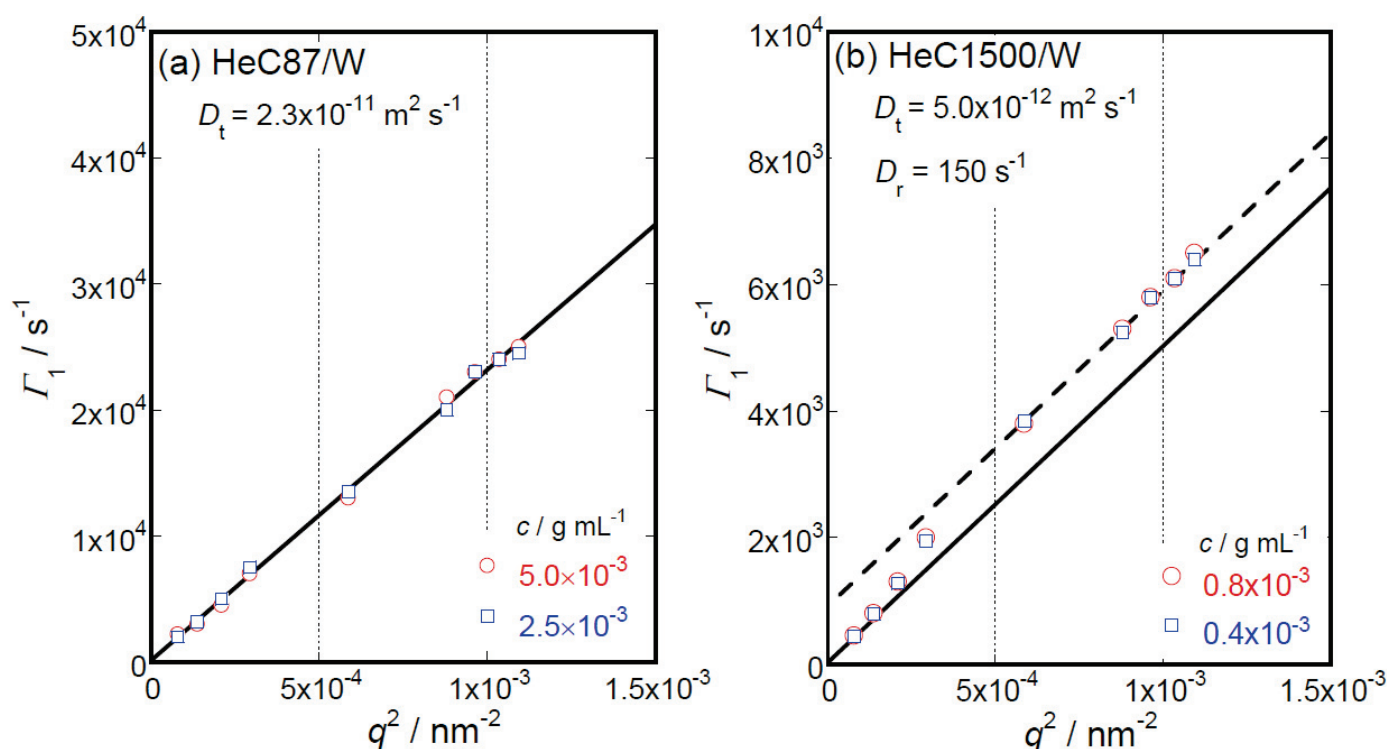


Figure 8. q^2 dependencies of the Γ_1 data for the shortest (HeC87, (a)) and the longest (HeC1500, (b)) samples. The broken line in (b) demonstrates the relationship $\Gamma_1 = 6D_r + D_t q^2$; thus, the D_r value can be evaluated from the intercept of the broken line at $q^2 = 0 nm^{-2}$.

The M_w dependencies of the obtained D_t and D_r data for the HeC samples in aqueous solution are shown in Figure 9a. The D_t data for the HeC samples appear at a glance to demonstrate the relationship $D_t \propto M_w^{-0.65}$, which is usually observed for flexible polymer chains in solution. However, because the SLS, S-WANS, S-WAXS and viscometric data strongly suggest that HeC molecules do not assume simple flexible polymer chain conformations and structures but rather appear as rigid rod-like structures in aqueous solution, we discuss the D_t and D_r data based on the rigid rod particle model [30]. According to Ortega and García de la Torre [30], D_r and D_t are theoretically expressed as

$$D_{t \text{ cal}} = \frac{k_B T \{ \ln(L_\eta / d_\eta) + C_t \}}{3\pi\eta_w L_\eta}, \quad (2)$$

$$D_{r \text{ cal}} = \frac{3k_B T \{ \ln(L_\eta/d_\eta) + C_r \}}{\pi \eta_w L_\eta^3}, \quad (3)$$

where k_B , T , C_t and C_r represent, respectively, the Boltzmann constant, the absolute temperature and numerical constants describing the contribution of hydrodynamic interactions for D_t and D_r . The values of C_t and C_r are given as L_η/d_η polynomials [30]. The solid lines shown in Figure 9a show the theoretical $D_{t \text{ cal1}}$ and $D_{r \text{ cal1}}$ calculated simply assuming $L_h = L$ and $d_h = d$. The broken lines labeled $D_{t \text{ cal2}}$ and $D_{r \text{ cal2}}$ in Figure 9a were obtained assuming $L_h = 0.87L$ and $d_h = d$, the same values that were used in the evaluation of the M_w dependency of the viscometric $[\eta]$ data. The observed differences between $D_{t \text{ cal1}}$ and $D_{t \text{ cal2}}$ and between $D_{r \text{ cal1}}$ and $D_{r \text{ cal2}}$ are not significant. As the agreement between the obtained D_t data and $D_{t \text{ cal1}}$ (and $D_{t \text{ cal2}}$) and between the D_r data and $D_{r \text{ cal1}}$ (and $D_{r \text{ cal2}}$) is reasonable, we might conclude that the rigid rod particle model can explain the M_w dependency of the D_t and D_r data for the HeC samples examined in this study. Consequently, the existence of a rigid rod-like conformation and structure of HeC molecules in aqueous solution is strongly supported from the viewpoint of diffusional behavior.

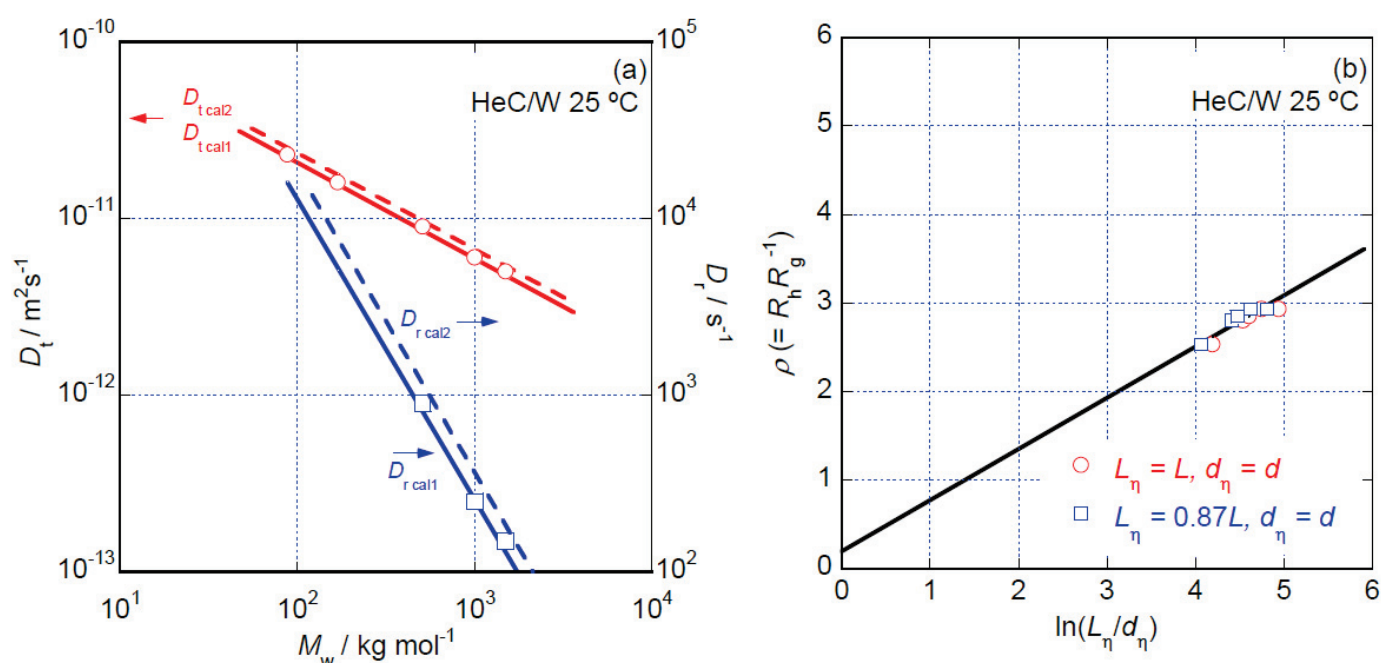


Figure 9. (a) M_w dependencies of D_t and D_r data for the HeC samples in aqueous solution at 25 °C. The solid lines show $D_{t \text{ cal1}}$ and $D_{r \text{ cal1}}$ calculated assuming $L_h = L$ and $d_h = d$, and the broken lines show $D_{t \text{ cal2}}$ and $D_{r \text{ cal2}}$ calculated assuming $L_h = 0.87L$ and $d_h = d$, as in the M_w dependencies of the $[\eta]$ data seen in Figure 7. (b) Dependence of $\rho (= R_h R_g^{-1})$ on $\ln(L_h d_h^{-1})$ for all the HeC samples in aqueous solution at 25 °C assuming the first condition, $L_h = L$ and $d_h = d$ (circular symbols), and the second condition, $L_h = 0.87L$ and $d_h = d$ (square symbols).

The conformations and structures of solute molecules can be considered based on the value of the so-called shape factor (ρ) of the molecules. This factor is defined as the ratio of R_g to the hydrodynamic radius (R_h) of the molecule given as $R_h = k_B T (6\pi D_t)^{-1}$. If the particles formed by HeC molecules simply assume flexible coil-like conformations and structures in aqueous solution, the particles have a shape factor of $\rho \sim 1.6$ irrespective of the M_w of the particles [30]. The reason for this constant ρ value is that an identical M_w exponent is usually observed for both the R_g and R_h ($\propto D_t^{-1}$) data for many flexible polymer chain systems. On the other hand, if the particles of HeC molecules have a rigid rod-like conformation, the shape factor should satisfy the relationship $\rho = 0.18 + 0.58 \ln(L_h d_h^{-1})$ for long particles [17,30] for which $L_h d_h^{-1} > 10$. Figure 9b shows the dependence of the ρ data on $\ln(L_h d_h^{-1})$ for all the HeC samples in aqueous solution evaluated using the simple

first condition, $L_h = L$ and $d_h = d$, and the second condition, $L_h = 0.87L$ and $d_h = d$, as assumed in the discussion of viscometric behavior. Here, we must note that the relationship $L_h d_h^{-1} > 10$ is well satisfied for all the HeC sample solutions examined according to the SLS data analysis in which the rigid rod particle form factor above is assumed. The ρ data present inconstant values significantly greater than 1.6 and proportional to $\ln(L_h d_h^{-1})$; they approximately follow the theoretically predicted solid line, $\rho = 0.18 + 0.58 \ln(L_h d_h^{-1})$, irrespective of the choice of conditions for L_h , as shown in Figure 9b. These observations regarding the ρ data also strongly suggest that a rigid rod conformation and structure reasonably describe the formed particle structure of HeC molecules in aqueous solution.

We very recently reported that commercially available methyl cellulose, MC, samples also assume long rigid rod-like conformations and structures in aqueous solution irrespective of their M_w values, similar to the HeC samples in this study [30]. Therefore, it is likely that commercially available water-soluble chemically modified cellulose ethers such as HeC and MC samples have a tendency to assume rigid rod-like conformations and structures in aqueous solution and that this is an essential characteristic of these compounds that results from the strong physicochemical features of cellulose.

4. Conclusions

The conformation and structure of hydroxyethyl cellulose, HeC, ether samples with molar substitution numbers, MS , ranging from 2.36 to 2.41 and weight average molar masses, M_w , ranging from 87 to 1.5×10^3 kg mol⁻¹, in aqueous solution were examined using the multiscattering techniques static and dynamic light scattering, neutron scattering and X-ray scattering and by viscometry. Although the form factors of flexible polymer chains and flexible cylinders cannot describe the obtained scattering vector, q , dependencies of the excess scattering intensity data obtained in the multiscattering measurements, the form factor of rigid rod particles of length L and diameter d dependent and the M_w of the molecular chains can reasonably explain the obtained scattering data. For HeC samples with $M_w < 2 \times 10^2$ kg mol⁻¹, the rigid rod-like particle model predicts L values close to half of the contour length l and $d \sim 1.4$ nm. Thus, the formation of rigid rod-like particles resulting from a hairpin-like conformation and structure of HeC molecules is strongly suggested. The finding that the determined L of the formed particles is not proportional to M_w and that the ratio, lL^{-1} , and the d values of the formed rigid rod-like particles increase with M_w for HeC samples with $M_w > 2 \times 10^2$ kg mol⁻¹ indicates that the formed rod-like particles become thicker with increasing M_w , maintaining their rigidity due to intramolecular attractive interactions such as hydrogen bond formation.

The translational and rotational diffusion coefficients determined using dynamic light scattering techniques and the intrinsic viscosities determined through viscometric measurements also strongly support the formation of rigid rod-like particles and an increase in rod diameter, d , with increasing M_w .

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Article

Room-Temperature Cholesteric Liquid Crystals of Cellulose Derivatives with Visible Reflection

Yuki Ogiwara, Tatsuya Suzuki, Naoto Iwata and Seiichi Furumi *

Department of Chemistry, Graduate School of Science, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku, Tokyo 162-8601, Japan

* Correspondence: furumi@rs.tus.ac.jp; Tel.: +81-3-3260-4271

Abstract: Hydroxypropyl cellulose (HPC) derivatives with alkanoyl side chains have attracted attention as bio-based cholesteric liquid crystal (CLC) materials with reflection colors. By taking advantage of the ability to change the reflection color in response to external stimuli, the thermotropic CLCs can be applied to a wide variety of photonic devices for a sustainable society of future generations. However, the thermotropic CLCs of HPC derivatives substituted with only one kind of alkanoyl group are not suitable for such applications because they do not exhibit visible reflection at room temperature. In this report, we describe a promising strategy to control the reflection colors of HPC derivatives at room temperature by introducing two kinds of alkanoyl groups with different lengths into the side chains of HPCs, which also enables the fine control of temperature dependence on the reflection wavelength. By chemically optimizing the side chain, we successfully prepared room-temperature thermotropic CLCs of HPC derivatives with visible reflection. This report would contribute toward the development of versatile photonic applications by CLCs produced from biomass.

Keywords: cellulose; cholesteric liquid crystal; thermotropic; side chain; visible reflection; room temperature

1. Introduction

Cellulose is a polysaccharide that consists of glucose as a repeating unit. Recently, cellulose and its derivatives are gaining attention as a biomass resource in today's world, where the realization of a sustainable society is a pressing issue [1]. This is because cellulose is one of the most abundant biopolymers on the Earth, and might not become depleted, unlike petroleum resources. Cellulose and its derivatives are widely used in many industries, not only as environment-friendly materials, but also as functional materials with excellent mechanical strength or liquid crystallinity [1–3]. For example, cellulose nanocrystals are often used as a filler to enhance the mechanical strength of resins as robust materials [4–6]. On the other hand, its aqueous dispersion exhibits liquid crystalline phases arising from its rod-like structure as a soft material [7,8].

Among the various liquid crystalline phases, some derivatives of cellulose can exhibit a cholesteric liquid crystal (CLC) phase, which is well known for having unique physical properties of light reflection [7–15]. Such CLC structures are commonly found in insects and other organisms, often leading to the emergence of metallic and brilliant reflection colors [16,17]. These colors of CLCs are derived from Bragg reflection. In CLCs, the layers, in which rod-shaped liquid crystalline molecules are oriented, accumulate with slight changes in the azimuthal orientation vectors to form a helical molecular structure. The rod-like molecules of CLCs exhibit anisotropy in the refractive index. Therefore, the helical molecular structure of CLCs brings about the periodic modulation of the refractive index, thereby resulting in the emergence of photonic band gap(s) as photonic crystals in the photon dispersion diagram, due to anomalous group velocity near the edge of the Brillouin zone [18]. The reflection peak wavelength (λ) of CLCs, corresponding to the region of

the photonic band gap, can be determined by the following equation suggested by de Vries [19]:

$$\lambda = n_{av}p \quad (1)$$

where n_{av} and p represent the average refractive index and helical pitch length of the CLC, respectively. Here, p means the geometric distance between layers until the orientation vectors rotate 360 degrees. Therefore, the p value is determined by a distance between the adjacent layers (d) and a helical twisting angle (φ) of the CLC helical structure, according to the following equation:

$$p = 360d / \varphi \quad (2)$$

The reflection peak at λ can be shifted by controlling the p value, which is related with CLC structural parameters of d and φ , according to Equation (2). The p value can be changed in response to various external stimuli, such as the addition of solvent [9,20], temperature [13,21], and so forth [22,23]. As a result of their unique properties, CLC materials have potential applications to a wide variety of photonic devices, such as mechanochromic sensors [24,25], temperature sensors [26–28], and wavelength-tunable lasers [26,29,30].

Hydroxypropyl cellulose (HPC), which is one of the derivatives of cellulose, is well known to exhibit a thermotropic CLC phase by chemically modifying the terminal hydroxy groups of HPC [12–14]. Cellulose nanocrystals are also known to possess the intrinsic capability to generate the CLC phase. However, they can exhibit a CLC phase only when they are dispersed in water at appropriate concentrations [8,11], corresponding to a sort of lyotropic CLC phase. Thus, thermotropic CLCs of HPC derivatives are easy to handle, rather than the lyotropic CLCs of cellulose nanocrystals. This is because the reflection properties of lyotropic CLCs of cellulose nanocrystals are quite vulnerable under dry atmospheric conditions due to concentration increase by natural evaporation of water as the dispersion medium. On the other hand, some kinds of HPC derivatives show sufficient stability in the thermotropic CLC phase, with visible reflection even under dry conditions which arises from the solvent-free system of thermotropic CLC materials. Another technological advantage is that the chemical modifications of HPC allow for on-demand control of the reflection properties of thermotropic CLCs.

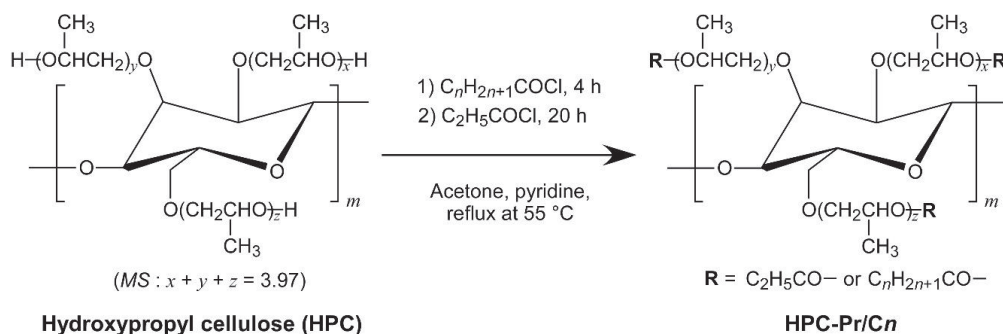
In this context, numerous studies have been reported on the thermotropic and lyotropic CLC materials of HPC derivatives from an optical perspective. For example, Watanabe and co-workers investigated the side chain effect of esterified HPC derivatives, with solely one kind of an alkanoyl group on the reflection properties of thermotropic CLCs. The reflection peak wavelength of HPC derivatives can be controlled by the side chain length, that is, the length of the alkanoyl group [21]. According to their report, the reflection peak tends to red-shift by increasing the side chain length. In other words, the thermally induced wavelength shift of the reflection peak is also crucially dependent on the side chain length. Moreover, the heating treatment of esterified HPC derivatives over $\sim 90^\circ\text{C}$ is requisite for the emergence of a reflection peak of thermotropic CLCs in a full visible wavelength range, by sweeping the temperature. As another strategy, the addition of solvent in HPC derivatives results in easy preparation of CLCs with visible reflection characteristics at room temperature, which are called lyotropic CLCs [20,31]. Such lyotropic CLCs are cumbersome to handle because their reflection properties are easily changed by unintended volatilization of the solvents in lyotropic CLCs. Taking this perspective in account, thermotropic CLCs of cellulose derivatives with visible reflection at room temperature are very attractive soft materials for sustainable photonic device applications.

In this study, we succeeded in the preparation of a series of esterified HPC derivatives as thermotropic CLCs with blue, green, or red reflection features, even at room temperature. For this objective, we designed HPC derivatives possessing two kinds of alkanoyl groups, with various chain lengths in their side chains. The reflection properties of HPC derivatives with alkanoyl groups of 3–18 carbons were investigated in detail. To the best of our knowledge, there are no reported cases of reflection properties of HPC derivatives with such long-chain alkanoyl groups [21,32,33]. As a result, an interesting phenomenon was

observed: as the length of the side chain increased, there was a tendency for changes in reflection properties. The chemical optimization of the side chains in esterified HPC derivatives enabled the preparation of room-temperature thermotropic CLCs with visible reflection. This study could contribute to the development of photonic devices utilizing cellulose for the realization of a sustainable society.

2. Experimental Section

We synthesized a series of HPC derivatives that possessed two different kinds of alkanoyl side chains through the esterification of all hydroxy groups in HPC with linear alkanoyl chlorides. The introduction ratio of two kinds of alkanoyl groups was controlled to be approximately 9:1 in a stoichiometric manner. Thereafter, we defined the HPC derivatives substituted with two different alkanoyl groups as HPC-X/C_n. The X means the abbreviation of the main alkanoyl group. In this study, X is -Pr, -Bu, or -Pe, where they refer to propionyl, butyryl, or pentanoyl groups, respectively. The C_n stands for the abbreviation of another alkanoyl group, where the value of *n* is the number of carbon atoms excluding that at position one. Note that the alkanoyl groups of Pr, Bu, and Pe are the same abbreviations as C2, C3, and C4, respectively. These three alkanoyl groups were denoted in the two ways, in order to make them easier to distinguish between the main alkanoyl group and the other alkanoyl group. For comparison, HPC derivatives modified by a single alkanoyl group, defined as HPC-X (X = -Pr, -Bu, and -Pe), were also synthesized. The typical synthesis procedure of HPC-Pr/C_n as an example is represented in Scheme 1.



Scheme 1. The synthesis procedure of HPC-Pr/C_n (*n* ≥ 3). HPC-Pr/C_n was prepared by esterifying hydroxy groups of pristine hydroxypropyl cellulose (HPC) with two kinds of alkanoyl chlorides of C₂H₅COCl and C_nH_{2n+1}COCl. The MS value denotes the average number of hydroxypropyl groups per anhydroglucose unit of HPC.

As a starting material, we used HPC (viscosity of 2.0 wt% aqueous solution: 2.0–2.9 mPa·s at 20 °C) of FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). The weight average molecular weight (*M_w*) and number average molecular weight (*M_n*) of HPC were determined to be 4.45×10^4 and 2.30×10^4 , respectively, by measurement with size-exclusion chromatography (HLC-8220 GPC, TOSOH, Tokyo, Japan), using tetrahydrofuran as the eluent and polystyrene standards as the calibration. The average number of hydroxypropyl groups per anhydroglucose unit, that is, the molar substitution (MS) value, was determined to be 3.97 by measuring the ¹H-NMR spectrum of pristine HPC in CDCl₃ according to a method in a previous report [34]. Therefore, the molecular weight of the anhydroglucose monomer unit of HPC was calculated from the MS value to be 392 on average. Super dehydrated acetone and anhydrous pyridine were used as the reaction solvent and base catalyst, respectively, and were also purchased from the FUJIFILM Wako Pure Chemical Co. Alkanoyl chlorides (C_nH_{2n+1}COCl; *n* = 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, and 17) and poly(vinyl alcohol) (PVA, *M_w* = $1.3\text{--}2.3 \times 10^4$, 87–89% hydrolyzed degree) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively. These reagents were used as received.

We synthesized 27 kinds of esterified HPC derivatives: HPC-Pr, HPC-Pr/C_n (*n* = 3, 4, 5, 6, 7, 9, 11, 13, 15, and 17), HPC-Bu, HPC-Bu/C_n (*n* = 2, 4, 5, 6, 7, 9, 11, 13, 15, and 17),

HPC-Pe, and HPC-Pe/ C_n ($n = 2, 3, 5$, and 6). The synthesis procedures were determined with reference to our previous study [35]. The amounts of alkanoyl chlorides used in each synthesis are listed in Table 1 for HPC-Pr/ C_n , Table 2 for HPC-Bu/ C_n , and Table 3 for HPC-Pe/ C_n . Here, the synthesis of HPC-Pr/C3 is described as an example.

Table 1. Feed amounts of alkanoyl chlorides used in the syntheses of HPC-Pr/ C_n , and the number of the hydroxy groups substituted by C_2H_5COCl and $C_nH_{2n+1}COCl$ per anhydroglucose unit, denoted as PrE or CnE , respectively, determined by 1H -NMR spectra. The amounts of reagents are expressed in the unit of equivalents relative to the number of anhydroglucose units in 2.00 g of HPC.

Sample	Amounts of Reagents (eq.)		PrE	CnE
	C_2H_5COCl	$C_nH_{2n+1}COCl$		
HPC-Pr	5.40	0.00	3.00	—
HPC-Pr/C3	5.10	0.30	2.72	0.33
HPC-Pr/C4	5.10	0.30	2.71	0.33
HPC-Pr/C5	5.10	0.30	2.71	0.30
HPC-Pr/C6	5.10	0.30	2.70	0.34
HPC-Pr/C7	5.10	0.30	2.67	0.35
HPC-Pr/C9	5.10	0.30	2.68	0.34
HPC-Pr/C11	5.10	0.30	2.69	0.34
HPC-Pr/C13	5.10	0.30	2.68	0.35
HPC-Pr/C15	5.10	0.30	2.69	0.33
HPC-Pr/C17	5.10	0.30	2.73	0.31

Table 2. Feed amounts of alkanoyl chlorides used in the syntheses of HPC-Bu/ C_n , and the number of the hydroxy groups substituted by C_3H_7COCl and $C_nH_{2n+1}COCl$ per anhydroglucose unit, denoted as BuE or CnE , respectively, determined by 1H -NMR spectra. The amounts of reagents are expressed in the unit of equivalents relative to the number of anhydroglucose units in 2.00 g of HPC.

Sample	Amounts of Reagents (eq.)		BuE	CnE
	C_3H_7COCl	$C_nH_{2n+1}COCl$		
HPC-Bu/C2	5.10	0.30	2.63	0.29
HPC-Bu	5.40	0.00	2.92	—
HPC-Bu/C4	5.10	0.30	2.48	0.31
HPC-Bu/C5	5.10	0.30	2.61	0.31
HPC-Bu/C6	5.10	0.30	2.69	0.32
HPC-Bu/C7	5.10	0.30	2.75	0.32
HPC-Bu/C9	5.10	0.30	2.59	0.32
HPC-Bu/C11	5.10	0.30	2.56	0.31
HPC-Bu/C13	5.10	0.30	2.56	0.32
HPC-Bu/C15	5.10	0.30	2.78	0.31
HPC-Bu/C17	5.10	0.30	2.61	0.32

Table 3. Feed amounts of alkanoyl chlorides used in the syntheses of HPC-Pe/ C_n , and the number of the hydroxy groups substituted by C_4H_9COCl and $C_nH_{2n+1}COCl$ per anhydroglucose unit, denoted as PeE or CnE , respectively, determined by 1H -NMR spectra. The amounts of reagents are expressed in the unit of equivalents relative to the number of anhydroglucose units in 2.00 g of HPC.

Sample	Amounts of Reagents (eq.)		PeE	CnE
	C_4H_9COCl	$C_nH_{2n+1}COCl$		
HPC-Pe/C2	5.10	0.30	2.71	0.32
HPC-Pe/C3	5.10	0.30	2.45	0.32
HPC-Pe	5.40	0.00	2.85	—
HPC-Pe/C5	5.10	0.30	2.44	0.31
HPC-Pe/C6	5.10	0.30	2.43	0.33

Before the synthesis, pristine HPC was dried under vacuum at room temperature for over 12 h in advance. In a 200 mL three-neck round-bottom flask filled with nitrogen, pristine HPC (2.00 g, 1.00 eq. to the number of anhydroglucose units in 2.00 g of HPC) was completely dissolved in anhydrous acetone (30.0 mL). Subsequently, anhydrous pyridine (2.42 g, 0.98 g/mL, 30.6 mmol, 6.00 eq. to the number of anhydroglucose units in 2.00 g of HPC) was added into the HPC solution. After that, butyryl chloride ($\text{C}_3\text{H}_7\text{COCl}$; 0.16 g, 1.02 g/mL 1.53 mmol, 0.30 eq. to the number of anhydroglucose units in 2.00 g of HPC) was portion-wise added into the reaction solution at room temperature. Successively, the esterification proceeded with heating at 55 °C under reflux for 4 h. As esterification occurred with remaining hydroxy groups of HPC and another alkanoyl group, propionyl chloride ($\text{C}_2\text{H}_5\text{COCl}$; 2.41 g, 1.06 g/mL, 26.0 mmol, 5.10 eq. to the number of anhydroglucose units in 2.00 g of HPC) was added dropwise into the reaction solution under continuous stirring and heating at the same temperature. After reacting for an additional 20 h, the reaction solution was purified by dropping the solution into 500 mL of ultrapure water. The precipitated white, sticky product was completely dissolved in a small amount of acetone, and then the solution was dropped into ultrapure water again. This purification process was repeated four times. Finally, the product was obtained after drying in an oven at 60 °C to remove the solvents. All HPC derivatives synthesized in this study were obtained as sticky polymer melts. These HPC derivatives exhibited a thermotropic CLC phase in the absence of any additives, such as solvents or chiral dopants, as will be discussed later.

The numbers of hydroxy groups substituted with $\text{C}_n\text{H}_{2n+1}\text{COCl}$ per anhydroglucose unit, denoted as *XE* or *CnE*, were determined by measuring ^1H -NMR spectra in CDCl_3 (Ultrashield 400 Plus spectrometer, Bruker, Billerica, MA, USA). The esterification was also confirmed from an FT-IR spectroscopy (FT-IR4700, JASCO, Tokyo, Japan) equipped with an attenuated total reflection (ATR) unit with a diamond prism (ATR PRO ONE, JASCO). The degradation of the HPC derivatives during the heating process was monitored using a thermogravimetric analysis (TGA) system (TGA 2010SA, NETZSCH, Selb, Germany). The TGA measurements of pristine HPC and HPC-Pr were conducted between 25 °C and 350 °C, at a heating rate of 10 °C/min under a nitrogen atmosphere.

Reflection properties of the HPC derivatives were investigated by measuring transmission spectra upon changing the temperature. To fabricate the CLC cell, an aqueous solution of PVA with 2.0 wt% was prepared on a glass substrate by spin-coating it at 800 rpm for 10 s, and then at 2000 rpm for 20 s using a spin-coater (ACT-220D II, Active, Saitama, Japan). After drying the glass substrates coated with a PVA thin film, their surface was uniaxially rubbed 50 times with a stick wrapped in a cupra. The HPC derivatives as sticky polymer melts were sandwiched between a pair of the substrates with a gap of ~200 μm on a hot stage at 100–120 °C. Then, the HPC derivatives were applied by shear orientation treatment, which was conducted by manually sliding the upper substrate along the uniaxially rubbing direction, in order to prepare a CLC alignment state with a relatively clear reflection peak. Importantly, the HPC derivatives were not solid-state films or powders but polymer melts. The detailed results are explained in Section 3.1. Transmission spectra of the CLC cells were acquired using a compact charge-coupled device (CCD) spectrometer (USB2000+, Ocean Optics, Orlando, FL, USA) and a tungsten halogen white light source (HL2000, Ocean Optics). The temperature of the CLC cell was precisely controlled using a temperature controller (HS1, Mettler-Toledo, Columbus, OH, USA) equipped with a hot stage (HS82, Mettler-Toledo). Reflection spectra of the CLC cells were recorded using the above-mentioned spectrometer and halogen light source. An optical fiber with a reflection probe was used for the measurements. All reflection spectra in this study were measured at room temperature of ~25 °C.

3. Results and Discussion

3.1. Synthesis and Characterization of HPC-X/Cn

The chemical structures of HPC-X/Cn were identified using both ATR FT-IR and ^1H -NMR spectral measurements. Here, the ATR FT-IR and ^1H -NMR spectra of pris-

tine HPC, HPC-Pr/C7, HPC-Bu/C7, and HPC-Pe/C3 are shown as the typical examples (Supplementary Materials, Figures S1 and S2).

When ATR FT-IR spectra of pristine HPC and esterified HPC derivatives were compared, a broad peak of the O-H stretching vibration around $3100\text{--}3600\text{ cm}^{-1}$ thoroughly disappeared after the esterification of pristine HPC. Furthermore, a sharp peak of C=O stretching vibration concomitantly appeared at 1700 cm^{-1} (Supplementary Materials, Figure S1). Such a change in the FT-IR spectrum suggests that the hydroxy groups of HPC were completely substituted with alkanoyl groups by the esterification [10].

Subsequently, ^1H -NMR spectra were measured to quantitatively determine the XE and CnE values, defined in the Experimental Section, by the integrated values of the peaks (Supplementary Materials, Figure S2). The detailed analysis procedures and assignment of the peaks in ^1H -NMR spectra are described in the Supplementary Materials. Both the XE and CnE values of HPC derivatives are summarized in Tables 1–3. The XE and CnE values were ~ 2.7 and ~ 0.3 , respectively. These values suggest that the mixed ratio of two kinds of alkanoyl groups can be controlled at approximately 9:1, and that hydroxy groups in HPC are completely substituted with alkanoyl groups because of the total XE and CnE values of ~ 3.0 .

The complete substitution is quite important for a fair comparison of reflection properties of HPC derivatives. This is because the substitution degree of HPC has a great impact on the reflection characteristics. For instance, according to the previous report on HPC-Bu with different BuE , their reflection peak wavelength red-shifted from 401 nm to 835 nm as BuE decreased from 2.96 to 2.20 [36]. In this study, the XE and CnE values were standardized at ~ 2.7 and ~ 0.3 , respectively, to elucidate the pure influence of the side-chain length on reflection properties. These values mean that hydroxy groups in pristine HPC were completely substituted with alkanoyl groups. It should be noted that the PeE values of some HPC-Pe/ Cn were ~ 2.4 due to the low esterification reactivity of pentanoyl chloride, probably due to relatively long alkyl chains.

TGA measurements also confirmed that the HPC derivatives did not degrade during the heating process up to $350\text{ }^\circ\text{C}$. As evidenced from the TGA curves of pristine HPC and HPC-Pr, their decomposition occurred above $200\text{ }^\circ\text{C}$ (Supplementary Materials, Figure S3). This temperature was much higher than the temperature range to measure the reflection properties of HPC derivatives, as described in Sections 3.2–3.4. It should be stressed that the thermal decomposition of HPC derivatives hardly occurs during their optical measurements.

3.2. Reflection Properties of HPC-X ($X = \text{-Pr, -Bu, and -Pe}$)

The reflection peak of HPC-X ($X = \text{-Pr, -Bu, and -Pe}$) shifted to a longer wavelength as the temperature increased. The thermally induced red-shift of reflection peak wavelength is consistent with a previous study [21]. Figure 1A shows the changes in the transmission spectrum of HPC-Pe as a function of temperature on the heating process. Although HPC-Pe exhibited a reflection peak at 611 nm, observed as a red reflection color, at $30\text{ }^\circ\text{C}$, the heating treatment resulted in a continuous red-shift of the reflection peak to the infrared wavelength region. For instance, when heated to $100\text{ }^\circ\text{C}$, the reflection peak reached 922 nm. Similarly, the reflection peak of HPC-Pr also red-shifted from 419 nm to 659 nm by heating from $100\text{ }^\circ\text{C}$ to $140\text{ }^\circ\text{C}$ (Supplementary Materials, Figure S4A), and HPC-Bu from 404 nm to 661 nm by heating from $40\text{ }^\circ\text{C}$ to $90\text{ }^\circ\text{C}$ (Supplementary Materials, Figure S4B). Such a red-shift in reflection peak wavelength during the heating process can be ascribed to an increase in the p value due to the thermotropic CLC property [21,37,38].

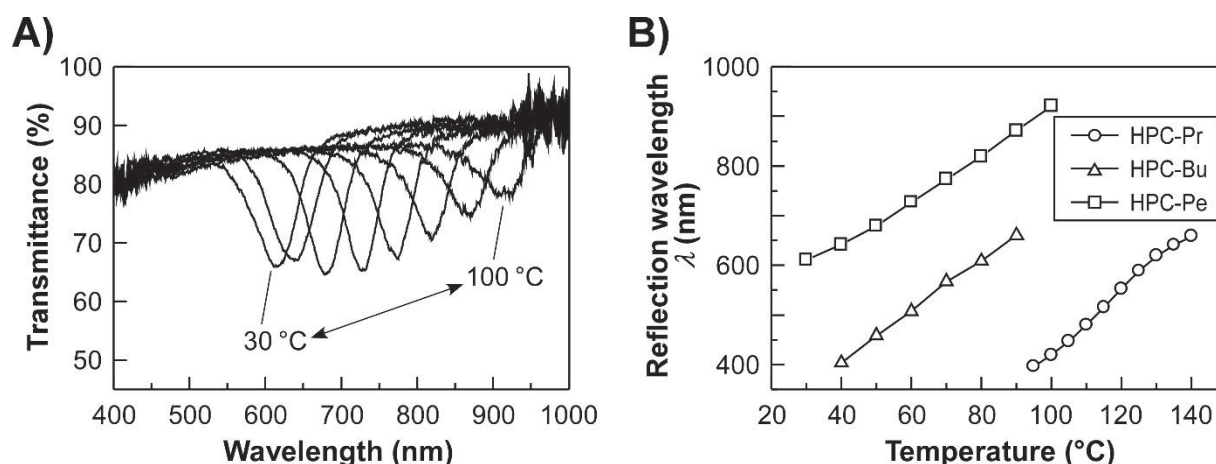


Figure 1. Reflection properties of HPC-Pr, HPC-Bu, and HPC-Pe by temperature. (A) Changes in the transmission spectrum of HPC-Pe as a function of the temperature from 30 °C to 100 °C at intervals of 10 °C. (B) Temperature dependences of reflection peak wavelengths of HPC-Pr, HPC-Bu, and HPC-Pe.

As the temperature of HPC-X approached the isotropic phase transition temperature (T_i), the reflection peak gradually broadened and attenuated. This is because the helical orientation of CLCs was disturbed, and the distribution of the p value widened by thermodynamic molecular motion when the temperature of CLCs approached the T_i . For instance, the reflection peak in the transmission spectrum of HPC-Pe at 100 °C was found to be broader and smaller than the peak at 30 °C (Figure 1A). This tendency was especially apparent for the transmission spectra of HPC-Pr (Supplementary Materials, Figure S4A), and can be ascribed to the relatively high T_i of HPC-Pr. The T_i of HPC-Pr is ~150 °C according to our previous study [39], in which the T_i of HPC-Pr was determined by polarized optical microscopic observation under crossed-Nicol upon the heating process. The T_i of HPC-Pr was the highest among the three kinds of esterified HPC derivatives with different alkyl chains of alkanoyl groups: HPC-Pr, HPC-Bu, and HPC-Pe [21,33]. It was anticipated that the effect of thermal motion of the molecules around T_i on the reflection property was the largest, resulting in the broadening of the reflection peak of HPC-Pr near its T_i .

The wavelength shifts of the reflection peaks of HPC-X (X = -Pr, -Bu, and -Pe), which were dependent on the temperature due to the thermotropic CLCs, were fully reversible unless the temperature did not exceed the T_i of HPC-X. It should be noted that even if the temperature exceeded T_i once, the reflection peak could be revived by applying mechanical stimuli, such as shear strain [40]. The wavelength shifts in the reflection peaks of HPC-Pr, HPC-Bu, and HPC-Pe upon the heating process, are summarized in Figure 1B. Thus, the reflection peak wavelengths and colors of HPC-X could be controlled by changing the temperature.

According to a previous study [21] and the results shown in Figure 1B, it was confirmed that CLCs with reflection peaks below ~600 nm, that is, blue and green reflection colors, at room temperature could not be fabricated when HPC derivatives with a single component of alkanoyl group in their side chains were used. Both HPC-Pr and HPC-Bu were expected to exhibit a reflection peak in ultraviolet wavelength region below 400 nm at room temperature. If the chain length of the alkanoyl group was further extended than the pentanoyl group, that is, an HPC derivative with a hexanoyl group, the reflection peak has been observed in the infrared wavelength region over 2000 nm at 20 °C [21]. Thus, the reflection peak of HPC-X tends to red-shift as the side chain length increases. This red-shift stemmed from an increase in the d value as well as a decrease in the φ value, which result in an increase in both the p and λ values, according to Equations (1) and (2). A previous study revealed that an introduction of a longer side chain leads to an increase in the d value and a decrease in the φ value [33]. This result implies that the long side chain induces con-

sequent weakening of the chiral intermolecular interaction, which induces the molecular helical structure. Considering these results, it is not possible to continuously control the reflection peak wavelength and color of HPC-X at room temperature. Thus, CLCs with visible reflection at room temperature cannot be produced by simply introducing one kind of alkanoyl group to HPC.

3.3. Side-Chain Length Effect of HPC-Pr/Cn, -Bu/Cn, and -Pe/Cn on Reflection Properties

When comparing the wavelength shift behavior of the reflection peak of HPC-Pr/Cn by changing the temperature, we found that the reflection peaks emerged at longer wavelengths at the same temperature when the HPC derivatives had long alkanoyl groups in the side chains, corresponding to an increase in the n value. For example, the reflection peak of HPC-Pr/Cn ($n = 2, 3, 4, 5, 6, 7, 9, 11$, and 13) at $95\text{ }^{\circ}\text{C}$ increased by $\sim 23\text{ nm}$ as n increased by one (Figure 2A).

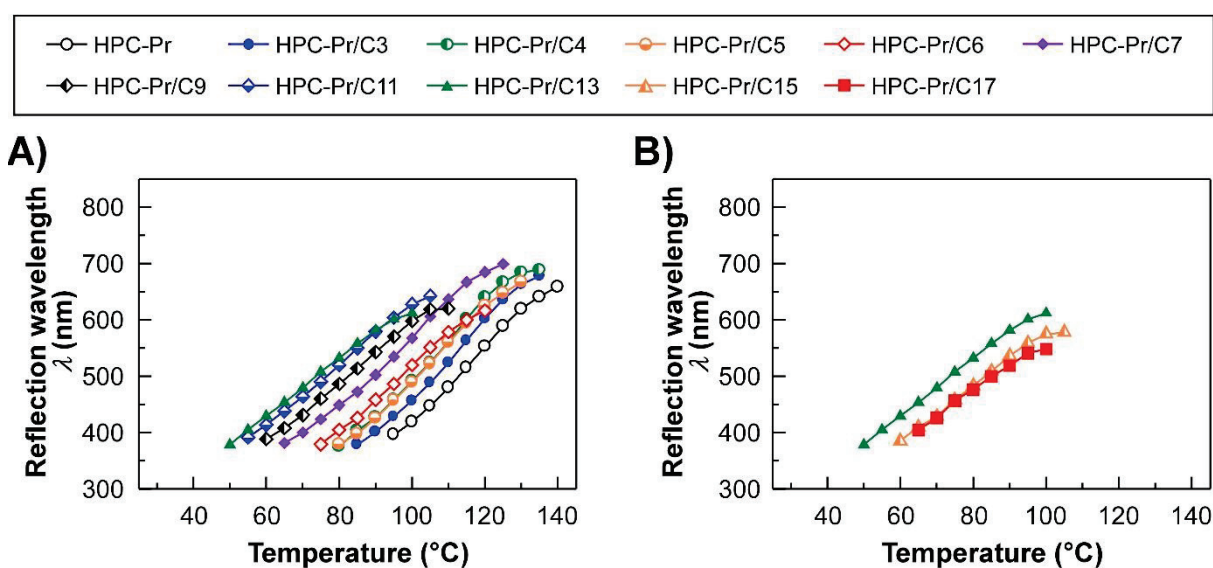


Figure 2. Temperature dependences of reflection peak wavelengths of HPC-Pr/Cn. (A) HPC-Pr ($n = 2$) and HPC-Pr/Cn ($n = 3, 4, 5, 6, 7, 9, 11$, and 13). The region of temperature at which HPC-Pr/Cn reflects visible light shifted toward lower temperatures as n increased. (B) HPC-Pr/Cn ($n = 13, 15$, and 17). The region of temperature at which HPC-Pr/Cn reflects visible light shifted toward higher temperatures as n increased.

This trend was no longer valid at temperatures above $110\text{ }^{\circ}\text{C}$: for instance, the reflection peak of HPC-Pr/C5 exceeded that of HPC-Pr/C6. This can be ascribed to the broadening of the reflection peak when approaching the temperature near T_i (Supplementary Materials, Figure S5). The same behaviors of reflection wavelength were also observed in HPC-Bu/Cn (Supplementary Materials, Figure S6) and HPC-Pe/Cn (Supplementary Materials, Figure S7).

The region of temperature range at which HPC-Pr/Cn ($n = 2, 3, 4, 5, 6, 7, 9, 11$, and 13) exhibited a reflection peak in a visible wavelength range shifted toward lower temperatures (Figure 2A), but that of the temperature range of HPC-Pr/Cn ($n = 13, 15$, and 17) with a reflection peak in a visible wavelength range shifted toward higher temperatures (Figure 2B). The mechanism of these shifts in the temperature range will be discussed in the following section.

To elucidate the relationship between side chain length and reflection wavelength in more detail, Figure 3A plots the temperature of HPC-Pr/Cn with a reflection peak at 500 nm . The reflection peak wavelength of 500 nm was chosen because the temperature of the reflection peak of 500 nm was far away from T_i . The CLC orientation is not considered to be disturbed, allowing for a fair comparison of the effects of side chains on the reflection

properties. The temperature was lowered from 113 °C to 74 °C as n increased from 2 to 13 (Figure 3A, blue arrow). This temperature lowering was caused by the enlargement of the p value. This is reasonable because the increase in the side chain length contributed to the increase in p value and the red-shift of the reflection peak wavelength, as described in Section 3.2. On the other hand, the temperature was raised from 74 °C to 85 °C by increasing the n value from 13 to 17 (Figure 3A, red arrow). This increase probably occurred from the enhancement in van der Waals interactions. When the alkyl chain length was extended even longer, van der Waals interactions among side chains were augmented, and intermolecular interactions intensified, resulting in a decrease in d value. As a result, the temperature required to reflect light at 500 nm began to increase at $n = 13$.

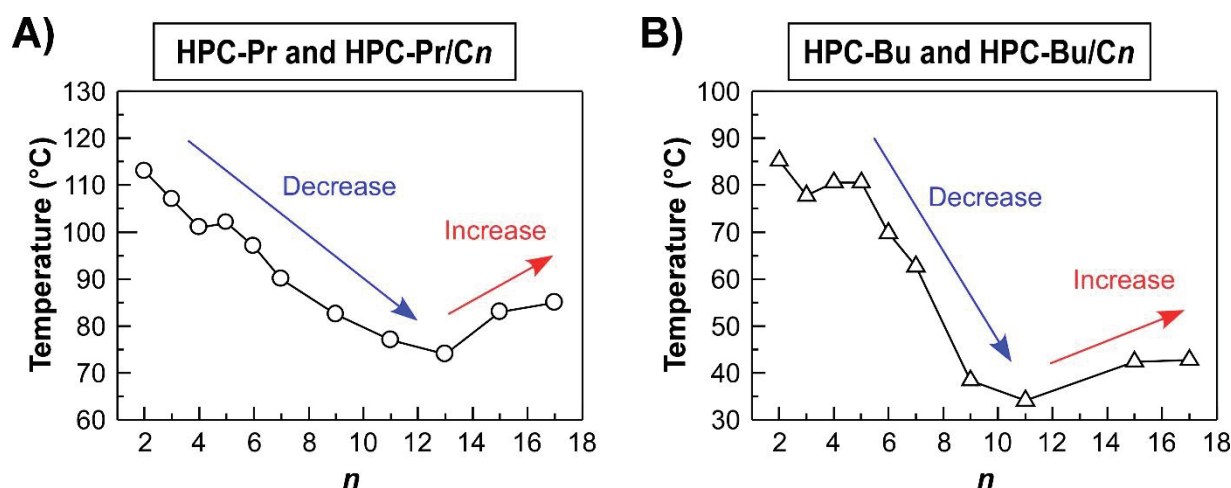


Figure 3. Effect of the side chain length (n) of HPC derivatives with propionyl (A) or butyryl group (B) on the temperature at which light of a certain wavelength is reflected. (A) The temperatures at which HPC-Pr ($n = 2$) and HPC-Pr/C n reflect a light at 500 nm. (B) The temperatures at which HPC-Bu ($n = 3$) and HPC-Bu/C n reflect a light at 600 nm. A plot at $n = 13$ was omitted because HPC-Bu/C13 did not exhibit any reflection colors (Supplementary Materials, Figure S8).

The same tendency was also observed for HPC-Bu/C n (Figure 3B). The temperature at which HPC-Bu/C n showed a reflection peak at 600 nm lowered from 85 °C to 34 °C as the n increased from 2 to 11 (Figure 3B, blue arrow), and then increased to 43 °C at $n = 17$ (Figure 3B, red arrow). Note that a plot at $n = 13$ is omitted because a reflection peak of HPC-Bu/C13 was not observed in the visible wavelength range. Although a very broad reflection peak at ~670 nm appeared in the transmission spectrum of HPC-Bu/C13 at 30 °C, no reflection peak appeared at both 40 °C and 50 °C (Supplementary Materials, Figure S8). Such broadening of the peak was also observed for HPC-Pr near its T_i ; it can be estimated that the T_i of HPC-Bu/C13 was probably ~40 °C, which prohibited the emergence of visible reflection at room temperature.

In the case of HPC-Pe/C n , a fair comparison of the temperature at which HPC-Pe/C n reflected a light of a certain wavelength was difficult because the PeE values were not standardized at ~2.7. Since the PeE value has a great impact on the reflection peak wavelength, as described in Section 3.1, the HPC-Pe/C n with different PeE values are not suitable to survey the effect of the side chain length on reflection properties. The temperature of HPC-Pe/C n with a reflection peak at 800 nm is summarized in Supplementary Materials, Figure S9. The reflection peak of 800 nm was determined because HPC-Pe/C6 showed a reflection peak over 800 nm above room temperature.

3.4. Side-Chain Length Effect of HPC-X/C2 on Reflection Properties

The temperature dependences of reflection peak wavelengths of HPC derivatives possessing propionyl groups (HPC-Pr, HPC-Bu/C2, and HPC-Pe/C2) are shown in Figure 4. The reflection peak of HPC-Bu/C2 red-shifted from 415 nm to 671 nm when heated from

50 °C to 100 °C. Similarly, HPC-Pe/C2 showed the wavelength shift in reflection peak from 568 nm to 872 nm when heated from 30 °C to 100 °C. The temperatures at which the reflection peak reached 650 nm were ~136 °C for HPC-Pr, ~95 °C for HPC-Bu/C2, and ~51 °C for HPC-Pe/C2. The temperatures at which the same reflection wavelength stepwise decreased by ~40 °C as the number of carbons in the main component of alkanoyl group increased by one. This is based on our experimental results that the temperature dependence of reflection peak wavelength of HPC-X/C2 is almost the same. In other words, since the slopes of profiles in Figure 4 are almost ~5 nm/°C for all the esterified HPC derivatives, the above value of 40 °C is expected to remain almost the same for any reflection peak wavelength.

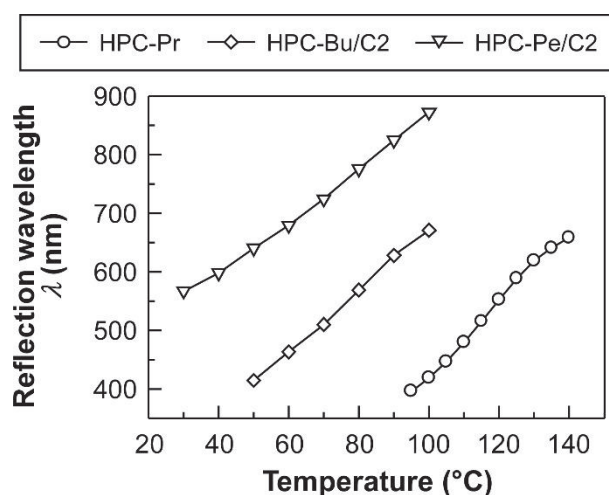


Figure 4. Temperature dependences of the reflection peak wavelengths of HPC-Pr, HPC-Bu/C2, and HPC-Pe/C2.

The difference of only one carbon number in the main alkanoyl group of esterified HPC derivatives had a significant influence on the reflection peak wavelength and the temperature range of visible light reflection. In the case of HPC-Pr/C n , when the n increased by one, that is, when the chain length of the minor alkanoyl group increased by one, the temperature at which HPC-Pr/C n reflected light at 500 nm decreased by ~5 °C in the n range between 2 and 4, as shown in Figure 3A. On the other hand, the temperature of HPC-X/C2 at which the same reflection wavelength decreased by ~40 °C as the number of carbons in the main-alkanoyl group increased by one, as shown in Figure 4. This experimental result implies that the structural change in the chain length of the alkanoyl group with a larger proportion of introduction has a more significant impact on the reflection properties of thermotropic CLCs of esterified HPC derivatives.

3.5. Room-Temperature Thermotropic CLCs of HPC Derivatives with Visible Reflection

Utilizing the above-mentioned reflection properties of the esterified HPC derivatives, it is possible to determine the conditions for preparing HPC derivatives with arbitrary reflection colors at room temperature.

For instance, HPC-Bu/C6, HPC-Bu/C7, HPC-Pe/C2, and HPC-Pe were available because their reflection peaks are covered within the full visible wavelength range from 492 nm to 684 nm, even at room temperature. The reflection spectra of these HPC derivatives at 25 °C are shown in Figure 5. The light reflection intensities and wavelengths of reflection peaks of HPC-Bu/C6, HPC-Bu/C7, HPC-Pe/C2, and HPC-Pe were found to be 5.5% at 492 nm, 3.9% at 523 nm, 10.3% at 619 nm, and 7.9% at 684 nm, respectively. The reflection colors of HPC-Bu/C6, HPC-Bu/C7, and HPC-Pe/C2, were observed as blue, green, and red, respectively, as seen in the inset of Figure 5. The reflection image of HPC-Pe showed as dark red or invisible, due to its reflection peak appearing in the near infrared wavelength of 684 nm.

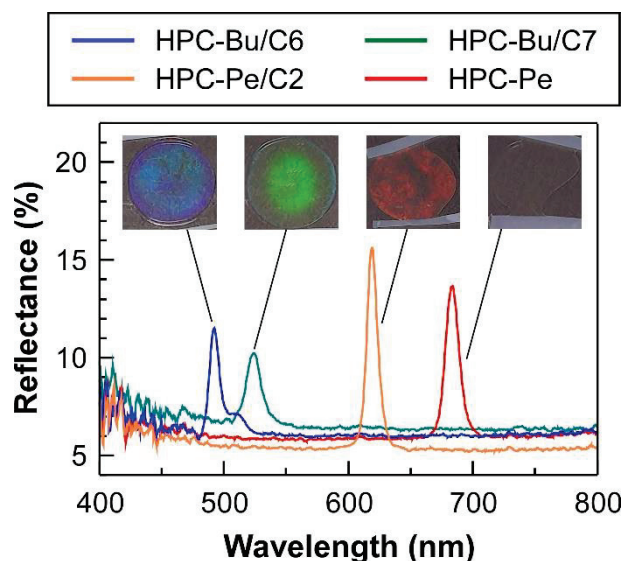


Figure 5. Reflection spectra of HPC-Bu/C6, HPC-Bu/C7, HPC-Pe/C2, and HPC-Pe at a room temperature of $\sim 25^{\circ}\text{C}$. The insets denote the reflection images of HPC derivatives at room temperature.

By changing not only the length of alkanoyl groups but also the ratio of the two alkanoyl groups introduced, the reflection peak wavelengths of HPC derivatives at room temperature could be seamlessly tuned. We evaluated the temperature dependences of the HPC derivatives possessing propionyl groups, butyryl groups, or mixed propionyl and butyryl groups (Supplementary Materials, Figure S10). The temperature range in which HPC-Pr reflected visible light was the highest, and the range shifted to the lower temperature side as the introduction ratio of butyryl group increased. These results mean that the reflection peak wavelength of an HPC derivative at a certain temperature can be modulated. Concretely, a change in the introduction ratio of two kinds of alkanoyl groups by 0.3 resulted in a spectral change in the reflection wavelength with ~ 44 nm. A similar result was also reported in a previous study [35]. In this way, by controlling the introduction ratio of the two kinds of alkanoyl groups, CLCs of HPC derivatives that reflect light at a desired wavelength at room temperature can be prepared. These CLCs are easy to handle because their reflection colors appear at room temperature without the aid of any solvents such as lyotropic CLCs, which can easily evaporate and critically affect the reflection properties of CLCs. Solvent-free CLC materials with reflection colors at room temperature are very attractive from the technological standpoint when the CLCs are used in functional devices such as external stimuli-responsive sensors, since they show stable reflection colors and are resistant to discoloration. Our findings would expand the possibilities for the technological applications of CLC materials to sustainable photonic devices using cellulose.

4. Conclusions

In this study, we successfully obtained CLCs that exhibit reflection colors at room temperature, without any solvents, by introducing two different lengths of alkanoyl groups into HPC. Furthermore, we found an interesting phenomenon, that the introduction of a long alkanoyl group as part of the side chain changes the temperature-dependent trend of the reflection property. This phenomenon is likely due to increased intermolecular interactions between alkanoyl side chains. Our findings pave the way for the preparation of room-temperature thermotropic CLCs of esterified HPC derivatives with visible reflection. This study can contribute to the development of versatile applications of CLCs utilizing cellulose for the realization of a sustainable society.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym15010168/s1>, Figure S1: ATR FT-IR spectra of HPC and HPC derivatives; Figure S2: ^1H -NMR spectra of HPC and HPC derivatives; Figure S3: TGA profiles of pristine HPC and HPC-Pr; Figure S4: Transmission spectral changes of HPC-Pr and HPC-Bu upon heating process; Figure S5: Transmission spectral change in HPC-Pr/C5; Figure S6: Temperature dependence of reflection wavelengths of HPC-Bu/Cn; Figure S7: Temperature dependence of reflection wavelengths of HPC-Pe/Cn; Figure S8: Transmission spectral changes of HPC-Bu/C13; Figure S9: The temperature at which HPC-Pe and HPC-Pe/Cn reflect a light at 800 nm; Figure S10: Temperature dependence of the HPC derivatives possessing propionyl groups, butyryl groups, or both; mathematical derivation of XE and CnE of HPC-X/Cn (PDF).

Author Contributions: Y.O. conducted experiments and analyzed the data and prepared a draft of the manuscript. T.S. also conducted experiments. N.I. analyzed the data and revised the manuscript with Y.O. S.F. conceived and supervised this project, and prepared the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Article

Low-Frequency Ultrasound Effects on Cellulose Nanocrystals for Potential Application in Stabilizing Pickering Emulsions

Louise Perrin ^{1,2,*}, Stephane Desobry ¹, Guillaume Gillet ² and Sylvie Desobry-Banon ¹

¹ Laboratory of Biomolecules Engineering (LIBio), University of Lorraine, 2 Avenue de la Forêt de Haye, BP 20163, 54500 Vandœuvre-les-Nancy, France; stephane.desobry@univ-lorraine.fr (S.D.); sylvie.desobry@univ-lorraine.fr (S.D.-B.)

² SAS GENIALIS Route d'Acheres, 18250 Henrichemont, France; g.gillet@genialis.fr

* Correspondence: louise.perrin@univ-lorraine.fr

Abstract: Cellulose, in the form of cellulose nanocrystals (CNCs), is a promising biomaterial for stabilizing Pickering emulsions (PEs). PEs are commonly formed using low-frequency ultrasound (LFU) treatment and impact CNC properties. The present study investigated the specific effects of LFU treatment on CNCs' chemical and physical properties. CNCs were characterized using dynamic light scattering, ζ -potential determination, Fourier transform infrared spectroscopy, X-ray diffraction, and contact angle measurement. CNC suspensions were studied using rheological analysis and static multiple light scattering. LFU treatment broke CNC aggregates and modified the rheological behavior of CNC suspensions but did not affect the CNCs' chemical or crystallographic structures, surface charge, or hydrophilic properties. During the storage of CNC suspensions and PEs, liquid crystal formation was observed with cross-polarized light. Hypotheses related to the impact of liquid crystal CNCs on PE stability were proposed.

Keywords: sustainable polymer; liquid crystals; properties; structure; rheological analysis

1. Introduction

Cellulose is a renewable, biodegradable, and biocompatible resource attracting growing interest in many fields. This non-toxic material is the most abundant biopolymer on earth, with an estimated production of between 10^{11} and 10^{12} tons each year. Cellulose is a linear homopolymer composed of several thousands of 1,4-anhydro-D-glucopyranose units (varying depending on cellulose sources). Cellulose chain assemblies, through hydrogen bonds, form nanofibrils and microfibrils [1]. Cellulose can be extracted from wood, plants, algae, marine animals, and fungi, as well as bacteria [2,3].

In plants and wood, cellulose microfibrils form a matrix with hemicelluloses and lignin. Cellulose extraction requires chemical treatments, such as bleaching with sodium chlorite (NaClO_2) or hydrogen peroxide (H_2O_2) to remove lignin and alkaline treatment with sodium hydroxide (NaOH) to remove hemicelluloses [4]. The resulting cellulose microfibrils can then be used to produce nanocelluloses, notably in the form of cellulose nanocrystals (CNCs).

CNCs are mainly extracted with acid hydrolysis using hydrochloric, sulfuric, phosphoric, or organic acids and also with enzymatic hydrolysis, ionic liquid treatment, and subcritical water hydrolysis. These methods can be used in combination with mechanical treatments such as ball milling, ultrasonication, high-pressure homogenization, or microfluidization [1]. CNCs are characterized by their high crystallinity and nanometric size. The diameter of CNCs varies from two to seventy nm, and the length can vary from one hundred to several hundreds of nanometers [3,5].

CNCs are easily produced at the industrial scale [6] with frequent use in many fields such as food, paper, textile, paint, packaging, cosmetic, biomedical, wastewater treatment, energy, and electronic applications [4,5,7,8]. Among their usages, CNCs can stabilize

Pickering emulsions (PEs) in cosmetic, food, and pharmaceutical products [9]. PEs are emulsions stabilized by solid particles [10], forming a mechanical barrier at the oil/water interface and preventing droplet coalescence. In addition, particles can form a three-dimensional network between droplets, preventing their displacement and leading to a highly stable emulsion [11]. Although the first works on PEs date back more than a century [12,13], interest in these emulsions has only developed since the early 2000s, and the annual research has increased each year [14–16].

The use of inorganic and synthetic particles to stabilize PEs is the most well-known approach, but current challenges are associated with the use of renewable, sustainable, and green colloidal particles. Many particles based on proteins, lipids, and polysaccharides can be used to stabilize PEs [14,17]; among them, CNCs appear to be a promising biomaterial [18]. CNCs are reported to have amphiphilic properties due to the crystalline organization of cellulose, which is responsible for their position at the oil/water interface [19]. PEs formulated with CNCs are stable for several months [20,21].

PEs are most frequently formed using low-frequency ultrasound (LFU) emulsification [22]. Cavitation forces during LFU treatment can impact CNC properties by reducing CNCs' size and modifying their surface charge or wettability [23–28], and these properties determine PE stabilization [29]. For example, a reduced CNC size and increased contact angle (i.e., hydrophobicity) during LFU treatment improved O/W PE stability [27,30]. However, these studies were carried out on non-commercial cellulose and are difficult to replicate, as CNC properties differ in terms of cellulose sources and extraction methods [18].

In a previous paper, we studied PE formation using commercial CNCs [31]. A question arose regarding the specific influence of the LFU process on CNC properties. The aim of the present work was to investigate how chemical and physical CNC properties, modified with LFU treatment, can influence their ability to formulate and stabilize PEs.

2. Materials and Methods

2.1. Materials

CNCs, produced from wood using sulfuric acid hydrolysis, were purchased from Celluloseforce (Montreal, Canada). The water used was deionized using an Aquadem purification system (Veolia, Aubervilliers, France) to reach a resistivity of 18 M Ω ·cm. Caprylic/capric triglycerides (Miglyol[®] 812N) were provided by IOI Oleochemical GmbH (Witten, Germany).

2.2. CNC Suspension Preparation and Low-Frequency Ultrasound Treatment

CNC suspensions at 0.5 and 5% (*w/w*) were formulated by stirring an appropriate CNC quantity in 200 g of water for 12 h at 20°C. The LFU conditions for treating the CNC suspensions were the same as those previously used for PE formation [31]: 125 g of CNC suspension was treated using LFU (20 kHz) with an amplitude of 40% and cycles of 25 s ON and 5 s OFF, using a Fisherbrand[™] sonicator 500 W (Fisher Scientific, Waltham, MA, USA). The treatment time was 45 min, which corresponded to a total energy of about 58 kJ. Energy was reported in kJ per gram of CNCs. The LFU treatment was carried out in a thermoregulated reactor to avoid an excessive temperature rise in the suspension (23 ± 1 °C). The CNC suspensions treated using LFU were named “CNCs-US”, with “US” for “ultrasounds”. The remaining untreated CNC suspensions were referred to as “CNCs-UT”, with “UT” for “untreated”.

2.3. Size and ζ -Potential Measurements

Size and ζ -potential of CNCs in suspension were measured using dynamic light scattering (DLS) at an angle of 173° with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a green laser at 532 nm. The DLS method considers particles as spheres, whereas CNCs have a rod-like shape. Thus, calculated data are given as apparent hydrodynamic size to compare CNC samples since measurements were carried out under the same conditions [32,33]. D10, D50, and D90 are size distribution parameters

where 10%, 50%, and 90% particles have a smaller apparent diameter, respectively. Analyses were performed in triplicate at 25 °C.

2.4. Chemical Structure Analysis

The CNC chemical structures were analyzed using Fourier transform infrared spectroscopy in attenuated total reflection mode (ATR-FTIR) using a TENSOR 27 spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector (Bruker, Germany) and an ATR top-plate (ATR Platinum, Bruker, Germany). The CNC suspensions were previously freeze-dried (Christ Beta 1-8 LD Plus, Martin Christ, Osterode am Harz, Germany) for 72 h at −30 °C and 0.37 mbar. The measurements were performed with 64 scans, 10 kHz scanning, from 4000 to 400 cm^{-1} , and with a resolution of 4 cm^{-1} . The raw data were processed using OPUS software, version 7.2 (Bruker, Germany). The air spectrum, measured as a reference, was subtracted. H_2O and CO_2 peaks were compensated, and absorbance caused by the diamond was removed by generating a straight line from 2250 to 2440 cm^{-1} . The ATR spectra were then converted to absorbance spectra, vector normalization was applied, and a baseline correction was performed. Three measurements for each sample were performed and averaged.

2.5. X-ray Diffraction Measurements

X-ray diffraction (XRD) patterns of CNC samples were determined with a D8 ADVANCE X-ray diffractometer (Bruker, Germany) using $\text{Cu K}\alpha_1$ radiation ($\lambda = 1.54056 \text{ \AA}$). The scattered radiation was detected in the range of 2θ range 5° to 100°. Diffractograms obtained were analyzed with DIFFRAC.EVA software, version 5.2.0.5 (Bruker, Karlsruhe, Germany).

2.6. Contact Angle Measurements

Static sessile drop contact angle measurements were carried out to determine CNC hydrophilicity. CNC films were formed using the casting method with 2.5% (w/w) CNC suspensions. The films were dried in atmosphere at 20 °C and 40% relative humidity for 4 days and then conditioned in a desiccator containing silica gel stored at 20 °C for at least 2 days prior to measurements. The contact angle measurements were performed using an OCA 15EC goniometer (DataPhysics, Filderstadt, Germany) and automatically calculated using the Young–Laplace method. The water contact angle was determined by depositing 4 μL of deionized water on the CNC film surface and measured 10 s after deposition. The results were reported as the average of five measurements.

2.7. Rheological Measurements

An HR20 discovery hybrid rheometer (TA Instruments, New Castle, DE, USA) using a 50 mm parallel plate geometry and a gap height of 250 μm between the two plates was used to perform rheological measurements. Before starting a measurement, an equilibration time of 180 s was set. Viscosity measurements were carried out over a shear rate range from 0.01 to 1000 s^{-1} in steady-state mode. The storage modulus (G') and loss modulus (G'') were determined with a dynamic frequency sweep in the angular frequency range from 1 to 100 rad.s^{-1} at a constant strain of 1.0% (previously determined to be in the linear viscoelastic range). All measurements were performed at 20 ± 0.1 °C on fresh CNC suspensions. The rheological results were reported as the mean of at least two measurements.

2.8. Observation under Cross-Polarized Light

The presence of isotropic and anisotropic phases in CNC suspensions was observed under cross-polarized light, using a method adapted from Browne et al. (2022) [34]. Briefly, 0.5 to 5% (w/w) CNC suspensions were prepared with successive dilution of the 5% w/w CNC suspension. Six grams of each CNC suspension were placed in a glass bottle. After 4 weeks of settling at 20 °C, the samples were placed between two crossed linear polarizers to be photographed. The anisotropic phase ratio (%) was determined as the ratio between

the height of the anisotropic phase and the total height of the suspension, measured with ImageJ software, version 1.53t (National Institutes of Health, Bethesda, MD, USA).

2.9. Suspension Stability Study

The CNC suspension stability was studied using static multiple light scattering measurements with a Turbiscan Classic MA2000 apparatus (Formulaction, Toulouse, France) using a pulsed near-infrared light source at 850 nm. Seven milliliters of CNC suspensions were placed in a specific glass cell, which was then scanned with a light source. Detection was controlled with a transmission detector, which measured light that passed through the sample at 180° to the incident beam. The measurements were carried out in duplicate for up to 56 days of storage at 20 °C.

2.10. PE Preparation and Characterization

PEs, containing 1 or 5% of CNCs (w/w in the final emulsion) and 10% oil, were formulated in two steps [31]. The CNCs-UT suspension and oil (caprylic/capric triglycerides) were pre-emulsified using high-speed homogenization for 5 min at 10,000 rpm, with an Ultra-turrax® T-25 equipped with a S 25N-18G dispersal tool (IKA-Werke, Staufen im Breisgau, Germany). Emulsification was then carried out using LFU under the same conditions as for CNC suspension processing (Section 2.2), i.e., a frequency of 20 kHz, an amplitude of 40%, and cycles of 25 s ON and 5 s OFF. The PE temperature was maintained at 23 ± 1 °C during LFU treatment.

The PE structure was observed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with an AxioCam ICc 1 CCD camera (Zeiss, Oberkochen, Germany).

2.11. Statistical Analysis

Data were analyzed with one-way analysis of variance (ANOVA) using RStudio software, version 1.3.959 (R-tools Technology, Richmond Hill, ON, Canada). Differences at $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. LFU Effect on CNC Size

The LFU treatment effects on CNC size were studied using DLS measurement. Figure 1a shows that the CNCs' apparent hydrodynamic D50 decreased rapidly from 350 to 80 nm when LFU was applied up to 30 kJ/g CNCs. This could be attributed to the disaggregation of large CNC particles due to LFU-induced cavitation [33]. For LFU treatments up to 93 kJ/g CNCs, a plateau was observed around 80 nm (Figure 1a). However, the CNCs' apparent hydrodynamic diameter distribution showed the emergence of 10 nm particles with increasing LFU energy (Figure 1b). This demonstrates the capacity of LFU treatment to break up CNCs in particles of 10 nm, as previously observed [33].

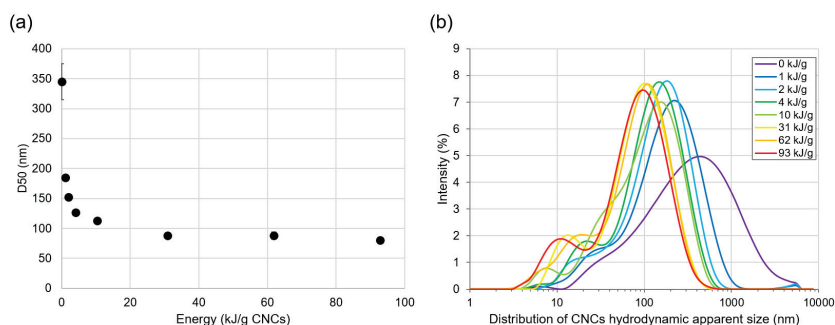


Figure 1. Size study of CNCs suspended at 0.5% (*w/w*) and treated using LFU for 45 min. (a) Apparent hydrodynamic D50 of CNCs and (b) mean distribution of the CNCs' apparent hydrodynamic diameter as a function of energy applied with LFU treatment. Low standard deviations of apparent hydrodynamic D50 are reported in (a).

The results reported in Figure 1 concern LFU treatment for the 0.5% (*w/w*) CNC suspension. Similar results were obtained for the 5% (*w/w*) CNC suspension. Size distribution parameters are presented in Table 1. Even if the energy per gram of CNCs was 10 times lower for the 5% (*w/w*) CNC suspension, D50 and D90 were slightly increased, while D10 remained unchanged. These results showed that very fine particle formation and CNC breakup were due to the energy increase. The agglomerated CNC breakage was more governed by the ultrasound power than by the energy supplied per gram of CNCs. These observations were in agreement with those obtained by Beuguel et al. (2018) [23].

Table 1. CNC apparent size distribution parameters (D10, D50, and D90) depending on the CNC concentration (0.5 or 5% *w/w*) and the energy applied (before or after 45 min LFU treatment).

CNC Suspension Concentration (%— <i>w/w</i>)	Energy (kJ)	Energy (kJ/g CNCs)	D10 (nm)	D50 (nm)	D90 (nm)
0.5	0	0	59 ± 9.3 ^a	345 ± 30.0 ^a	1350 ± 155 ^a
	58	93	13 ± 1.2 ^b	80 ± 2.1 ^b	203 ± 22.7 ^b
5	0	0	57 ± 2.6 ^a	366 ± 7.6 ^a	1370 ± 260 ^a
	58	9.3	12 ± 1.7 ^b	100 ± 5.6 ^c	269 ± 19.8 ^c

Different superscript letters (^{a,b,c}) in the same column indicate significant differences ($p < 0.05$).

3.2. LFU Effect on the Chemical and Crystallographic Properties of CNCs

An FTIR analysis was carried out to investigate the CNCs' chemical composition and possible chemical changes due to LFU treatment (Figure 2). From all the identified peaks (Table 2), those observed at 1029 cm^{-1} (C-O-C deformation) and 1367 cm^{-1} (C-H asymmetric stretching and C-O symmetric stretching) were characteristic of pyranose rings of glucose units [35,36]. Glycosidic linkages between glucose units were highlighted by peaks at 1159 and 1105 cm^{-1} corresponding to C-O-C wagging and twisting and C-O-C asymmetric stretching, respectively [32,35]. The peak at 898 cm^{-1} was assigned to C₁-H rocking and O-H bending [36]. The peak at 1203 cm^{-1} (S=O stretching) referred to the presence of sulfate groups [36], which originate from acid hydrolysis of cellulose in CNCs using sulfuric acid [37].

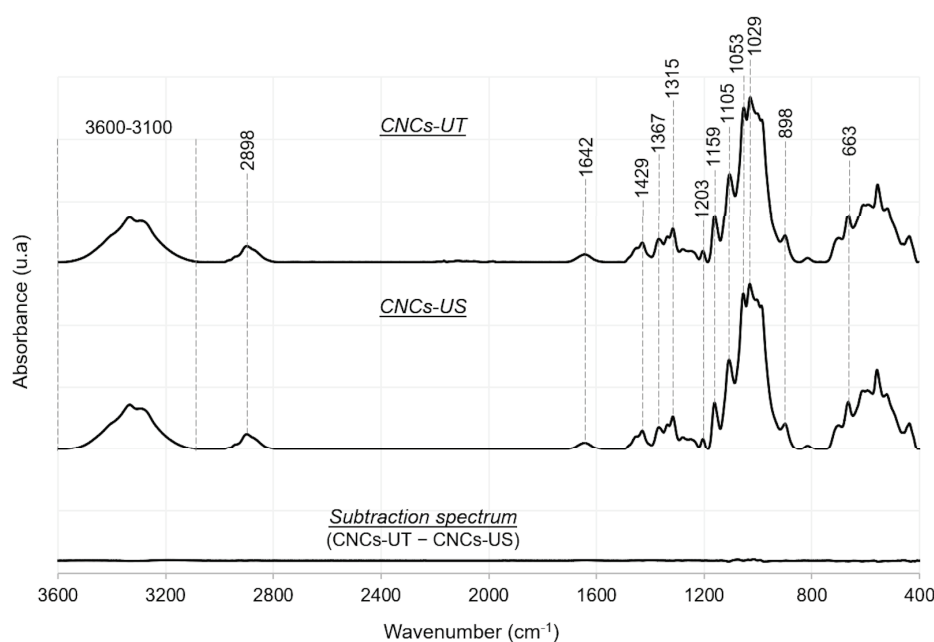


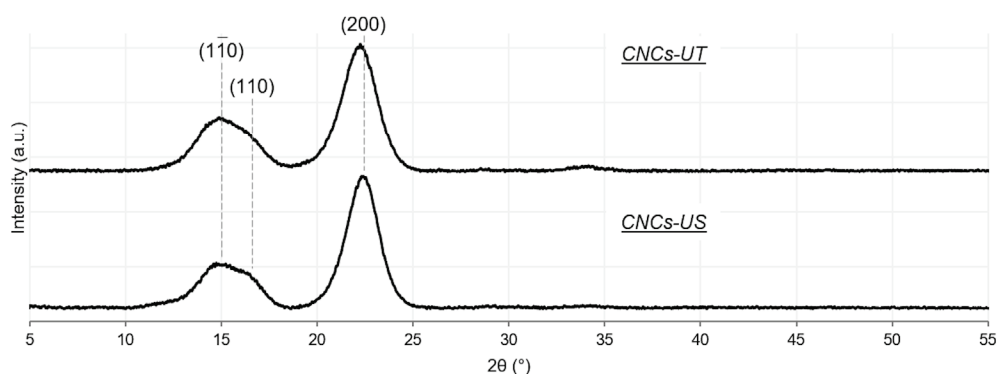
Figure 2. FTIR spectra of CNCs-UT and CNCs-US, and subtraction spectrum (CNCs-UT – CNCs-US).

Table 2. Peak wavenumber and assignment of corresponding chemical groups, identified with FTIR measurements.

Wavenumber (cm ⁻¹)	Assignments	References
Between 3100 and 3600	O-H stretching of primary and secondary hydroxyl groups	[32,36,38]
2898	C-H stretching	[32,35]
1642	O-H bending of adsorbed water	[32,35,36]
1429	In-plane bending vibration of H-C-H and O-C-H bonds	[38]
1367	C-H asymmetric stretching and C-O symmetric stretching	[35]
1315	C-H ₂ wagging	[39]
1203	S=O stretching	[36]
1159	C-O-C asymmetric stretching	[32,36]
1105	C-O-C wagging and twisting	[35]
1053	C-O stretching	[38]
1029	C-O-C deformation	[36]
898	C-H rocking and O-H bending	[36,40]
663	Out-plane hydrogen bonded O-H groups twisting	[32]

The FTIR spectra were similar for CNCs-UT and CNCs-US, showing that no change in the CNCs' chemical structure was caused by LFU treatment, particularly with regard to the presence of sulfate groups. Even if CNC suspensions were thermo-regulated during LFU treatment, hot-local points might rise and cause the de-esterification of sulfate groups [41]. The FTIR results ensured that LFU treatment had no significant effect on the CNCs' chemical structure. In addition, the absence of a peak shift also showed that LFU treatment did not change the cellulose polymorphism [42].

XRD measurements were carried out to investigate the treatment effect of LFU on the CNCs' structure. The CNCs-UT diffractogram revealed three peaks around $2\theta = 15^\circ$, 16.5° and 22.8° corresponding to $(1\bar{1}0)$, (110) and (200) crystallographic planes, respectively (Figure 3). These planes were characteristic of cellulose I [43]. The CNCs-US diffractogram was similar to that of CNCs-UT, showing that the CNCs' structure was not altered with LFU treatment, in agreement with the results of Zakani et al. (2022) [33]. The (200) crystallographic plane would be composed solely of CH groups, conferring hydrophobic properties, while the other $(1\bar{1}0)$ and (110) planes would be hydrophilic [19]. The XRD results not only confirmed the FTIR results but also made it possible to identify the different crystallographic planes of the CNCs.

**Figure 3.** X-ray diffraction patterns of CNCs-UT and CNCs-US.

3.3. LFU Effect on CNC Charge

The LFU effect on CNC charge was studied using ζ -potential measurements. CNCs-UT and CNCs-US exhibited a negative ζ -potential of -50.25 ± 1.33 mV and -53.20 ± 3.22 mV, respectively, due to the presence of sulfate groups, as identified with the FTIR measurements. ζ -potential values above $|30 \text{ mV}|$ suggested that CNC colloidal suspensions were stable over time, as charges are sufficient for CNC particles to repel each other [4,32]. Although

Chen et al. (2014) and Csiszar et al. (2016) observed a slight increase in ζ -potential value with increasing sonication time [44,45], no significant change in ζ -potential was observed between both CNC samples, in accordance with Beuguel et al. (2018) [23]. This suggested that the LFU treatment did not break covalent ester bonds between the sulfate groups and cellulose, supporting the FTIR results (Section 3.2.). Moreover, the pH of the CNC suspensions (close to 6.3 for 0.5% (*w/w*) CNC suspensions) was not significantly altered with LFU treatment, confirming that no sulfuric acid molecule was released into the medium.

3.4. LFU Effect on CNC Hydrophilicity

Particle wettability is a key factor in PE stability and is characterized by contact angle measurements [46]. The contact angles for CNCs-UT ($\theta_{\text{CNCs-UT}}$) and CNCs-US ($\theta_{\text{CNCs-US}}$) were determined using the sessile drop method on CNC films. It can be noticed that this method did not consider the CNCs' position at the oil/water interface since in PEs, free CNCs could orient themselves at the interface. However, the sessile drop method provided information on a possible CNC surface hydrophilicity modification with LFU.

Contact angle measurements around 30° revealed the hydrophilic nature of CNCs. No significant difference between CNCs-UT ($\theta_{\text{CNCs-UT}} = 31.9 \pm 2.1^\circ$) and CNCs-US ($\theta_{\text{CNCs-US}} = 29.1 \pm 4.2^\circ$) demonstrated that the LFU treatment did not modify the CNCs' wettability. According to the FTIR and XRD results, neither sulfate groups nor crystalline organization were modified with the LFU treatment. Thus, as expected, hydrophilicity was found to be similar for CNCs-UT and CNCs-US. Our results differed from those obtained by Ni et al. (2021, 2022) who observed an increase in contact angle (hydrophobicity) with LFU treatment time, probably due to CNC fragmentation exposing more hydrophobic crystal planes [27,30]. The CNCs used in this study differed in terms of origin and production methods from those used by Ni et al. (2021, 2022). Thus, the absence of modification in the CNC contact angle confirmed that the LFU treatment did not break CNCs but released aggregated CNC units in our suspensions.

3.5. LFU Effect on Rheological Properties

The rheological properties of 5% (*w/w*) CNCs-UT and CNCs-US suspensions were analyzed by measuring viscosity and viscoelastic parameters. The viscosity of the CNC suspensions decreased with increasing shear rate, showing that CNC suspensions had a shear-thinning behavior (Figure 4a). The CNCs-US suspension had a lower viscosity than the CNCs-UT suspension. These results were previously observed and explained in terms of water and ion release during the disruption of CNC aggregates with LFU treatment. Firstly, the entrapment of water molecules in CNC agglomerates would lead to an increase in the CNCs' apparent concentration, and hence to an increase in viscosity [23,47]. Secondly, the breakage of CNC agglomerates would release ions in the bulk and consequently compress the CNCs' electrical double layer, reducing electroviscous effects and decreasing the viscosity [23,48].

The viscosity curve of the CNCs-US suspension showed three different phases representative of CNC aqueous suspensions and characteristic of a lyotropic liquid crystalline polymer [49]. At low shear rates, the viscosity decreased due to the progressive alignment of liquid crystal (LC) domains (Phase I). The alignment of all LC domains in the shear direction may correspond to the viscosity stabilization at an intermediate shear rate (Phase II). At higher shear rates, the LCs' destruction and individual CNC particles' alignment explained the decrease in viscosity (Phase III). Although the LCs' presence in 5% (*w/w*) CNCs-US suspension was suggested, the rheological profile did not explain if this suspension contained only a liquid crystalline phase or a biphasic phase (i.e., a liquid crystalline phase and an isotropic phase) [50].

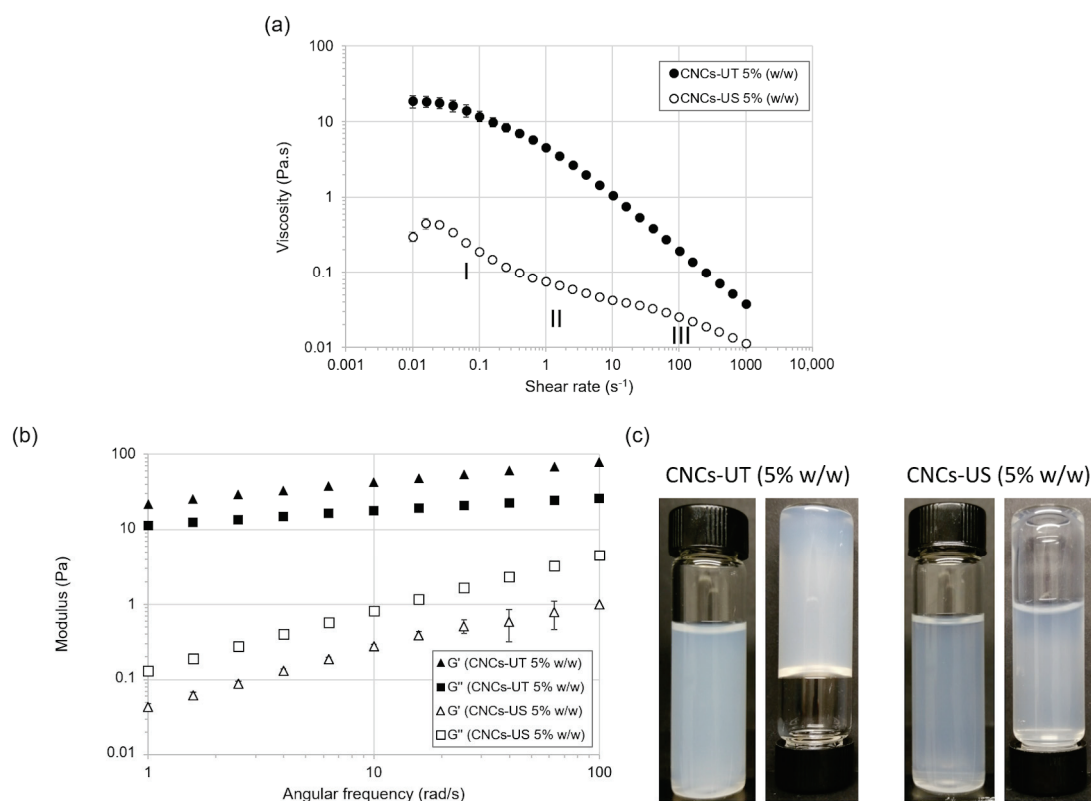


Figure 4. Effect of LFU on the (a) viscosity and (b) viscoelastic properties of the 5% (*w/w*) CNC suspension. (c) Visual observations of the CNCs-UT and CNCs-US suspensions.

LFU treatment also had a strong effect on viscoelastic properties (Figure 4b). For CNCs-UT at 5% (*w/w*), the storage modulus (G') was higher than the loss modulus (G''), over the whole angular frequency range studied, which is characteristic of a viscoelastic solid. CNCs-US at 5% (*w/w*) behaved like a viscoelastic liquid, with G' lower than G'' (Figure 4c).

The rheological data showed that LFU favored the dispersion of CNC aggregates and prevented gelation. LFU provided favorable conditions for LC formation. This change in rheological behavior caused by the LFU treatment had already been observed for highly concentrated suspensions [24]. For lower CNC concentrations, suspensions that were not treated with LFU had a viscoelastic liquid behavior [49].

3.6. LFU Effect on Liquid Crystal Formation

The LCs' presence in CNCs-US was suggested by the rheological measurements (Figure 4a) in the 5% (*w/w*) CNC suspension. Indeed, CNCs have the property of forming chiral nematic LCs above a critical concentration [51]. LC formation was then analyzed over a wide range of CNC concentrations using observations under cross-polarized light. Anisotropic LCs possess specific optical properties that enable them to change the light polarization [52]. As CNC suspensions were observed under cross-polarized light, the LCs were bright, and the isotropic phase appeared as dark [53]. Figure 5a,b highlights LC formation in both CNCs-US and CNCs-UT suspensions. The rheological analysis did not reveal the presence of LCs in CNCs-UT, probably due to the gel-like structure of these suspensions, or possibly due to a lower LC content in the CNCs-UT suspension. Indeed, CNCs-UT formed aggregates (Figure 1) that could disrupt the LCs' organization.

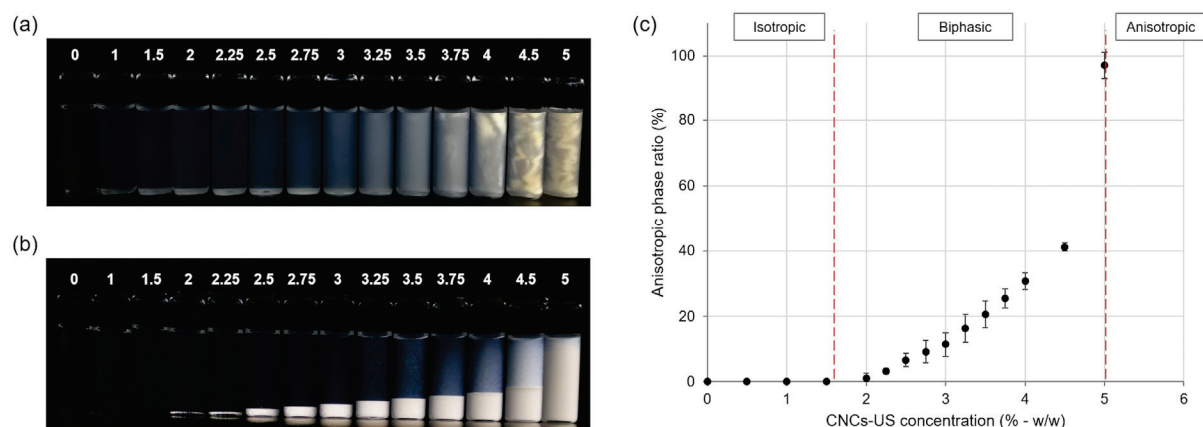


Figure 5. Observation of LCs in CNC suspensions under cross-polarized light. Photographs of (a) the CNCs-UT and (b) CNCs-US suspensions at different concentrations (in % w/w) and after 28 days of storage. (c) Phase diagram showing evolution in the anisotropic phase ratio versus CNCs-US concentration.

LCs have the particularity of having a higher density than isotropic phase [54]. After 28 days of storage, they sedimented in the CNCs-US suspensions. In the CNCs-UT suspensions, LCs were dispersed in the whole suspension, due to the gel-like behavior of highly concentrated suspensions, preventing sedimentation. LC formation increased with CNC concentration. The LCs' sedimentation for CNCs-US enabled macroscopic measurement of the anisotropic phase ratio for each CNC concentration and phase transition diagram build-up (Figure 5c). The isotropic phase was observed up to a critical CNC concentration between 1.5 and 2% (w/w), where the biphasic suspension was formed with isotropic and anisotropic phases. The 5% (w/w) CNCs-US suspension was mostly anisotropic.

3.7. LFU Effect on CNC Suspension Stability

The static multiple light scattering method was used to compare the stability of the CNCs-UT and CNCs-US suspensions at 5% (w/w) during storage (Figure 6). When the CNC suspension at 5% (w/w) was treated using LFU, the transmitted light intensity doubled compared with CNCs-UT. This is in accordance with the CNC particle size reduction observed after LFU treatment (Table 1) [47] and consistent with results obtained by Csiszar et al. (2016), who observed that a 3.4% (w/w) CNC suspension was less opaque after LFU treatment [45].

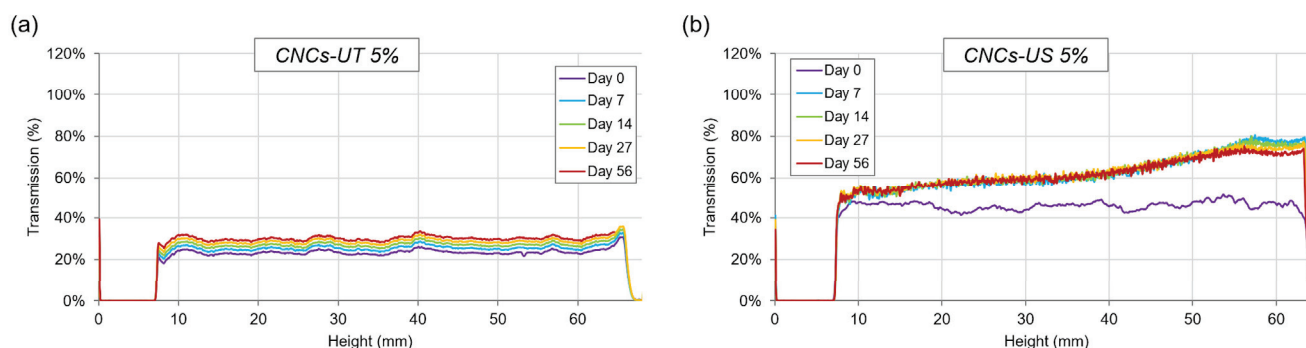


Figure 6. Static multiple light scattering analysis showing transmission data versus sample height (7 to 64 mm) and storage time for the (a) CNCs-UT and (b) CNCs-US suspensions at 5% (w/w).

Uniform transmittance from the bottom to the top of the CNCs-UT suspension (Figure 6a) showed that the suspension was stable in relation to its gel structure (Figure 4). The gel aging explained the slight up-translation in transmittance during storage. In contrast

with CNCs-UT, the transmitted light intensity of CNCs-US increased significantly from the bottom to the top of the tube at 7 days of storage and would reflect the LC sedimentation and LC concentration gradient over the entire height of the suspension. Multiple light scattering analyses provided additional information on LC gradient within suspension that was not observable under polarized light.

3.8. LFU Effect on CNCs and Impact on PE Properties

After studying the LFU effect on CNC properties, the link between CNC properties and PE formation and stabilization, was studied to evaluate how LFU-induced CNC modifications impact PE properties.

3.8.1. Effect of CNC Particle Size and Shape on PEs

CNC particle size plays a role in PE formation and stability. The small particles adsorbed faster at the oil/water interface and formed a denser layer at the interface than larger particles [55]. By reducing the CNCs' size (Figure 1), the LFU treatment favored CNC adsorption and organization at the oil/water interface and PE formation.

If particles are considered as spheres, particle desorption energy ($\Delta E_{\text{Desorption}}$) corresponds to Equation (1) [56]:

$$\Delta E_{\text{Desorption}} = \pi R^2 \gamma_{\text{ow}} (1 + \cos \theta)^2, \quad (1)$$

where R is the particle radius, γ_{ow} is the oil–water interfacial tension, and θ is the contact angle of particles measured in the aqueous phase. Although decreasing particle size would favor desorption, Berton-Carabin and Schroën, (2015) highlighted that PEs remained stable because $\Delta E_{\text{Desorption}}$ remained high with a value superior to thermal energy at 25 °C [14]. So, decreasing the sphere-like particle size would have little impact on PE destabilization.

Desorption energy also depends on particle shape. For non-spherical particles, length and width have to be considered [57]. The commercial CNCs used in this study have a rod-like shape [34,58]. Rod-like particles are expected to present more physical interactions at interfaces than volume-equivalent spherical particles. LFU, favoring the dispersion of CNC units with rod-like shapes, would act positively on PE stabilization.

3.8.2. Effect of the CNC Particles' Amphiphilic Properties and Charge on PEs

The XRD results showed that CNCs were composed of hydrophobic and hydrophilic planes (Figure 3) and explained the amphiphilic properties of CNCs and their potential use for stabilizing PEs [19]. LFU treatment would not affect the CNCs' amphiphilic properties and would not modify the CNCs' abilities to form a layer at the oil/water interface to stabilize PEs. Although CNCs had amphiphilic properties, contact angle measurements (around 30°) revealed that CNCs are hydrophilic and better stabilize O/W PEs.

Particle surface charge is a key parameter in PE stability and promotes electrostatic repulsion between droplets, preventing droplet coalescence. A high particle surface charge can induce electrostatic repulsion between particles and alter their absorption at the oil/water interface. On the contrary, a low surface charge favors particle aggregation and droplet attraction, leading to coalescence and PEs' destabilization [59]. According to the literature, the range of ζ -potential values efficient on PE stability is large and depends on CNC origin and PE formulation and processing. PEs with 20% (w/w) corn oil and 0.6% (w/w) CNCs (from cotton) were produced using high-speed homogenization for CNC ζ -potential adjusted to -38 mV and -24 mV with NaCl addition [60]. CNCs, extracted from palm-pressed fiber with ζ -potential values around -60 mV, were able to stabilize up to 14-day high-pressure homogenized PEs composed of 20% (w/w) palm kernel olein and 0.15 or 0.30% CNCs (w/w) [61]. With a ζ -potential around -50 mV, Celluforce CNCs (as used in this study) stabilized PEs with salt addition [62–66] or without salt [31,67]. As an example, PEs composed solely of water, caprylic/capric triglycerides, and CNCs were shown to be stable for up to 56 days of storage without creaming when the CNC content was above 3% (w/w) and the oil mass fraction was below 50% (w/w). PEs with reversible

creaming were obtained when the CNC content and the oil mass fraction were less than 3% (w/w) and 40% (w/w), respectively [31].

3.8.3. LC Effect on PE Stability

The LCs' presence in the CNC suspensions influenced the PEs' formation and stabilization. After LFU processing, the PEs' structure was observed using a polarized optical microscope (Figure 7). As LCs did not form below 2% CNCs (w/w) (Figure 5), the PE containing 1% (w/w) CNCs (Figure 7a) did not reveal any LC bright structures (Figure 7b). On the contrary, in the PE containing 5% (w/w) CNCs (Figure 7c), bright stains were observed, highlighting the LCs' presence (Figure 7d). Bright LCs appeared dispersed in a continuous phase, suggesting that CNC concentration was greater than 2% (w/w). Moreover, the PE was very stable during 56 days of storage without creaming [31].

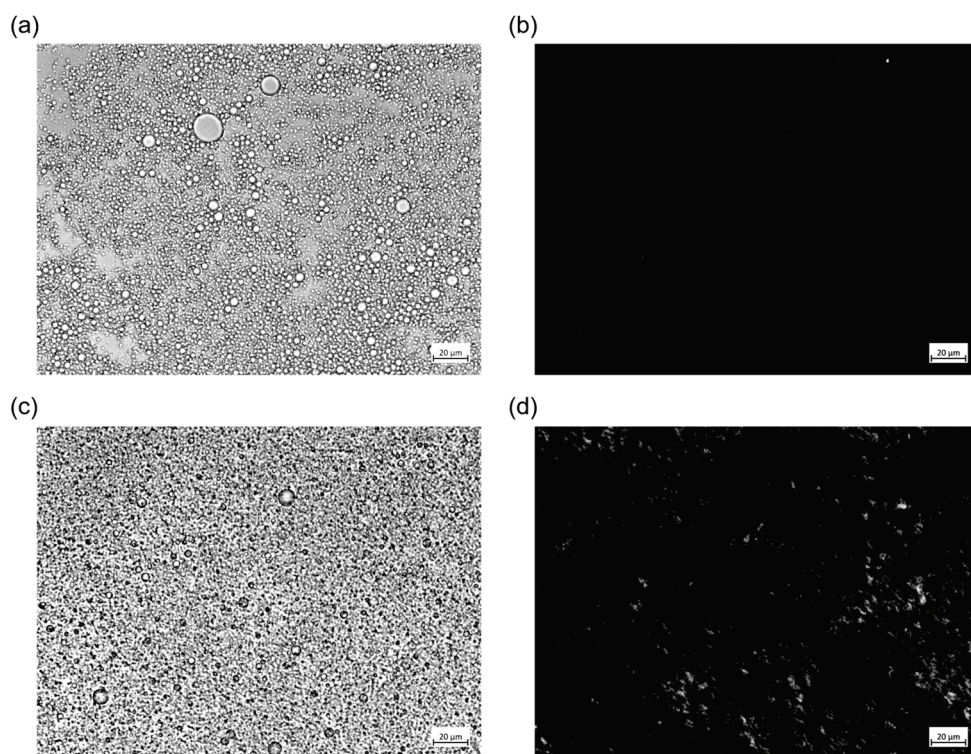


Figure 7. Optical microscope images of PEs containing (a,b) 1% CNCs (w/w) and 10% (w/w) oil and (c,d) 5% CNCs (w/w) and 10% (w/w) oil under (a,c) unanalyzed polarized light and (b,d) analyzed polarized light. The scale bar corresponds to 20 μm .

LCs have already been observed in PEs containing CNCs [68] and other solid particles [69]. The relationship between CNC structuration in LC form and PE stabilization may be relevant. According to the phase diagram of PEs [31] and the present results, a phenomenological model can be proposed, enhancing the LCs' positive influence on PE formation and stabilization.

During LFU processing, CNC units formed a layer at the oil/water interface and stabilized oil droplets [11]. The unadsorbed CNCs (in excess) remained in an aqueous continuous phase (Figure 8a), and their self-organization formed LCs. The LCs were dispersed in the continuous aqueous phase (Figure 8b), and/or adsorbed at the oil/water interface (Figure 8c). The LCs' formation could favor the droplet stabilization by producing steric repulsion at the droplet interface. It can be supposed that for a high volume fraction (oil and CNCs), a weak LC network could be produced within an aqueous continuous phase.

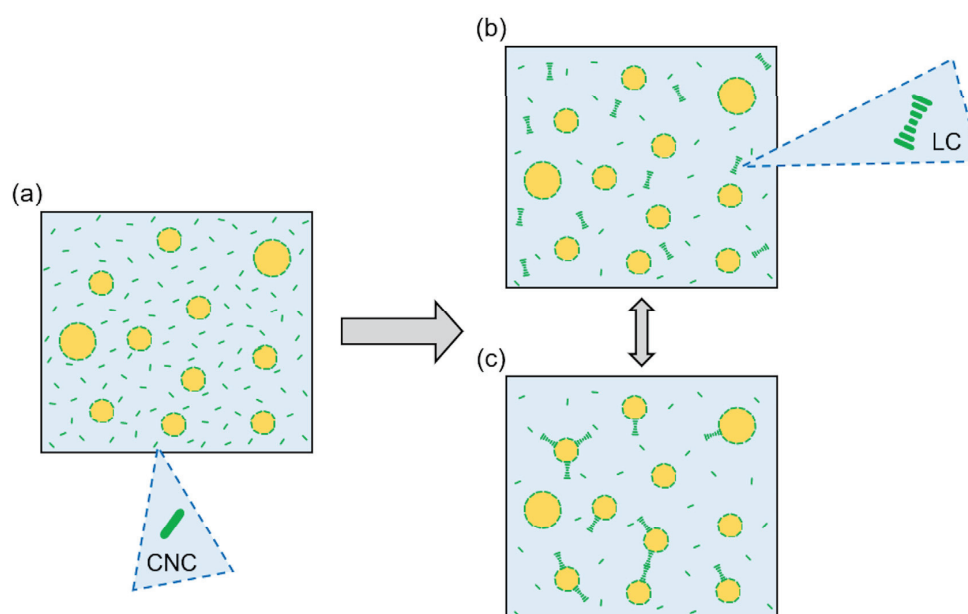


Figure 8. Phenomenological model on the organization of high CNC concentration in PEs. (a) Oil droplets prevent LC formation, and CNCs are dispersed in a continuous phase. (b) CNCs form LCs dispersed in a continuous phase. (c) LCs are dispersed in a continuous phase and adsorb at the oil/water interface.

In our previous study [31], it was shown that PEs containing less than 3% (*w/w*) CNCs were unstable with creaming occurring during storage, whereas PEs containing more than 3% (*w/w*) remained stable for up to 56 days of storage. Better PE stability with increasing CNC content was assumed to be due to an increasing continuous phase viscosity. The presence of LCs in PEs containing large CNC concentrations acted as an additional stabilizing agent.

4. Conclusions

In this study, the LFU treatment effects on CNC properties were investigated with the aim of using CNCs to stabilize PEs. CNC suspensions were treated (or not) using LFU, and their chemical and physical properties were studied and compared.

The CNCs' size showed that CNC aggregates were dissociated with LFU treatment above 20 kJ/g CNCs. The FTIR results revealed the presence of sulfate groups due to CNC hydrolysis conditions. These sulfate groups provided a high CNC surface charge. In PEs, droplet coalescence was prevented by efficient electrostatic repulsion between them. The XRD results highlighted the presence of a hydrophobic crystallographic plane, conferring amphiphilic properties to CNCs and enabling CNCs to adsorb at the oil/water interface to stabilize PEs. No chemical and crystallographic changes were observed with FTIR and XRD measurements after LFU treatments of CNC suspension. The surface charge and hydrophilic properties of CNCs were not altered with LFU treatment.

However, the LFU treatment modified the rheological properties of CNC suspensions, breaking down gel structures and promoting LC formation and sedimentation. In PEs, when CNCs were in excess in a continuous phase, LCs were formed and participated in PE stabilization. A phenomenological model was proposed to describe LCs' role in PE stabilization.

The LFU treatment, as a common emulsification process, would not alter CNC properties and could even improve CNCs' capabilities to adsorb at the oil/water interface and stabilize PEs. Further investigation will be required to validate these hypotheses using LFU-treated CNCs and also CNCs subjected to other emulsification processes like high-pressure homogenization or microfluidization.

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Conflicts of Interest: Authors Louise Perrin and Guillaume Gillet were employed by the company GENIALIS. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Bacterial Nanocellulose from *Komagataeibacter Medellinensis* in Figue Juice for Activated Carbons Production and Its Application for Supercapacitor Electrodes

Juliana Villarreal-Rueda ¹, Zulamita Zapata-Benabithé ^{2,*}, Laia Posada ³, Estefanía Martínez ³, Sara Herrera ³, Stiven López ³, Ana B. J. Sobrido ⁴ and Cristina I. Castro ³

¹ Semillero de Termofluidos y Conversión de la Energía, Ingeniería Química, Escuela de Ingenierías, Universidad Pontificia Bolivariana, Medellín 050031, Colombia

² Grupo de Energía y Termodinámica, Ingeniería Química, Escuela de Ingenierías, Universidad Pontificia Bolivariana, Medellín 050031, Colombia

³ Grupo de Investigación sobre Nuevos Materiales, Ingeniería en Nanotecnología, Escuela de Ingenierías, Universidad Pontificia Bolivariana, Medellín 050031, Colombia; cristina.castro@upb.edu.co (C.I.C.)

⁴ School of Engineering and Materials Science, Queen Mary University of London, London E1 4NS, UK; a.sobrido@qmul.ac.uk

* Correspondence: zulamita.zapata@upb.edu.co; Tel.: +57-300-4705147

Abstract: This paper presents the results obtained from the chemical activation of bacterial nanocellulose (BCN) using figue juice as a culture medium. BNC activation (BNCA) was carried out with H₃PO₄ and KOH at activation temperatures between 500 °C to 800 °C. The materials obtained were characterized morphologically, physicochemically, superficially, and electrochemically, using scanning electron microscopy, X-ray photoelectron spectroscopy (XPS), the physisorption of gases N₂ and CO₂ at 77 K and 273 K, respectively, cyclic voltammetry, chronopotentiometry, and electrochemical impedance spectroscopy (EIS). The samples activated with H₃PO₄ presented specific surface areas (S_{BET}) around 780 m² g^{−1}, while those activated with KOH values presented specific surface areas between 680 and 893 m² g^{−1}. The XPS analysis showed that the P_{XPS} percentage on the surface after H₃PO₄ activation was 11 wt%. The energy storage capacitance values ranged between 97.5 F g^{−1} and 220 F g^{−1} by EIS in 1 M H₂SO₄. The samples with the best electrochemical performance were activated with KOH at 700 °C and 800 °C, mainly due to the high S_{BET} available and the accessibility of the microporosity. The capacitance of BNCA was mainly improved by electrostatic effects due to the S_{BET} rather than that of pseudocapacitive ones due to the presence of phosphorus heteroatoms.

Keywords: activated carbon; chemical activation; bacterial nanocellulose; supercapacitors; energy storage

1. Introduction

The increasing of global population is followed by a marked rise in energy consumption, primarily from fossil fuels, which has a detrimental effect on environment. In order to struggle these issues, there is a current trend toward adopting alternative technologies to produce “green” energy through the promotion of renewable energy sources such as solar and wind. However, implementing these alternatives is not trivial, mainly because of their intermittency. Therefore, it is critical to guarantee their effective large-scale distribution and storage. In other words, if a large part of the energy comes from renewable sources, there must be a suitable way of storage it and its surpluses, paired with a distribution network with the capacity to transport all the energy generated [1].

Energy storage systems (ESS) come in a variety of forms, such as mechanical (hydro-electric power plants), electrical, thermal (solar thermal power plants), and electrochemical (supercapacitors (SCs) and batteries). Essential parameters in electrochemical ESS are energy density (Wh kg^{−1}) to determine how much electrical energy can be stored, and

power density (W kg^{-1}) to resolve the maximum power the device will deliver. From the Ragone diagram [2], it can be concluded that SCs can store more energy than conventional electrolytic capacitors can, although their power density is lower. On the other hand, the power density of SCs is higher than that of batteries, but the former exhibit lower energy density. SCs store energy by adsorbing ions by a double-layer mechanism [3] and are currently used in applications where fast charging and small amounts of energy are required.

Currently, electrochemical storage systems are getting more attention and having relevance due to the interest in replacing fossil fuels as a primary energy source with more environmentally friendly alternatives, which is why the world is in an “energy transition” process. Due to the low cost, outstanding lifetime and low maintenance of SCs, they are one of the most promising energy storage systems in cases where high power density is needed [4]. SCs are formed by two conductive electrodes spaced apart by an insulating dielectric (usually a polymer) impregnated with a conductive electrolyte (usually a polymer). Applying a voltage to the electrodes causes an accumulation of opposite charges on the material’s surface; those charges are kept apart by the dielectric function, producing an electric field that allows SCs to store electrical energy [5]. There are three different forms of electrochemical energy storage for SCs: (a) pseudocapacitors, which store electrochemical energy transferring electrons between the electrode and an electrolyte; (b) double-layer capacitors, which store energy through the phenomenon of electrostatic charge; and (c) hybrid capacitors, which combine the advantages of the previous two to generate higher energy densities and increase their lifetime [6].

In commercial SCs, carbonaceous materials from non-renewable sources such as graphite or coke carbon serve as primary sources for electrode production. Therefore, it is necessary to look for novel renewable alternatives to replace them and satisfy the currently high demand for sustainability. Activated carbons, carbon nanotubes, and carbon nanofibers are some of the most common materials used to prepare SCs electrodes, each of them presenting different properties and characteristics. For instance, activated carbons with high specific surface areas can be produced from several sources, such as banana peel, palm kernel, sawdust, and others [7]. Additionally, other materials such as aerogels, xerogels, carbon nanotubes, and nanofibers are able to achieve the desired physical and chemical properties for electrodes of SCs, such as moderate conductivity, high thermal resistance, a large specific surface area, and a relatively low cost [8].

There is great interest in using and transforming “green” materials for energy applications. Among these materials is cellulose, one of the most abundant renewable biopolymers in nature, with an outstanding production of around 100 billion metric tons per year, and cellulose-derived carbon materials are ideal for SCs electrodes owing to their high carbon content and tridimensional structure [9,10]. Bacterial nanocellulose (BNC) is a renewable, degradable, and safe-to-use material, as it is a non-toxic material with an interconnected three-dimensional morphology and carbonaceous chemistry. BNC offers significant advantages over vegetable cellulose, with the possibility of large-scale production using raw materials from agroindustrial activity in a relatively straightforward process, which results in a low-cost and ecofriendly material. Additionally, BNC exhibits high crystallinity and mechanical properties of industrial interest, such as a high Young’s modulus (100–130 GPa) and the property of being lightweight (density of 1.6 g cm^{-3}), which makes it interesting for applications in portable devices [11]. BNC also offers other physical properties that make it attractive for application as an active material in SCs, including a hierarchical fibrillar structure that promotes a high liquid absorption capacity, high chemical stability, and high mechanical strength [12]. Furthermore, the BNC morphology facilitates the production of active materials with increased interaction between the electrolyte and surface, guaranteeing continuity in ion transmission, which translates into high material performance [13].

Moreover, BNC has attractive properties for application in energy storage devices, such as its high carbon content, ease of surface modification and doping, high specific

surface area, and high thermal and structural stability [14]. Some authors have reported the obtention of BNC from bacterial strains such as *Acetobacter xylinum* [15–17] or food sources such as nata de coco [18], and its chemically (KOH and H_3PO_4) and physically (CO_2) activation to improve the BNC-specific surface area values (S_{BET}) between 340 and $1754 \text{ m}^2 \text{ g}^{-1}$. The active material has been used as electrochemical double-layer electrodes [18] and catalyst support for the catalytic dehydration of ethanol [16]. For example, Lee et al. [18] reported energy storage capacity values ranging from 26 to 42 F g^{-1} using 0.5 M K_2SO_4 as an electrolyte in a potential range between -0.2 V and 0.2 V in a three-electrode cell configuration. Usually, Hestrin-Schramm (HS), a nutrient culture medium obtained via oxidative fermentation, is employed to obtain BNC. However, figue juice is an agroindustrial waste with high levels of nitrogen and carbon in the form of glucose, and it is therefore considered an excellent substrate for BNC production [19]. This work presents the results from the activation of BNC obtained by *Komagataeibacter Medellinensis* using figue juice as a culture medium. The activated carbons chemically obtained are evaluated as supercapacitor electrodes in a two-electrode electrochemical cell in an aqueous medium.

2. Materials and Methods

2.1. Chemical Activation of the Bacterial Nanocellulose

The BNC was produced by the *K. Medellinensis* strain using figue juice, from the northeast region of Antioquia, Colombia, as a culture medium in accordance with the methodology presented by Castro et al. [20], and dried by lyophilization [19]. The chemical activation of BNC (BNCA) was carried out by impregnation with an activating agent (H_3PO_4 85% or KOH, supplied by Merck (Colombia/Bogotá)) for 24 h at a 1:1 dry weight ratio. The impregnated BNC was dried at 100°C for 24 h and then heat-treated in a horizontal tube oven in a nitrogen environment at a flow rate of 100 mL min^{-1} at different temperatures: 500 and 600°C for H_3PO_4 (BNCA-P), and 600, 700 and 800°C for KOH (BNCA-K). The heat treatment was carried out at two heating ramps: the first one was carried out at 3°C min^{-1} from room temperature to 300°C for 60 min, and the second one was carried out at 5°C min^{-1} from 300°C up to the final temperature for 60 min. Finally, the activated BNC was successively washed with distilled water until it reached a constant pH, and then it was dried in an oven at 100°C for 24 h.

2.2. Morphological, Superficial, Porous, and Physicochemical Characterization

The morphological and compositional characterization of the obtained materials was carried out by scanning electron microscopy (SEM-EDX) using Thermo Fisher Scientific (Colombia/Bogotá) model Apreo 2 S LoVac 5 kV Field Emission Electron Microscope (FESEM).

The superficial and porous characterization was determined by using the physical adsorption/desorption technique for gases (N_2 at 77 K and CO_2 at 273 K) in ASAP 2020 Plus equipment (Micromeritics, Colombia/Medellín) after degassing the samples for 8 h at 120°C in high vacuum conditions. The specific surface area (S_{BET}) was calculated by applying the BET model [21], micropore volume (V_{N_2} and V_{CO_2}) determined by the Dubinin–Radushkevich model [22], the micropore size (D_{micro}) which was calculated by applying the Stoeckli equation [23], the mesopore volume (V_{meso}) which was calculated by the difference in the volume of nitrogen adsorbed at P/P_0 of 0.98 and the V_{N_2} (Gurvich's rule). The pore distribution was determined by the density functional theory (DFT) model and Barrett–Joyner–Halenda (BJH) model from the N_2 isotherm at 77 K. The true density was determined using an Accupyc II 1340 helium pycnometer (Micromeritics, Colombia/Medellín).

The surface chemistry was determined by confocal Raman spectroscopy on Horiba Jobin Yvon equipment (Colombia/Bogotá), the Labram HR model with a high resolution, a 633 nm laser and 15 s exposure time, and by X-ray photoelectron spectroscopy (XPS) X-ray photoelectron spectroscopy (NAP-XPS) with a PHOIBOS 150 1D-DLD analyzer, using a monochromatic $\text{Al-K}\alpha$ (1486.7 eV, 13 kV, 100 W) with a step energy of 90 eV for general

spectra and 20 eV for high-resolution spectra. The high-resolution spectra were recorded for the C_{1s}, O_{1s}, and P_{2p} elements. The C_{1s} peak at 284.6 eV was used as a reference.

2.3. Electrochemical Characterization

The active material was mixed with a polymeric binder (60 wt% polytetrafluoroethylene, PTFE, dispersed in water, supplied by Merck (Colombia/Bogotá)) and a conductive material (acetylene black, Químicos JM (Colombia/Medellín)) in an 80/10/10 weight ratio. The mixture was left to dry in an oven at 100 °C for 24 h. Then, the electrodes were assembled with 7 mg of the mixture on 12.2 mm diameter graphite paper. The electrodes were impregnated with a 1 M H₂SO₄ solution (supplied by Merck (Colombia/Bogotá)) for 72 h. The electrodes were characterized in a Swagelok two-electrode cell in a symmetric arrangement and in a three-electrode configuration between 0 and 0.75 V. The gravimetric capacitance was calculated from the capacitance (F) by different techniques (electrochemical impedance spectroscopy, cyclic voltammetry, and chronopotentiometry) [24,25] and normalized by the mass (g) of the active material. The electrochemical characterization was carried out in a Multi Autolab/M204 (Metrohm, Colombia/Bogotá).

- Electrochemical impedance spectroscopy (EIS) is a technique used to characterize the frequency response of a device. The Nyquist plot was carried out in a frequency range between 1 mHz and 100 kHz with a sinusoidal amplitude of ± 10 V. The C_{EIS} (F g⁻¹) is the maximum gravimetric capacitance, which was determined according to Equation (1) at the minimum frequency, and the interfacial capacitance IC (μF cm⁻²) is the gravimetric capacitance normalized by the specific surface area according to Equation (2).

$$C_{EIS} = \frac{-Z''}{m\omega|Z|^2} \quad (1)$$

$$IC = \frac{C_{EIS}}{S_{BET}} \quad (2)$$

where Z'' is the imaginary impedance, $|Z|$ is the complex impedance, ω is the angular frequency and m is the total active material weight (g).

Additionally, important parameters on supercapacitor behavior, such as the equivalent series resistance (ESR), charging time (τ) and phase angle (φ), could be determined from the EIS.

- The cyclic voltammetry (CV) technique applies a potential perturbation (scan rate) in a specific potential range. The cyclic voltammetry curves were carried out between 0 and 0.75 V at different scan rates (V_b): 0.5, 1, 2, 5, 10, 25, and 50 mV s⁻¹. The kinetic or diffusion limitation can be detected from CV curves [24]. The C_{CV} (F g⁻¹) is the gravimetric capacitance, and it was determined from the voltammetry curves according to Equation (3).

$$C_{CV} = \frac{2\sum i(t)}{m\Delta V} \quad (3)$$

where $i(t)$ is the change in the current as a function of time, ΔV is the potential window and, m is the total active material weight (g). The capacitance value decreases as the scan rate increases because the number of active sites diminish inside the material and the redox reaction is not complete with a higher potential.

The capacitance retention percentage (R_{CV}) was determined from the C_{CV} values at different sweep ratios with respect to the C_{CV} calculated at 2 mV s⁻¹ (Equation (4)).

$$R_{CV}(\%) = \frac{C_{CV,smVs^{-1}}}{C_{CV,2mVs^{-1}}} \times 100 \quad (4)$$

- The chronopotentiometry (CP) technique applies a constant current density and measures the potential (E) with respect to time, allowing it to determine the device's stability. The charge and discharge curves were performed between a potential window from 0 to 0.75 V at different current densities: 0.156, 0.313, 0.625, and 0.938 A g⁻¹. The gravimetric capacity (C_{CP}) was calculated from the discharge curve according to Equation (5). Additionally, the best samples underwent cyclability analysis at the 0.33 A g⁻¹ current density (5000 charge–discharge cycles).

$$C_{CP} = \frac{4I_c \Delta t}{m \Delta V} \quad (5)$$

where I_c is the current density (A g⁻¹), Δt is the discharge time (s), ΔV is the potential range (V) and m is the total active material weight (g).

3. Results and Discussion

The yield of activated carbons after the BNC activation process ranged from 5% to 38%. BNC activated with H₃PO₄ are represented by the letter P followed by the heat treatment temperature ($T = 500$ and 600 °C), PT, and those activated with KOH are represented by the letter K followed by the heat treatment temperature ($T = 600$, 700 and 800 °C), KT.

3.1. Morphological Characterization of BNCA's

Figure 1 shows SEM micrographs of freeze-dried BNC. The BNC showed a long fiber structure with diameters between 70 and 100 nm and a true density of 1.09 ± 0.01 cm³ g⁻¹. After activation, the fibrous structure disappeared and the fibers collapsed. The EDX spectra are presented in the Supplementary Information (SI).

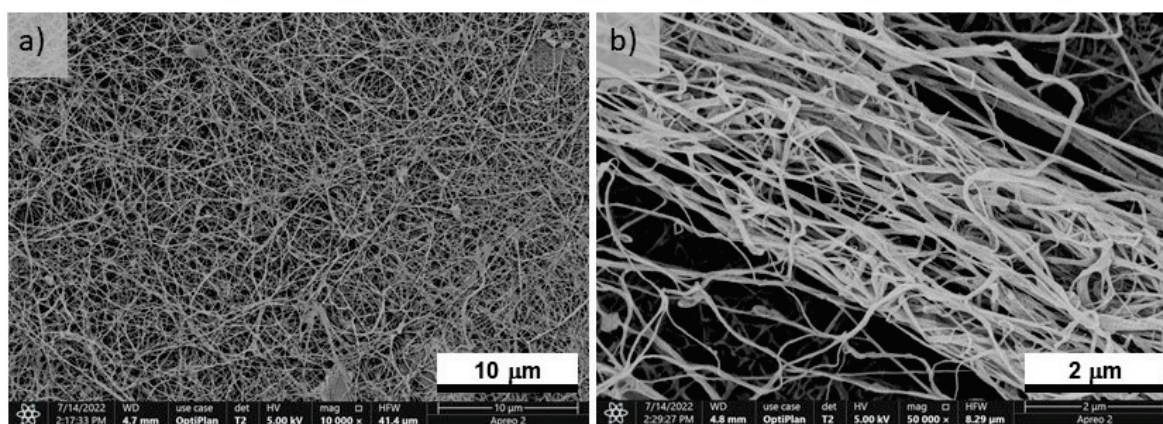


Figure 1. SEM micrographs of freeze-dried BNC at (a) $\times 10,000$ and (b) $\times 50,000$.

SEM micrographs of H₃PO₄-activated carbons (P500 and P600) are presented in Figure 2. Primary particles in the form of microspheres constituted some of the surface morphology of P500 (Figure 2a), but in other regions of this sample, these particles merged into a continuous network (Figure 2b). The primary particles in Sample P600 were also microspheres but smaller than those in P500 (Figure 2c). However, in some areas (P area in Figure 2d), there was a cluster of different morphology particles in the network that was identified by EDX as phosphorus particles (Figure S1c).

In the case of BNC activated with KOH, sample K600 exhibited an interconnected structure formed by open pores in some sections (Figure 3a) and a compact surface with macropores (Figure 3b). Sample K700 presented a nanostructured network with variously sized accessible pores (Figure 3c,d). Sample K800 showed a heterogeneous morphology (Figure 3e,f); in one section, the carbon network was composed by primary particles in small microspheres, and, in another section, a three-dimensional network was formed by accessible pores of a smaller size than those of K700. The rise in the temperature

significantly affected the morphology of the activated carbons, especially at 800 °C where K800 apparently showed a weaker structure, similarly to the findings reported by Boongate and Phisalaphong [15].

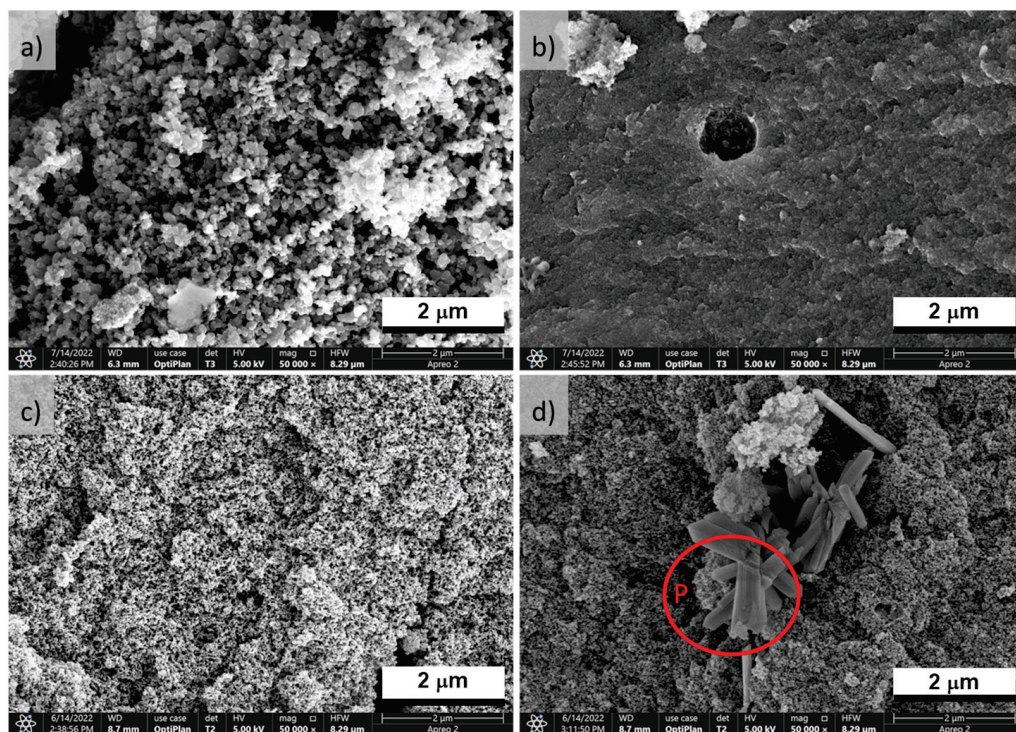


Figure 2. SEM micrographs at $\times 50,000$ of BNCA-Pat 500 °C (a,b) and at 600 °C (c,d). P area: clusters of phosphorus particles.

3.2. Superficial and Porous Characterization of BNCA

N_2 adsorption/desorption isotherms at 77 K are shown in Figure 4a,b, and CO_2 adsorption isotherms at 273 K are shown in Figure 4c. Samples P500 and P600 present type IV isotherms (Figure 4a) with a type H3 hysteresis cycle according to IUPAC, which corresponds to that of materials with heterogeneous micropore distribution and the presence of wider micropores (~ 4 nm). Samples K600 and K700 are classified as type Ia, corresponding to the properties of essentially microporous materials, while sample K800 can be classified as type Ia-IV due to the H4 hysteresis cycle, suggesting the presence of both micropores and mesopores (Figure 4b) [26].

The pore size distribution for samples P500 and P600 (Figure 4d) presented a similar distribution of around 1.2 nm and a wide distribution of mesopores of between 2–20 nm. Activated samples with KOH (Figure 4e) exhibited a similar micropore size distribution in the microporous region (< 2 nm), with a strong peak at 0.5 nm, for K800.

Table 1 presents the structural properties of the activated materials. The S_{BET} values ranged from 718 to 893 $m^2 g^{-1}$, values slightly higher than those found in the literature for this type of material [16–18]. The temperature did not have a significant effect on microporous development when H_3PO_4 was selected as the activating agent; however, the volume of mesopores increased when using H_3PO_4 . Additionally, the activated samples with KOH showed a substantial increase in the S_{BET} , particularly the sample treated at 800 °C, which can be explained by the intercalation of K between the walls of the carbonaceous structure which promotes changes in the microstructure (including defects, edges, morphology, and pore size) [27], and the formation of hierarchical pores. The micropore size of activated samples with H_3PO_4 was 1.26 nm and for the activated samples with KOH, the micropore size was 1 nm. The micropore size distribution was broad and heterogeneous because $V_{N_2} > V_{CO_2}$; the N_2 could fill all the micropores during its

adsorption at 77 K, and the CO₂ could only fill the ultramicropores (<0.7–0.8 nm wide) [28]. Figure 4f shows that the activation with H₃PO₄ mainly promoted the development of mesopores. P500 presented an average pore diameter of 2.8 nm, while P600 presented an average pore diameter of 7 nm with a wider distribution.

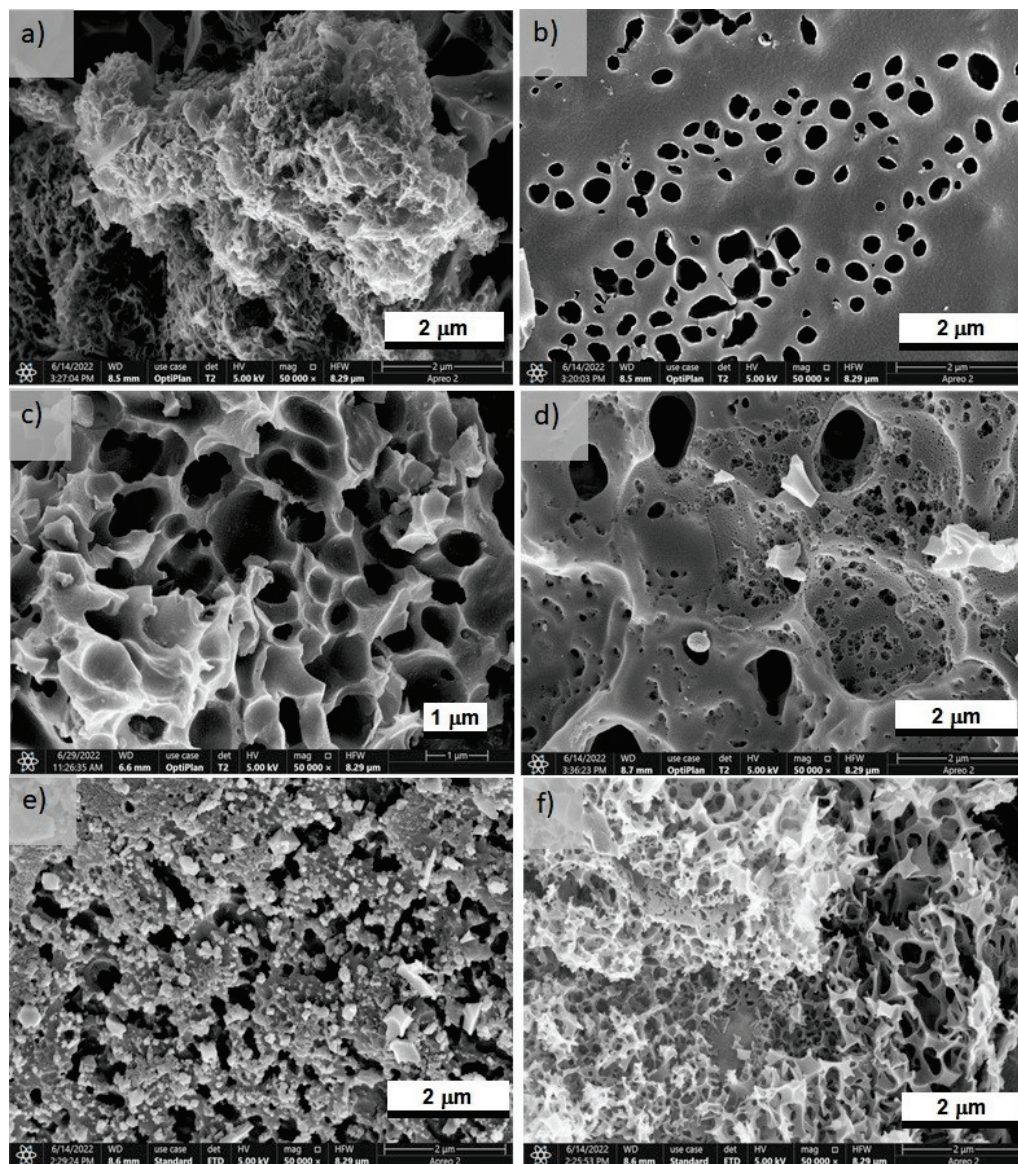


Figure 3. SEM micrographs at $\times 50,000$ of BNCA-K (a) at 600 °C (a,b), at 700 °C (c,d), and at 800 °C (e,f).

Table 1. Structural properties of BNCA.

BNCA	S_{BET} ($\text{m}^2 \text{g}^{-1}$)	D_{micro} (nm)	V_{N_2} ($\text{cm}^3 \text{g}^{-1}$)	$V_{\text{total},0.985}$ ($\text{cm}^3 \text{g}^{-1}$)	V_{meso} ($\text{cm}^3 \text{g}^{-1}$)	V_{CO_2} ($\text{cm}^3 \text{g}^{-1}$)
P500	718	1.27	0.234	0.73	0.50	0.084
P600	791	1.26	0.258	1.72	1.46	0.045
K600	806	0.91	0.263	0.33	0.06	0.245
K700	761	1.00	0.236	0.35	0.12	0.177
K800	893	1.22	0.315	0.69	0.38	0.169

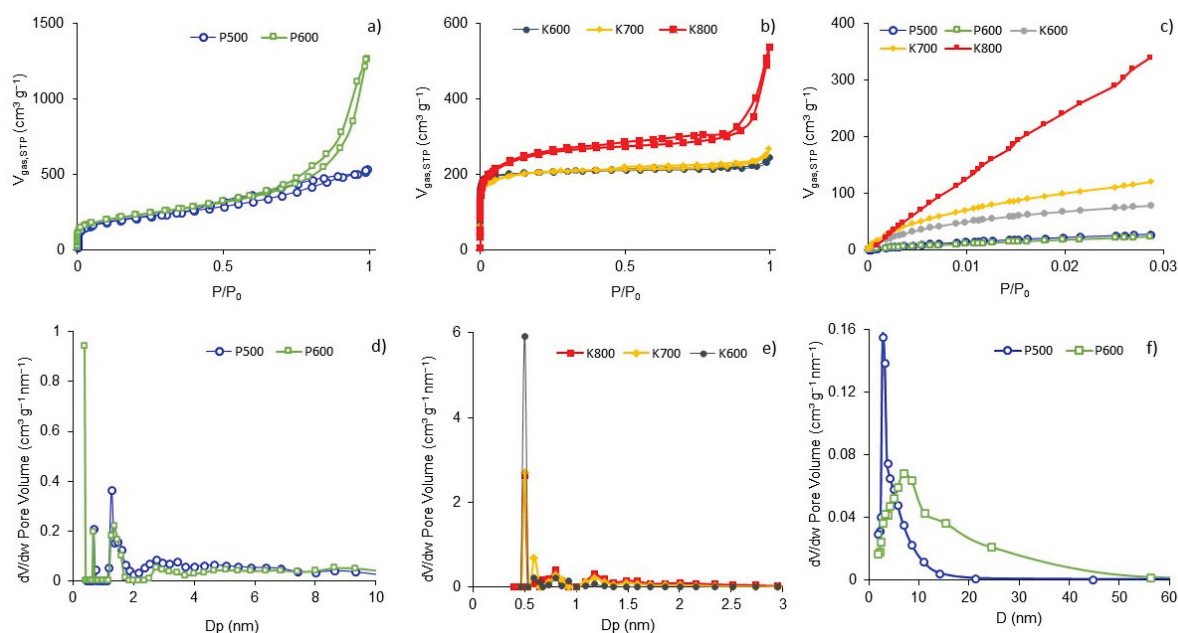


Figure 4. N_2 isotherms at 77 K for (a) BNCA-P and (b) BNCA-K; (c) CO_2 isotherms at 273 K, with pore size distribution by DFT for (d) BNCA-P and (e) BNCA-K, and (f) pore size distribution by BJH for BNCA-P.

3.3. Physico-Chemical Characterization of BNCAs

Figure 5a shows Raman spectra of BNCAs which were used to evaluate the degree of graphitization of surfaces.

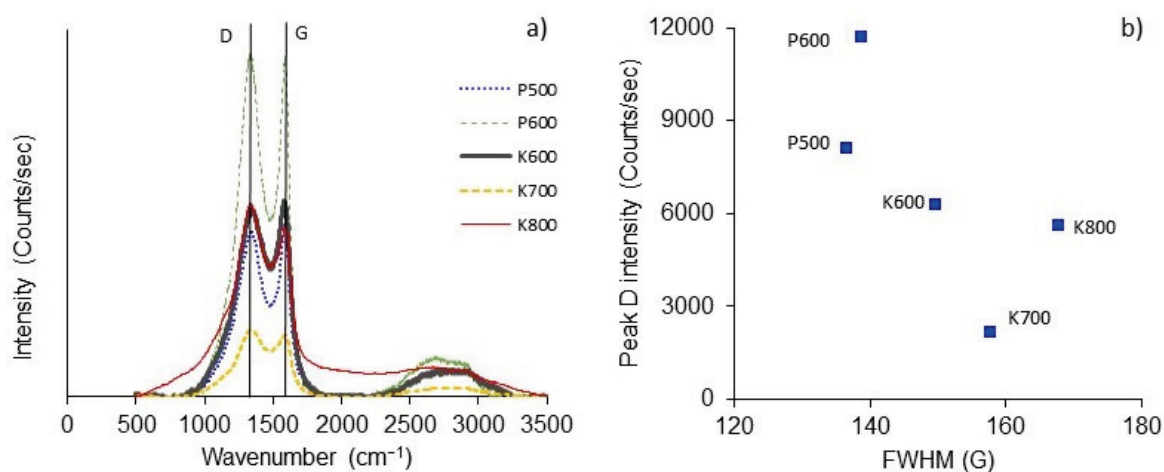


Figure 5. (a) Raman spectra of BNCAs, and (b) the intensity of I_D as a function of the FWHM of I_G . D-peak: intensity (I_D); G-peak: intensity (I_G).

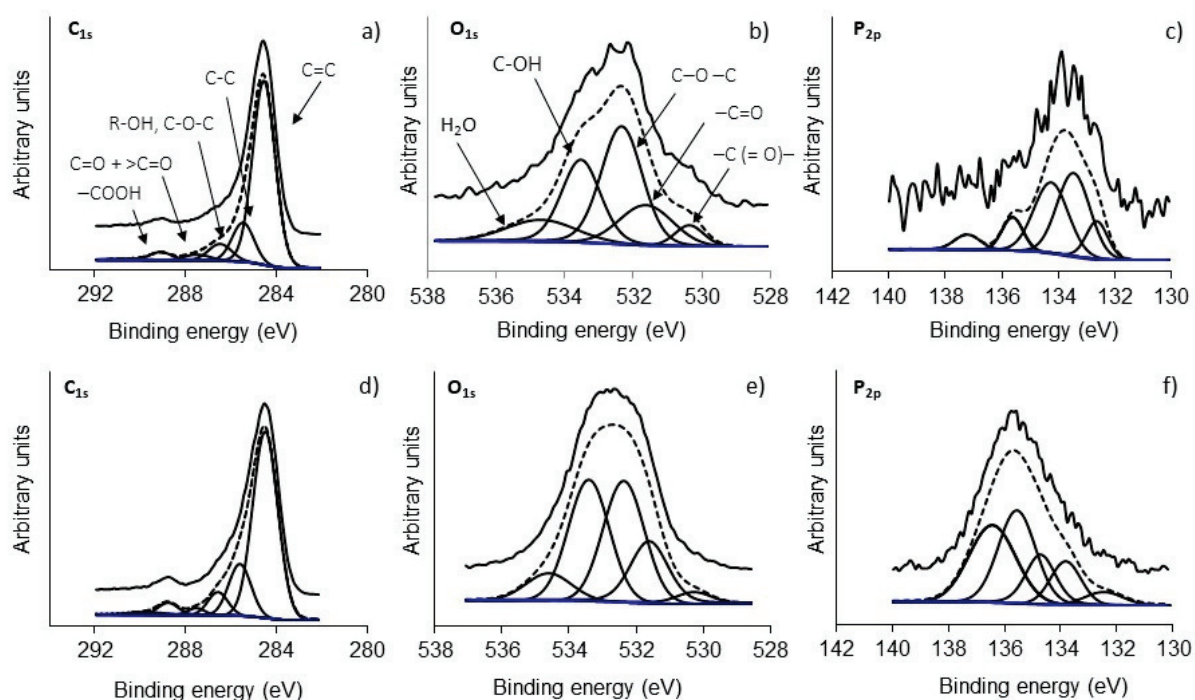
Three prominent peaks were observed; The first signal intensity (I_D) of the D-peak at ~1335 cm⁻¹, corresponding to the vibrations of carbon atoms with sp^3 bonding assigned to defects and disorder of the lattice and to the low symmetry carbon structure of graphite. The second signal intensity (I_G) of the G-peak at ~1590 cm⁻¹ (I_G), which is characteristic of graphite, corresponding to the sp^2 stretching vibration of carbon atoms in the graphite phase. The third peak was observed at 2710–2724 cm⁻¹, which can be attributed to second-order phonons [29]. Table 2 compiles the main physicochemical properties of BNCAs determined by XPS, SEM-EDX and Raman.

Table 2. Physicochemical properties of BNCA by XPS, SEM-EDX and Raman.

BNCA	XPS (wt%)					EDX (wt%)				Wavelength (cm ⁻¹)		I _D /I _G
	C _{1s}	O _{1s}	N _{1s}	P _{2p}	O/C (%)	C	O	N	P	D	G	
P500	84.7	11.7	0.5	3.1	13.8	31.2	49.6	-	19.2	1336.5	1585.6	2.00
P600	68.2	20.4	0.2	11.1	29.9	40.1	5.1	-	54.7	1333.6	1585.6	2.03
K600	81.9	17.5	0.6	-	21.4	82.4	17.6	-	-	1342.3	1583.7	1.87
K700	84.8	15.0	0.16	-	17.7	70.0	30.0	-	-	1345.2	1590.5	1.86
K800	82.9	16.88	0.21	-	20.4	67.0	33.0	-	-	1337.5	1590.5	1.89

The graphitization degree is determined from the ratio of I_D/I_G peak areas, and it is related to the carbon material structure as a measure of the extent of disorder in a carbon layer [30]. The I_D/I_G ratio is slightly lower for BNCA-K (~1.87) than BNCA-P (~2.0), indicating that graphitic carbon formation is associated with a loss of dissociable functional groups. An increased in the I_D/I_G ratio promotes a loose fibrous form, as shown by the SEM images of BNCA-P (Figure 2) [18], caused, in this case, by H₃PO₄ activation. The disorder in carbon surface distortions could be related to the microporous structure or disorganized regions near the edges, possibly as a result of the introduction of phosphorus functional groups to the surface [31]. Additionally, P600 showed a high I_D intensity and a low full width at the half maximum (FWHM) of the I_G (Figure 5b), which suggests that structural disorder is caused by the presence of oxygen and hydrogen atoms.

Figure 6 shows XPS high-resolution spectra of C_{1s}, O_{1s}, and P_{2p} for samples P500 and P600, and Figure 7 shows the C_{1s}, and O_{1s} spectra of samples K600 (a and b), K700 (c and d), and K800 (e and f). These peaks were deconvoluted according to the binding energy, corresponding to a specific functional group presented in Table S1 [31–33]. The BNCA samples presented O/C ratios between 13% and 30%, where P600 presented the highest percentage, suggesting that at 600 °C, surface oxidation came from functional groups of phosphors [32].

**Figure 6.** XPS high-resolution spectra of (a) C_{1s}, (b) O_{1s} and (c) P_{2p} regions for P500 and (d) C_{1s}, (e) O_{1s}, and (f) P_{2p} regions for P600.

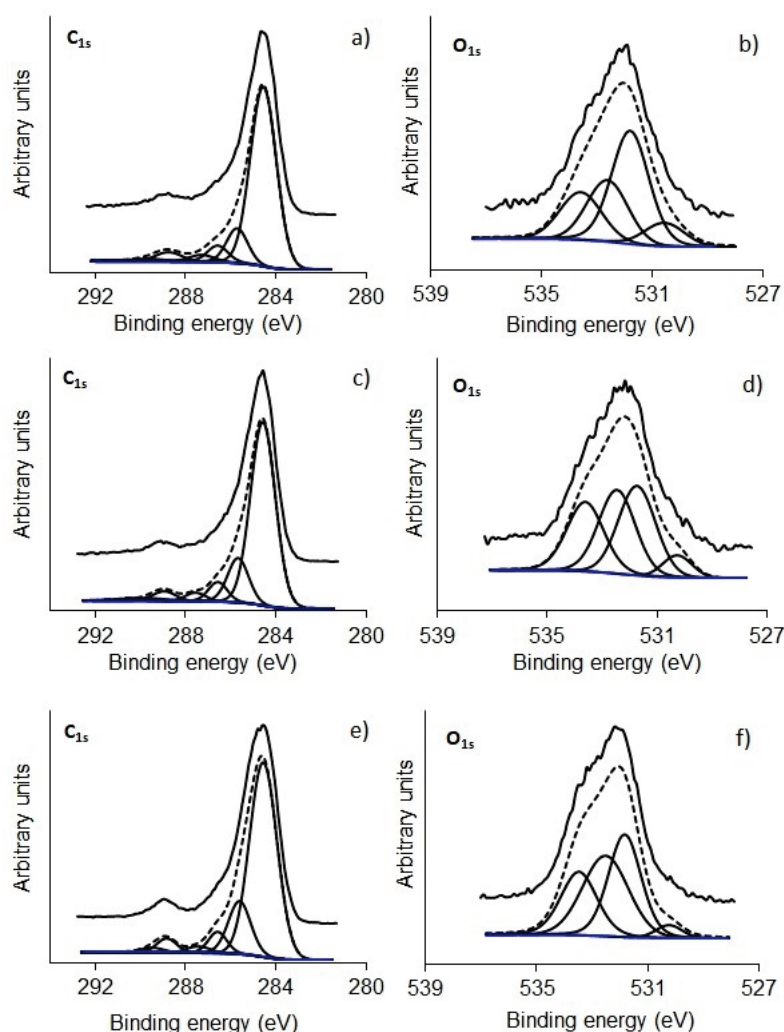


Figure 7. XPS high-resolution spectra of (a) C_{1s} and (b) O_{1s} regions for K600, (c) C_{1s} and (d) O_{1s} regions for K700 and (e) C_{1s} and (f) O_{1s} regions for K800.

The percentage of nitrogen on the surface was lower than 0.6 wt% (0.16–0.6 wt%), which may be associated with the residual impurities. However, samples treated at the lowest temperatures showed the highest percentage (P500 and K600). The phosphorus percentage on the surface was 3.1 wt% and 11.1 wt% for P500 and P600, respectively, which are higher than other biomasses activated with H_3PO_4 [34]. The P500 sample showed, in the P_{2p} region, a higher percentage (38%) at ~ 133.5 eV, which corresponds to the $C-PO_3$ or C_2-PO_2 functional groups. However, the P600 sample presented an increase in pyrophosphates functional groups at ~ 135.2 eV (33.5 wt%), and an increase in P_2O_5 at ~ 136 eV (33.7 wt%), while $C-PO_3$ or C_2-PO_2 groups at ~ 133.5 eV decreased (12 wt%). This suggests that activation temperature plays a key role in the formation of functional groups at the surface of the resulting carbons.

3.4. Electrochemical Characterization of BNCAs

Figure 8a,d show the Nyquist plots for the BNCAs. A semicircle in the high-frequency region is observed, followed by a gradual change in the slope from 45° to 90° , which represents Warburg diffusive behavior and is attributed to the diffusive effects of chemical species along the electrode [35]. In the low-frequency region (<1 Hz), a vertical line is observed and represents capacitive behavior. The P600, K700 and K800 samples presented the best capacitive behavior, while the P500 and K600 samples presented a distorted semicircle associated with difficulty in electrolyte diffusion inside the electrode in the mid-frequency region [36].

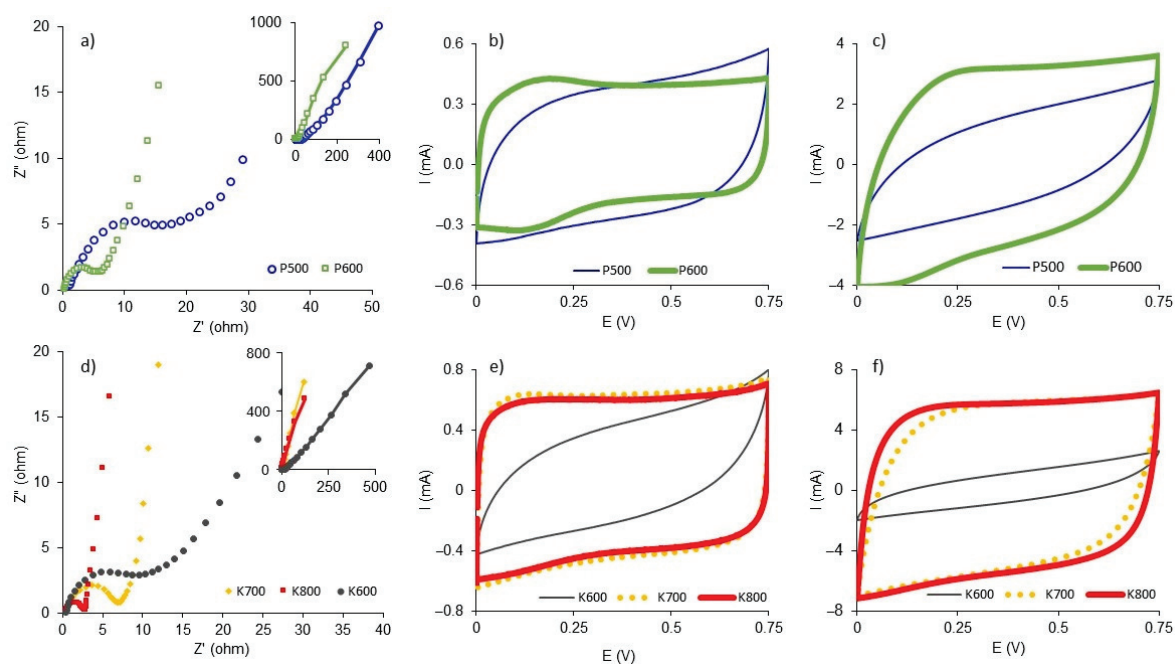


Figure 8. (a) Nyquist plot, cyclic voltammetry curves at (b) 2 mV s^{-1} and (c) 25 mV s^{-1} for samples BNCA-P, and (d) Nyquist plot and cyclic voltammetry curves at (e) 2 mV s^{-1} and (f) 25 mV s^{-1} for samples BNCA-K at $1 \text{ M H}_2\text{SO}_4$.

The CVs of the K700 and K800 samples showed a quasirectangular, horizontal and symmetrical shape at low (Figure 8e) and high (Figure 8f) scan speeds, which is similar behavior to that of an ideal electrochemical double-layer capacitor (EDLC). The P500 (Figure 8b,c) and K600 (Figure 8e,f) samples presented CVs with a nonrectangular shape and that were deviated from being horizontal, which was related to the diffusion limitations of electrolyte ions in the micropores. The P600 sample presented ideal behavior at 2 mV s^{-1} (Figure 8b) with an additional shoulder at a lower potential, which was associated with pseudocapacitive effects, most likely due to the presence of phosphorus heteroatoms [37,38] on the surface (11.1 wt%). However, at 25 mV s^{-1} (Figure 8c), ideality was lost due to the diffusive limitations of the electrolyte inside the carbonaceous matrix. Part of the electrode surface was inaccessible, and just its external surfaces interacted.

Table 3 presents the main electrochemical properties of the BNCAs in terms of the CV and EIS. The K700 and K800 samples presented the highest values of gravimetric capacitance (C_{EIS}), 181 F g^{-1} and 219.5 F g^{-1} , respectively, values higher than those reported in the literature for materials produced under similar conditions [18], but slightly lower than those reported for some electrodes prepared with doped materials mainly with nitrogen, phosphorus and sulfur heteroatoms ($43\text{--}509 \text{ F g}^{-1}$).

Table 3. Electrochemical properties of BNCAs determined by cyclic voltammetry and electrochemical impedance spectroscopy.

BNCA	$C_{CV} (\text{F g}^{-1})/V_b (\text{mV s}^{-1})$				C_{EIS} (F g^{-1})	ESR (Ω)	$-\varphi$ ($^\circ$)	τ (s)	IC ($\mu\text{F cm}^{-2}$)
	2	5	10	25					
P500	112	84	64	43	99.8	23.2	68.1	159.2	13.9
P600	107	103	94	78	129.9	7.4	73.3	5.8	16.4
K600	119	79	57	32	111.6	11.0	56.8	159.2	13.8
K700	192	182	169	143	181.6	8.8	78.8	2.3	23.9
K800	180	179	169	151	219.5	2.9	75.7	0.9	24.6

The equivalent series resistance (ESR) value can be calculated in the high-frequency area of the Nyquist plot. The ESR represents the sum of the contact resistance, charge transfer resistance, and diffusion resistance in electrolyte pores during the electrochemical process. The ESR is calculated from the vertical line extrapolation at medium-low frequencies of the Nyquist diagram and its cut on the abscissa [39]. The K800 sample presented the lowest ESR value ($2\ \Omega$), which benefits electrochemical double-layer formation, while P500 showed the highest ESR value ($23\ \Omega$) mainly due to electrolyte diffusion limitations. The relaxation time (τ) is how quickly a capacitor can be charged or discharged. The K800, K700 and P600 samples exhibited the lowest relaxation times due to their low ESR in the order $K800 < K700 < P600$. The phase angle (φ) is obtained from the bode diagram at the lowest frequency. K700 showed the lowest phase angle value near -90° and the lowest I_D/I_G value, properties that suggest better conductivity due to high graphitization, and the behavior of an ideal supercapacitor [40,41].

From the EIS and CV curves (Figure 8), no supercapacitor works entirely as an ideal capacitor. Therefore, the electrical equivalent circuit could be represented by a “Randles circuit” that is conformed for a double layer capacitance (C_{dl}) component parallel to the Faradaic charge (R_F) and impedance Warburg (W) components, and these are in series with an electrolytic resistance (R_s) [24]. The K800 sample presented the highest C_{EIS} and IC value ($24.6\ \mu F\ cm^{-2}$). This last value is slightly higher than that reported for a clean graphite surface [42] and indicates deeper electrolyte accessibility to micropores. The electrochemical behavior of the P600 sample suggests that the presence of phosphorus functional groups improves surface conductivity. However, phosphorus superficial functional groups (135 eV and 136 eV) did not improve electrode surface wettability by electrolytes or the formation of EDLC. Additionally, the P500 sample had a P_{XPS} of 3.1 wt% and an S_{BET} that was like that of other samples. Nevertheless, it presented low electrochemical performance, which may have been related to its compact external morphology, making it difficult for the electrolyte access the micropores.

Microporosity seems to have the largest effect on capacitance (Figure 9a). According to the pore distribution (Figure 4e), the BNCA-K samples presented narrow pore distribution below 2 nm, with an average micropore size between 0.9 and 1.27 nm, which is higher than the optimal size for hydrated molecules SO_4^{2-} , which is 0.7 nm [43]. Figure 9b,c show that capacity increases as ESR decreases as a consequence of the presence of oxygen and phosphorus surface functional groups, mainly C—O single bonds (532.3 eV). Oxygen superficial functional groups are important for the introduction of pseudocapacitive effects that influence the increase in IC .

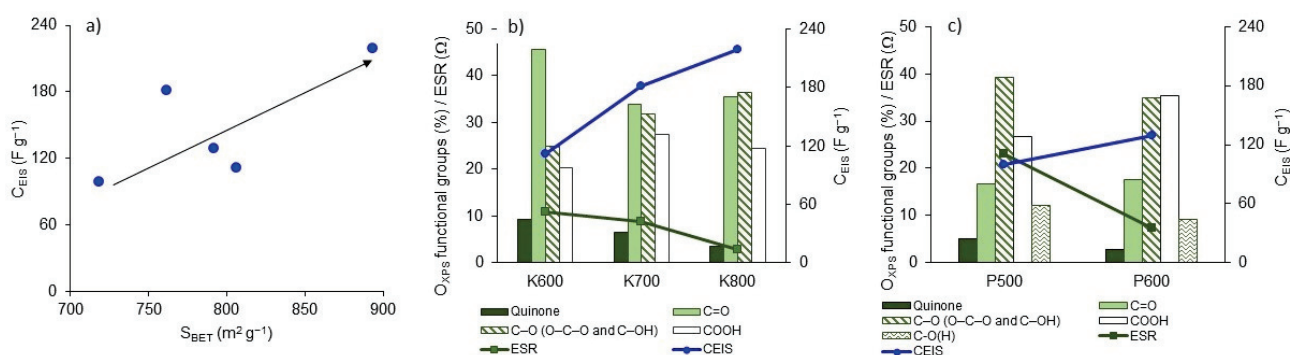


Figure 9. Relationship of gravimetric capacitance (C_{EIS}) to (a) S_{BET} , ESR and oxygen superficial functional groups of (b) BNCA-K and (c) BNCA-P.

Based on the data shown in Table 3, Figure 10a depicts a reduction in capacitance retention as a function of the scan rate increasing. The K800 sample presented the highest R_{CV} (84%) at $25\ mV\ s^{-1}$, followed by K700 (75%) and P600 (73%). The low behavior of the P500 and K600 samples is attributed to the presence of functional groups, which hinder

electrolyte ion diffusion. Figure S2 shows the electrochemical performance of the K700 sample characterized in a three-electrode cell configuration. The cyclic voltammetry curves show a quasirectangular, horizontal, and symmetrical shape, with the presence of redox peaks in -0.3 V and 0.3 V being assigned to quinone/hydroquinone groups. Additionally, the charge and discharge curves were symmetrical, suggesting supercapacitor behavior. The capacitance values were higher than those shown in Table 4, verifying that these materials could be considered excellent for electrodes for supercapacitor devices.

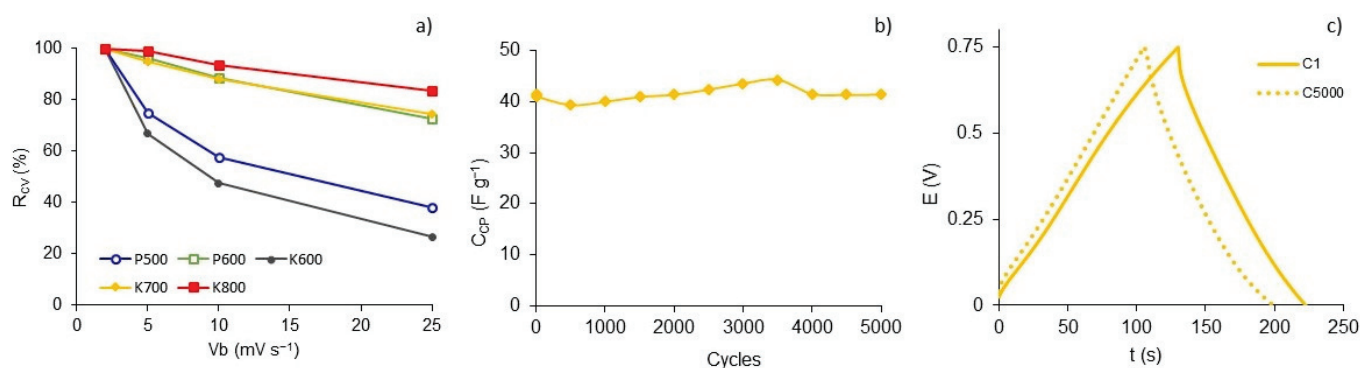


Figure 10. (a) Capacitance retention as a function of different scan rates, (b) gravimetric capacitance (C_{CP}) evolution during 5000 charge and discharge cycles at 0.313 A g^{-1} in $1 \text{ M H}_2\text{SO}_4$ of K700, and (c) charge–discharge curves between 0 and 0.75 V of the first and last cycle of K700.

Table 4. Gravimetric capacities of electrodes from doped BNCs. 3E: three-electrodes configuration; 2E: two-electrodes configuration.

Reference	Cellulose		Dopant Agent	S_{BET} ($\text{m}^2 \text{ g}^{-1}$)	Capacitance (F g^{-1})
Wang et al. [44]	Hydrolyzed cotton	N:	Urea	123–366	220–275 3E 6 M KOH
Li et al. [45]	BC, Hainan Yide Food Industry Co.	N, P, and S:	$(\text{NH}_4)_2\text{HPO}_4$, $(\text{NH}_4)_2\text{SO}_4$ $(\text{NH}_4)_2\text{HPO}_4 / (\text{NH}_4)_2\text{SO}_4$	296–498	80–255 2 M H_2SO_4
Liu et al. [46]	BC, Guilin Qi Hong Technology Co.	N, P, and S:	Thiourea ($\text{CH}_4\text{N}_2\text{S}$) Rogor ($\text{C}_5\text{H}_{12}\text{NO}_3\text{PS}_2$, 40 wt%) Phoxim ($\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_3\text{PS}$, 40 wt%)	261–589	132–509 3E and 2E 0.5 M H_2SO_4
Fang et al. [47]	BC, Hainan Yide Food Industry Co.	O:	Juglone (5-hy-droxy-1,4-naphthalenedione) Carbon nanotubes	95–102	74.8–461.8 3E 1 M H_2SO_4
Wang et al. [48]	BC, Hainan Yeguo Food Company	N:	Polypyrrole Carbon nanotubes (20–50 μm)	—	$0.15\text{--}0.5 \text{ F cm}^{-2}$ Gel PVA/ H_2SO_4
Zhang and Chen [49]	BC, Hainan Yide Food Co.	N:	Polydopamine	191–464	26–260 2E and Coin CR2032 1 M H_2SO_4
Bai et al. [13]	BC. Yeguo Foods Co.	O:	Poly(ethylene-co-vinyl alcohol)	747–2189	420 3E 6 M KOH
Wannasen et al. [50]	BC. <i>Gluconacetobacter xylinum</i> en D-glucose	N, P, Co:	Cobalt(II) nitrate hexahydrate Phosphoric acid	12–44	43.3 (158.5 mF cm^{-2}) 3E 3 M KOH
Xu et al. [51]	BC. Hainan Yide Foods Co., Ltd.	N:	1 M Pyrrole	—	459.5 3E 1 M NaCl

Figure 10b presents high stability after the 5000 charge and discharge cycles at 0.313 A g^{-1} for the K700 sample. The charge and discharge curves of the first and last cycles are presented in Figure 10c, which have a quasitriangular and symmetrical shape that indicates ideal conservation. The capacitance retention after 5000 cycles was 66% compared

to the first cycle at 0.313 A g^{-1} . The increase in capacitance around cycle 3500 was related to the contribution of pseudocapacitance by surface functional groups [52].

4. Conclusions

BNC obtained from *K. Medellinensis* using fique juice as a culture medium allowed the obtention of chemically activated carbons with high specific surface areas. The materials were evaluated by different electrochemical techniques, obtaining the highest gravimetric capacitances between 180 and 220 F g^{-1} , corresponding to those of the KOH-activated materials at 700°C and 800°C . These materials showed higher capacitance retention, and after 5000 cycles, a retention of 66% was still present. Temperature is a crucial parameter for developing microporosity and thus improving electrochemical double-layer formation. H_3PO_4 activation allowed a high content of phosphorus heteroatoms to be incorporated into the surface; however, the functional groups formed did not show any significant contribution to the pseudocapacitive effects that could have increased the overall electrode performance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym15071760/s1>. Figure S1: SEM images and EDX spectra of BNCA-P at 500°C X10000 (a) and 600°C X50000 (b and c); Table S1: Binding energies of functional groups of XPS high-resolution spectra C_{1s} , O_{1s} , and P_{2p} ; Figure S2: (a) Cyclic voltammetry curves at 0.5 mV s^{-1} , (b) Charge/Discharges curves at 0.125 A g^{-1} , and (c) Capacitance as a function of different scan rates for the K700 sample.

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Article

Lithium Iron Phosphate/Carbon (LFP/C) Composite Using Nanocellulose as a Reducing Agent and Carbon Source

Macarena Kroff ¹, Samuel A. Hevia ^{2,3}, James N. O'Shea ⁴, Izaskun Gil de Muro ⁵, Verónica Palomares ^{5,6}, Teófilo Rojo ⁵ and Rodrigo del Río ^{1,3,*}

¹ Departamento de Química Inorgánica, Facultad de Química y de Farmacia, Pontificia Universidad Católica de Chile, Santiago 7820244, Chile; makroff@uc.cl

² Instituto de Física, Pontificia Universidad Católica de Chile, Santiago 6904411, Chile; sheviaz@uc.cl

³ Centro Investigación en Nanotecnología y Materiales Avanzados UC (CIEN-UC), Pontificia Universidad Católica de Chile, Santiago 7820244, Chile

⁴ School of Physics and Astronomy, University of Nottingham, Nottingham NG7 2RD, UK; j.oshea@nottingham.ac.uk

⁵ Departamento de Química Orgánica e Inorgánica, Facultad de Ciencia y Tecnología, Universidad del País Vasco UPV/EHU, 48940 Leioa, Spain; izaskun.gildemuro@ehu.eus (I.G.d.M.); veronica.palomares@ehu.eus (V.P.); teo.rojo@ehu.eus (T.R.)

⁶ BCMaterials, Parque Científico de la UPV/EHU, 48940 Leioa, Spain

* Correspondence: rdelrioq@uc.cl; Tel.: +56-22-354-1522

Abstract: Lithium iron phosphate (LiFePO_4 , LFP) is the most promising cathode material for use in safe electric vehicles (EVs), due to its long cycle stability, low cost, and low toxicity, but it suffers from low conductivity and ion diffusion. In this work, we present a simple method to obtain LFP/carbon (LFP/C) composites with different types of NC: cellulose nanocrystal (CNC) and cellulose nanofiber (CNF). Microwave-assisted hydrothermal synthesis was used to obtain LFP with nanocellulose inside the vessel, and the final LFP/C composite was achieved by heating the mixture under a N_2 atmosphere. The resulting LFP/C indicated that the NC in the reaction medium not only acts as the reducing agent that aqueous iron solutions need (avoiding the use of other chemicals), but also as a stabiliser of the nanoparticles produced in the hydrothermal synthesis, obtaining fewer agglomerated particles compared to synthesis without NC. The sample with the best coating—and, therefore, the best electrochemical response—was the sample with 12.6% carbon derived from CNF in the composite instead of CNC, due to its homogeneous coating. The utilisation of CNF in the reaction medium could be a promising method to obtain LFP/C in a simple, rapid, and low-cost way, avoiding the waste of unnecessary chemicals.

Keywords: lithium-ion batteries; lithium iron phosphate (LFP); nanotechnology

1. Introduction

The development of new generations of Li-ion batteries (LIBs) is in constant growth for their use as the energy sources for electric vehicles (EVs) [1,2], as well as for energy storage for sustainable energies, contributing to their intermittence [3–5]. Lithium iron phosphate (LiFePO_4 , LFP) cathodes are widely used for these purposes because they have the advantages of low cost, environmental friendliness, thermal stability, and low toxicity, unlike the most widely used cathode material LiCoO_2 [6]; however, they have the disadvantages of low electric conductivity and poor lithium-ion diffusivity, limiting their use by decreasing their practical capacity [7,8]. Many researchers have studied the diminution of the particle size to improve the diffusion of lithium ions, due to the particle reduction shortening the distances to cover [8–10]. Consequently, the synthesis conditions are vital for obtaining a small particle size; hence, there exist many methods to obtain these compounds: solid-state, hydrothermal, solvothermal, etc. [11]. Hydrothermal synthesis is the simplest method to obtain nanoparticles, because it uses simple compounds such as

water [12,13]; thus, the use of toxic and harmful chemicals is avoided. Unfortunately, this method faces the difficulty of iron-ion oxidation in aqueous media; hence, it is necessary to include an auxiliary compound that prevents the oxidation. Previous works have shown that hydrothermal synthesis combined with microwave methodology can be used to obtain a homogeneous particle distribution, shorter reaction time, and small particle size, which could be beneficial for cathodes in LIBs [10,14].

On the other hand, to improve the low electric conductivity of LFP, the most widely used method is coating the nanoparticles with carbon compounds. Many different compounds have been studied successfully, including graphene, carbon nanotubes, and more sustainable sources such as glucose and sucrose. Researchers have shown that organic sources of carbon compounds can cover the particles homogeneously [15–17], unlike inorganic sources such as carbon nanotubes, whose cylindrical shape makes it difficult for them to cover the nanoparticles completely [18–20]. Kanagaraj et al. investigated the incorporation of multi-walled carbon nanotubes (MWCNTs) using an *ex situ* pathway, obtaining large particles with a lack of electron conduction, leading to a poor specific capacity close to 22 mAhg^{−1} at 0.2 C-rate, using an inorganic compound [21]. Conversely, Meng et al. used *in situ* coating with an organic source to obtain an LFP material with 1–2 nm of carbon coating and a specific capacity close to 162 mAhg^{−1} at 0.1 C-rate [22].

Therefore, the use of organic sources for the carbon compounds in LFP/C composites seems to be a promising approach that needs to be improved, because one of the main problems is the low quality of the final carbon obtained—since it requires an additional heat treatment [23], in contrast to inorganic compounds—and also, typically, larger particles (attributed to precursors with large dimensions, such as glucose or sucrose [24]) are obtained. For these reasons, it is necessary to search for new materials to obtain LFP/C composites with a smaller particle size.

Nanocellulose (NC), a nanoscale material of cellulose fibre, is the most abundant natural polymer in the world. It can be classified into different types, of which cellulose nanocrystal (CNC) and cellulose nanofiber or nanofibrillated cellulose (CNF) are the most important [25,26]. They differ in the methodology used to obtain them: chemical treatment for the CNC, and delamination of the wood using mechanical procedures in the case of CNF [27–29]. Using this kind of material as the carbon source can avoid the use of inorganic, expensive, and toxic compounds such as graphene or carbon nanotubes, and they can cover LFP nanoparticles homogeneously due to their organic nature. NC can also act as the reducing agent [30,31] that iron ions need in hydrothermal synthesis; thus, this nanomaterial is an encouraging compound due to its small particle size in contrast to other organic sources that have been used. Few investigations have used some type of NC to form LFP/C composites. Park et al. [32] reported a mixture of CNF with graphene oxide and LFP that, after heat treatment, formed a composite in which the carbon was produced from the carbonisation of CNF and reduced graphene. This cathode exhibited good performance, with a discharge capacity of 168.9 mAhg^{−1} at 0.1 C-rate, and the researchers attributed these good results to the CNF because it can prevent the GO stacking, act as an extra carbon source, and avoid agglomeration of LFP during annealing. This methodology mixes carbon compounds after the synthesis of LFP—an *ex situ* pathway. In contrast, in this work, we present a simple methodology using only the reagents for LiFePO₄, NC, and water as the solvent to obtain the final LFP/C composite—a much more efficient and environmentally friendly methodology that avoids the use of harmful reagents and multiple steps. Moreover, we report an easy method to obtain the LFP/C composite, using NC in the hydrothermal reaction medium as a carbon source, a reducing agent for the iron ions, and a stabiliser to avoid grain size growth, creating a simple and new methodology to create sustainable LFP/C materials. For this purpose, we mixed the precursors of LFP with CNC and CNF separately for further comparisons, in deionised water, and incorporated them into a microwave reactor vessel to heat at different temperatures and for different reaction times. Then, the dry mixture of LFP/NC was carbonised in a tube furnace at 600 °C for 1 h in a N₂ atmosphere to obtain the LFP/C composite.

2. Materials and Methods

Lithium hydroxide (LiOH 98%), iron(II) sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), and phosphoric acid (H_3PO_4 85%) were purchased from Merck. CNC (8 wt. %) was obtained from Blue Goose Biorefineries Inc., Canada, and CNF (15% solids) was purchased from the Process Development Center, the University of Maine, USA. As solvents, technical ethanol (EtOH 96%) and Milli-Q water ($18.2 \text{ M}\Omega\text{cm}^{-1}$) were used for all experiments. Lithium hexafluorophosphate in ethylene carbonate and dimethyl carbonate solution (1.0 M LiPF_6 in EC/DMC 50/50 *v/v*, battery grade) was used as an electrolyte; poly(vinylidene fluoride) $M_w \sim 534,000$ (PVDF), carbon Vulcan XC-72C, and 1-methyl-2-pyrrolidinone (NMP) were purchased from Merck and were used to create the electrodes. All reagents were used without further purification.

The synthesis of LFP and its composites was carried out as follows: In a microwave vessel, the precursors for LFP were mixed (LiOH, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and H_3PO_4 , at a molar ratio of 3:1:1) as described in the literature [12,33]. To this mix, we added different weight percentages of CNF and CNC separately for further comparisons, until a homogeneous suspension was formed. First, the LiOH was put into the vessel with ultrapure water until full dissolution, and then H_3PO_4 was added to form Li_3PO_4 . The next step was to incorporate the NC and mix it for a few minutes. Finally, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was added until full dissolution; ultrapure water and ethanol were then used to fill the total vessel volume (25 mL maximum) and fix the pH of the solution between 6.5 and 8.1, since at this pH a pure LFP phase can be obtained [34,35]. For synthesis without NC, the incorporation of this material in the vessel was omitted. The vessel with the mixed precursor was put into the CE Corporation Discover SP microwave, where 200 W and 17.2 bars were the experimental power and pressure conditions to control the reaction, respectively. Different temperatures and reaction times were selected to analyse the formation of LFP through this route. After the microwave reaction, the mixture was washed and centrifuged several times with deionised water, before being left to dry overnight and obtain LFP or LFP/NC powder. To obtain the LFP/C composites, the powder obtained in the previous step (LFP/NC) was put into a tube furnace for heat treatment at 600°C for 1 h, with a N_2 atmosphere (99.9% yield, from Indura, Chile).

Morphological characterisations were carried out with field-emission scanning electron microscopy (FE-SEM) using an FEI microscope (model: Quanta FEG 250) and transmission electron microscopy (Philips CM200) with an accelerating voltage of 200 kV and a point resolution of 0.235 nm. FT-IR spectroscopy was carried out using a Nicolet iS10 (Thermo Scientific) between 4000 and 400 cm^{-1} wavelengths, using KBr pellets at 1:100% wt. Raman spectra were used to analyse the presence of carbon in the created composites using a WITec Alpha 300-RA spectrometer with a 532 nm laser; the D and G bands were deconvoluted with Origin software using Lorentzian curves. XRD diffractograms were measured with a Bruker D8 Advance, with $\text{CuK}\alpha_1 = 1.5604 \text{ \AA}$ between 2θ angles of 10° and 80° , with 0.20° intervals. Rietveld refinements were analysed using FullProf Suite software. The XPS characterisation was performed with a Devi-sim (SPECS) near-ambient-pressure X-ray photoelectron spectroscopy instrument operating under ultrahigh vacuum, comprising a Phoibos 150-NAP hemispherical analyser and a monochromatic Al $\text{K}\alpha$ X-ray ($h\nu = 1486.6 \text{ eV}$) source. A flood gun was used for charge compensation. Binding energies were calibrated to the C 1s peak at 284.8 eV; for background subtraction, a Shirley background was used, and for data treatment CasaXPS software was used to fit the spectral curves to pseudo-Voigt lineshapes.

The cathode formation was accomplished as follows: A mix of active material (LFP/C), PVDF, and conductive carbon (Vulcan XC-72) at a weight proportion of 8:1:1 was added to NMP in order to form a homogeneous solution through stirring for 1 h. Then, the slurry was placed onto the aluminium foil with a specific thickness of 100 μm , using the Dr Blade technique. Then, the foil was dried and cut into electrodes of active material of 11 mm in diameter. Coin cell assemblies were prepared inside a glovebox using metallic Li of 13 mm in diameter for the counter and reference electrodes, two drops of electrolyte were

used per cell (1.0 M LiPF_6 in EC/DMC 50/50 *v/v*), and polyethylene (PE) was employed as the separator. Cyclic voltammetry was carried out using an Ivium Vertex X.S, between 2.8 and 4.1 V versus Li/Li^+ at 0.1 mVs^{-1} . Charge and discharge curves were measured at a 0.1 C-rate between 2.8 and 4.1 V versus Li/Li^+ in a Biologic MPG-2 battery cycler.

3. Results and Discussion

3.1. Synthesis of LFP/C Composites

Table 1 shows the microwave-assisted hydrothermal synthesis conditions investigated for LFP using CNF and CNC as reducing agents and carbon sources. The time and temperature of the reaction were previously investigated for this synthesis using ethanol as a reducing agent (without carbon), as shown in Figure S1. From this analysis, we concluded that 150°C and 15 min of reaction are the minimum conditions to form LFP using the proposed synthesis method.

Table 1. Microwave-assisted hydrothermal synthesis conditions of LFP using different types and amounts of NC (CNF and CNC).

Sample	Type NC	% wt. NC	Temperature ($^\circ\text{C}$)	Time (min)
N1	CNC	0.15	150	15
N2	CNC	1	150	15
N3	CNF	0.15	150	15
N4	CNF	1	150	15
N5	CNC	0.15	150	30
N6	CNC	1	150	30
N7	CNF	0.15	150	30
N8	CNF	1	150	30
N9	CNC	0.15	205	15
N10	CNC	1	205	15
N11	CNF	0.15	205	15
N12	CNF	1	205	15
N13	CNC	0.15	205	30
N14	CNC	1	205	30
N15	CNF	0.15	205	30
N16	CNF	1	205	30

The characterisations were performed after heat treatment at 600°C for one hour in a tube furnace with an inert atmosphere, in order to carbonise the NC into a conductive material and form LFP/C composites, due to NC being a non-conductive material.

Figure 1 shows the diffraction peaks of LFP/C composites obtained with different percentages of NC and under different synthesis conditions; for purposes of comparison, compounds without NC and with and without heat treatment (HT) were added (samples E1–E4). The figure shows how the diffraction peak changes according to the synthesis conditions— 150°C and 15 min (Figure 1A), 150°C and 30 min (Figure 1B), 205°C and 15 min (Figure 1C), and 205°C and 30 min (Figure 1D)—and the presence of NC in different amounts (0.15% wt. and 1% wt.). All of the conditions exhibited the diffraction peaks of LFP with an olivine structure and *Pnma* space group [34]; no other peaks of iron species were observed [36], except for sample E4 (Figure 1D), where other diffraction peaks around 22° and 32° were observed and could be attributed to a typical reaction intermediate such as Li_3PO_4 ; however, when NC was used in any form, no other peaks were observed (N samples, Figure 1D).

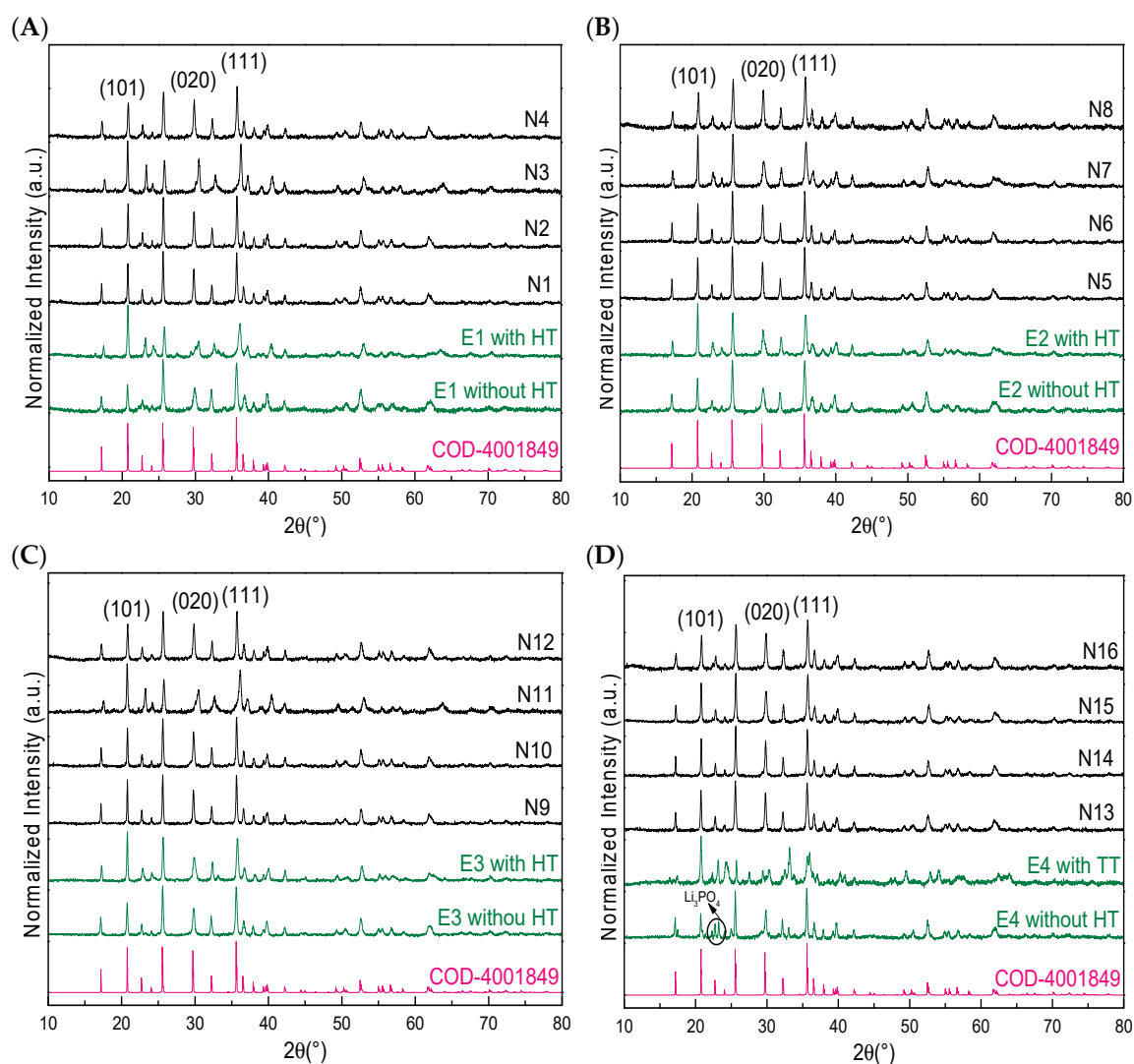


Figure 1. XRD diffractograms of LFP obtained with ethanol before and after the heat treatment (E samples) and with NC (N samples) to form LFP/C composites under different synthesis conditions: (A) 150 °C and 15 min; (B) 150 °C and 30 min; (C) 205 °C and 15 min; (D) 205 °C and 30 min.

It can be observed that after the heat treatment of the samples without NC (E samples), the signal of the crystalline phase located at 20.7° was the peak that increased the most in intensity, followed by the signal at 25.6°. This means that after the heat treatment, the crystalline face (101) became preferential, followed by (111). The crystalline face (020), which is the crystalline face responsible for the lithium-ion diffusion [34], increased in intensity when NC was used in the synthesis in all cases but did not increase in the samples without carbon and heat treatment. Therefore, it can be said that the incorporation of NC in the synthesis medium helps to improve the intensity of this crystalline face where the lithium diffusion is located—something that heat treatment does not do.

In general, with the lowest reaction time (Figure 1A,C), low-intensity diffraction peaks of the composites were observed, in contrast to the longer reaction times (Figure 1B,D); the same happened with the E samples (i.e., those without NC). These results show that the reaction time is crucial for obtaining LFP with crystalline and stable behaviour in order to avoid oxidation and the appearance of undesirable diffraction peaks when HT is implemented, independent of the incorporation of NC.

Regarding the NC concentrations, we can usually see a higher intensity with a higher amount of NC. It is possible to notice a decrease and the appearance of unwanted signals close to the lithium-ion diffraction peak (020) located at 29.7° with the sample created

with 0.15% wt. of CNF, especially with the shortest reaction time (N3; Figure 1A and N11; Figure 1C), which does not occur with a higher percentage of CNF (N4; Figure 1A and N12; Figure 1C). This difference may be due to the heat treatment used, because the amount of NC incorporated in the composite is carbonised; this process could lead to the LFP material being unrecovered when a low amount is used (like 0.15% wt.), whereas using a higher amount of NC (1% wt.) makes this less likely.

When we compare CNC and CNF, we can observe wider peaks when using CNF rather than CNC; thus, we can expect smaller crystallites with CNF and, consequently, smaller particles. In order to evaluate this parameter, FE-SEM images were taken.

Figure 2 shows the FE-SEM images obtained with CNC and CNF (samples N1 to N16). The average particle size was calculated with 200 particles as n-observations, using ImageJ software; Table S1 and Figure S2 show the values obtained and the particle sizes of the E samples for comparisons. In all cases, using CNC or CNF in the synthesis of LFP decreased the average particle size compared to synthesis without NC, demonstrating that the NC acts as an anti-agglomerate agent, helping to create different growing nuclei, as shown in previous studies with other compounds, such as ethylene glycol or urea [37,38]. The particle size differences depend not only on the amount and type of NC, but also on the synthesis conditions of the LFP compound; the largest differences in average particle size were obtained under synthesis conditions involving a longer reaction time (30 min; Figure 2E–H,M–P).

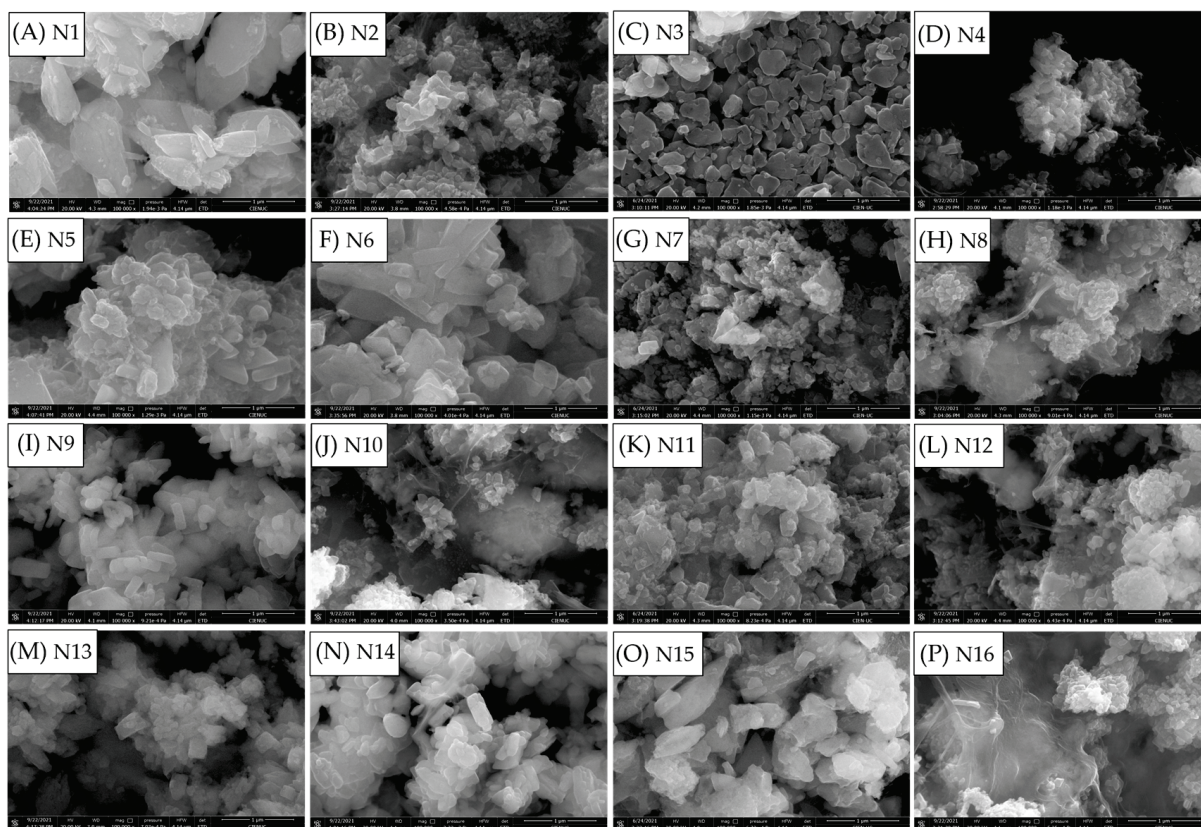


Figure 2. FE-SEM images of LFP/C composites with NC (N samples): (A) N1; (B) N2; (C) N3; (D) N4; (E) N5; (F) N6; (G) N7; (H) N8; (I) N9; (J) N10; (K) N11; (L) N12; (M) N13; (N) N14; (O) N15; (P) N16.

In most cases, smaller average particle sizes were obtained when CNF was used in the reaction medium instead of CNC, which is consistent with what was previously seen in the XRD results; hence, the particles obtained using this NC were smaller. Small particle size could be achieved with CNF, because it is known that its fibres are longer than those of CNC [39,40]. Therefore, CNF fibres could avoid agglomeration during hydrothermal synthesis in the microwave vessel better than the shorter CNC fibres. Finally, using a

higher amount of NC (1% wt.), smaller particles were obtained; the biggest difference was obtained under the synthesis conditions of 150 °C for 30 min: 147 nm with CNC (Figure 2B) vs. 92 nm with CNF (Figure 3B). This indicates that working with a lower percentage of NC (0.15% wt.) is not sufficient to avoid agglomerations and improve the growth nuclei, leading to larger particle sizes in comparison with when a higher percentage of NC is used. Because of this, it is better to work with 1% wt. NC in the microwave vessel instead of lower percentages; on the other hand, the synthesis conditions of 150 °C for 30 min are the most promising for obtaining LFP, due to the better cell occupancy values with Rietveld refinement (Table S2), since this is enough time for the crystalline structure to be rearranged (unlike the 15 min synthesis condition).

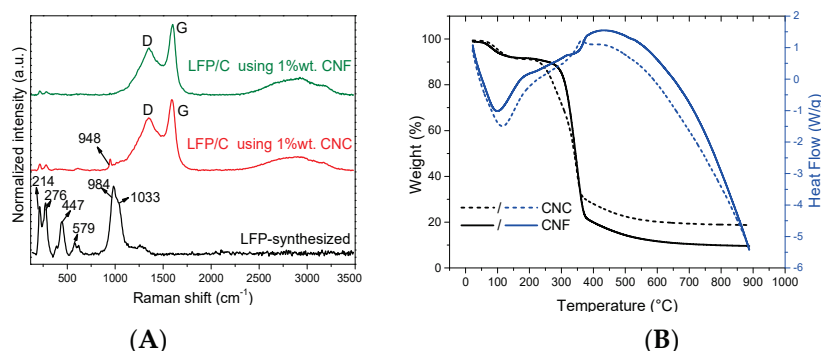


Figure 3. (A) Raman spectra of LFP/C composites produced with 1% wt. CNC (red line) and 1% wt. CNF (blue line); LFP synthesised without NC is added for comparison (black line). (B) TGA–DSC analysis of CNC and CNF.

To characterise the carbon obtained in the LFP/C composites, RAMAN spectroscopy was performed on the samples with higher % wt. NC (Figure 3). It can be observed that the typical D and G signals associated with defects in carbon species were observed only in composite materials, and not with the LFP synthesised without NC, indicating the presence of carbon derived only from NC in the synthesis.

The vibrations at 950, 1000, and 1070 cm⁻¹ correspond to the three characteristic bands of the phosphate group reported previously in LFP [24], which were not observed in the LFP compound synthesised by the proposed route. Instead, two vibrations were present at 984 and 1033 cm⁻¹, which were attributed to the decomposition of LiFePO₄ into γ -Li₃Fe₂(PO₄)₃, and the bands at 214 and 276 cm⁻¹ were attributed to the oxidation of the compound to α -Fe₂O₃ as a result of the laser intensity used in the Raman technique [21,41,42]. These vibrations were weakly observed in the composites formed with both CNC and CNF, confirming that the use of a carbon coating on the LFP particles protects against the decomposition or oxidation of the materials. This can also be confirmed by detecting the 948 cm⁻¹ vibration of the sample with CNC (Figure 3A; red line), because this band corresponds to the characteristic bands of the phosphate group in LFP.

To evaluate the quality of the carbon obtained in the composite, the ratios of the D- and G-band intensities (I_D/I_G) were measured, where the D band is attributed to disordered carbon bands and the G band to graphitic carbon [43]. A low band ratio is expected to be beneficial for good LFP cathode performance [44], as it would mean that there are more graphitic species (G bands) than amorphous species (D bands). The ratio values for CNF and CNC were 0.72 and 0.84, respectively; therefore, with CNF, we can obtain major graphitic species due to the carbon derived from NC starting to decay during thermal treatment, leaving the material unprotected. Thus, as CNC fibres are shorter than CNF fibres, the graphitic species of the composite decline more significantly in CNC compared to CNF. These results confirm that CNF is a better option for obtaining LFP/C composites with smaller particle size and major graphitic carbon species.

TGA–DSC analysis in a nitrogen atmosphere was carried out for both NCs (Figure 3B) to understand the heat treatment that was used to carbonise them. It was observed that

both NCs had the same pattern: a weight loss between 40 and 250 °C that could be attributed to internal water evaporation following glass transition, due to its endothermic behaviour in DSC [45,46]. A second weight loss took place between 250 and 450 °C for the carbonisation of NC into carbon compounds, followed by a continued weight loss due to the decomposition of the carbon material, indicating that carbonisation at 600 °C causes a continuous decomposition of carbon to occur, validating the results of the RAMAN and XDR analyses.

In order to determine the percentage of carbonised carbon after the thermal treatment in the LFP/C powder composites created in the 150 °C and 30 min reaction, an elemental analysis was carried out. Table 2 shows the weight percentages used in the microwave vessel to form the LFP material, along with the weight percentages of carbon obtained in the final LFP/C powder composites.

Table 2. Percentages of NC used in the synthesis, and percentages of carbon obtained after heat treatment in the LFP/C powder composites.

Sample	Type of NC	% wt. of NC Used in Vial Synthesis	% wt. Carbon Obtained in LFP/C Powder
N6	CNC	1	6.8
N8	CNF	1	12.6

The carbon obtained after heat treatment was 6.8% wt. with CNC and 12.6% wt. with CNF, confirming that CNC decomposes faster than CNF due to its shorter fibres [47]. This difference in carbon also explains the variation in particle size observed: 369 nm with CNC and 95 nm with CNF, because having less carbon in the composite could lead to LFP particles increasing their size faster than with a higher amount of carbon in the composite, indicating that CNF is a better material to be used for obtaining LFP compounds. In order to confirm this, an equal percentage of carbon (4% wt.) in an LFP/C powder composite was synthesised. Figure 4A shows the diffractograms of both composites, with peaks of only LFP, proving the presence of an olivine structure with the space group *Pnma* formed with the same percentage of carbon (4% wt. C).

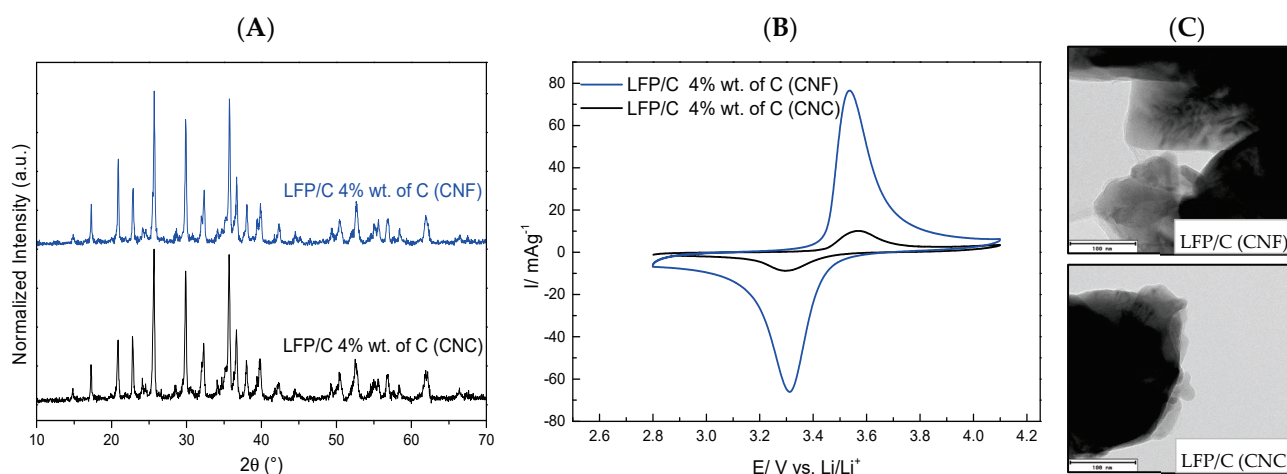


Figure 4. (A) XDR of both composites. (B) Cyclic voltammetry of LFP/C composites obtained with CNC and CNF at the same weight percentage of carbon (4 % wt.) in LFP/C, measured at 0.1 mV s⁻¹ between 2.8 and 4.1 V versus Li/Li⁺ in 1 mol L⁻¹ LiPF₆ with EC/DMC 1:1 *v/v*, using the coin cell CR-2032. (C) TEM images of LFP/C composites created with CNF (above) and CNC (below).

Then, an electrochemical characterisation by cyclic voltammetry (CV) was carried out to estimate their performance as cathodes for lithium-ion batteries. Figure 4B shows the typical oxidation peak at 3.5 V and reduction peak at 3.3 V, indicating the presence of LFP in both samples. However, higher current density per weight was obtained in LFP/C

composites using CNF as a carbon source (Figure 4B, blue line), which would indicate that the large fibres of CNF could recover the LFP particles better than the short fibres of CNC. The TEM images (Figure 4C) show that a more homogeneous coating is observed using CNF, which is consistent with the cyclic voltammetry results.

Finally, the performance of an LFP/C composite obtained with 1% wt. CNF (12.6% wt. final carbon) was evaluated. Figure 5A shows the cyclic voltammetry (black line) of the composite and of LFP synthesised without NC (i.e., carbon; red line). An improvement in electrochemical response and current density per weight can be observed in the composite material as a result of the incorporation of carbon from the NC carbonisation process, enhancing the reversibility of the LFP electrode. Electrical conductivity was measured using a four-point conductivity measurement of prefabricated electrodes of each compound (i.e., synthesised LFP and LFP/C composite), formed as follows: 80% wt. LFP or LFP/C, 10% PVDF, and 10% carbon Vulcan XC-72C, obtaining results of 11.7 kS/m and 49.9 kS/m for the synthesised LFP and the LFP/C composite, respectively, demonstrating that the incorporation of carbon improves the electrical conductivity and enhances the electrochemical performance of this material.

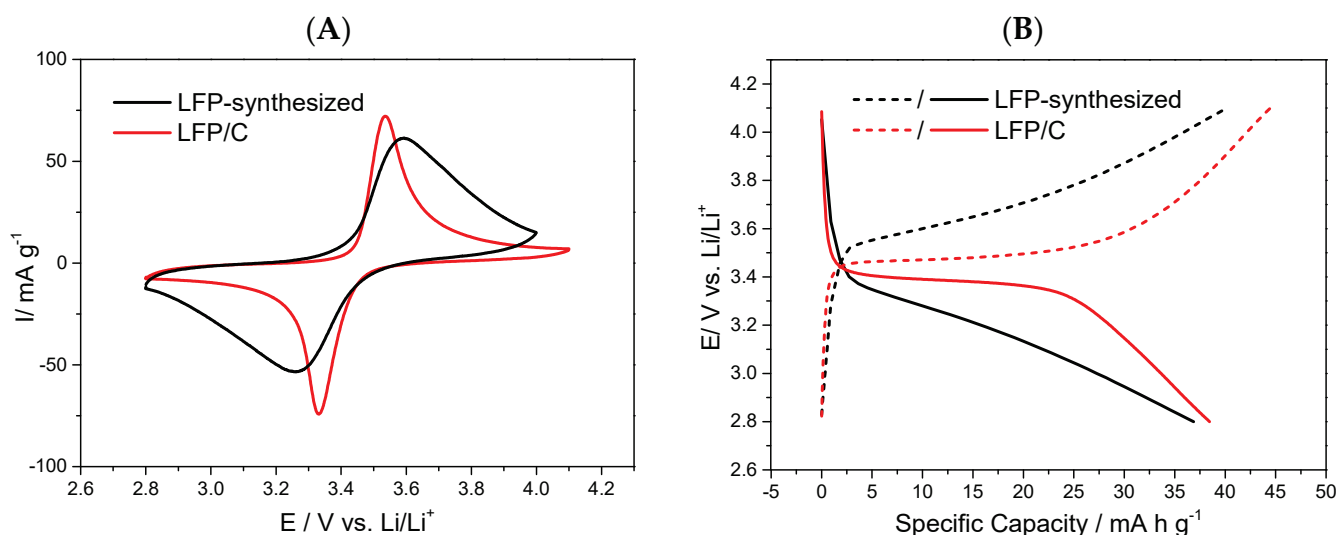


Figure 5. (A) Cyclic voltammetry of an LFP/C composite obtained with 12.6% wt. carbon derived from CNF and LFP synthesised without NC, measured at 0.1 mV s^{-1} between 2.8 and 4.1 V versus Li/Li^+ in 1 mol L^{-1} LiPF_6 with EC/DMC 1:1 *v/v*; second cycle. (B) Charge–discharge curve at 0.1 C-rate between 2.8 and 4.1 V versus Li/Li^+ using the coin cell CR-2032; second cycle.

The charge–discharge curves at 0.1 C-rate were derived for the LFP/C composite and the LFP synthesised without carbon materials to evaluate their performance as cathodes. Better behaviour and specific capacity were obtained in the proposed composite compared with LFP without the incorporation of NC during its synthesis, indicating that it is possible to obtain a better performance of LFP via the synthetic route in this work compared with LFP synthesised without the incorporation of NC.

The specific capacity of the proposed LFP/C composite was near 40 mAhg^{-1} at 0.1 C, indicating that an improvement of the carbon coating must be investigated to achieve better capacities; however, the incorporation of carbon derived from NC during its synthesis improved its response compared with LFP synthesised without this material.

Figure 6 shows different TEM images of the proposed LFP/C composite to evaluate carbon coating and particle size. An accumulation of smaller particles (near 500 nm, Figure 6A), isolated carbon, and covered and uncovered particles can be observed (Figure 6B,C), indicating that NC and LFP particles do not form a homogeneous mixture before heat treatment, so we can observe carbonised NC (conductive carbon) isolated from LFP particles that agglomerate after this heat treatment. Nevertheless, we can also observe particles with a homogeneous carbon coating (Figure 6C).

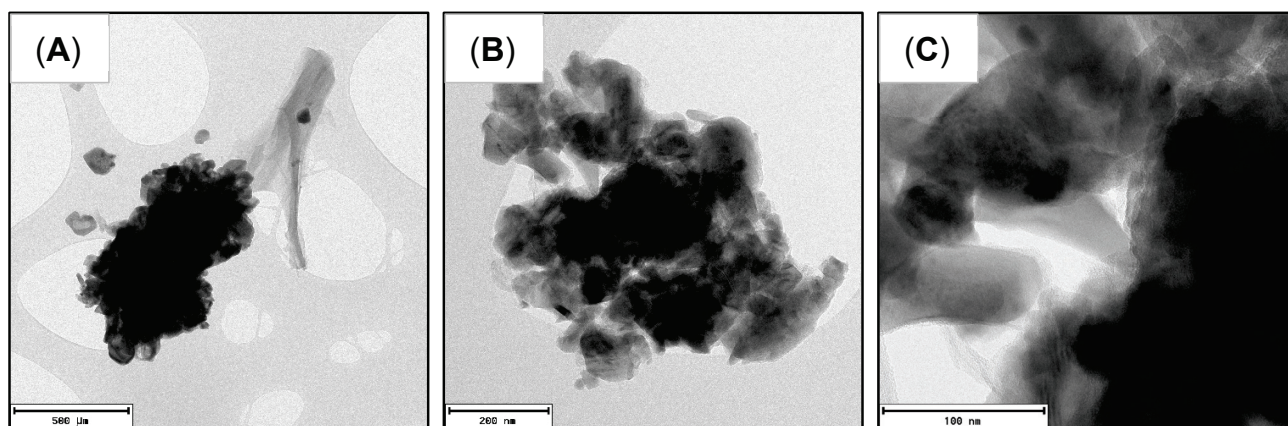


Figure 6. TEM images of composites formed with CNF, at different size scales: (A) 500 nm; (B) 200 nm and (C) 100 nm.

On the other hand, considering the composite created with 4% wt. carbon (from CNF, Figure 4C), we also observed a homogeneous coating in some particles and less isolated carbon than in the composite created with 12.6% wt. carbon, indicating that an enhancement of the mix of LFP and NC before heat treatment would be beneficial to obtain LFP/C composites through this route with homogeneous coatings of all particles, avoiding excessive amounts of carbon.

3.2. X-ray Photoelectron Characterisation of LFP/C Composite

To investigate the material created via the synthetic route, X-ray photoelectron spectroscopy (XPS) characterisation was carried out.

Figure 7A shows the high-resolution Fe 2p spectra of the LFP/C composite and of LFP synthesised without carbon (from CNF) as a powder. Through these spectra, it is possible to identify whether the samples contain iron in the 2+ or 3+ oxidation state: signals at 710 eV are attributable to iron 2+ in both samples, and signals near 715 and 729 eV corresponds to satellite peaks that commonly appear in this spectrum when iron 2+ is the predominant species [48–50]. Meanwhile, signals attributable to iron 3+ from spin 3/2 that appear at 711–712 eV are not observed. Finally, a cycled coin cell of LFP/C composite for 10 cycles at 0.1 C-rate was investigated in contrast with its cathode without cycling. Thus, an LFP/C composite cathode was opened in a glove box after cycling and then maintained in open-circuit potential to dry the remaining electrolyte, after which it was placed inside the sample holder of the XPS instrument outside the glove box.

The C 1s spectrum (Figure 8A) shows the same signals identified in the cathode (below) as in the cycled coin cell (above), but with greater intensity—mainly because the cycled cathode was dried inside the glove box, so it might contain remains of the used electrolyte (LiPF_6 , in EC/DMC solvents) that contain C–C, C–O, and C=O bonds, which would influence the intensities observed in the cathode after cycling. The same tendency can be observed in the high-resolution O 1s spectrum (Figure 8B), with the C–O functional groups; the peak near 531 eV can be attributed to the phosphate group in the LFP material [51]. On the other hand, the appearance of a second signal in the F 1s spectrum (Figure 8C) when the coin cell is cycled indicates the presence of LiF ionic bonds [52] formed for the reduction of anions in carbonate electrolytes [53] (e.g., the LiPF_6 electrolyte in the EC/DMC solvent that may have remained once the cell was opened to characterise the cathode).

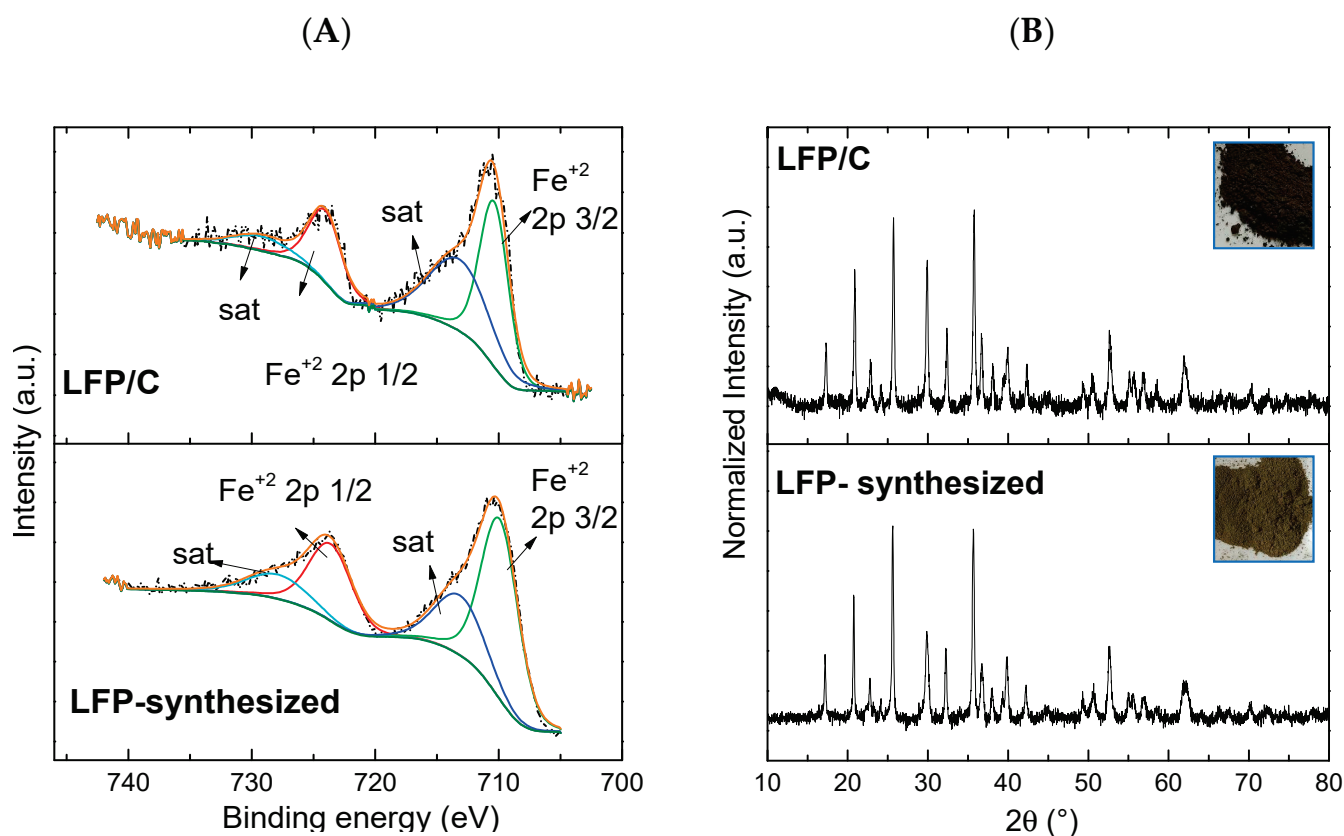


Figure 7. (A) High-resolution XPS Fe 2p spectra of synthesised LFP and LFP/C. (B) XRD diffractograms of the samples, along with their respective pictures.

In the high-resolution Fe 2p spectrum (Figure 8D), a clear change in the signals was observed before and after cycling the compound for 10 charge/discharge cycles at a rate of 0.1 C-rate. Before cycling, the iron 2+ species could be clearly identified, but after cycling additional signals appeared that could be attributed to different oxidation states of iron: 0, 2+, and 3+, due to their respective binding energies of 707, 710, and 713 eV. The presence of iron 3+ is expected for the charging and discharging process of a coin cell. The iron changes its oxidation state from 2+ to 3+; therefore, when the cycling is ended and open-circuit potential is achieved, the amounts of iron 2+ and 3+ should be very similar [52,54], since it is not fully charged (Fe³⁺) and not fully discharged (Fe²⁺). These results indicate that the composite loses the reversibility of the Fe²⁺/Fe³⁺ signals after cycling, appearing with other species such as FeOOH or Fe (0) [55], since these elements do not actively participate in the intercalation process of the LFP, which could be the main reason for the specific capacity achieved.

If we compare these results with those of previous works (Table S3), the synthesis conditions reported in this work facilitates obtaining LFP using low temperatures, without unnecessary reagents to prevent iron oxidation (e.g., ascorbic acid), and an in situ carbon coating with organic and sustainable materials such as NC to easily obtain an LFP/C composite. Other synthesis routes use higher temperatures that are detrimental to the synthesis, such as solid-state, ex situ coatings with inorganic and toxic compounds. However, the incorporation of insufficient carbon coating in the LFP/C composite, which improves the reincorporation of lithium ions inside the LiFePO₄ structure, could lead to iron 3+ or iron 0 species, affecting the specific capacity observed. Therefore, subsequent investigations of homogeneous coatings will be crucial to improve the specific capacity of LFP materials created through this proposed synthesis route.

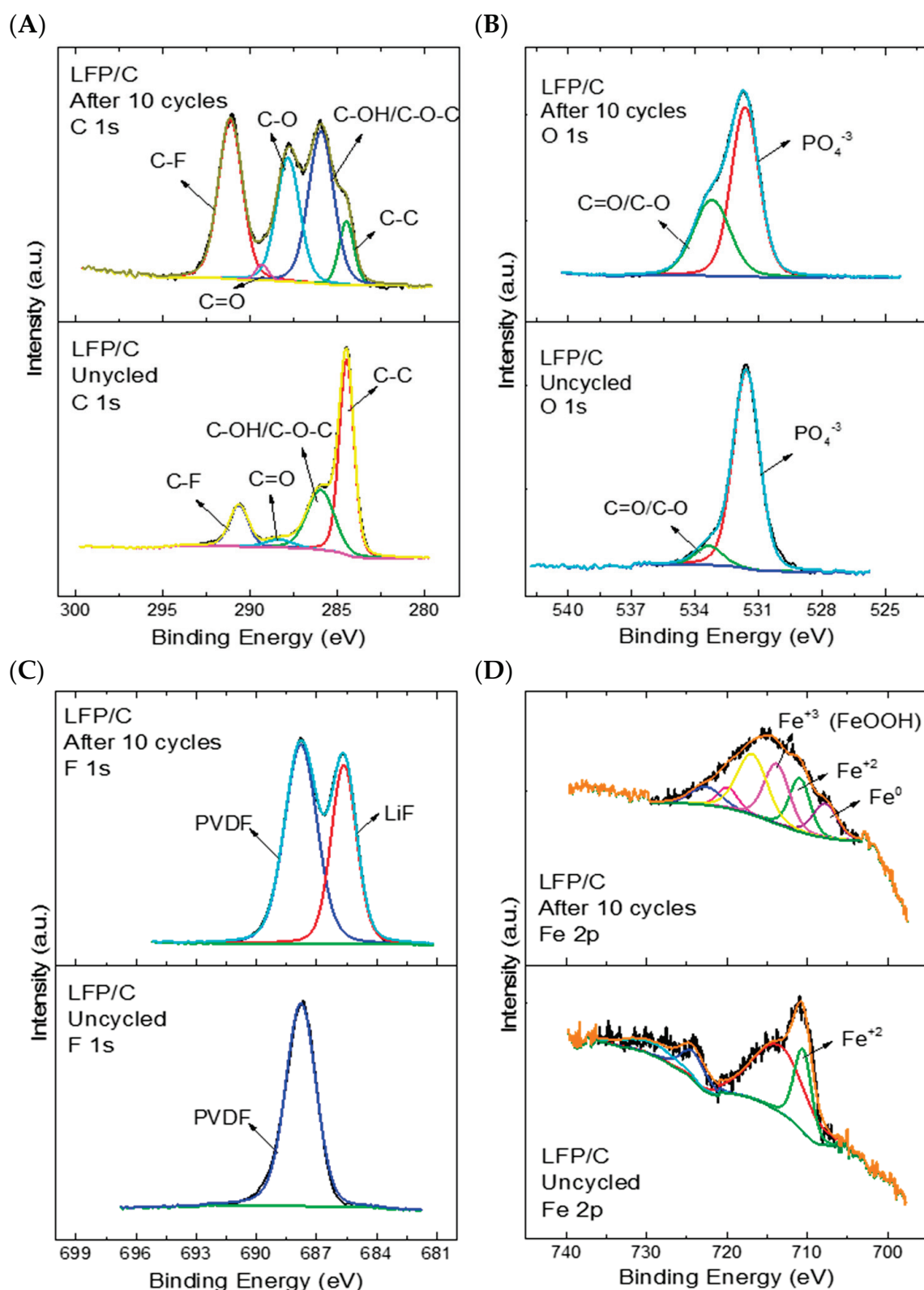


Figure 8. High-resolution XPS spectra of the LFP/C composite before cycling and after cycling for 10 cycles at 0.1 C-rate: (A) C 1s spectrum; (B) O 1s spectrum; (C) F 1s spectrum; (D) Fe 2p spectrum.

4. Conclusions

When adding NC to the synthesis process, the resulting composites (LFP/C) exhibit better intensity of the crystalline face responsible for lithium diffusion, smaller particle

size (with both types of NC), and better electrochemical performance in comparison to LFP synthesised without carbon, due to the carbon coating created by the NC after heat treatment. Between the two NCs used, CNC exhibited better diffraction peaks in XRD than CNF, but we observed wide peaks with CNF, indicating smaller crystallites and, consequently, smaller particles, as evidenced by the FE-SEM images. Comparing the different percentages of NC used, it should be noted that small percentages (i.e., 0.15% wt. NC) can lead to unwanted diffraction peaks due to the possibility of iron oxidation when a small amount of NC is carbonised. In contrast, with a high percentage of NC (i.e., 1% wt.), no unwanted diffraction peaks were observed, but this does not guarantee a proportional decrease in average particle size, and in some cases with CNC an increase in particle size was observed.

Both NCs carbonised to carbon in an inert atmosphere, but through Raman and elemental analysis of the carbon we concluded that CNC decomposes faster than CNF, leading to increase in particle size and a decrease in the final amount of carbon obtained (% wt. carbon in LFP/C composite) when using the same percentage of each NC in the reactor vessel, which is consistent with the results of CV experiments with the same amount of final carbon. As a consequence, the use of CNF instead of CNC in the LFP synthesis shows promising characteristics for use as a cathode: the composite created at 150 °C for 30 min with 1% wt. CNF had 12.6% wt. carbon after heat treatment.

The electrochemical characterisations of the composite showed improved behaviour compared with the LFP synthesised without carbon, confirming that the coating of the LFP particles improves the Li^+ intercalation behaviour, while a reduced particle size improves the voltammetry profile and the charge–discharge curves.

Through the XPS spectra, it can be seen that the synthesised composite (LFP/C) exhibits only Fe^{2+} species, while after cycling a coin cell of this compound, other iron species such as Fe^{3+} and Fe^0 can be observed, indicating that inactive species are formed once the coin cell is cycled, which may be responsible for the specific capacity achieved.

Through this proposed synthesis route, we can easily obtain an LFP/C composite, avoiding unnecessary chemicals and multiple steps, and the resultant material shows promise for use as a cathode due to its small particle size (95 nm), carbon coating, and good electrochemical behaviour according to cyclic voltammetry.

Homogeneity of the carbon coating might be the main issue to achieve better capacity, and this should be investigated in further research in order to obtain particles that are fully covered by conductive material.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym15122628/s1>, Figure S1: FT-IR characterisation of different synthesis conditions of LFP by the microwave-assisted hydrothermal method; Table S1: Average particle size of the N samples, as measured using ImageJ software; Figure S2: FE-SEM images of LFP samples synthesised with ethanol as a reducing agent under different synthesis conditions: (a) 150 °C for 15 min; (b) 150 °C for 30 min; (c) 205 °C for 15 min. The particle distribution is shown as an insert in each image; Table S2: Rietveld values obtained for LFP synthesised using ethanol as a reducing agent, Table S3: Comparison of LFP materials obtained in this study and previous works.

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Article

Electrical Conduction Mechanisms in Ethyl Cellulose Films under DC and AC Electric Fields

Jesús G. Puente-Córdova ^{1,*}, Juan F. Luna-Martínez ^{1,*}, Nasser Mohamed-Noriega ¹ and Isaac Y. Miranda-Valdez ²

¹ Facultad de Ingeniería Mecánica y Eléctrica, Universidad Autónoma de Nuevo León, Av. Universidad s/n, Cd. Universitaria, San Nicolás de los Garza 66455, Mexico; nasser.mohamednr@uanl.edu.mx

² Department of Applied Physics, Aalto University, P.O. Box 11000, FI-00076 Espoo, Finland; isaac.mirandavaldez@aalto.fi

* Correspondence: jesus.puentecr@uanl.edu.mx (J.G.P.-C.); juan.lunamrt@uanl.edu.mx (J.F.L.-M.)

Abstract: This work reports the dielectric behavior of the biopolymer ethyl cellulose (EC) observed from transient currents experiments under the action of a direct current (DC) electric field ($\sim 10^7$ V/m) under vacuum conditions. The viscoelastic response of the EC was evaluated using dynamic mechanical analysis (DMA), observing a mechanical relaxation related to glass transition of around ~ 402 K. Furthermore, we propose a mathematical framework that describes the transient current in EC using a fractional differential equation, whose solution involves the Mittag-Leffler function. The fractional order, between 0 and 1, is related to the energy dissipation rate and the molecular mobility of the polymer. Subsequently, the conduction mechanisms are considered, on the one hand, the phenomena that occur through the polymer-electrode interface and, on the other hand, those which manifest themselves in the bulk material. Finally, alternating current (AC) conductivity measurements above the glass transition temperature (~ 402 K) and in a frequency domain from 20 Hz to 2 MHz were carried out, observing electrical conduction described by the segmental movements of the polymeric chains. Its electrical properties also position EC as a potential candidate for electrical, electronics, and mechatronics applications.

Keywords: ethyl cellulose; electric conduction; fractional calculus; Schottky effect; AC conductivity

1. Introduction

Ethyl cellulose (EC) is a biopolymer cellulose derivative whose main applications are in the pharmaceutical and food industries [1–4]. From the electrical point of view, EC has applications as a binder in printing pastes, electrets, and thermoelectric materials [5–7]. The macromolecular structure of EC consists of repetitive units of anhydroglucose. EC synthesis proceeds through the reaction between cellulose and ethyl chloride under alkaline conditions. The properties of the resulting EC will depend on the degree of substitution of the ethyl ether group. Among the diverse types of EC synthesized, the most used in industrial sectors is that with a degree of ethyl ether substitution between 2.4 and 2.5 [2,8]. This substitution degree provides important properties, for example, water resistance and solubility in polar and non-polar solvents, and certain azeotropic mixtures. The difference in size and electronic configuration of the atoms of EC induces an asymmetric spatial distribution of electric charge carriers, where each chemical group of the polymer chains is associated with an electric dipole moment. The molecular mobility of electric dipoles is of great interest for the characterization of the polymer structure, i.e., dielectric analysis and infrared spectroscopy.

The study of the electrical properties of organic polymeric materials is a fundamental research topic for the development of new devices and technologies. Polymeric materials exhibit two distinct electrical behaviors: they can store electric charge as a dielectric, and they can conduct electricity due to the movement of charge carriers. To fully understand the electrical properties of polymers, it is important to study the electric polarization and

space charge phenomena that occur within them. By characterizing these phenomena, we can gain valuable insights into the behavior of polymeric materials in various electrical applications. The presence of space charge enhances the local electric field; in the absence of an external electric field, this electric field is different from zero, which seriously affects the dielectric properties of polymers and accelerates aging processes. Therefore, identifying the conditions and conduction mechanisms is fundamental to unraveling why they accumulate electrical charges on the polymer surface or as a bulk. In this sense, Khare et al. obtained thermograms by thermally stimulated discharge currents (TSDC) for EC, which consist principally of two relaxation peaks located at 333 K and 413 K. The first of these is attributed to the disorientation of ethoxy groups of the glycosidic units and the second to the diffusion of the space charge on the electrodes or to their thermal release from traps [9]. In addition, the authors performed current–voltage measurements, interpreting that the Schottky–Richardson mechanism dominates the electrical conduction process [10]. Bidault et al. [11] carried out dielectric spectroscopy measurements for EC, which exhibited three secondary relaxations at temperatures below its glass transition (~ 403 K). The comparison of the relaxation time and activation energy with other polymers allowed them to attribute the relaxation to the side groups and local movements of the main chain. Kumanek et al. [5] have reported the synthesis of multi-walled carbon nanotubes and the preparation of composite films with an ethyl cellulose matrix, which have potential applications in new energy technologies. They evaluated the thermoelectrical properties of the films, tuning the electrical response with heteroatoms, so that the material simultaneously demonstrated the highest possible value of electrical conductivity and Seebeck coefficient while restricting the thermal conductivity value to its minimum. More recently, Reyes-Melo et al. [2] fabricated and synthesized hybrid films with silver nanoparticles into an ethyl cellulose matrix. The authors studied the mechanical and dielectric relaxations of these films and found a remarkable interaction between the nanoparticles and the chemical groups of the organic matrix.

Although there are several reports in the literature about the electrical response of EC and its composites, there are still questions to be resolved regarding the space charge, charge transport, and the relationship between the structure and electrical properties of EC. This work evaluates the structure and morphology of EC using dynamic mechanical analysis and X-ray diffraction, and the measurement of transient currents at different DC electrical fields under vacuum conditions. Our goal is the application of fractional calculus for the analysis of the transient currents in polymers as an innovative tool. The analysis of the electrical conduction mechanisms of EC films is covered by several physical models in order to obtain an insight on the generation and transportation of carriers. Finally, AC conductivity at temperatures above their glass transition temperature was carried out. Experimentation and mathematical models were used in this study to understand the polymer's electrical behavior, explain potential failures in service, and optimize its properties for insulator and dielectric applications.

2. Electrical Conduction in Polymers

Polymers that are free of chemical impurities and structural defects have no valence electrons that can exhibit an electronic current like in metals. According to the energy band theory, polymers typically have a bandgap energy higher than 5 eV but does not explain the origin of the electric currents produced, in the order of the picoamperes (pA). Therefore, to describe the conduction mechanisms of polymers at medium and high electric fields, the type and density of traps (potential wells) must be considered, in addition to the atomic structure. In this sense, several authors have modified the energy band theory to adapt it to the study of the electrical behavior of polymers [12–14]. One way to address conduction mechanisms in polymeric materials is through the experimental measurement of transient currents, monitoring the evolution of the electric current over time. Two main contributions make up the transient currents: the first is associated with the polarization current, which depends on the number of electric dipoles oriented in the direction of the electric field, and

the second is related to a conduction current, which is assembled in two groups. The first corresponds to electric currents that are a function of the injection of electrons or holes in the polymer through the polymer–electrode interface (extrinsic charge carriers). The second group corresponds to electric currents associated with the movements of the electric charge carriers that are in the volume of the polymer (intrinsic charge carriers).

The conduction mechanisms controlled by the bulk are complex and have several origins. The orientation of electrical dipoles (permanent or induced) by the effect of an external electrical field produces a current that can be measured [15,16]. Additionally, the injection of electrons can be evaluated through an electric current. After the injection of electrons, these can be trapped in two ways, producing an accumulation of electrical charge in the polymer, considered as space charge. The mobility of the electric charge carriers is significantly restricted when they are trapped or occupy the localized energy states. The energy associated with trapping charge carriers also depends on the temperature, electric field, and energy depth of the traps. The first type of trapping is identified as physical or shallow traps and corresponds to changes in the conformational states of the polymer; the energy associated with this trapping mechanism is around 0.1–0.5 eV. The second trapping mechanism is identified as a chemical or deep trap, and is related to the formation of free radicals, chemical cross-linking, or breakage of covalent bonds; the energy associated with these traps has been reported as >1 eV. Several physical models are proposed in the literature for describing and understanding the nonlinear behavior and conduction mechanisms in polymeric materials: conduction mechanisms through the electrode–polymer interface (Schottky and Fowler–Nordheim) and those corresponding to the bulk (Poole–Frenkel, Space Charge Limited Current SCLC and Ionic-Hopping) [17–19].

Regarding the injection mechanisms through the electrode–polymer interface, the Schottky mechanism considers that electrons with sufficient energy will be able to overcome the potential barrier ϕ_0 , where the required energy source is associated with an increase in temperature T or an external electric field E . Under a high electric field, the decrease of the potential barrier is related to an image force, due to an electrostatic field created by the electron that has left the electrode, and the charge induced by the opposite sign in the electrode [12]. This attraction produces a gradual change of the potential barrier and is considered the potential energy of the electron. Therefore, the current density J is given by Equation (1). It is important to remark that in this equation a trap-free material was assumed, without physical or chemical defects.

$$J = A^* T^2 \exp \left[-\frac{(\phi_0 - \beta_S E^{\frac{1}{2}})}{kT} \right] \quad (1)$$

where A^* is Richardson's constant. If the electric charge carrier is an electron, this constant has a value of $120 \text{ A/cm}^2 \cdot \text{K}^2$. β_S is the Schottky constant, T is the absolute temperature in K, m is the mass of the electron, q is the charge of the electron, k is the Boltzmann constant and h is the Planck constant.

When the electron cannot overcome the potential barrier, there is the possibility that, due to the wave–particle duality, it can cross it, and thus inject from the electrode into the polymer. This phenomenon, known as the tunneling effect or Fowler–Nordheim mechanism, is approached by the principles of quantum mechanics [20]. The deduction of the equation that governs this mechanism considers a triangular potential barrier, which is created due to the potential energy of the electron. Equation (2) defines the current density, which is independent of temperature. This phenomenon typically occurs in insulators and dielectrics at high electric fields in the order of 10^8 – 10^9 V/m .

$$J = \frac{q^3 E^2}{8\pi h \phi_0} \exp \left(-\frac{8\pi \sqrt{2m} \phi_0^{\frac{3}{2}}}{3hqE} \right) \quad (2)$$

Under low electric fields, Ohm's law can describe the conduction mechanisms in the bulk polymer. At high electric fields, the voltage–current curves deviate from a linear relationship. If the electrons are injected into the polymer and if the relative dielectric permittivity, ϵ_r , is high (greater than 10), a high concentration of electrical charge is generated within the material. Consequently, more electrical charge is injected into the polymer than can be released. It is generally assumed that the injected electrical charge is evenly distributed spatially throughout the material; however, various structural phenomena prevent the charges from being distributed symmetrically. In this context, the space charge-limited current (SCLC) is a measure of the rate of injection and extraction of electric charge carriers into the bulk material [12,15,21]. This electrical behavior depends on the concentration of charge carriers, the type of electrical charge, its mobility, the nature of the electrodes and the trapping characteristics. Equation (3), also known as the Mott–Gurney equation, gives the current density. It considers a polymer without traps and thermal generation of charge carriers. Having an ohmic contact, which involves injecting a single type of charge carriers, is also desirable.

$$J = \frac{9}{8} \epsilon_0 \epsilon_r \mu \frac{E^2}{d} \quad (3)$$

In Equation (3), ϵ_r is the relative permittivity of the material, ϵ_0 is the dielectric permittivity of the vacuum, d is the thickness of the polymer film, and μ is the mobility of the electric charge carriers. It is important to note that J is proportional to the square of the electric field, and inversely proportional to the thickness. On the other hand, for the case of a polymeric material in which the single energy level trap density is assumed, a modified expression, given by Equation (4), is obtained for J . Equation (4) considers the factor θ , denoting the proportion between free and trapped carriers.

$$J = \frac{9}{8} \epsilon_0 \epsilon_r \mu \theta \frac{E^2}{d} \quad (4)$$

Another bulk effect is the Poole–Frenkel mechanism, analogous to the Schottky mechanism. Considering an energy potential well associated with a trap, the Poole–Frenkel effect is the result of the reduction of the trapping energy under the combined effect of the Coulomb potential and the electric potential associated with the external electric field. In polymeric materials in thin film form, the trapping centers are considered fixed, therefore, conduction is carried out only by electrons that have crossed the lowered potential barrier. The expression of the Poole–Frenkel current density is given by Equation (5), where J_0 is a pre-exponential factor, and β_{PF} is the Poole–Frenkel constant.

$$J = J_0 \exp \left[- \frac{(\phi_0 - \beta_{PF} E^{\frac{1}{2}})}{kT} \right] \quad (5)$$

Ions exist in large amounts in polymers and can originate from impurities, depending on the synthesis and manufacturing procedures. These can also be produced by ionization processes, such as radiation absorption, material breakdown, and contaminant absorption. The motion of ions results in mass transport, and their size is too large compared with the size of the electrons, hence ionic mobility is several orders of magnitude lower than electron mobility. In this case, the physical transport mechanism occurs through a series of jumps, where λ represents the average distance between jumps over the potential barriers, allowing the ions to move from one place to another through the bulk [22]. The complex structure of the polymer creates potential barriers and can be modified by applying an electric field or by increasing temperature. An expression that defines the current density for the ionic conduction mechanism is Equation (6), following the hypothesis of uniformly distributed traps.

$$J = J_0 \sinh \left[q\lambda E / 2kT \right] \quad (6)$$

3. Materials and Methods

Samples were manufactured in the form of a thin film from solutions of ethyl cellulose EC with an average molecular weight of 135,000 g/mol (Dow Chemicals, Rochester, NY, USA) in tetrahydrofuran (THF, 99%, Sigma-Aldrich, St. Louis, MO, USA) as a solvent. A concentration of 10% by weight was used, ensuring an appropriate film manufacture [2]. To guarantee the dissolution process, the EC–THF mixture was stirred at 700 rpm for 1 h, at 313 K. The solutions obtained were subjected to a tape casting process (this process controls the film thickness) on a polytetrafluoroethylene (PTFE) surface, to separate the solvent by natural convection, at room temperature for 24 h. The EC films obtained were stored in a desiccator for later use. The thickness of the obtained films was measured with a micrometer, obtaining a value of $\sim 20 \pm 2 \mu\text{m}$.

The viscoelastic response of the EC was analyzed by DMA, using a Perkin Elmer DMA8000 (Waltham, MA, USA), under isochronous conditions (0.1, 1 and 10 Hz), in tension mode, with a heating ramp of 2 K/min, from 298 to 453 K [8]. EC was analyzed by X-ray diffraction (XRD) to determine the presence of amorphous or crystalline phases. The XRD analysis was undertaken using a PANalytical diffractometer Empyrean model, with $\text{CuK}\alpha$ radiation at the wavelength of $\lambda = 0.154 \text{ nm}$, over the range from 5 to $80^\circ (2\theta)$; the sample was previously pulverized to perform the XRD analysis.

The EC films were subjected to a constant direct current (DC) voltage for the electrical characterization and measurement of transient currents. The experimental device consists of two flat and parallel copper electrodes of 25 mm diameter. The polymer sample was placed between the electrodes to obtain a capacitor configuration. The external electrical circuit of the configuration was connected to a Keithley 6517B electrometer (Beaverton, OR, USA), which has a resolution of 10^{-14} A . The electrical characterization was carried out in two stages. The measurements of $I(t)$ were recorded at different step voltages for a time of 1000 s (polarization current), and the applied voltage was then reduced to zero for a time of 1000 s (depolarization current). After this, the previous protocol was repeated, but applying a step voltage of greater magnitude. In total, curves $I(t)$ were recorded for DC voltages of 100, 200, 300, 400 and 500 V.

This experimental procedure was carried out at room temperature (298 K) and constant pressure, where the electrode–polymer set was placed in a vacuum chamber, with the aim to reduce the effect of water molecules on the measurements. The experimental data were used to identify the conduction mechanisms. Because the physical models (Section 2) relate an electric current density ($J = I/A$) with an electric field ($E = V/d$), the observed I – V were transformed into data pairs J – E . For this purpose, the geometry of the sample analyzed was considered (area A and thickness d).

Additionally, alternating current (AC) conductivity measurements were carried out using the electrode–polymer set. An Agilent E4980A electrometer (Santa Clara, CA, USA) was used in a frequency range from 20 Hz to 2 MHz. The applied voltage follows an oscillating signal between -1 and 1 V ; the measurements were performed above the glass transition temperature, from 403 K to 473 K.

4. Results and Discussion

4.1. Dynamic Mechanical Analysis

The viscoelastic response of the EC was carried out using DMA, to determine the mechanical manifestation of the relaxation phenomena. The DMA results are presented in Figure 1a,b and correspond to the temperature dependence of storage modulus E' and $\tan \delta_m$, respectively, for three frequencies of the applied mechanical stimulus (0.1, 1 and 10 Hz). Figure 1b shows that the T_g (defined as the peak position in $\tan \delta_m$) is a function of frequency as expected in viscoelastic materials [2,8]. The magnitude of the T_g for EC at the frequency of 1 Hz corresponds to 402 K, which is comparable to that reported by Davidovich-Pinhas et al. ($T_g = 403 \text{ K}$) [23]. Additionally, through a DSC measurement for the EC at a heating rate of 5 K/min, a value of 397 K was obtained for the T_g . A thermal event associated with the fusion of crystals in the polymer was also observed, at a temperature of 451 K, suggesting a semicrystalline behavior in the polymer.

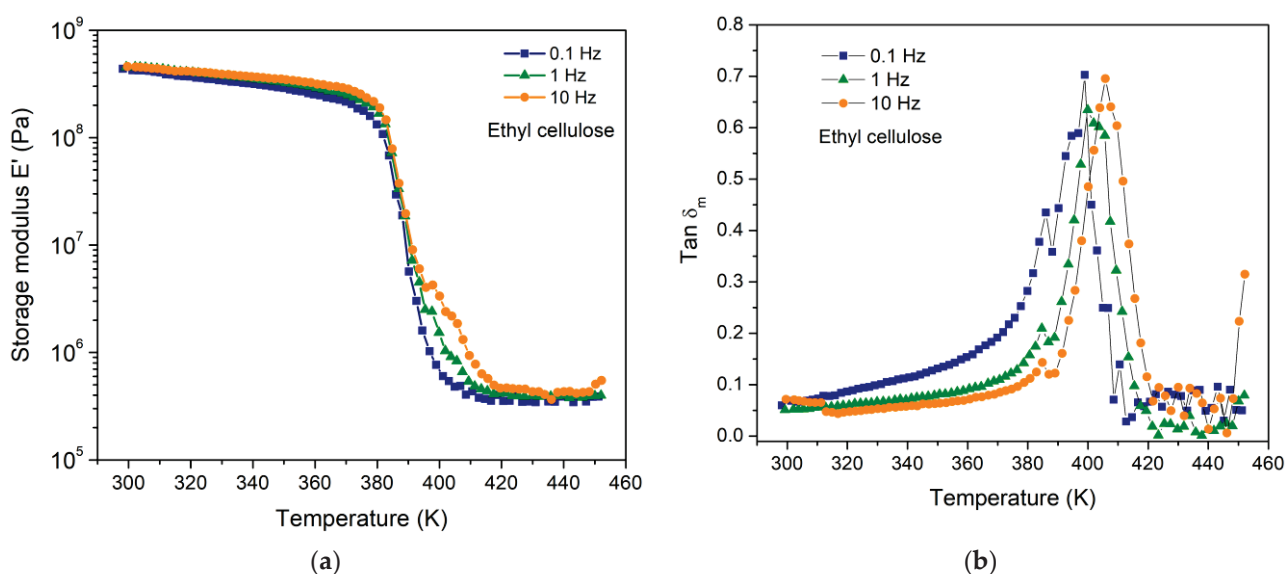


Figure 1. (a) E' and (b) $\tan \delta_m$, as a function of temperature and three different frequencies for EC.

4.2. X-ray Diffraction Analysis

X-ray diffraction pattern for EC powder is presented in Figure 2. This measurement is important because the electrical properties of polymers are very sensitive to structure and morphology [15]. The diffraction pattern shows two scattered peaks; this means that EC has a specific order within the amorphous background (short-range order). In the literature it is reported that EC presents an intimate mixture of amorphous and crystalline phases [1,23]. The first peak is centered at $2\theta = 8.5^\circ$ ($d = 10.39 \text{ \AA}$), which is associated with the distance between the ordered structures of the polymer, and the second peak at $2\theta = 20.3^\circ$ ($d = 4.37 \text{ \AA}$) gives information about the interchain distance. Another two weak peaks are detected, at $2\theta = 35.4^\circ$ ($d = 2.53 \text{ \AA}$) and $2\theta = 44.5^\circ$ ($d = 2.03 \text{ \AA}$). These distances, d , were calculated using Bragg's equation ($2d \sin \theta = \lambda$). The degree of crystallinity was calculated considering the area of crystalline and amorphous peaks, obtaining a value of 53.77%. The crystallite size was calculated using the Scherrer equation, obtaining a value of $10.08 \text{ \AA}$, the order of magnitude of the cellulose dimer length [23]. From an electrical point of view, amorphous regions function as traps with different energy levels, generating a complex electrical behavior in the polymer.

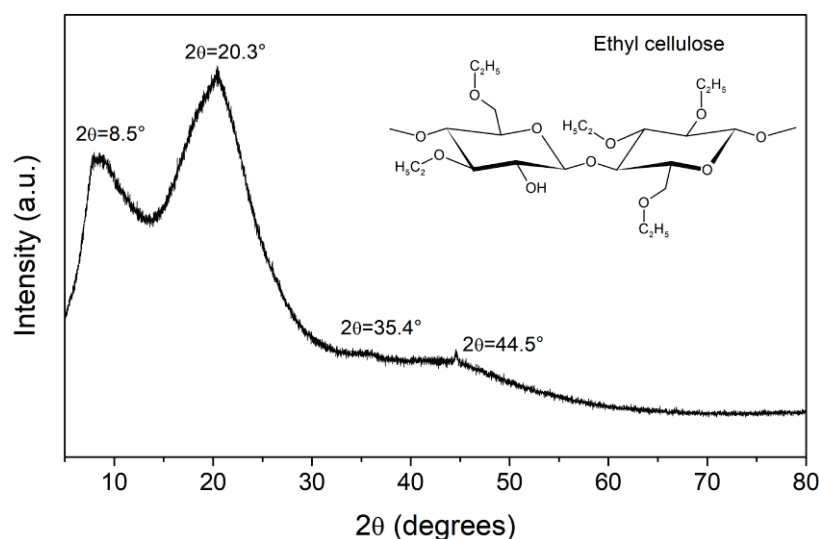


Figure 2. XRD results for EC powder. In the inset of the figure the chemical structure of EC is presented.

4.3. Transient Currents

Figure 3 shows the isothermal curves $I(t)$ obtained for the applied DC voltages. The experimental curves describe electrical currents in an order of magnitude of the pA, and increase globally as the applied DC voltage increases, but each curve shows a decrease in current as time increases. The shape of the curves does not seem to be affected significantly by the applied voltage; this behavior can be considered as a qualitative indicator of the movements induced by electric charge carriers because it is a relaxation phenomenon. The transient currents are analogous to stress relaxation experiments, within the linear viscoelastic regime. In a polymer, under an external electric field, the formation of new electric dipoles is induced. These dipoles, as well as the existing dipoles, orient in the direction of the applied field, with a rate that depends mainly on the viscoelastic nature of the polymer [11,24]. This dipole orientation process occurs macroscopically as a polarization current, which is a function of the number of dipoles and the rate with which they are oriented. When the electrical stimulus is removed, an electric current of the opposite sign is obtained (depolarization current), with a tendency to a zero value, which is related to the random redistribution of the electric dipoles caused by thermal agitation, which in turn tends to return to its initial conformational state. One electrical charge can be stored (capacitive response), and the other is dissipated as electric current (resistive response). Considering the chemical groups that make up the molecular structure of the EC, the permanent dipoles hydroxyl, pyranose ring, ethoxy and methoxy [11], dipolar orientation is established as a conduction mechanism that contributes to the shape of the transient currents $I(t)$.

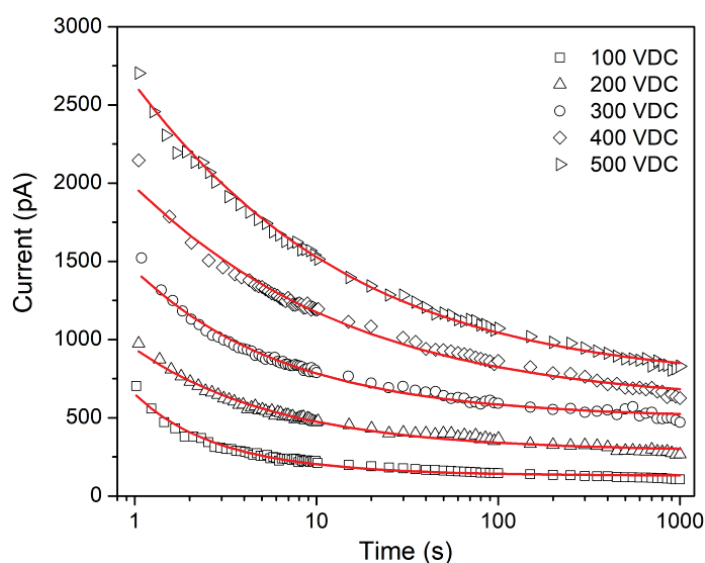


Figure 3. Comparison between isothermal transient currents for EC (symbols) and continuous lines represents the best fitting result.

In the literature it has been established that the transient currents follow an empirical function, the well-known Curie-von Schweidler law, $I(t) = At^{-n}$. However, this only considers a unique relaxation process or conduction mechanism, ignoring a steady-state conduction. As a first approximation, the curves $I(t)$ for several insulators have been modeled empirically considering a negative exponential function, obtained from an equivalent resistor–capacitor circuit [25,26]. The resistor represents a conductive behavior in the material, and the capacitor represents a dielectric behavior. The classical expression for this circuit is $I(t) = I_0 \exp(-t/\tau)$, where I_0 is a pre-exponential factor, $\tau = RC$ is the relaxation time in seconds, R is the resistance in Ohms and C is the capacitance in Farads. According to this model, and if only the orientation of permanent or induced electric dipoles is presented, it can be concluded that, as the applied DC voltage increases, the

global increase of the curves $I(t)$ is a direct consequence of many electric dipoles that are oriented when the electric field increases. To obtain the best fit to experimental data, using a generalized model of resistors and capacitors is an alternative, with a higher number of RC elements, but an increase in the number of parameters. The poor description obtained when we use exponential function to fit experimental data can be improved using a Mittag–Leffler function [27,28]. The Mittag–Leffler function E_α of one parameter α is defined as Equation (7):

$$E_\alpha(z) = \sum_{k=0}^{\infty} \frac{z^k}{\Gamma(\alpha k + 1)} \quad (7)$$

where $\Gamma(\cdot)$ is the gamma function, $\alpha > 0$ and $z \in \mathbb{C}$. If $\alpha = 1$, we obtain the exponential function $\exp(z)$. In this sense, Mittag–Leffler function is considered a generalization of the exponential function. A fractional differential equation for an RC circuit is obtained using the fractional calculus approach [29], Equation (8), which relates the voltage $V(t)$ and the current $I(t)$. The fractional derivatives are described by Caputo derivative definition.

$$C\tau^{\alpha-1} \frac{d^\alpha V(t)}{dt^\alpha} = \tau^\alpha \frac{d^\alpha I(t)}{dt^\alpha} + I(t) \quad (8)$$

The solution for Equation (8) is obtained using the Laplace transform, considering $V(t) = V_0 u(t)$, where $u(t)$ is the unit step function, and an initial condition $I(0) = 0$:

$$I(t) = I_0 \cdot E_\alpha \left[-\left(\frac{t}{\tau} \right)^\alpha \right] \quad (9)$$

where α is a fractional order that takes values between 0 and 1. Equation (9) is useful for fitting experimental data considering the polarization current and relaxation phenomena. If $\alpha = 1$, we recover the classical expression for transient current (exponential function). The Mittag–Leffler function presents two interesting asymptotic approximations, at small times ($t \rightarrow 0$), $E_\alpha(-z^\alpha) \sim \exp[-z^\alpha / \Gamma(1 + \alpha)]$, and at large times ($t \rightarrow \infty$), $E_\alpha(-z^\alpha) \sim z^{-\alpha} / \Gamma(1 - \alpha)$. This means that $E_\alpha(\cdot)$ interpolates between the stretched exponential function (Kohlrausch–Williams–Watts function) and a negative power-law (Curie–von Schweidler law) [28,30].

According to the nature of the transient currents, in this work the total current is expressed as the sum of the polarization current I_p and the conduction current I_c .

$$I(t) = I_p + I_c = I_0 \cdot E_\alpha \left[-\left(\frac{t}{\tau} \right)^\alpha \right] + I_c \quad (10)$$

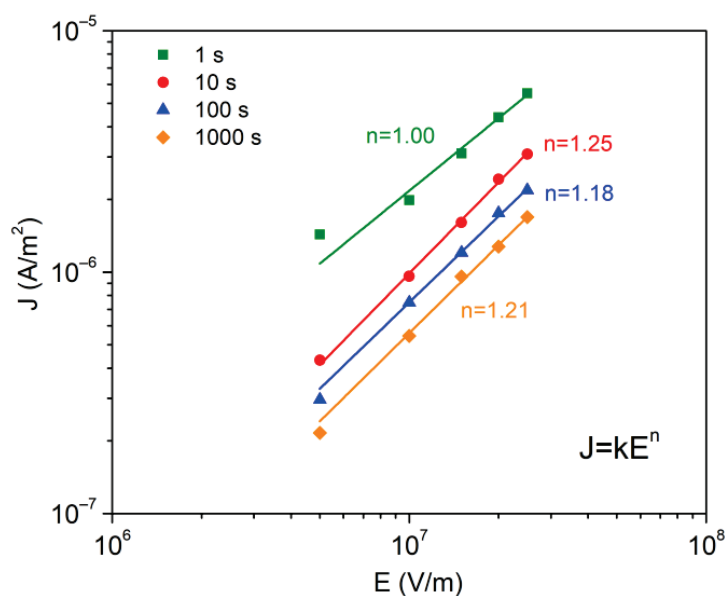
Figure 3 compares the experimental data and the modeling, which is considered acceptable. The fitting procedure was performed in MATLAB using a routine proposed by Podlubny [31]. Table 1 shows the values of the parameters involved in Equation (10). I_c presents an increase as the voltage or electric field increases, which in other polymer systems is observed [15,32]. For I_0 , a relationship as a function of voltage is not presented, which is due to the manifestation of different conduction mechanisms at very short times. The obtained values for the fractional order α are between 0 and 1. The fractional order α can be interpreted as a measure of the rate of energy dissipation in the polymeric material. When the voltage increases, the fractional order decreases. This can be associated with a greater storage of electric charge, mainly due to the number of electric dipoles that are oriented under the electric field. However, dissipative phenomena are still present, coupled with the different conduction mechanisms that are manifested. In addition, the relative average absolute deviations (AAD) present lower values as the voltage increases, which refers to a better fit compared with the classic exponential function.

Table 1. Fit results from experimental data.

Parameter	100 V	200 V	300 V	400 V	500 V
I_c (pA)	129.61	286.06	497.81	591.51	726.03
I_0 (pA)	6289	11,532	16,290	7055	9120
τ (s)	0.1519	0.0159	0.0135	0.0707	0.0866
α (-)	0.75	0.53	0.52	0.41	0.41
AAD (%)	6.41	3.63	2.49	2.67	1.88

4.4. Analysis of Conduction Mechanisms

The transient currents $I(t)$ of Figure 3 were analyzed using the physical models summarized in Section 2. From these curves $I(t)$ for each of the applied voltages, a data set J – E was constructed for several values of time t (1, 10, 100 and 1000 s), and Figure 4 was constructed. The experimental data were adjusted to the expression $J = kE^n$, which allows one to characterize whether or not there is an ohmic contact between the electrodes and the sample under analysis. Only for $t = 1$ s is the ohmic contact satisfactory because $n = 1$, and for the others times the values of index n present a slight deviation from unity. The DC conductivity of the EC was calculated at $t = 1$ s, $\sigma = 2.5 \times 10^{-13}$ S/m, which is a function of the density of electric charge carriers present in the EC, and its respective mobility; this calculated value is in the order of magnitude reported for other polysaccharides [11,33].

**Figure 4.** Current density as a function of the electric field, at different times of analysis.

In the literature it is accepted that the presence of space charge modifies the electric field at the electrode–polymer interface, where there is a competition between the polarization charges and the charges injected into the polymer. In this sense, we proceed with the analysis of the conduction currents that can be controlled by the electrode–polymer interface. The two main injection mechanisms will be considered: Fowler–Nordheim (Equation (2)) and Schottky (Equation (1)). The Fowler–Nordheim effect (quantum mechanical phenomenon) controls the current if the plot $\ln(J/E^2)$ versus $(1/E)$ is linear and with a negative slope. This condition is not satisfied in our case, because this phenomenon requires thin insulating barriers that can only be achieved at high electric fields ($\sim 10^9$ V/m). Concerning the Schottky model, this is generated by the reduction of potential barrier in the interface due principally to external electric field, which implies that electrons can be injected into the polymer. The data were calculated from Figure 4, taking $\ln(J)$ versus $E^{1/2}$, as given by Figure 5.

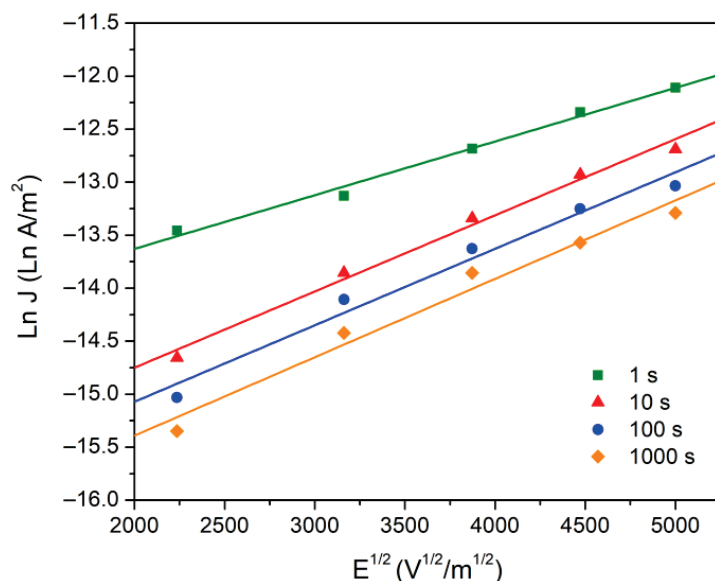


Figure 5. Ln J versus $E^{1/2}$, at different times of analysis.

Furthermore, Poole–Frenkel (Equation (5)) is also considered with respect to bulk conduction mechanisms. According to the Poole–Frenkel effect, the conduction current is induced by a decrease of the potential barrier of the traps, under the combined effect of the Coulomb potential and the electric potential originated by the external electric field. The equations for Schottky and Poole–Frenkel are similar, and in consequence it is not easy to identify the mechanisms responsible for the conduction (if the electrons are compensated, $\beta_{PF} = 2\beta_S$). From the linear fit for data of the Figure 5, the slopes were calculated to obtain β_{PF} and β_S , which are presented in the Table 2. Miranda-Valdez et al. [34] report a relative permittivity of $\epsilon_r = 5$ at 1 MHz for EC, with this value used to calculate theoretical $\beta_{theo} = 2.71 \times 10^{-24} \text{ J m}^{1/2}/\text{V}^{1/2}$. This result agrees with the values obtained for the Schottky mechanism (Table 2), suggesting that, at voltages greater than 100 V, there is a probability that charge carriers are injected into the polymer through the interface. The height of the energy barrier was $\sim 1.07 \text{ eV}$, which agrees with that typically observed in electrode–polymer–electrode systems, from 1–1.5 eV [15,32].

Table 2. Fit results for the Schottky and Poole–Frenkel constants from experimental data.

Parameter	1 s	10 s	100 s	1000 s
$\beta_S (\text{J m}^{1/2}/\text{V}^{1/2})$	2.08×10^{-24}	2.96×10^{-24}	2.97×10^{-24}	3.04×10^{-24}
$\beta_{PF} (\text{J m}^{1/2}/\text{V}^{1/2})$	4.17×10^{-24}	5.91×10^{-24}	5.94×10^{-24}	6.09×10^{-24}

For the case of the conduction currents controlled by the polymer bulk, the SCLC model was also considered (Equation (4)). Figure 6 was also constructed from Figure 4, taking $\text{Ln}(J)$ vs $\text{Ln}(E)$. The purpose of this graph is to observe a slope change (denoted as “m”). With $m = 1$ it is said that the conduction mechanism follows an ohmic behavior, and if $m = 2$, the mechanism is associated with the presence of space charge [21]. The calculated values for m vary from 0.84 to 1.28, as time increases. If we consider in this case a material with a trapping density, which is a consequence of the complex semicrystalline structure, there is a tendency to accumulate electric charge.

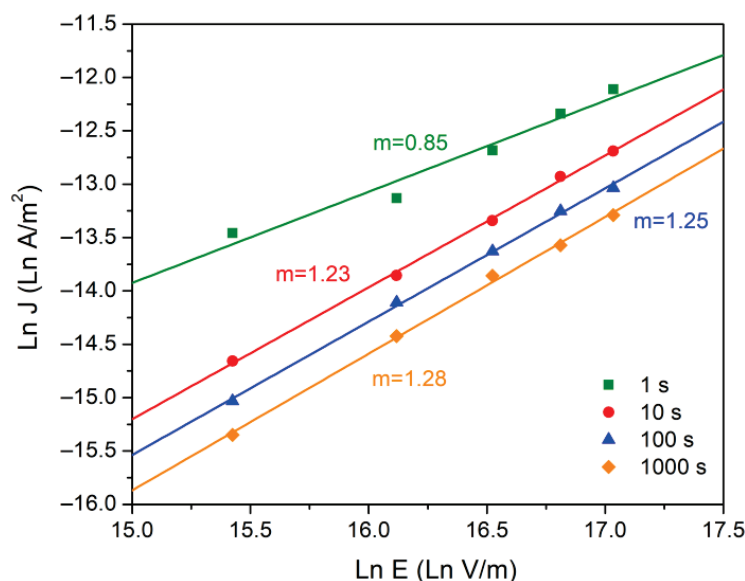


Figure 6. $\ln J$ versus $\ln E$, at different times of analysis.

Additional analysis of conduction mechanism is very important considering the current in terms of mobile ions, defect sites and a carrier hopping process. A model initially developed for ionic conduction (Equation (6)) leads to a density current proportional to $\sim \sinh(q\lambda E/2kT)$, where λ is the jump distance between potential wells. To obtain the fit with the data, the method used by Guillermin et al. was used [32]. The values obtained are presented in Figure 7 and are consistent with others reported for different polymers [22,35]. At $t = 1$ s, a value of $\lambda = 2.6$ nm is calculated, while for the other times values of $\lambda \sim 2.8$ – 2.9 nm were calculated. The tendency for the jump distance to remain principally unchanged in the glassy state is related to the small change in chain arrangement caused by the frozen micro-Brownian motion [22].

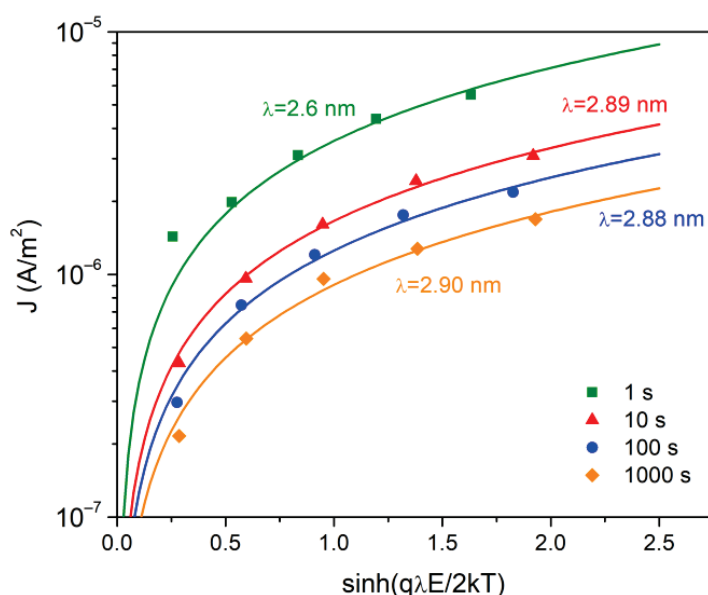


Figure 7. Current density versus $\sinh(q\lambda E/2kT)$, at different times of analysis.

Based in these models, it is established that the isothermal curves $I(t)$ obtained for the EC are the result of the contribution of electron injection, dipolar orientation, and ionic conduction. On the other hand, there is no evidence of SCLC under the conditions analyzed. This is not enough to establish that the injected electrons have not restricted

their mobility due to the traps (shallow or deep) in the bulk of the polymer, which would produce an accumulation of electrical charge. However, the presence of space charge can be stimulated by a considerable increase in temperature, or the injection of electrons at high electric fields.

4.5. AC Electrical Conductivity

AC electrical conductivity, σ_{AC} , measurements at temperatures above glass transition temperature ($T_g = 397$ K from DSC) were carried out. Figure 8 shows σ_{AC} as a function of frequency, from 20 Hz to 2 MHz, and at temperatures from 403 to 473 K. At low frequencies, an increase in σ_{AC} is denoted as the temperature increases; this suggests that the σ_{AC} is a thermally activated process and can be associated with an increase of the mobility of charge carriers. This is favored due to the increment in free volume and an increase in segmental motions of polymer chains. A nonlinear dependence on conductivity with frequency is observed, where this dependence is presumed to be due to hopping transport between the localized states. Additionally, it is possible that electrode polarization is present due to a charge accumulation near the electrodes, which is observed at low frequencies and in the temperature range of 403–433 K. Jonscher proposed an equation relating AC conductivity as a function of frequency, $\sigma_{AC} = A\omega^s$, which follows the form of a power law [36], where A is a constant and the exponent s takes values typically between 0 and 1. This behavior has been observed in disordered solids and amorphous polymers at high frequencies [37,38]. The equation did not fit adequately to the data in Figure 8 in the analyzed frequency spectrum. A possible explanation for this is the presence of various conduction mechanisms, such as the Maxwell–Wagner–Sillars (MWS) interfacial relaxation, which originates from the heterogeneous structure of the semicrystalline polymer [39].

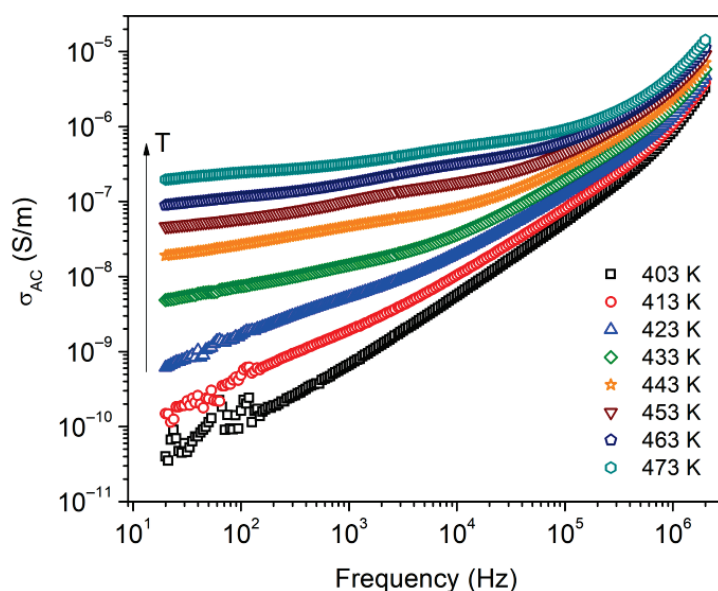


Figure 8. AC conductivity as a function of the frequency, at different temperatures.

From the data in Figure 8, an Arrhenius equation $\sigma_{AC} = \sigma_0 \exp(-E_a/kT)$ was used to calculate the activation energy E_a , where σ_0 is a pre-exponential factor, k is Boltzmann's constant, and T is the absolute temperature. The $\ln \sigma_{AC}$ vs. $1000/T$ has been plotted, for which the activation energy has been calculated for six different frequency values, shown in Figure 9a. The results are presented in Figure 9b, where one can observe a decrease in the activation energy as the frequency increases.

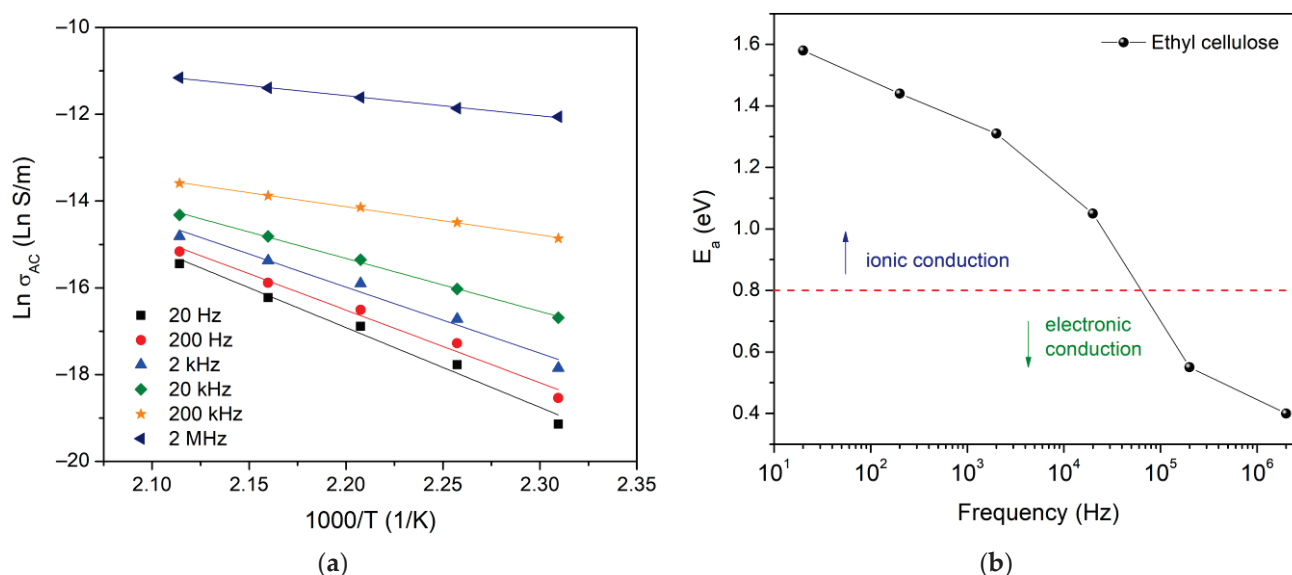


Figure 9. (a) $\ln \sigma_{AC}$ versus $1000/T$ and (b) activation energy as a function of frequency.

In the literature, the approach proposed by Jonscher and Ansari has been used for the identification of conduction mechanisms. For this approach, E_a being higher than 0.8 eV indicates a dominant ionic conduction, whereas E_a being less than 0.8 eV indicates that the dominant conduction mechanism is through the motion of electrons [40]. The value obtained in this work $E_a = 1.6$ eV, at low frequency, suggests a conduction mechanism governed by the motion of the macromolecular chains of ethyl cellulose. However, the presence of space charge and the phenomenon of interfacial relaxation should not be ruled out.

5. Conclusions

This study has confirmed the semicrystalline structure of EC, which consists of an intimate mixture of amorphous and crystalline phases. The XRD tests revealed a crystal size of about 10 Å and a degree of crystallinity of 54%. From DMA it was observed that the EC presents a main mechanical relaxation at 402 K, which is related to cooperative molecular movements of the glass transition phenomenon. The transient currents presented an exponential decay as a function of time. The comparison between experimental data and the solution from the fractional differential equation for the RC circuit shows good agreement. The fractional order takes values between 0 and 1, and it has been found that this parameter lowers as the voltage increases, indicating increased electric charge storage.

The interpretation of the voltage–current data has given a positive result to the Schottky mechanism, with injection of charge carriers through the electrode–polymer interface at voltages greater than 100 V. At the level of the bulk polymer, the electric current generated is the result of the orientation of the electric dipoles and ionic-hopping process. Under the voltage and temperature conditions employed in this work, space charge was not detected. AC conductivity measurements yield an activation energy of 1.6 eV, at high temperatures and low frequencies, indicating that electric conduction is governed by the mobility of EC chain segments.

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Article

Superhydrophobic, Magnetic Aerogels Based on Nanocellulose Fibers Derived from Harakeke for Oily Wastewater Remediation

Yitong Zhai and Xiaowen Yuan *

Future Fibers Laboratory, School of Engineering, Computer and Mathematical Sciences,
Auckland University of Technology, Auckland 1010, New Zealand; yitong.zhai@autuni.ac.nz

* Correspondence: xiaowen.yuan@aut.ac.nz; Tel.: +64-9-921-9999 (ext. 7320)

Abstract: Cellulose-based aerogels have been seen as a promising sorbent for oil and organic pollutant cleaning; however, their intrinsic hydrophilicity and difficulty of recycling has hindered their practical application. In this work, a superhydrophobic, magnetic cellulose-based aerogel was fabricated as a highly efficient sorbent for the adsorption of oils and organic solvents. The aerogel was prepared via a simple freeze-drying method, followed by chemical vapor deposition (CVD). The incorporation of Fe_3O_4 nanoparticles into the aerogel not only makes it responsive to external magnetic field, but also contributes to the better hydrophobicity of the aerogel, in which the water contact angle (WCA) was about 20° higher than the aerogel without loading with Fe_3O_4 nanoparticles. The adsorption test showed that the resultant aerogel can selectively adsorb a wide range of oils and organic solvents from oil/water mixtures with a high adsorption capacity (up to 113.49 g/g for silicone oil). It can retain about 50% of its adsorption capacity even after 10 adsorption–squeezing cycles, which indicates its outstanding reusability. Moreover, the aerogels can be easily controlled by an external magnet, which is preferred for the adsorption of oily contaminants in harsh environments and enhanced the recyclability of the aerogel. We believe that this study provides a green and convenient approach for the practical fabrication of cellulose-based oil sorbents.

Keywords: aerogel; cellulose; harakeke; magnetic; oil adsorption; reusability

1. Introduction

It is widely recognized that there is an urgent need to address the issue of water pollution caused by petroleum and organic solvents [1]. Various methods, such as in situ burning [2], flotation [3], centrifugation [4], and bioremediation [5] have been applied to solve this problem. However, those methods show many disadvantages, such as secondary pollution, high-cost, and high-energy consumption. Owing to the high efficiency and ease of operation, physical adsorption has been considered to be a preferable approach to other methods. Traditionally used synthetic polymer-based sorbents, including polyurethane foam, melamine foam, and polypropylene, have been modified through various methods, including particle deposition and polymerization, to alter their surface wettability to achieve the selective adsorption of oil [6,7]. However, those synthetic polymer-based oil sorbents not only show low-efficiency and non-biodegradability, but also suffer from complicated preparation processes and non-satisfactory adsorption capacities [8]. Nowadays, the concept of sustainable development has attracted much attention from researchers. The demand for renewable carbohydrate polymers to replace petroleum-based polymers has continuously grown [9,10]. As a renewable, biocompatible, biodegradable, and most abundant natural polymer, cellulose can be extracted from plants, animals, tunicates, algae, and some bacteria. Owing to its abundance and low cost, cellulose is now receiving widespread attention from researchers as a promising alternative to fossil fuel-based polymers [11].

Since first being reported by Kistler in 1931 [12], aerogels have developed a broad variety of different types, ranging from the first generation of silica-based to the third generation of cellulose-based types [13]. Aerogels are ultralight 3D porous materials with many fascinating properties, such as low density, high porosity, and expansive specific surface area. Aerogels have been extensively studied for a wide range of applications from thermal insulation and drug delivery to the provision of catalyst carriers [14]. Furthermore, the high volume-to-weight ratios of aerogels make them ideal for sorbent applications [8]. Different types of aerogels have been investigated and have shown promising results for oil sorption, such as synthetic silicone [15], synthetic polymer-based [16], and graphene-based aerogels [17]. However, the silica aerogels suffer from poor mechanical strength and fragility [18], and synthetic polymer-based aerogels are not biodegradable and their accumulation in the ecosystem makes the problem more serious [19]. The high cost and complicated fabrication process are the main drawbacks of graphene-based aerogels. Recently, considerable attention has been paid to developing novel cellulose-based aerogels for oil sorption and oil/water mixture separation. For example, Zhang et al. [20] modified microfibrillated cellulose with Vinyltrimethoxysilane (VTMO) and prepared hydrophobic aerogel via freeze-drying. The modified microfibrillated cellulose aerogel was used as an oil sorbent for oil/water mixture separation and exhibited a high adsorption capacity up to 130.1 g/g. Chen et al. [21] immersed the cellulose aerogel into hydrolyzed silicone alcohols, in which the silanol reacted with the cellulose hydroxyl groups to prepare hydrophobic cellulose-based aerogels for oil sorption.

However, the difficulty of recycling oil-adsorbed aerogels hinders their practical application. Preparing magnetic and hydrophobic cellulose aerogel provides a promising solution to this issue. Herein, a facile and cost-effective method was developed to prepare Fe_3O_4 nanoparticle-loaded, low-density, high-porosity, superhydrophobic, and magnetic cellulose aerogel (HMCA) from harakeke (a New Zealand native plant). With the incorporation of Fe_3O_4 nanoparticles, the HMCA can be controlled to move by an external magnet, which greatly facilitates the recycling of oil saturated aerogels and the application of aerogels in harsh environments. The prepared HMCA exhibited outstanding adsorption and retention capacity towards a variety of oils and organic solvents and excellent oil adsorption selectivity in oil/water mixtures. The adsorption capacity of HMCA is the highest for silicone oil of 113.49 g/g. The aerogel also exhibited good reusability, where about 50% of its original adsorption capacity can be retained after 10 adsorption–squeezing cycles. Moreover, the aerogel only showed an about 10% reduction in the adsorption capacity if the adsorbed oils were removed via washing. Therefore, the HMCA prepared in this work has the potential to become an efficient and sustainable oil absorbent for treating oil pollution in water.

2. Materials and Methods

2.1. Materials

Pure cellulose fibers (a SEM image of the fibers is shown in Figure S1) were extracted from harakeke according to the method from our previous work [22]. Glutaraldehyde solution (25%, Alfa Aesar, Haverhill, MA, USA) was used. Fe_3O_4 nanoparticles, methyl-trichlorosilane (MTCS), sulfuric acid (H_2SO_4 , 1.0 vol%), and ethanol were obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). All chemicals were analytical grade, and distilled water was used throughout.

2.2. Synthesis of Pristine Cellulose Aerogel (CA)

In total, 0.5 g of the extracted cellulose was magnetically stirred in 99.5 g of distilled water at 25 °C for 48 h to allow the aerogels to completely swell. The mixture was then put into a homogenizer (SHW400R, Shanghai Weisheng Electrical Instrument Co., Ltd., Shanghai, China) at 10,000 rpm for 2 h, followed by ultrasonication treatment (UP400St, Hielscher, Teltow, Germany) at an output power of 600 W for 2 h in an ice/water bath so that a homogeneous cellulose microfibril suspension of 0.5 wt% was formed. Subsequently,

150 μL of glutaraldehyde (25 wt%) and 50 μL of sulfuric acid (2 vol%) were added dropwise into 30 g of CNF suspension (0.5 wt%) via magnetic stirring for 1 h, followed by heating in an oven at 75 $^{\circ}\text{C}$ for 3 h to promote crosslinking. Next, the solution was frozen at -196°C using liquid nitrogen and freeze-dried (LGJ-12S, Songyuan Freeze Dryer, Songyuan, China) at -50°C for 48 h to produce cellulose aerogel.

2.3. Preparation of Magnetic Fe_3O_4 Nanoparticle-Coated Cellulose Aerogels (MCA)

In order to attain the optimal addition of Fe_3O_4 nanoparticles for MCA, different amounts of Fe_3O_4 nanoparticles (0.2, 0.4, 0.6, and 0.8 wt%) were added to the glutaraldehyde and cellulose suspension to prepare MCAs with different Fe_3O_4 loadings. The Fe_3O_4 /cellulose mixture suspension was magnetically stirred for 1 h at room temperature, followed by ultrasonication in the ice/water bath for another 20 min to remove any bubbles and uniformly disperse the Fe_3O_4 nanoparticles. The resultant bubble-free solution was then placed in a vacuum oven at 75 $^{\circ}\text{C}$ for 3 h to promote crosslinking. Subsequently, the crosslinked mixture was poured into a brass mold and frozen using liquid nitrogen at -196°C and then freeze-dried for 48 h at -50°C .

2.4. Preparation of Super-Hydrophobic Magnetic Cellulose Aerogels (HMCA)

The pristine MCA was hydrophilic because of the presence of large amounts of surface hydroxyl groups on the cellulose fiber. Therefore, hydrophobization was needed to modify the MCA, making it more suitable for oil adsorption. Silanization was carried out through chemical vapor deposition, using MTCS as the precursor. The obtained MCA was placed in a desiccator, and an open glass vial containing 5 mL of MTCS was placed next to the MCA in the same desiccator. Then, the desiccator was tightly sealed and heated in an oven at 50 $^{\circ}\text{C}$ for 12 h.

2.5. Characterization

The morphology of the aerogels was observed via scanning electron microscopy (SEM) (ZEISS Sigma 300, Carl Zeiss, Oberkochen, Germany) at a working voltage of 5 kV. The SEM was equipped with an energy-dispersive X-ray (EDX) spectrometer for elemental analysis. The chemical structure of the aerogels was characterized using a Fourier transform infrared spectrometer (FT-IR) (IRTracer-10; Shimadzu Co. Ltd., Tokyo, Japan) within 600–4000 cm^{-1} , with a resolution of 4 cm^{-1} , and X-ray diffraction (D8 Advance, Bruker, Billerica, MA, USA) operating with $\text{Cu-K}\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$) at a scan rate of $4^{\circ}/\text{min}$ at 40 kV/20 mA, ranging from 5 to 60° . The surface elemental analyses of the aerogels were characterized via X-ray photoelectron spectroscopy (XPS) with Al $\text{K}\alpha$ X-ray radiation (Thermo Fisher, Waltham, MA, USA). The thermal stability of the sample was characterized via thermogravimetric analysis (TG) using a TG analyzer (Q600, STA, New Castle, DE, USA). The sample was heated in air at a heating rate of 10 $^{\circ}\text{C min}^{-1}$ from room temperature to 800 $^{\circ}\text{C}$. The surface wettability of the aerogels was determined by measuring static water contact angle using a contact angle goniometer (OCA20, Dataphysics, Filderstadt, Germany) at ambient temperature. The magnetic measurement was performed using a vibrating sample magnetometer (VSM, LH-3, Nanjing NanDa Instrument Co., Ltd., Nanjing, China). Hysteresis loops were generated for the determination of the aerogel saturation magnetization (M_s) and coercivity (H_c).

The density of the aerogels was calculated via Equation (1), where the mass of the sample was measured using an electronic balance. Three parallel tests were carried out on each sample to find the average result [23].

$$\rho = \frac{m}{v} \quad (1)$$

where ρ is the density of the aerogel, and m and v are the mass (g) and volume (cm^3) of the sample, respectively.

The porosity of the sample was calculated via Equation (2).

$$P(\%) = \frac{V - \left(\frac{m}{\rho_c}\right)}{V} \times 100\% \quad (2)$$

where P is the porosity of the aerogel, m and v are the mass (g) and volume (cm^3) of the sample, and ρ_c is the bulk density of the cellulose scaffold, which is the bulk density of cellulose ($\rho_c = 1.528 \text{ g/cm}^3$) [24].

The adsorption capacity of HMCA was determined based on the method reported in a previous work. In brief, a measured weight of HMCA was immersed in different oils and solvents at room temperature for sufficient time to reach adsorption saturation. We removed the sample from the oil to drain the surface excess and weighed the sample again. The oil adsorption capacity of the HMCA was calculated based on Equation (3).

$$K = \frac{W - W_o}{W_o} \times 100\% \quad (3)$$

where K is the adsorption capacity of HMCA, and W_o and W are the weight before and after oil adsorption, respectively [25].

3. Results and Discussion

3.1. Fabrication and Structural Characterization of CA, MCA, and HMCA

Figure 1 is a schematic illustration of the preparation process of HMCA, and the corresponding photographs are of each intermediate aerogel. The cellulose fibers were crosslinked through the addition of glutaraldehyde, and Fe_3O_4 was loaded into the aerogel by dispersing Fe_3O_4 nanoparticles into the cellulose suspension through ultrasonication. The hydrophobization of magnetic cellulose aerogel was performed via chemical vapor deposition (CVD), using MTCS as the gaseous precursor. The color of HMCA changed from black to yellow–green after CVD, probably due to the formation of a small amount of iron(II) chloride tetrahydrate, which is the result of the reaction of Fe_3O_4 nanoparticles with HCl, a byproduct of MTCS hydrolysis. To optimize the concentration of Fe_3O_4 , MCAs with different amounts of Fe_3O_4 nanoparticles were prepared, starting from 0.2 wt% and gradually increasing to 0.8 wt% using increments of 0.2 wt%. It was found that as the concentration of Fe_3O_4 increased, the adsorption capacity of MCA for pump oil decreased from 96.74 ± 0.89 to $36.89 \pm 1.23 \text{ g/g}$. However, for the MCA of 0.2 wt% Fe_3O_4 , the aerogel cannot be properly controlled by the external magnet; therefore, by comprehensively considering the magnetic properties and adsorption properties, aerogels with 0.4 wt% Fe_3O_4 were chosen as the optimal composition. Figure 2 shows the SEM images of CA (a,b), MCA (c,d), and HMCA (e,f). It can be seen that the pristine CA (Figure 2a,b) possesses a highly porous and staggered interconnected three-dimensional microstructure with the presence of sheet-like cellulose plates. The sheet-like cellulose plates composed of entangled cellulose fibrils form during the freezing process, in which the cellulose fibers are compressed by the growing ice crystals accumulating in front of them. After the addition of Fe_3O_4 nanoparticles, the color of the aerogel changed from white to black. Figure 2c shows that the staggered network structure with sheet-like plates does not change after the loading of Fe_3O_4 nanoparticles. The higher magnification image (Figure 2d) shows that Fe_3O_4 nanoparticles are uniformly dispersed on the surface of cellulose sheets. The strong hydrogen bonds formed between the cellulose structure and Fe_3O_4 nanoparticles are believed to be the main reason for this uniform distribution [26]. After CVD treatment with MTCS, a thin layer of polysiloxane particles forms (Figure 2e,f); therefore, the aerogel shows more particles on the surface. Although more particles are deposited on the cellulose sheets surface, the porous cellulose skeleton with cellulose sheets remains. The distribution of Fe_3O_4 and silane particles is characterized via EDX. As shown in Figure 1g, the Fe and Si elements are uniformly distributed on the surface of the cellulose sheet, which is in

agreement with the SEM images. Table 1 shows the density of CA, MCA, and HMCA, respectively. All of the aerogels show low density and high porosity (>95%).

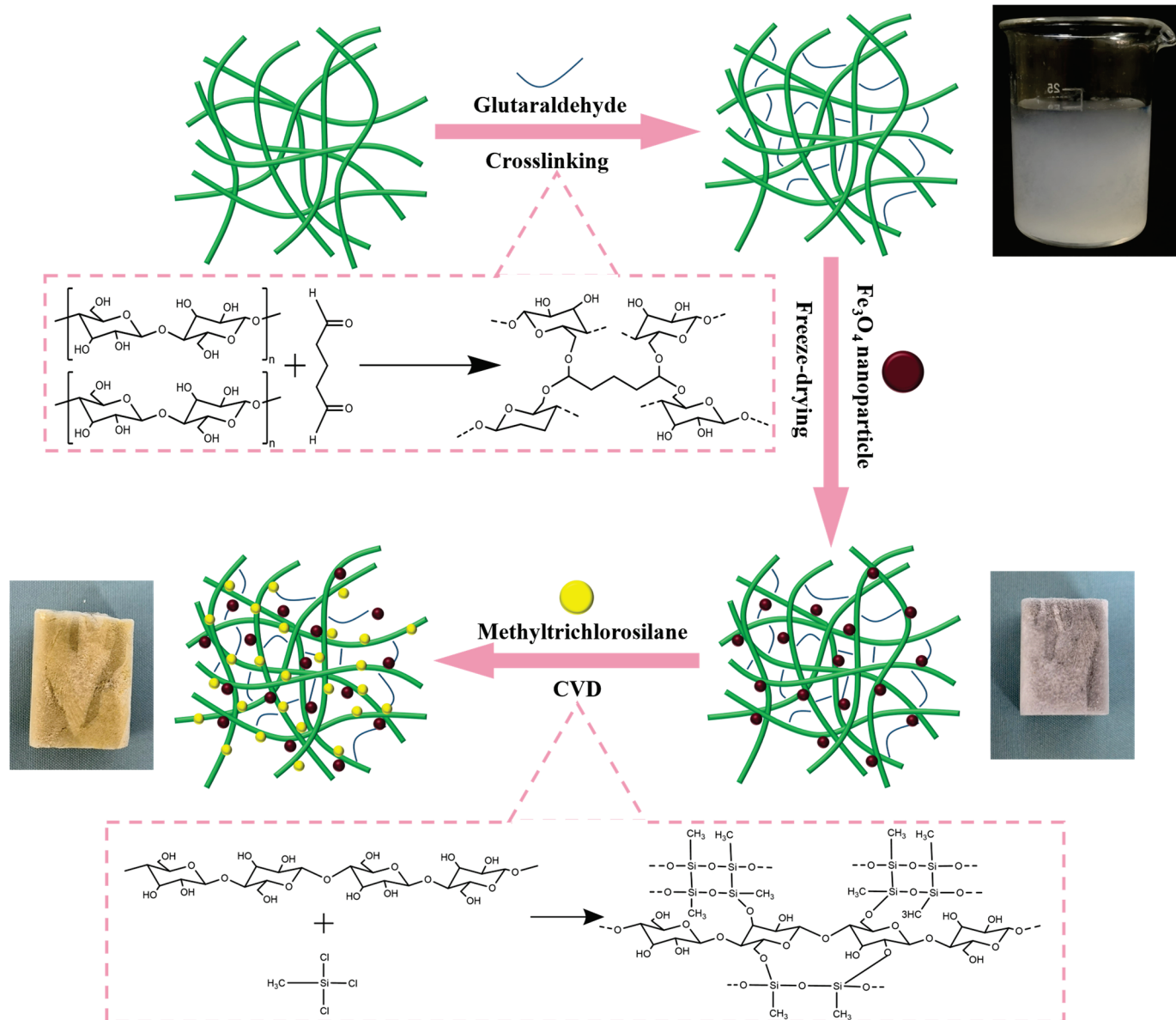


Figure 1. The schematic illustration of the preparation process of superhydrophobic magnetic cellulose aerogels.

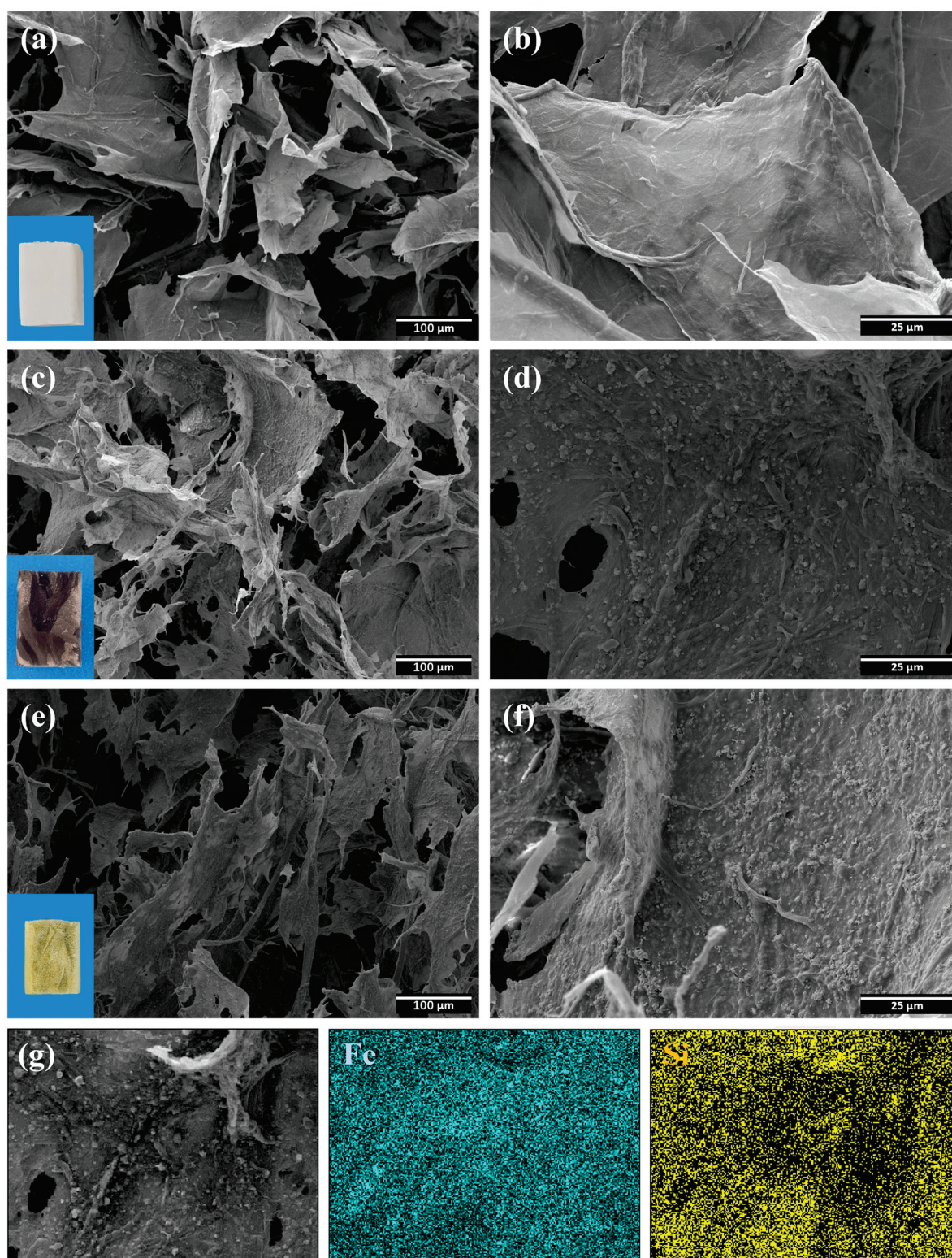


Figure 2. SEM images of (a,b) CA at different magnifications. (c,d) MCA at different magnifications, and (e,f) HMCA at different magnifications. The corresponding digital camera photos of each aerogel are at the bottom-left corner of (a,c,e). (g) EDX elemental mapping of HMCA, with blue and yellow corresponding to Fe and Si, respectively.

Table 1. Density and porosity of CA, MCA, and HMCA.

	CA	MCA	HMCA
Density (mg/cm ³)	9.47	14.63	18.25
Porosity (%)	99.38	99.04	98.80

3.2. Characterization of CA, MCA, and HMCA

The thermogravimetric analyses of CA, MCA, and HMCA are shown in Figure 3. All three samples underwent a small weight decrease (<10%) from the initial room temperature to 200 °C, which is ascribed to the evaporation of the absorbed water. The main weight loss that happened from 200 to 350 °C was mainly due to the thermal degradation of cellulose polysaccharide [27]. The residual weights for CA, MCA, and HMCA are 19%, 38% and 52%, respectively, indicating the weight percentages of the Fe₃O₄ nanoparticles and coated polysiloxane particles in the aerogels.

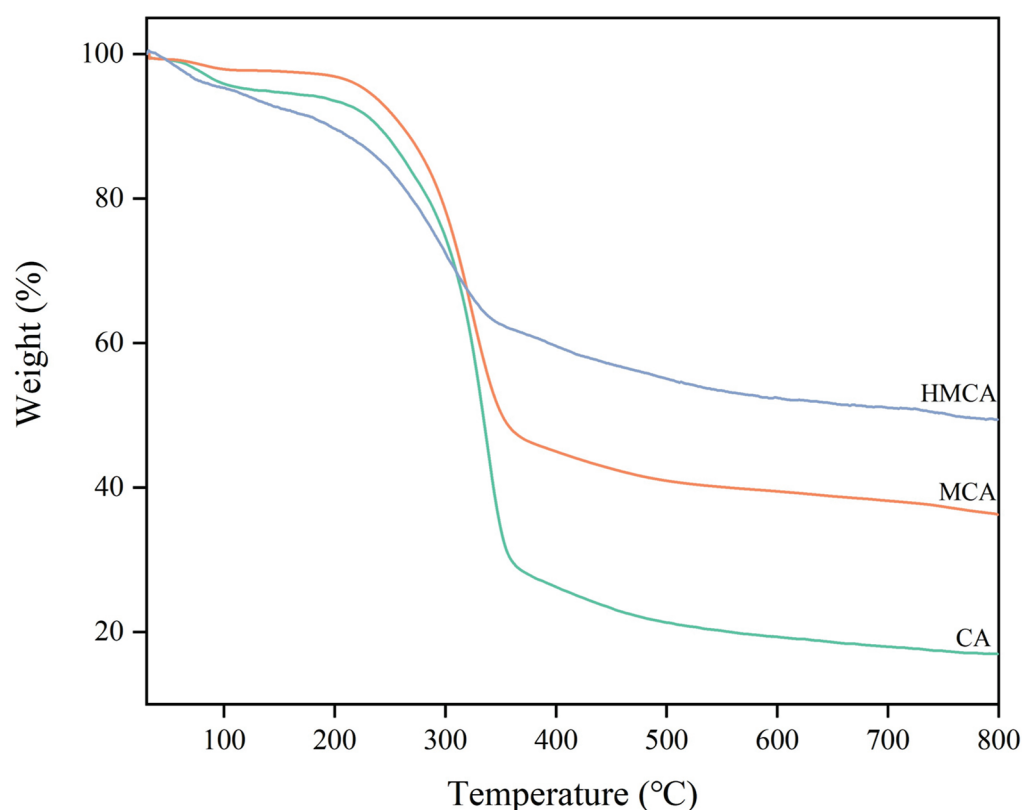
**Figure 3.** The thermogravimetric analyses of CA, MCA, and HMCA.

Figure 4a shows the XRD pattern of three aerogels. The unmodified CA exhibited two obvious peaks at $2\theta = 16.2^\circ$ and 22.5° , which are the characteristic peaks of cellulose, attributed to the (110) and (200) reflections [28]. After the addition of Fe₃O₄ nanoparticles, five new peaks at 30.1° , 35.6° , 43.2° , 57.0° , and 62.5° , corresponding to (220), (311), (400), (511), and (440) plane diffractions of Fe₃O₄, appeared on the MCA and HMCA XRD patterns [24]. Those characteristic Fe₃O₄ diffraction peaks confirmed the successful incorporation of Fe₃O₄ nanoparticles into the cellulose aerogel. No new peak or peak shift were seen from the silane modified aerogel, indicating that no new phase was introduced via this silanization process.

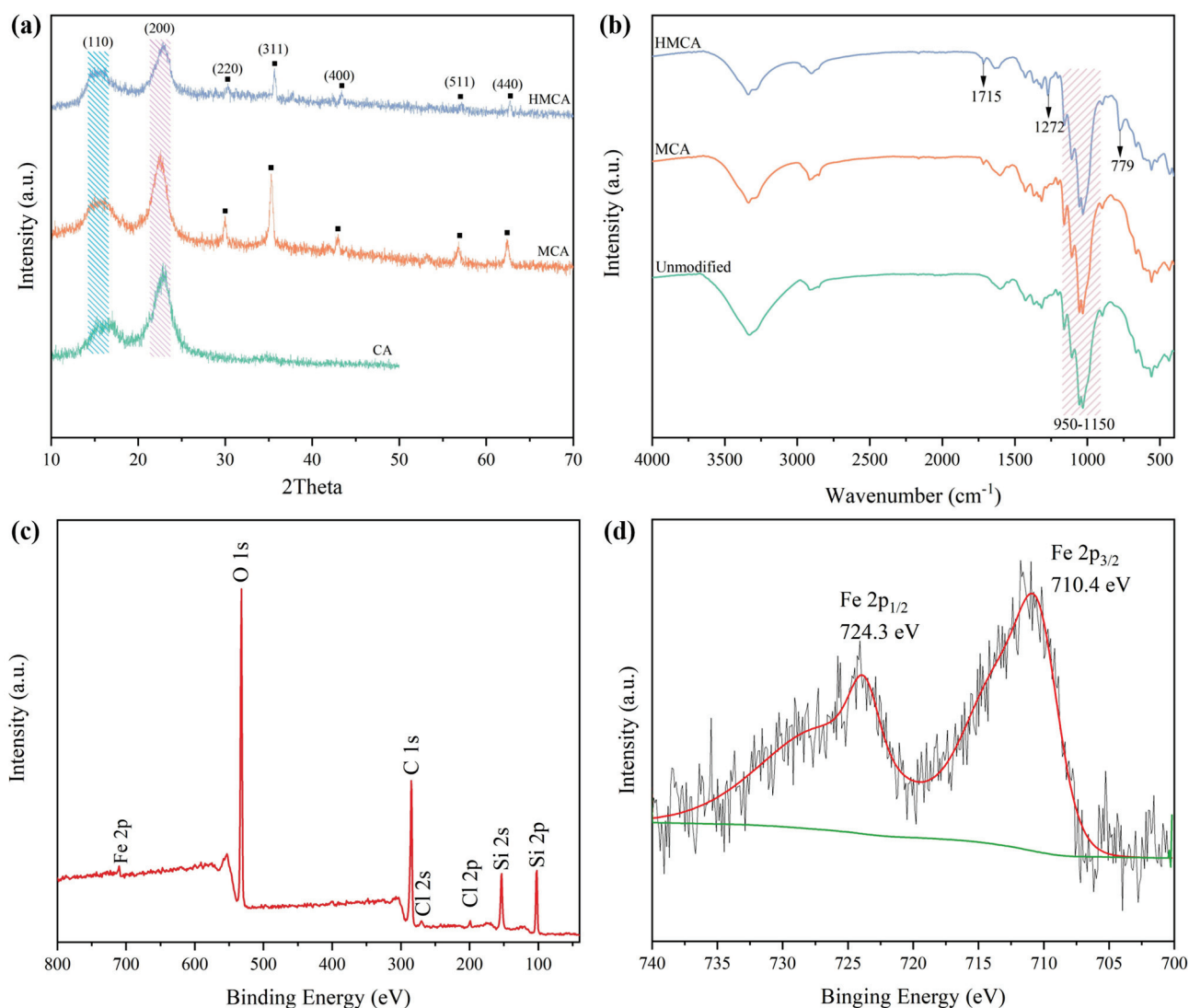


Figure 4. (a) XRD pattern of CA, MCA, and HMCA. (b) The FTIR spectra of unmodified cellulose aerogel, MCA, and HMCA. (c) Full-scan XPS spectra of HMCA. (d) XPS high-resolution Fe 2p scan of HMCA.

FTIR was applied to study the effects of the glutaraldehyde crosslinking of the cellulose aerogel. As shown in Figure 4b, all three samples exhibit the characteristic peaks of cellulose, including a wide band around $3700\text{--}2900\text{ cm}^{-1}$, with a broad peak around $3300\text{--}3350\text{ cm}^{-1}$, which is attributed to the O–H stretching; a peak at 2890 cm^{-1} , which is assigned to the C–H stretching vibration; and a peak at 1029 cm^{-1} , which is due to the C–O vibration of cellulose [29,30]. Compared to the unmodified cellulose aerogel, the FTIR spectrum of glutaraldehyde crosslinked aerogels (MCA) showed a higher peak intensity at $950\text{--}1150\text{ cm}^{-1}$, which is ascribed to C–O stretching. This is attributed to the successful crosslinking of glutaraldehyde, in which its C=O double bonds are open [31]. In addition, a new aldehyde peak appearing at 1715 cm^{-1} also confirmed the crosslinking of glutaraldehyde [32]. After hydrophobic modification via MTCS, two new peaks were seen on the FTIR spectra of HMCA at 779 cm^{-1} and 1272 cm^{-1} . These two absorption peaks were attributed to the Si–C bond vibration and the $-\text{CH}_3$ vibration of the polysiloxane particles, respectively. The appearance of those two new peaks indicates the successful MTCS modification of the aerogels. XPS analysis was carried out on the HMCA to study the chemical composition. The full-scan spectra (Figure 4c) shows seven peaks associated with Si 2p, Si 2s, Cl 2p, Cl 2s, C 1s, O 1s, and Fe 2p, which is in agreement with the EDX

results. In the full-scan spectra, the peaks associated with chlorine are due to the formation of a small amount of FeCl_2 during the MTCS CVD process, and the weak intensity indicates its content is very low. The high-resolution Fe 2p scan is shown in Figure 4d, and two peaks at 710.4 eV and 724.3 eV attributed to Fe 2p_{3/2} and Fe 2p_{1/2} spin orbit peaks of Fe_3O_4 , respectively, are seen, which indicate that the Fe_3O_4 has been successfully incorporated into the aerogel [33].

3.3. Surface Wettability

The surface wettability of oil sorbent is crucial for achieving selective adsorption of oil. CA and MCA are hydrophilic due to the presence of a large number of hydroxyl groups. A straightforward chemical vapor deposition (CVD) process, using MTCS as a gaseous precursor, was carried out on the MCA to alter its surface wettability (Figure 1). Following CVD, the hydrophilic $-\text{OH}$ groups were substituted with the polysiloxane particles, which contain the hydrophobic $-\text{CH}_3$ terminal groups that rendered the aerogels superhydrophobic. The enhanced surface roughness resulting from the presence of polysiloxane particles further enhanced the HMCA's hydrophobicity. As shown in Figure 5a, after MTCS modification, HMCA could float on the surface of the water, but CA and MCA sunk under the water surface. Figure 5b shows that when pushing HMCA under water, a mirror reflection was seen on the surface of the aerogel, which is due to the entrapped air between the aerogel surface and surrounding water. This mirror-reflection also refers to the Cassie–Baxter surface wetting model [34]. Figure 5c shows that water droplets (dyed with methylene blue) stood spherically on the surface of HMCA, while vegetable oils (dyed with Sudan red) were adsorbed and penetrated the aerogel. The wettability difference indicates the excellent oil adsorption selectivity of MTCS modified HMCA. The static water contact angle was measured to characterize the surface wettability of HMCA. As shown in Figure 6a, the water droplet stood spherically on the surface of HMCA with a contact angle of 150.3° , which illustrated its superhydrophobicity. The water contact of the MTCS-treated cellulose aerogel (without the addition of Fe_3O_4 nanoparticles) was also measured to study the effect of Fe_3O_4 nanoparticles on aerogel's wettability. It was found that without the addition of Fe_3O_4 nanoparticles, the water contact angle of the MTCS-treated aerogel was 130° (Figure 6b), which was lower than the HMCA. This is attributed to the increased surface roughness of the Fe_3O_4 nanoparticles coating the aerogel surface.

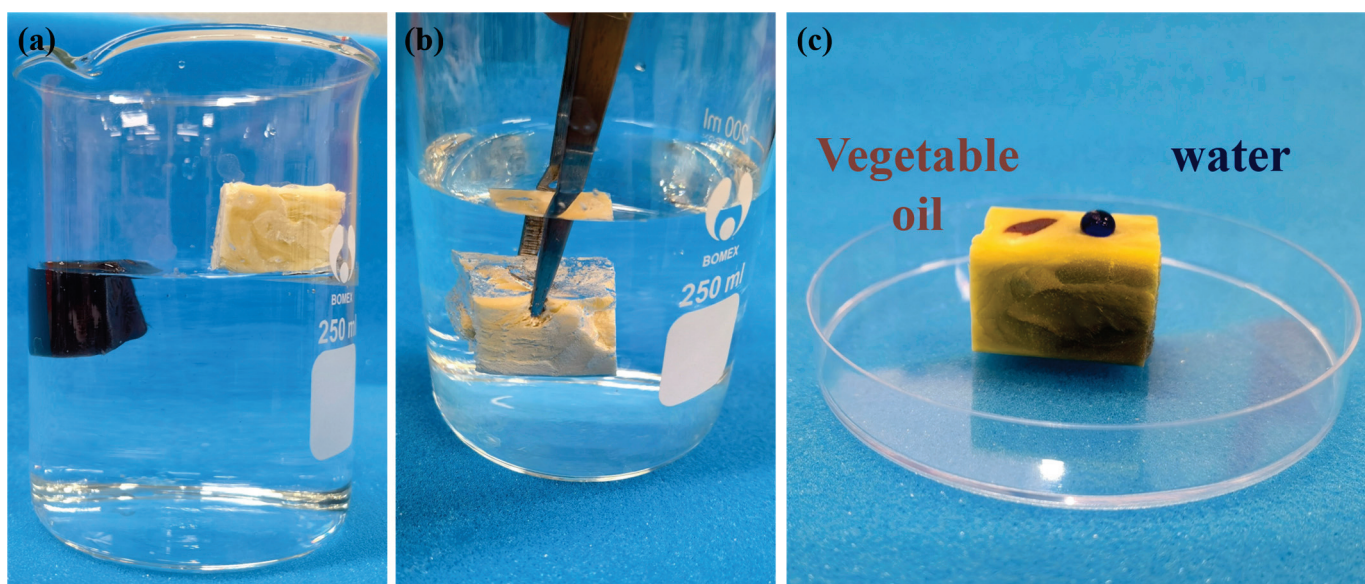


Figure 5. (a) MCA is sinking into the water, while HMCA is floating on the water surface. (b) Mirror-reflection phenomenon occurred when pushing HMCA under water. (c) Vegetable oil and water droplets on the surface of HMCA.

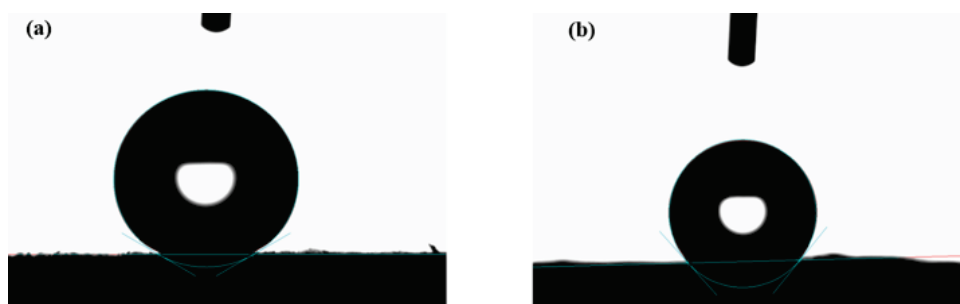


Figure 6. (a) Static water contact angle of HMCA. (b) Static water contact angle of MTCS-treated cellulose aerogel without addition of Fe_3O_4 nanoparticles.

3.4. Magnetic Property of HMCA

Figure 7a shows that after the incorporation of Fe_3O_4 nanoparticles, the HMCA can be easily lifted by external magnets, which demonstrates its great response to an external magnetic field. Since the magnetic properties of HMCA are critical for its application, the magnetic properties of the aerogel were further investigated using VSM at room temperature, with a magnetic field strength of $\pm 18,000$ Oe, and the resultant hysteresis loops are shown in Figure 7b,c. From Figure 7b, the saturation magnetization (M_s) of HMCA is determined as 15.75 emu/g , and the remanence (M_r) and coercivity (H_c) are determined as 1.12 emu/g and 40.42 Oe , respectively, in Figure 7c. Due to the small values of M_s and H_c , HMCA is determined to have superparamagnetic behavior, and the high M_s value ensured the good magnetic response of HMCA to external magnetic fields.

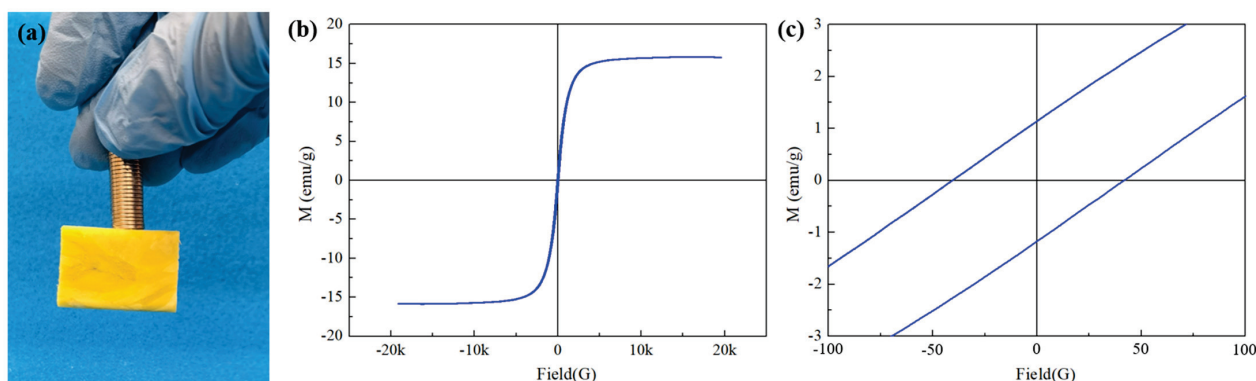


Figure 7. (a) Lifting HMCA with magnets. (b) Vibration sample magnetometer (VSM) measurement diagram of HMCA. (c).

3.5. Adsorption Capacity and Adsorption Kinetics of HMCA

To investigate the performance of modified cellulose aerogels for oil absorption in water and assess their suitability for addressing marine oil pollution, laboratory tests were conducted to determine their oil-removal efficacy. As shown in Figure 8a,b, HMCA was placed in a beaker containing a mixture of motor oil (dyed with Sudan III) and water. Upon contact with the oil/water mixture, the aerogel began to soak up the red motor oil while floating on the water surface. This demonstrated the rapid absorption rate of HMCA and outstanding oil sorption selectivity. Figure 8(a₂,a₃) shows that HMCA can be controlled to move across the water surface using external magnets. Furthermore, after the aerogel reached its sorption saturation, it still floated on the water surface, and no oil flow back was observed from the aerogel, which indicates its excellent oil sorption capacity and oil retention. Figure 8b shows that when pushing HMCA under the water, it could also quickly absorb the chloroform (dyed with Sudan III). The adsorption process of non-polar oils and organic solvents can be divided into three steps: (1) the diffusion of oil molecules onto the aerogel surface, (2) the retention of organic liquid due to the capillary force, and (3) the

accumulation of the adsorbed liquid into the pores of the aerogel [25,35]. Besides motor oil and chloroform, a variety of oils and organic solvents were selected to test the adsorption capacity of HMCA. The results are presented in Figure 8c. It was seen that HMCA showed a high adsorption capacity for all of the tested oils and organic solvents, and the maximum adsorption capacity for silicone oil was the highest at 113.49 g/g. This is mainly attributed to their superhydrophobicity and highly porous structure, which provides lots of space for 335 oils and organic solvents. Previous researchers have developed various oil sorbent materials with different oil adsorption capacities, including coir fiber-reinforced PVA aerogel (25–34 g/g) [36], silane-coated chitin sponge (29–58 g/g) [37], polydimethylsiloxane/carbonized bacterial cellulose sponge (3–9 g/g) [38], TiO₂-coated nanocellulose aerogels (20–40 g/g) [39], polyurethane foam (62–65 g/g) [40], calcium stearate-coated kapok fibers (up to 59.69 g/g) [41], graphene aerogels (260–570 g/g) [42], etc. The magnetic cellulose aerogel prepared in this work has the potential for practical oil adsorption due to the (i) outstanding oil adsorption capacity of many types of oils and organic solvents, (ii) low-cost and feasible preparation process compared to the graphene-based oil sorbents, and (iii) environmental friendliness compared to the synthetic polymer-based oil sorbents, such as polyurethane and polydimethylsiloxane. Moreover, the oil adsorption capacity of this HMCA is also better than those of many of other cellulose-based aerogels. A comparison of the oil adsorption capacity of our HMCA with those of other cellulose-based aerogels is shown in Table 2.

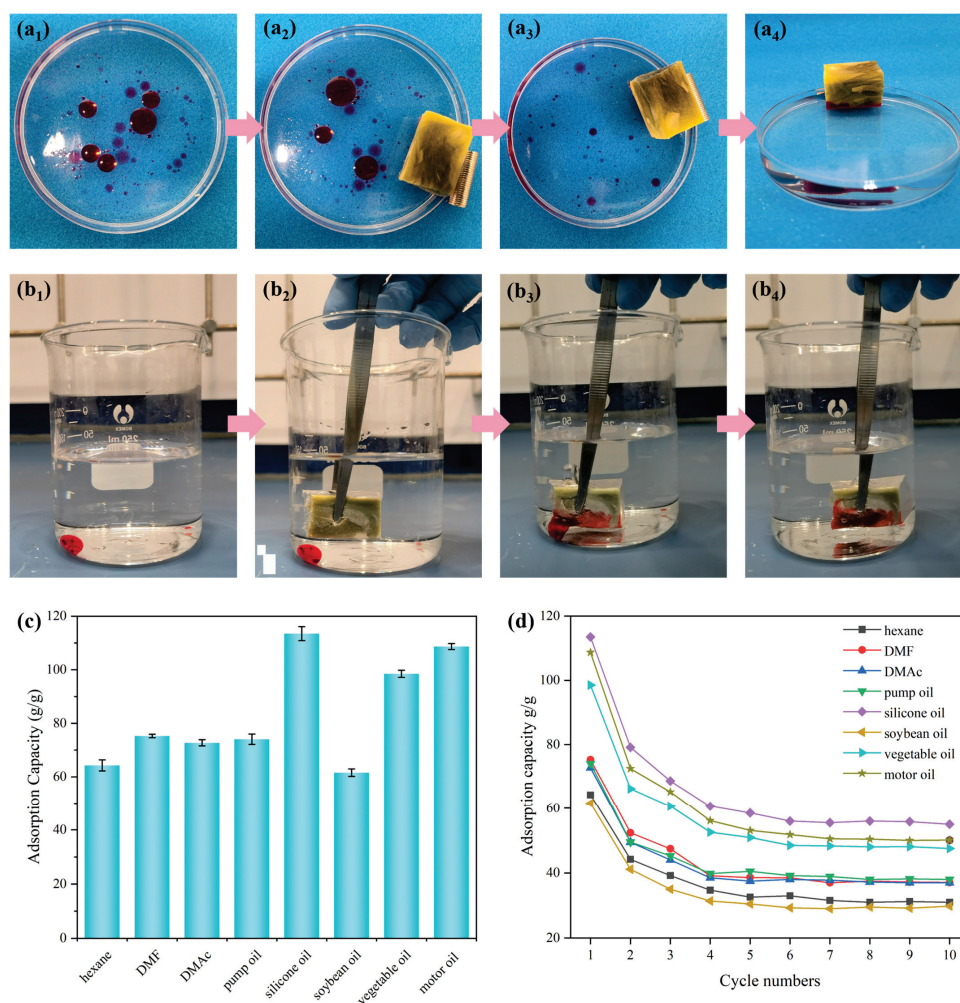


Figure 8. (a) Adsorption of vegetable oil at the surface of water. (b) Adsorption of chloroform below the water. (c) Adsorption capacity of HMCA for different types of oils and organic solvents. (d) Reusability of HMCA.

Table 2. Comparison of the oil adsorption capacity of our work with other cellulose-based aerogels.

Material	Adsorption Capacity (g/g)	Ref.
Cellulosic aerogel from water hyacinth	40.40–43.3	[43]
TiO ₂ -coated nanocellulose aerogels	20–40	[39]
Cellulose/tannic acid/castor oil aerogels	53.2–113.8	[44]
Nanoalumina-loaded nanocellulose aerogel	64.83–117.64	[45]
Hybrid silica–cellulose aerogel	24.8	[46]
Cellulose nanofibers/alginate aerogels	88.91	[47]
HMCA	61.56–113.49	This work

The adsorption kinetics of HMCA was determined by dipping equal-sized samples of HMCA into pump oil for different times and measuring their weight differences. Pseudo-first-order (Equation (4)) and pseudo-second-order models (Equation (5)) were used to simulate the oil sorption kinetics of s-HNAs.

$$\ln(q_m - q_t) = \ln q_m - k_1 t \quad (4)$$

$$\frac{t}{q_t} = \frac{1}{q_m} t + \frac{1}{k_2 q_m^2} \quad (5)$$

where q_m and q_t (g/g) are the maximum sorption capacity and the sorption capacity at time t , respectively. t (s) is the time. k_1 and k_2 are the sorption rate constants [48].

For the adsorption of pump oil using HMCA at room temperature, Figure 9a,b shows the plots of $\ln(q_m - q_t)$ versus t , and t/q_t versus t , corresponding to the pseudo-first-order and pseudo-second-order models, respectively. Figure 9c shows the experimental adsorption data and the fitted curves of the two adsorption models. The sorption constants are calculated based on the fitted curves, and the results are $k_1 = 0.06355$ and $k_2 = 0.00351$. The higher correlation factor (R^2) value shown in Figure 9b indicates that the pseudo-second-order model is better for the prediction of the adsorption behavior of HMCA.

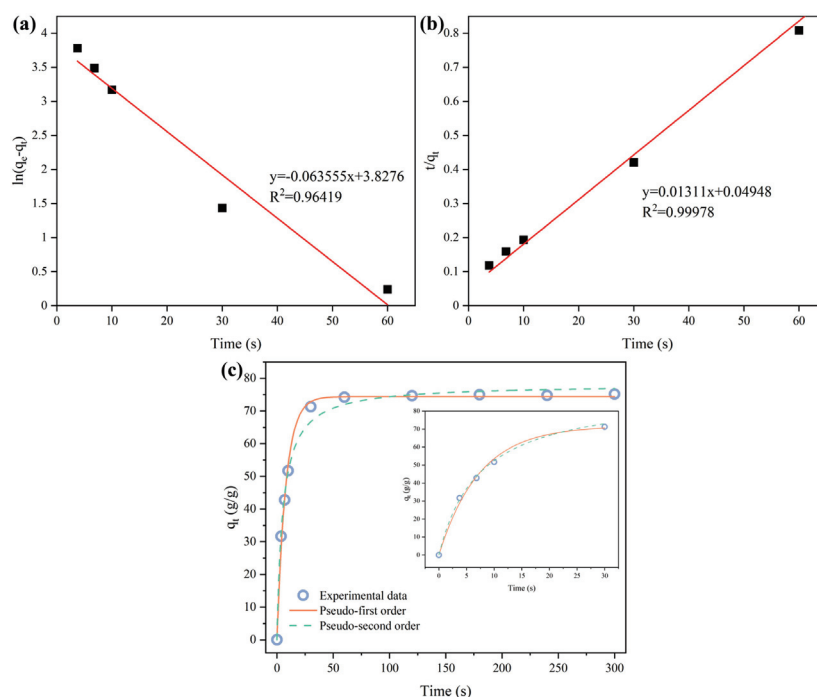


Figure 9. (a) Pseudo-first-order adsorption linear fitting of HMCA adsorb pump oil. (b) Pseudo-second-order adsorption linear fitting of HMCA adsorb pump oil. (c) Experimental data fitted with pseudo-first-order and pseudo-second-order models for the adsorption kinetics of HMCA-adsorbing pump oil.

Furthermore, the reusability of HMCA is another critical factor for its practical application as an oil sorbent. The reusability of HMCA was characterized via the cyclic adsorption–squeezing method, and the corresponding adsorption capacity after each cycle is shown in Figure 8d. In a typical adsorption–squeezing test, the saturated aerogel was removed from the organic solvent and squeezed between two parallel glass slides, whereby >90% strain was applied. Then, the aerogel was immersed in the same solvent to reach adsorption saturation. The weight of the aerogel after each adsorption cycle was measured. The recyclability measurement results show that the adsorption capacity of HMCA has a big deterioration (loss about 38% maximum adsorption capacity) after the first squeeze and gradually reduces to a stable value after the fourth cycle. The reduction in the adsorption capacity is attributed to the permanent collapse of the porous structure caused by mechanical compression, which results in the loss of pore volumes for retaining the organic solvent. However, about 50% of the initial adsorption capacity of HMCA can be retained after five adsorption–squeezing cycles, which indicates its good reusability.

Since the mechanical squeezing led to the permanent deformation of the internal structure of the aerogel, another rinsing method was applied to characterize the reusability of HMCA. In this method, the pump oil-saturated HMCAs were rinsed in ethanol to release the adsorbed oil. The sample was subsequently dried in a vacuum oven for 8 h at 70 °C, and the oil adsorption capacity was tested again. Almost no weight difference was observed between the ethanol rinsing, vacuum oven-dried HMCA, and HMCA before oil adsorption, indicating that this rinsing method can completely wash out the adsorbed oil. This adsorption–washing test was repeated 10 times, and the results are shown in Figure 10. It is found that the maximum adsorption capacity only decreased by about 10.5% after 10 adsorption–washing cycles, and the adsorption capacity tended to be stable.

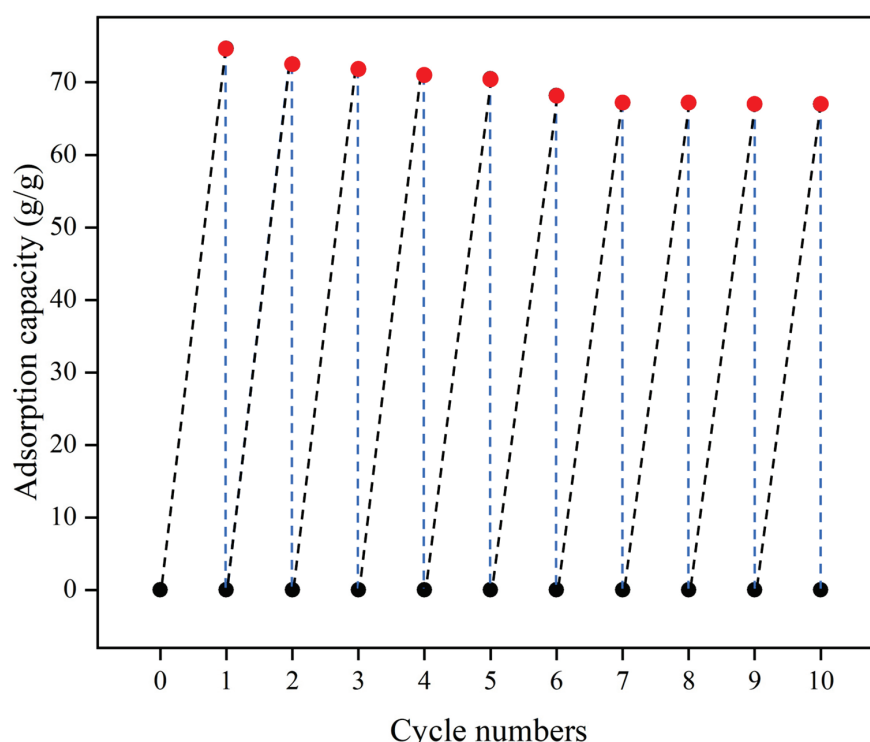


Figure 10. Reusability test of HMCA using adsorption–washing method.

4. Conclusions

A superhydrophobic magnetic cellulose-based aerogel was prepared in this work for the selective adsorption of oils and organic solvents from oily wastewater. The aerogel was prepared via an environmentally friendly and cost-effective method. It was found that the incorporation of Fe_3O_4 nanoparticles not only endowed the aerogel with super-

paramagnetic properties, but also contributed to better hydrophobicity. The water contact angle was surprisingly increased for the aerogel with Fe₃O₄ nanoparticles, and this was believed to be the result of higher surface roughness caused by the incorporation of Fe₃O₄. VSM testing shows that the HMCA is superparamagnetic, with a good response to external magnetic fields. The highly porous structure and hydrophobic modification were the main reasons for the outstanding oil adsorption capacity of HMCA (up to 113.49 g/g for silicon oil). The recycling test showed that the prepared HMCA can preserve about 50% of its maximum adsorption capacity after 10 adsorption–squeezing cycles. Moreover, the aerogel only showed an about 10.5% decline in the adsorption capacity if the washing method was applied to release the adsorbed oils, which indicates its great reusability. Combining these outstanding properties, this material has great potential to be used as an environmentally friendly and economical adsorbent for the treatment of oily wastewater.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym15193941/s1>, Figure S1. SEM image of the cellulose nanofibers isolated from harakeke.

Author Contributions: Conceptualization, Y.Z. and X.Y.; methodology, Y.Z.; validation, X.Y.; formal analysis, Y.Z.; investigation, Y.Z. and X.Y.; resources, X.Y.; writing—original draft preparation, Y.Z.; writing—review and editing, X.Y.; supervision, X.Y.; project administration, X.Y. All authors have read and agreed to the published version of the manuscript.

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Article

The Evaluation of Cellulose Acetate Capsules Functionalized for the Removal of Cd(II)

Irma Pérez-Silva *, Gladis D. Canales-Feliciano, José A. Rodríguez, Luis H. Mendoza-Huizar, Salvador Pérez-Estrada, Israel S. Ibarra and M. Elena Páez-Hernández *

Academic Area of Chemistry, Autonomous University of Hidalgo State, Carr. Pachuca-Tulancingo Km. 4.5, Mineral de la Reforma C.P. 42184, Hidalgo, Mexico

* Correspondence: iperez@uaeh.edu.mx (I.P.-S.); paezh@uaeh.edu.mx (M.E.P.-H.)

Abstract: Cellulose acetate is derived from cellulose and has the characteristics of biodegradability and reusability. So, it has been used for the elimination of toxic compounds capable of producing different diseases, such as cadmium, that result from human and industrial activity. For this reason, capsules functionalized with Cyanex 923 were prepared and characterized by FTIR spectroscopy, Energy Dispersive X-ray Spectroscopy (EDX), and SEM. The functionalized capsules were used for removing and recovering Cd(II) by modifying variables such as HCl concentration in the extraction medium and carrier content in the capsules, among others. The extraction of cadmium from battery leachates and the three isotherm models, Langmuir, Freundlich, and Dubinin Radushkevich, were also tested to model the cadmium removal process. The results showed a favorable physical sorption with a good capacity for extraction and the possibility of reusing the capsules for up to seven cycles without a decrease in the percentage of cadmium recovery.

Keywords: acetate cellulose; capsules; cadmium; removal; reusability

1. Introduction

Cellulose is a semi-rigid, linear, white polymer in a crystalline or amorphous state. It is an abundant polymer in nature presented in different types of plants, agricultural residues, wood, etc.; some characteristics are chemical resistance, biodegradability, durability, biocompatibility, thermal stability, and renewability [1,2]. Despite its advantages, cellulose has a low solubility in organic compounds, limiting its application. For this reason, cellulose has been modified by different processes to obtain some derivatives, such as cellulose sulfate, cellulose nitrate, methyl and ethyl cellulose, carboxymethyl cellulose, and cellulose acetate [3].

Cellulose acetate (CA) produces the acetylation of cellulose with acetic anhydride and acetic acid in the presence of sulfuric acid as a catalyst. It is a non-toxic, reusable, biodegradable, mechanical strength, and hydrophobic polymer [4,5]. Some uses of cellulose acetate are plastics production, fibers, textiles, and analytical applications such as membrane filters employed for preconcentration and sorbents [6]. For example, CA has been employed with graphene oxide [7] and sulfonated graphene oxide in beds [8], chitosan [9], and lemongrass [10] for the elimination of dyes such as crystal violet and methylene blue. Additionally, membranes [11,12], nanofibers impregnated with hydroxyapatite [13], dithi-zone nanosponges [14], and microbeads with layered double hydroxides [15] have been used for the adsorption of heavy metals such as lead, chromium, and cadmium.

Cadmium, a highly toxic metal, has been associated with osteoporosis, renal lesions, cardiovascular disease, and, in some cases, cancer. Because of this, the International Agency for Research on Cancer (IARC) has classified cadmium as a human carcinogen (group I) [16], while the US Environmental Protection Agency (EPA) considers Cd as one of the 13 metals from the priority pollutant list [17].

Cadmium is used for the synthesis of pigments, plastic stabilizers, metal coatings, alloys, electronics, and mainly in the manufacturing of batteries [18]. The use of these types of batteries (in open or closed configuration) has increased due to their versatility, low maintenance, long life, and low cost. For this reason, recycling this type of material is necessary to prevent environmental damage and economic losses [19]. However, some processes for cadmium spent batteries recovery demand high energy consumption. For example, pyrometallurgical processes require using a closed furnace, where metal distillation happens at temperatures between 800 and 900 °C, or a vacuum distillation furnace [20]. Other methods employed for the removal of cadmium from wastewater consist of mechanic and physic separation in acid or elementary solutions followed by ion exchange, solvent extraction (generally organophosphorus such as Cyanex 272, Cyanex 923, D₂EHPA, TBP), adsorption (activated carbon), precipitation (with sulfates or sodium hydroxide), electrodeposition (complement of other techniques) [21], silica nanoparticles with hydrophilic (OH) or hydrophobic groups (CH₃) [22], and magnetite or maghemite nanoparticles [23]. Furthermore, for this purpose, some materials based on cellulose have been developed. Because of that, the cellulose has been modified physically by sizes and forms or chemically by functional groups to increase adsorption capacity [24]. The cellulose has been physically (sizes and form) or chemically (functional groups) modified to increase adsorption capacity [24]. A chemical change occurs via substituting hydroxyl groups for other groups by etherification, oxidation, silanization, esterification, cross-link, and graft copolymerization [25]. For instance, cellulose has cheating properties for introducing -C=S, -NH₂, and -NH groups [26] and for using dibenzo-18-crown 6 [27].

According to the above, easy and environmentally friendly methods for the recovery of cadmium from the acid leachates of the waste that permit the reduction of contamination are necessary. As a result, in this review, functionalized capsules were developed using cellulose acetate and Cyanex 923 as extractant agents [28,29]. These capsules have served as metal extractants due to their large specific interfacial area, high selectivity, and stability. Moreover, these capsules are easy to prepare (only a few seconds are required to form them via phase inversion), with minimal use of organic solvent [30]. The influence of different parameters over the cadmium extraction was evaluated to obtain cellulose acetate capsules functionalized with Cyanex 923, which could become a viable option for the recovery of Cd(II) from aqueous and leached battery solutions with good performance and stability, allowing the extraction of cadmium even from acidic media and with the advantage of using the extracted agent.

2. Materials and Methods

2.1. Materials

Organic reagents, dimethylformamide (DMF, St. Louis, MO, USA), cellulose acetate (CA) (Mn ~50,000, 39.7 wt.% acetyl, Milwaukee, WI, USA), and hydrochloric acid purchased from Sigma-Aldrich (A.C.S grade, St. Louis, MO, USA); ethanol was purchased from J.T. Baker (ACS, Xalostoc, Edo. Mex, Mexico). The ion carrier trialkyl-phosphine oxides (Cyanex 923) were obtained from Cytec Industries Inc. (Mexico), and Cd(NO₃)₂•4H₂O was obtained from Alyt. All aqueous solutions were prepared in Milli Q water (18.2 MΩ cm resistance, Millipore, Bedford, MA, USA).

2.2. Preparation of Functionalized Capsules (FC)

Capsules were prepared during the phase inversion precipitation technique caused by an 18% wt/v CA solution in DMF. Then, different proportions of Cyanex 923 (Cy) (extractant) were added to the previous solution, and the mixture was stirred for 15 min at room temperature. Later, the CA-Cy-DMF solution was placed in a syringe with a needle (o.d. 0.36 mm) and added dropwise into an ethanol–water solution (3:10). In this step, the Cyanex dripped into the non-solvent solution and was trapped in the cellulose acetate due to its low affinity for the aqueous medium. The obtained functionalized capsules FC were

washed with deionized water several times and left separately, so any surplus water was drained away overnight [30,31].

2.3. Cd(II) Extraction and Recovery Experiments

Different amounts of FC were mixed with a Cd(II) solution of 10 mL and an initial concentration of 10 mg L⁻¹ in HCl. The total of Cd(II) extracted (q) was calculated according to the following Equation (1):

$$q = \frac{(C_0 - C_{AE}) \times V}{112.41 \times m_{FC}} \quad (1)$$

q is the cadmium extracted amount (mmol Kg⁻¹), C₀ is the Cd(II) concentration (mg L⁻¹) in the initial solution, C_{AE} is the Cd(II) concentration (mg L⁻¹) in the solution after the extraction step, V is the aqueous solution volume (L), 112.41 is cadmium atomic weight, and m_{FC} is the amount of functionalized capsules (Kg).

The percentage of extracted Cd(II) (%E_{Cd(II)}) was calculated according to the following Equation (2):

$$\%E_{Cd(II)} = \left(1 - \frac{C_{AE}}{C_0}\right) \times 100 \quad (2)$$

C_{AE} is the cadmium concentration in the solution after the extraction step (mg L⁻¹), and C₀ is the initial concentration of Cd(II) (mg L⁻¹). The operating variables studied were the mass of FC, the carrier concentration in the casting solution for FC preparation, contact time, the concentration of Cd (II), and cadmium recovery from battery-leaching samples.

For the cadmium recovery studies, FC from the extraction step (loaded with cadmium) was shaken with 10 mL of H₂SO₄ 0.1 mol L⁻¹ for 0.5 h. The percentage of Cd(II) recovery (%R_{Cd(II)}) was calculated according to Equation (3):

$$\%R_{Cd(II)} = \frac{C_{RS}}{C_0 - C_{AE}} \times 100 \quad (3)$$

C_{RS} is the concentration of cadmium (mg L⁻¹) in the sulphuric acid solution after the recovery process, and the other variables have the meaning described before.

All the experiments for the extraction and recovery were accomplished in triplicate at room temperature; Cd(II) concentration in the aqueous solutions was quantified using flame atomic absorption spectrophotometry (AAS) (SpectrAA 880, Varian®).

2.4. Battery-Leaching Process

Ni–Cd batteries were bought in a local market and dismantled to obtain the cell components separately. One gram of the rolled electrodes was in contact with a mixture of 2 mL of 32 wt% of H₂SO₄, 6 mL of deionized water, and 1 mL of H₂O₂ 30 vol% and heated for 2 h at 70 °C. The resulting solution was filtered and transferred to a 500 mL volumetric flask, completing the volume with deionized water [32].

3. Results

3.1. Evaluation of the Amount of Extractant

Functionalized capsules (FC) were prepared by adding Cyanex 923 (extractant) to a cellulose acetate/dimethylformamide solution (casting solution). The amount of extractant added to the solution varied from 0 to 15.5 wt/vol%, being 0% for capsules without Cyanex. The resulting capsules, prepared by phase inversion precipitation technique after dropping the above solution into an ethanol–water mixture, were analyzed by Energy Dispersive X-ray Spectroscopy (EDX) in a Scanning Electron Microscopy, JEOL JSM-5600LV model to confirm the presence of Cyanex 923 (Figure 1), and to estimate the phosphorus atomic percentage. The results presented in Table 1 show an increase in the percentage of phosphorus in capsules as Cyanex content increased in the casting solution. This increase in carrier content in FCs is also reflected in a gradual growth in cadmium extracted (Figure 2) when capsules are used to extract Cd from aqueous solutions.

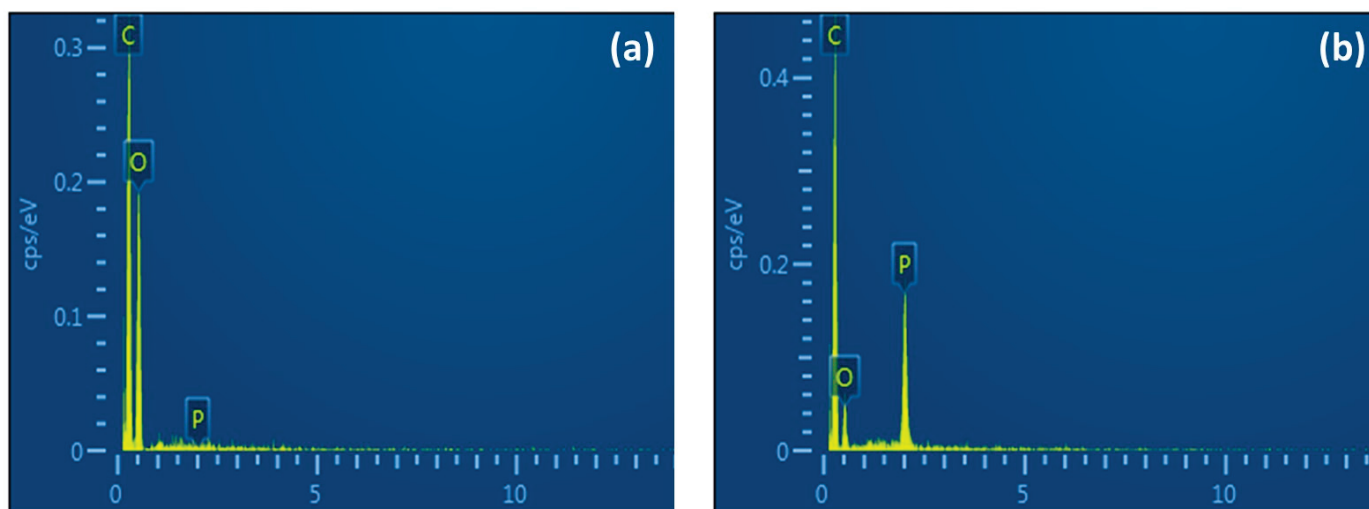


Figure 1. EDX spectrum of (a) capsules without Cyanex 923 and (b) capsules functionalized with 15.5% of Cyanex 923.

Table 1. Phosphorus atomic percentage in Cyanex functionalized capsules.

Cyanex 923 in Casting Solution (wt/vol%)	Phosphorous Atomic Percentage (%)
0.00	0.00
0.50	0.08
1.50	0.11
2.00	0.19
6.00	1.75
9.50	2.24
12.50	2.42
15.50	2.67

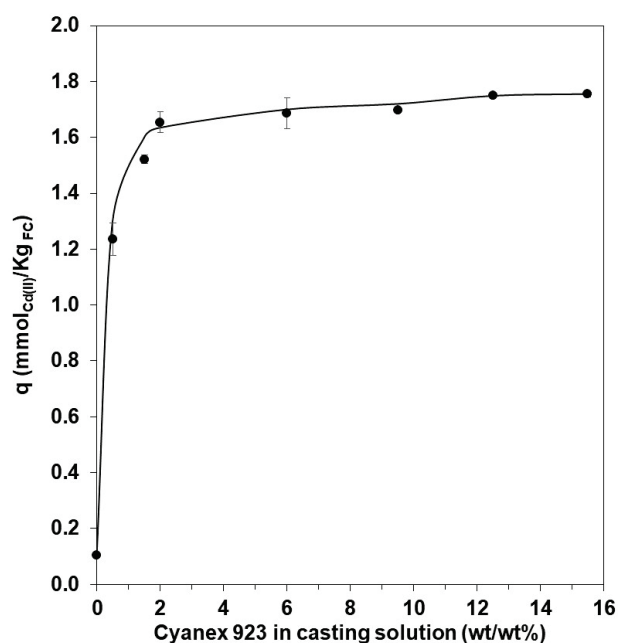


Figure 2. Effect of variation of the concentration of Cyanex 923 used for obtaining the capsules (FC). Experimental conditions: 10 mg L^{−1} of Cd(II) in aqueous solution in 2 mol L^{−1} of HCl; 0.5 g of FC. Values obtained after 2 h were counted from the start of the experiment.

Between these two variables, a directly proportional relationship was demonstrated [33]. Although with higher percentages of Cyanex, better results were obtained, the 1.5% value for future experiments observed the effect that other variables could have on the extraction of cadmium. Nonetheless, it is possible to achieve an extraction close to 100% of cadmium by increasing the content of Cyanex in the casting solution.

The presence of extractant in cellulose acetate capsules was determined by IR spectroscopy with a Perkin Elmer System 2000 with Fourier transform (Waltham, MA, USA). The CA spectrum (Figure 3a) shows a band at 1045 cm^{-1} of C-O stretching, a band at 1246 cm^{-1} corresponding to asymmetric stretching of the acetate C-C-O bond, a band at 1370 cm^{-1} for the C-CH₃ group, and a similar band to C=O bond at 1757 cm^{-1} [34,35]. In the case of Cyanex 923 (Figure 3b), 810 cm^{-1} and 1148 cm^{-1} characteristic bands of P-CH₃ stretching vibration and P=O in each group are identified [36,37]. These same bands and the characteristics of CA and Cyanex 923 are presented in the spectrum of Figure 3c, demonstrating that the extractant immobilized into the FC.

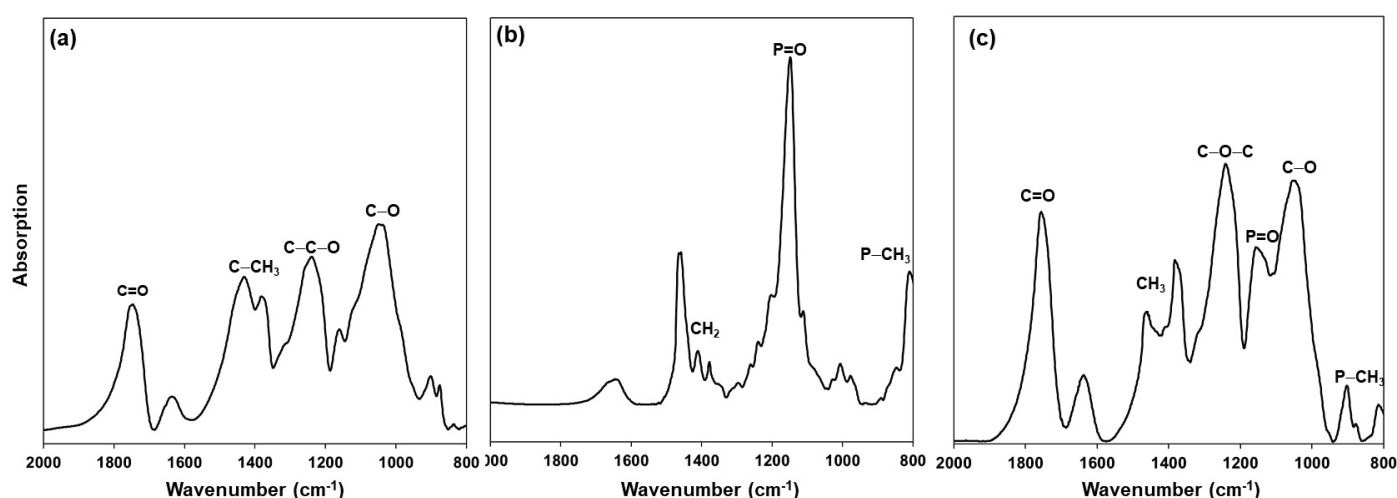


Figure 3. FTIR spectra. (a) Cellulose acetate (CA); (b) Cyanex 923 extractant agent; (c) functionalized capsules (FC).

3.2. Variation of the Number of Capsules Used during Extraction Experiments

The relationship between the mass of FC and the cadmium extraction percentage was examined (Table 2). As expected, increasing the mass of FC provides a greater contact growing area in the extraction percentage [38]. With these results, a plot of the logarithm of Cyanex 923 content (phosphorus atomic percentage obtained by EDS analysis) versus the logarithm of distribution ratio, D (ratio of the total amount of cadmium in the FC phase to its total amount in the aqueous phase), gives a slope value around one (0.8575) ($R^2 = 0.9716$). It indicates a 1:1 stoichiometric ratio between cadmium and the Cyanex extractant.

Table 2. Effect of variation of the mass of FC used in the cadmium extraction ¹.

FC Mass (g)	Cadmium Extraction in Percentage ² (%)
0.05	18.63 (3.56)
0.20	59.10 (3.84)
0.30	79.07 (1.19)
0.40	79.51 (6.13)
0.50	85.50 (1.18)
1.00	96.49 (0.03)

¹ Experimental conditions: 10 mg L^{-1} of Cd(II) in aqueous solution in 2 mol L^{-1} of HCl; FC prepared with 1.5% of Cyanex 923. Values were obtained after 2 h counted from the start of the experiment. ² Percentage of relative standard deviation in parenthesis.

3.3. Evaluation of the Effect of Chlorhydric Medium on Cadmium Extraction

The extraction of a cation such as Cd(II) with Cyanex depends on neutralizing its charge with an adequate counter ion. Thus, hydrochloric acid was added to cadmium solutions to allow the formation of chlorocomplexes neutralized by the presence of protons. The amount of cadmium extracted in Figure 4 shows the increase in HCl concentration due to the formation of neutral species such as CdCl_2 , HCdCl_3 , and H_2CdCl_4 [29] extracted by the solvation mechanism. Nevertheless, at higher HCl concentrations, the mineral acid can also be extracted, competing with cadmium and decreasing the percentage of metal adsorbed [39].

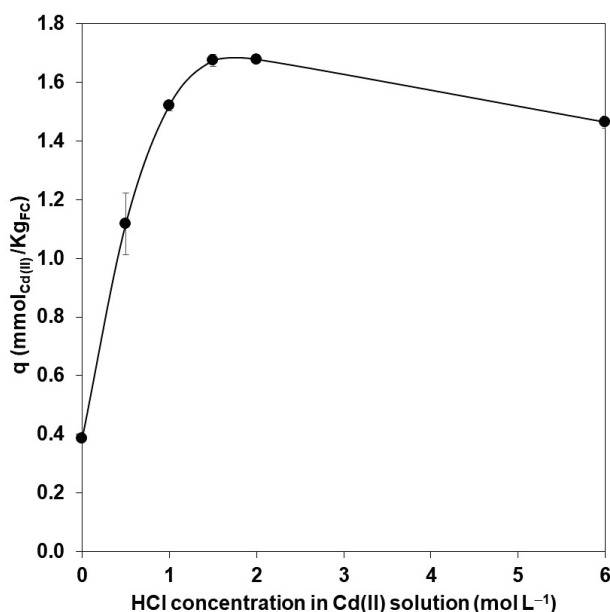


Figure 4. Effect of variation of the HCl concentration in the cadmium extraction. Experimental conditions: 10 mg L^{-1} of Cd(II) in aqueous solution of HCl; 0.5 g of FC prepared with 1.5% of Cyanex 923. Values obtained after 2 h were counted from the start of the experiment.

According to the Fraction Diagram ($f_{\text{Cd(II)}}$ vs. $\log \text{Cl}^-$) obtained through the MEDUSA program [40] at -0.7 pH value and $8.9 \times 10^{-5} \text{ mol}_{\text{Cd(II)}} \text{ L}^{-1}$ concentration, the predominant cadmium chemical species is CdCl_2 . With this information and knowing the stoichiometric relationship between Cd and Cyanex (previous section), the following reaction can be considered representative of the extraction process, where the upper line refers to the chemical species in the FC organic phase (Equation (4)):



3.4. Extraction Isotherms

Extraction isotherms describe the change between the initial and final amount of metal presented in a solution due to the FC at equilibrium [41]. To perform the extraction isotherms, the time the system needed to reach equilibrium was first determined (Table 3). According to this, the selected time was 5 h; although it is a prolonged time of agitation, it did not harm the extraction of Cd(II).

The experimental equilibrium data were fitted to three isotherm models: Langmuir, Freundlich, and Dubinin Radushkevich (D-R) [42,43]; this is in a range of cadmium concentrations from 10 to 150 mg L^{-1} . On one side, the experimental results and linearized forms of different models are shown in Figure 5. On the other side, Table 4 predicted a monolayer coverage of the adsorbate on the outer surface of the FC with favorable sorption ($0.01 < R_L < 0.15$) [44].

Table 3. Effect of the contact time in the extraction percentage ¹.

Contact Time (h)	Cadmium Extraction in Percentage ² (%)
0.25	59.58 (2.10)
0.50	76.41 (2.83)
1.00	81.76 (3.94)
2.00	97.46 (0.67)
5.00	98.15 (0.17)
7.00	98.23 (0.68)

¹ Experimental conditions: 10 mg L⁻¹ of Cd(II) in aqueous solution 1.5 mol L⁻¹ of HCl; 0.5 g of FC prepared with 1.5% of Cyanex 923. ² Percentage of relative standard deviation in parenthesis.

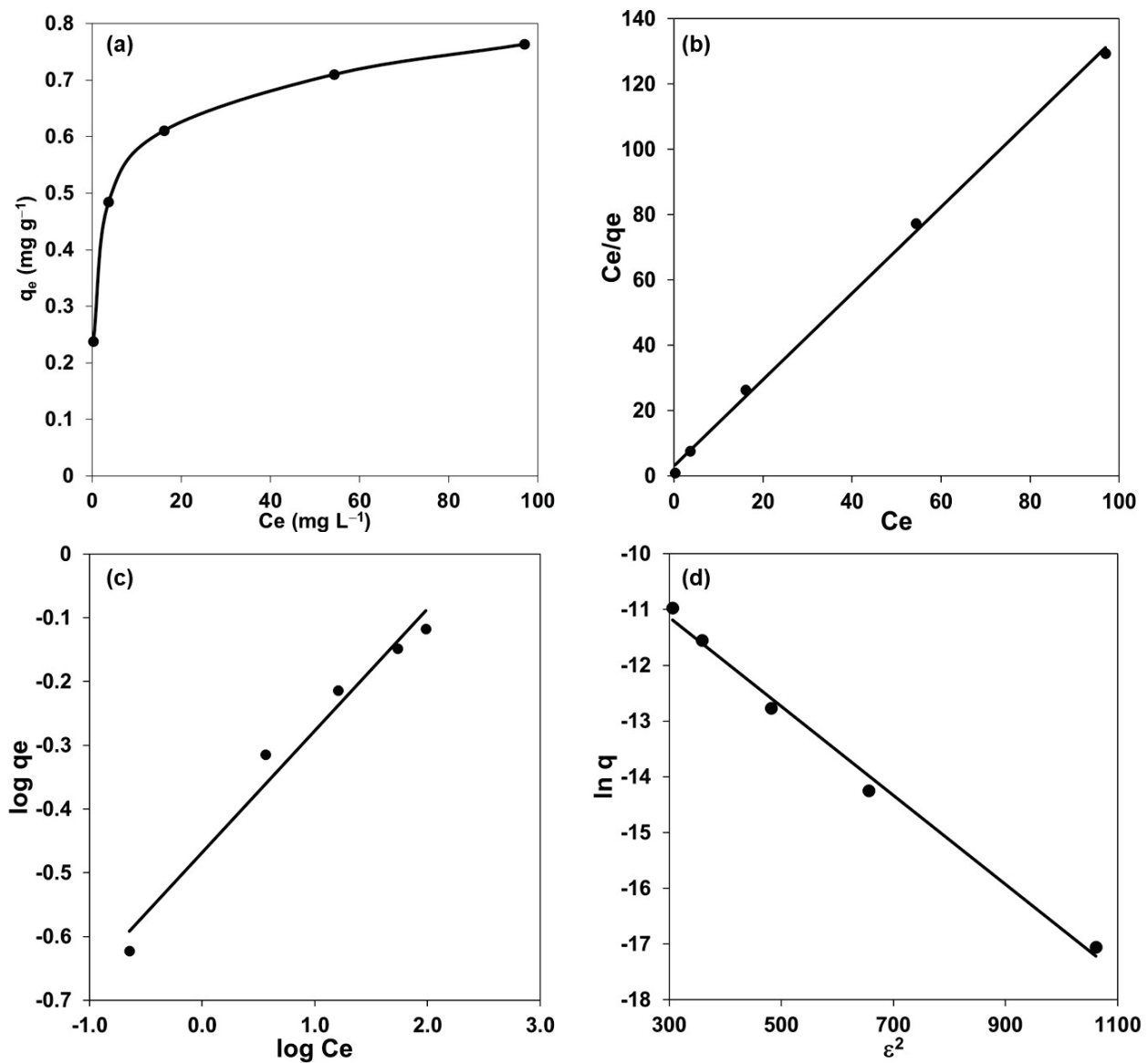


Figure 5. Isotherms curves: (a) experimental data and linearized forms of (b) Langmuir isotherm, (c) Freundlich isotherm, and (d) Dubinin Radushkevich isotherm. Experimental conditions: different amounts of Cd(II) in aqueous solution of 1.5 mol L⁻¹ HCl; 0.5 g of FC prepared to 1.5% of Cyanex 923.

Table 4. Dubinin Radushkevich, Freundlich, and Langmuir constants for sorption of Cd(II) onto FC.

Model	Estimated Isotherm Parameters ¹	
Langmuir $\frac{C_e}{q_e} = \frac{1}{q_{\max} K_L} + \frac{C_e}{q_{\max}}$	Q_o (mg g ⁻¹)	0.77
	K_L (L mg ⁻¹)	0.52
	R^2	0.998
Freundlich $\log q_e = \log K_f + \frac{1}{n} \log C_e$	K_F (L g ⁻¹)	0.34
	n_o	5.24
	R^2	0.972
Dubinin Radushkevich $\ln q_e = \ln q_{\max} - \beta \epsilon^2$	β (mol ² J ⁻²)	0.008
	ϵ (KJ mol ⁻¹)	7.91
	R^2	0.993

¹ Maximum extraction capacity, Q_o (mg g⁻¹); Langmuir constant, K_L (L mg⁻¹); Freundlich constant, K_F (L g⁻¹); free strength of sorption, n_o ; Dubinin Radushkevich constant, β (mol² J⁻²); Polanyi potential, ϵ (KJ mol⁻¹); coefficient of correlation, R^2 .

Referring to the D-R isotherm allowed us to identify the nature of the interaction between Cd(II) and the extractant as a physical sorption [45], which agrees with the characteristics of a solvation process.

Although the cadmium extraction capacity (Q_o) obtained for FC is lower than those reported for other types of polymeric materials, such as solvent-impregnated resins (SIR) and alginate capsules (Table 5), in this work, the FC has a smaller amount of extractant (22.5 mg_{Cyanex} g_{FC}⁻¹). Moreover, the amount of Cyanex used in this work is enough to reach extraction percentages above 95%, as well as those reported in papers with similar cadmium concentrations.

Table 5. Efficiency in the cadmium extraction obtained with impregnated materials with different extracting agents.

Q_o Cd(II) (mg _{Cd(II)} g ⁻¹)	$q_{\text{extractant}}$ ⁴ (mg _{extractant} /g _{material})	Extractant	Reference
1.56	77 (XAD2) ¹	Cyanex 301	[46]
11.5	138 (XAD7) ¹	Cyanex 301	
4.89	99	Cyphos [®] IL-101 ²	[47]
36.57	416	Cyphos [®] IL-101 ²	
13.54	271	DEHPA ³	[48]
41.06	500	DEHPA ³	
13.1	366	Cyanex 921	[49]
17.5	528	Cyanex 921	
15–42	94–290	Cyanex 301	[50]
14–34	204–481	Cyanex 302	

¹ Amberlite XAD-2 and XAD-7; ² phosphonium salt (tetradecyl(trihexyl)phosphonium chloride); ³ Di(2-ethylhexyl) phosphoric acid; ⁴ $q_{\text{extractant}}$ = amount of extractant.

Notwithstanding the above, it is relevant to highlight that, to observe the effect of different variables on the percentage of cadmium extraction, the maximum concentration of Cyanex in the casting solution was not used, so increasing the FC adsorption capacity is achievable, as is the extraction percentage, by modifying this variable during its preparation.

3.5. Kinetic Studies

Kinetic studies describe the amount of analyte in the capsules concerning the residence time, considering that the sorption depends on the characteristics of the sorbent [51]. For this reason, pseudo-first-order and pseudo-second-order kinetic models were evaluated [52]. Figure 6 and Table 6 show the curves and parameters of the two kinetic models considered. The experimental data provided the best correlation with the pseudo-second-order model, which indicates that the sorption mechanism depends on the amount of

Cd(II) near active sites, showing that chemisorption is the rate-determining step of the sorption [53].

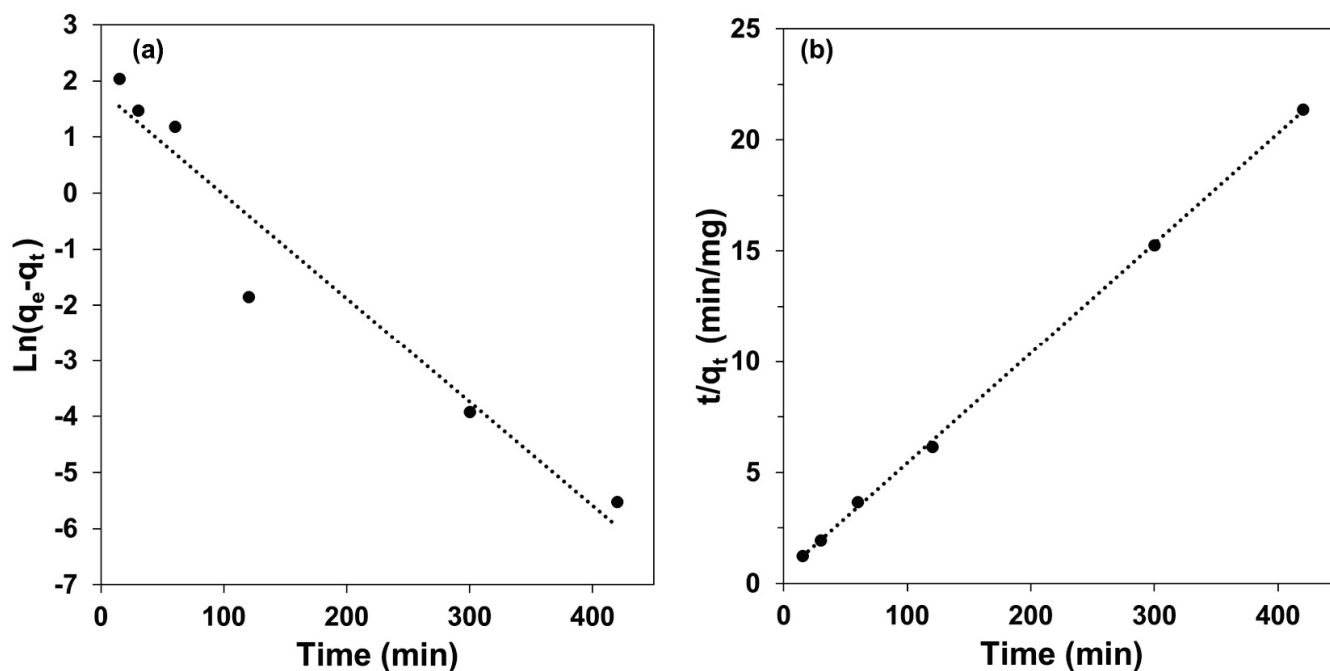


Figure 6. Curves of the two kinetic models evaluated: (a) pseudo-first-order and (b) pseudo-second-order. Experimental conditions: 10 mg L^{−1} of Cd(II) in an aqueous solution of 1.5 mol L^{−1} HCl; 0.5 g of FC prepared with 1.5% of Cyanex 923.

Table 6. Kinetic parameters for sorption of Cd(II) onto FC.

Model	Estimated Kinetic Parameters ¹	
Pseudo-first-order	q _e (mg g ^{−1})	6.203
ln(q _e − q _t) = lnq _e − K ₁ t	K ₁ (min ^{−1})	−4.40 × 10 ^{−5}
	R ²	0.943
Pseudo-second-order	q _e (mg g ^{−1})	20.20
$\frac{1}{q_t} = \frac{1}{K_2 q_e^2} + \frac{t}{q_e}$	K ₂ (g mg ^{−1} min ^{−1})	0.005
	R ²	0.999

¹ Cadmium extracted at equilibrium, q_e; constant of the pseudo-first-order, K₁ (min^{−1}); constant of the pseudo-second-order, K₂.

3.6. FC Reusability Study

Sulphuric acid is an effective re-extracting agent because it can form more stable complexes with cadmium in aqueous medium than chloride [49]. Thus, H₂SO₄ was used to recover cadmium from the functionalized capsules (10 mL of H₂SO₄ 0.1 mol L^{−1} in each experiment). Subsequently, the cadmium-free FCs were used again for extracting Cd(II), completing a new cycle. With these results, it was possible to evaluate the stability and durability of the FC, inferring a loss of its extraction capacity after the seventh cycle (Figure 7).

At the beginning of the experiments, the photomicrographs of FC were obtained to support this observation (Figure 8, on the left). On the contrary, Figure 8, on the right, was obtained after ten cycles of use. Unlike when they were freshly prepared, the used FCs show a rough and weathered surface because of structural damage. From the previous results, it might be that the process of efficiency is expressed as cadmium extraction decreases after seven cycles. Moreover, this sorbent material is made of cellulose acetate, a biodegradable biopolymer. It represents a phenomenal advantage over other functionalized

polymeric materials, such as SIR [47] or polysulfone microcapsules [54], which maintain their extraction performance only for five cycles.

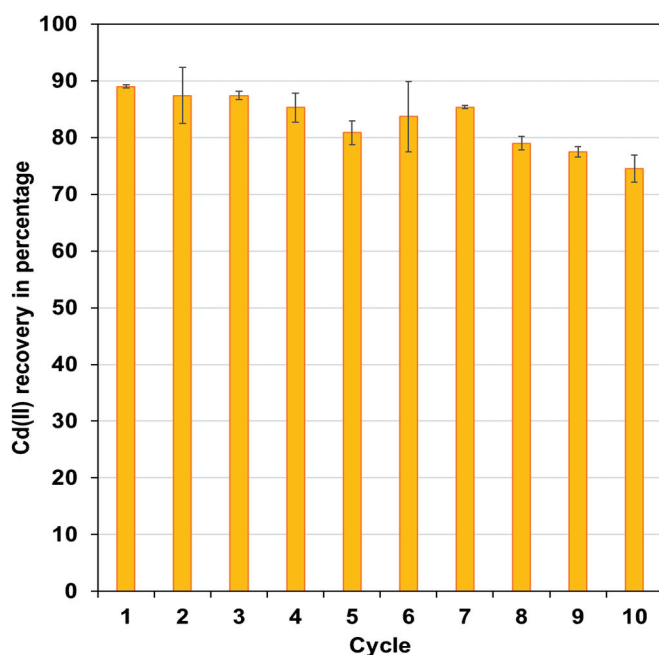


Figure 7. Percentage of cadmium recovery after re-extraction process. Experimental conditions: 10 mg L^{-1} of Cd(II) in aqueous solution of 1.5 mol L^{-1} of HCl; 0.5 g of FC prepared with 1.5% of Cyanex 923. Values obtained after 2 h were counted from the start of the experiment for extraction and 0.5 h for recovery.

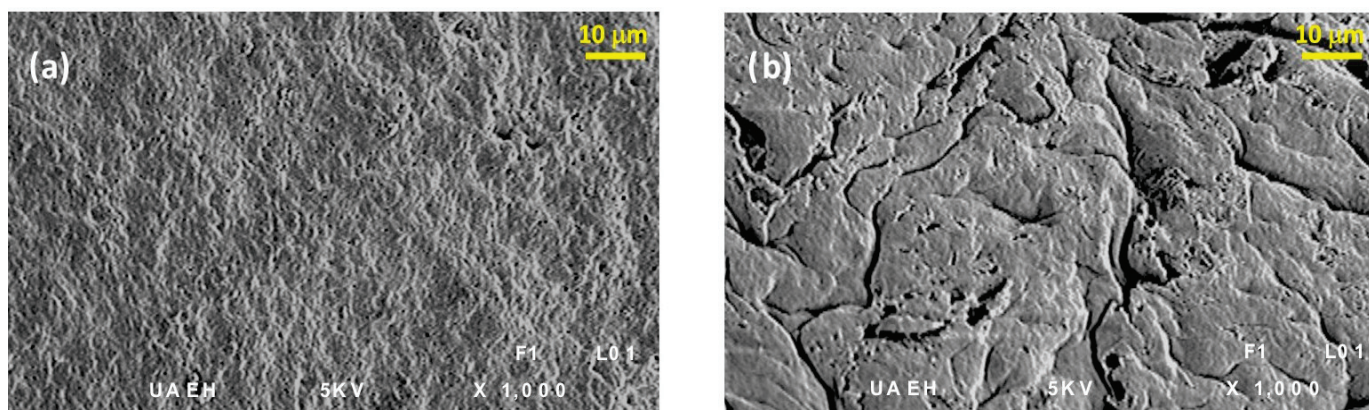


Figure 8. Scanning electron micrograph ($1000\times$) of different FC: (a) cellulose acetate with 1.5% Cyanex 923 and (b) cellulose acetate with 1.5% Cyanex 923 after 10 extraction–recovery cycles.

3.7. Recovery of Cd(II) from a Real Sample

Cadmium recovery studies from actual samples were carried out with a battery-leaching solution prepared according to the Battery-leaching Process section (see Section 2.4). From this stock solution, a 10 mg L^{-1} solution was prepared and used for the extraction and recovery of Cd(II) with FC. The obtained results ($q_{\text{Cd}} = 2.06 \text{ mmol Kg}^{-1}$, $\% \text{ExtractionCd} = 88.28\% \pm 0.76\%$, and $\% \text{RecoveryCd} = 79.59\% \pm 1.80\%$) are lower than those obtained with synthetic aqueous solutions. It can be attributed to the presence of nickel that competes to a certain degree with cadmium during the extraction with Cyanex. Although it interferes negatively with the extraction of cadmium, the nickel extraction percentage is less than 8% ($7.27 \pm 2.63\%$), indicating a good selectivity of the developed method.

4. Conclusions

The recovery of Cd(II) from aqueous HCl solutions with cellulose acetate capsules functionalized with Cyanex 923 was successful. The transfer mechanism is based on physical sorption and obeys a 1:1 stoichiometry between carrier and metal.

The best conditions for the extraction of cadmium were achieved using a 1.5 mol L⁻¹ HCl solution during a stirring time of 2 h with 0.5 g of functionalized capsules prepared with 1.5% Cyanex 923. An 88% percentage of cadmium recovery from cadmium-loaded FC using 10 mL of a 0.1 mol L⁻¹ H₂SO₄ solution during a stirring time of 0.5 h. Thereby, functionalized capsules can be reused up to seven times without significant changes in their cadmium extraction capacity.

The above demonstrates that it is possible to use a biopolymer such as cellulose acetate for the recovery of cadmium from battery leachates with good selectivity through a simple and environmentally friendly process.

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Article

Stiffness-Controlled Hydrogels for 3D Cell Culture Models

Arto Merivaara ^{1,*}, Elle Koivunotko ^{1,†}, Kalle Manninen ^{1,†}, Tuomas Kaseva ², Julia Monola ¹, Eero Salli ², Raili Koivuniemi ¹, Sauli Savolainen ^{2,3}, Sami Valkonen ^{1,4} and Marjo Yliperttula ^{1,*}

¹ Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, 00014 Helsinki, Finland

² HUS Medical Imaging Center, Radiology, University of Helsinki and Helsinki University Hospital, 00290 Helsinki, Finland

³ Department of Physics, University of Helsinki, 00014 Helsinki, Finland

⁴ School of Pharmacy, University of Eastern Finland, 70210 Kuopio, Finland

* Correspondence: arto.merivaara@helsinki.fi (A.M.); marjo.yliperttula@helsinki.fi (M.Y.); Tel.: +358-294-159-577 (A.M.); +358-294-159-141 (M.Y.)

† These authors contributed equally to this work.

Abstract: Nanofibrillated cellulose (NFC) hydrogel is a versatile biomaterial suitable, for example, for three-dimensional (3D) cell spheroid culturing, drug delivery, and wound treatment. By freeze-drying NFC hydrogel, highly porous NFC structures can be manufactured. We freeze-dried NFC hydrogel and subsequently reconstituted the samples into a variety of concentrations of NFC fibers, which resulted in different stiffness of the material, i.e., different mechanical cues. After the successful freeze-drying and reconstitution, we showed that freeze-dried NFC hydrogel can be used for one-step 3D cell spheroid culturing of primary mesenchymal stem/stromal cells, prostate cancer cells (PC3), and hepatocellular carcinoma cells (HepG2). No difference was observed in the viability or morphology between the 3D cell spheroids cultured in the freeze-dried and reconstituted NFC hydrogel and fresh NFC hydrogel. Furthermore, the 3D cultured spheroids showed stable metabolic activity and nearly 100% viability. Finally, we applied a convolutional neural network (CNN)-based automatic nuclei segmentation approach to automatically segment individual cells of 3D cultured PC3 and HepG2 spheroids. These results provide an application to culture 3D cell spheroids more readily with the NFC hydrogel and a step towards automatization of 3D cell culturing and analysis.

Keywords: freeze-drying; nanofibrillated cellulose; 3D cell culture; hydrogel; biomaterials; convolutional neural network

1. Introduction

Drug development is an expensive and time-consuming business. Current estimations for an approved drug product are 12–15 years and \$1000 million accounting for the costs of failed trials and investments [1,2]. Accurate and reliable *in vitro* assays are required to identify toxic or ineffective drug candidates already in the early phases of the drug development process to reduce costs, and to follow the rule of 3R (replacement, reduction, refinement) for animal testing. Furthermore, the need for fast, reliable, and efficient drug efficacy and toxicity assays, for example, primary cell-based assays, is increasing in the emerging era of personalized medicine [3].

Currently, a variety of *in vitro* assays, such as cell-based assays (cell lines, primary cells), microfluidics (immobilized-enzyme-micro-reactors, organ-on-a-chip), microsomes, and tissue extracts, are included in early drug discovery and the preclinical phase to evaluate drugs' efficacy, toxicity, metabolism, pharmacokinetic profile, and delivery. Each method has its advantages and disadvantages [4–7]: Microfluidic systems facilitate a low volume of sample but require special equipment for the fabrication of chips and collecting the cells for analysis after experiments is difficult. Human liver microsomes provide a simple and relatively inexpensive method to study drug metabolism and they

contain the metabolic enzymes of liver endoplasmic reticulum (such as cytochrome P450, Uridine 5'-diphospho-glucuronosyltransferase, and flavin monooxygenases), but they lack certain metabolizing enzymes, such as sulfotransferases. Tissue extracts provide accurate *in vivo* prediction of drug metabolism but they are expensive, sparsely available, and can show high biological variability. Cell cultures, for example, cancer cell lines or primary hepatocytes, provide powerful tools for drug efficacy, toxicity, and metabolism experiments. However, cell lines can show differences in the metabolizing enzymes, and the primary hepatocytes are expensive and sparsely available. Yet, developing cell culturing methods further can improve the advantages of cell cultures in drug discovery.

Traditionally, cells are used as two-dimensional (2D) tissue cultures in drug discovery. However, they represent living tissues poorly [8–10]. For example, cancer cells have different proliferation rates and metabolic activity, and they lack tumor tissue hypoxia in 2D cell cultures [11–13]. Three-dimensionally (3D) cultured cells show more *in vivo*-like gene transcription and morphologies than 2D cultured cells [14]. Furthermore, 3D cell cultures can provide a better prediction of drug behavior [8–10]. False conclusions about the drug efficacy and safety that result from using too-simple 2D cell culture models could be potentially reduced by using 3D cultured cells in the early phases of drug discovery. In addition, implementing 3D cultured cells into high-throughput screening (HTS) in early drug discovery would improve *in vitro*–*in vivo* correlation [3]. Eventually, this can reduce the costs of drug development and the need for test animals.

Subia et al. (2021) recently reviewed organ-on-a-chip applications for breast tumor research [5]. They summarized that 2D and 3D cell cultures, as well as microfluidic systems, are suitable for HTS. In another review, Brancato et al. (2020) concluded that scaffold-free, hydrogel, and microcarrier 3D cell systems are suitable for HTS while scaffold-based systems, bioreactors, and tumor-on-a-chip approaches have limited HTS options [10]. In accordance with Brancato et al., (2020), we believe that hydrogel-based 3D cell culture systems provide versatile *in vitro* tools suitable for HTS and implementable for drug discovery. Thus, in this research, we focus on hydrogel-based 3D cell cultures.

In general, hydrogels provide potential 3D cell culturing applications with their high content of water, porous structure, and polymer network that together facilitate the diffusion of oxygen, nutrients, and metabolic products. A wide range of natural or synthetic hydrogels has already been used for 3D cell culturing including, but not limited to, PuraMatrix™, AlgiMatrix™, HydroMatrix™, Matrigel, hyaluronic acid, collagen, gelatin, polyethylene glycol, and polyacrylamide [15–20]. One important parameter of hydrogel-based 3D cell culturing platforms is stiffness, i.e., the mechanical resistance that the cells sense. Stiffness of the tissue or artificial extracellular matrix (hydrogel) affects, for example, cell signal transduction, differentiation, morphogenesis, proliferation, and marker expression [19,21–23].

Certain deficiencies hinder the usability of hydrogels. First, especially animal-origin hydrogels, such as Matrigel, collagen, and gelatin, have inconsistency and batch-to-batch variation that cause problems with reproducibility [24,25]. Second, handling the hydrogels might be laborious. For example, some hydrogels require gelation with salts before they are suitable for cell seeding [15], and, if considering HTS, the viscous nature of hydrogels can be hard for automated pipetting systems [26]. Third, hydrogels that have limited transparency complicate observation, especially in high-content imaging, and require special setups for analysis [10]. Taken together, the hydrogel used in the high-throughput drug screening should be signal-free, have no human or animal origin, be easy to handle, transport, and store, and facilitate in-plate and off-plate analysis. To solve these challenges, a hydrogel that is stable at room temperature, supports cell seeding without multistep processing, is applicable for (high-content) imaging, and permits the collection of 3D cultured cell samples for off-plate analysis would be invaluable.

The platform used for 3D cell cultures is not the only aspect that hinders the implementation of cell spheroids more closely into drug discovery and development. Segmentation of cells in 3D cultures is necessary when performing, for example, drug efficacy and toxi-

city assays. Manual segmentation is often too overly tedious and time-consuming to be practical. In addition, although manual segmentation is often considered the gold standard, manual segmentation is subjective and the results are non-repeatable. For these reasons, various semi-automatic [27] and automatic approaches [28] for segmenting cells have been developed. However, the semi-automatic segmentation of 3D data still requires quite a lot of human work, and the results are non-reproducible. The developed automatic methods are typically problem-specific, i.e., developed for a certain type of cell. To our knowledge, there are no software or machine learning models available, at least in open-source repositories, that could automatically produce high-quality segmentations for the cells used in our studies. In addition, even when the method is suitable for a given application, clumped cells and weak imaging quality can degrade the segmentation results considerably.

We have previously shown the suitability of wood-derived nanofibrillated cellulose (NFC) hydrogel for 3D cell culturing and that the cells form spheroids in certain tissue-specific NFC concentrations [29,30]. NFC hydrogel is a shear-thinning, biocompatible, enzymatically degradable biomaterial. In addition, signaling between NFC hydrogel and cells is unspecific mechanical signaling, i.e., no specific interactions between the cells and the NFC hydrogel occur [31]. The mechanical force (stiffness) required by cells of different soft-tissue origins can be easily accessed by controlling the fiber concentration of the NFC hydrogel. Furthermore, freeze-dried NFC hydrogel forms highly porous structures, which can be stored and transported in a dry form at room temperature, and subsequently reconstituted rapidly in one step, as we have recently shown [32,33].

In this study, we aimed to freeze-dry 1.5% NFC hydrogel, reconstitute dry NFC formulations in one step with a mixture of water, cell medium, and cells into higher- or lower-fiber concentrations, and subsequently 3D culture prostate cancer (PC3), hepatocellular carcinoma (HepG2), and human adipose stem/stromal cells (hASCs) in the freeze-dried and reconstituted NFC hydrogels with the desired fiber concentration, i.e., stiffness. We aimed to show that regardless of the original NFC fiber concentration, freeze-dried and reconstituted NFC hydrogel could be used for 3D cell culturing of different cell types and tackle the challenges introduced earlier. Furthermore, to enable the segmentation of 3D cultured cells in practice, we aimed to show that the convolutional neural network (CNN)-based automatic nuclei segmentation approach, a type of supervised machine learning, is a viable option to automatically segment individual cells of PC3 and HepG2 spheroids. We hypothesized that the hydrogels obtained by reconstitution of dry NFC samples would provide a potential platform for 3D cell culturing of different cell types for applications in drug screening as a human-on-a-well-plate approach, and in the future preferably in automated format, for example in HTS.

2. Materials and Methods

2.1. Freeze-Drying and Reconstitution of NFC Hydrogel with Sucrose

Prior to freeze-drying, NFC hydrogel with 1.5% (m/V) fiber concentration (GrowDex®, UPM Kymmene, Lappeenranta, Finland) was mixed with sucrose (Sigma Aldrich, St. Louis, MO, USA) by syringe mixing, as described earlier by Paukkonen et al. (2017), to obtain the final formulation of 1.5% NFC hydrogel with 300 mM sucrose [34].

The freeze-drying cycle was programmed according to the glass transition temperature of the maximally freeze-concentrated sample (T_g') of the NFC hydrogel formulation. T_g' was determined with differential scanning calorimetry (TA instruments, New Castle, DE, USA) by decreasing the temperature from +40 °C to −80 °C and then heating 10 °C/min to +20 °C with a cell purge gas flow (N_2) of 50 mL/min. Temperature and heat flow were calibrated with indium. T_g' measurements were conducted in triplicate and results were analyzed with TRIOS software version 4.5.0.52598 (TA Instruments, New Castle, DE, USA).

The 1.5% NFC hydrogel with sucrose was freeze-dried in 6 mL ISO Clear Type I Tubular Glass Vial 2 -freeze-drying vials (Adelphi, Haywards Heath, UK) with a laboratory-scale freeze-dryer, LyoStar II (SP Scientific Inc., Warminster, PA, USA). The freeze-drying was performed by first decreasing the shelf temperature by 1 °C/min until −47 °C (freezing

step). The shelf temperature was kept at $-47\text{ }^{\circ}\text{C}$ for 2 hrs. During the primary drying, the temperature was increased to $-42\text{ }^{\circ}\text{C}$ ($1\text{ }^{\circ}\text{C}/\text{min}$) and the pressure was set to 50 mTorr (capacitance manometer). The endpoint of the primary drying was determined by comparing the pressure values of Pirani and capacitance manometer vacuum sensors. During the secondary drying, the temperature was raised $1\text{ }^{\circ}\text{C}/\text{min}$ from $-42\text{ }^{\circ}\text{C}$ to room temperature after which the vials were closed with rubber Daikyo D Sigma freeze-drying stoppers (Adelphi, Haywards Heath, UK) in a dry nitrogen atmosphere (400,000 mTorr). The cycle lasted approximately 100 hrs. A graphical presentation of the used freeze-drying cycle can be found in the Supplementary Materials (Figure S1). The closed vials were sealed manually with All-Aluminium Crimp seals (West Pharmaceutical Services, Exton, PA, USA). Freeze-dried samples were stored in closed vials, protected from light, at room temperature for up to two weeks before being processed.

Samples were reconstituted by using three different approaches depending on the desired application. (1) In the first method, freeze-dried NFC samples with sucrose were reconstituted by direct addition of milliQ (mQ)-water either gravimetrically into the NFC fiber concentration as before freeze-drying or into 3% (m/V) of fiber concentration. (2) In the second method, freeze-dried NFC samples with sucrose were reconstituted with a mixture of mQ-water and Dulbecco's modified eagle medium (DMEM) with high glucose and L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) into hydrogels with 0.125%, 0.4%, 0.6%, 0.8%, and 1.0% (m/V) of NFC fiber concentrations. The amount of water in the mixture was adjusted by weighing the fresh and dry NFC formulations and media was added to obtain the correct dilution. (3) The third reconstitution method was performed similarly to method 2, but the media also contained the correct concentration of cells. This method is explained in detail in the Section 2.4. describing the cell culture protocol.

2.2. Morphology of Freeze-Dried NFC Hydrogel

The porosity of the freeze-dried NFC hydrogel with sucrose was analyzed with scanning electron microscope (SEM). First, the freeze-dried NFC hydrogel cake was manually cut with tweezers. Then, the sample was carefully placed on a two-sided carbon tape with silver glue. Finally, the samples were sputtered with platinum for 25 s with an Agar sputter instrument (Agar Scientific 160 Ltd., Stansted, UK) and subsequently imaged in a high vacuum with 4–7 kV and 2–4 spot size with FEI Quanta 250 Field Emission Gun SEM.

2.3. Physicochemical and Rheological Properties of Reconstituted NFC Hydrogel Formulation

A coulometric Karl Fischer titrator (899 Coulometer, Metrohm, Herisau, Switzerland) was used to analyze the residual water content of freeze-dried samples to ensure successful drying. First, the dry samples were weighed to facilitate the calculation of the mass percent of water. Before the actual measurements, the background residual water of the methanol used to dissolve the samples was determined. For the analysis, the samples were dissolved in 1 mL of anhydrous methanol (Merck, Darmstadt, Germany), the samples were mixed vigorously, and 700 μL of the methanol was transferred to the measuring vessel.

The osmolality of the fresh and freeze-dried and reconstituted NFC hydrogel formulation with or without DMEM was measured with a manual freezing point depression osmometer (Osmomat 3000, Gonotech, Berlin, Germany). The osmometer was calibrated according to the manufacturer's instructions with ultrapure water for 0 mOsmol/kg and 100 to 850 mOsmol/kg with calibration standards bought from the manufacturer (NaCl, Gonotech, Berlin, Germany). After calibration, 15 μL of the sample was pipetted into the measuring vessel. Triplicates were used in all measurements. The pH of the reconstituted samples was determined with pH paper by dipping the pH paper carefully into the hydrogel (Macherey-Nagel, Düren, Germany).

Storage moduli (G') of freeze-dried and reconstituted NFC hydrogels were measured with the HAAKE Viscotester iQ Rheometer (Thermo Scientific, Karlsruhe, Germany) at a controlled temperature ($25\text{ }^{\circ}\text{C}$). Storage modulus was determined with oscillatory frequency sweep analysis. NFC hydrogels with 3.0%, 1.5%, and 1.0% (m/V) fiber contents were

measured with 2° cone geometry with a 0.1 mm gap, and NFC hydrogels with 0.8%, 0.6%, 0.4%, and 0.125% fiber contents were analyzed with double gap geometry with a 4 mm gap and a plate diameter of 25 mm. Before the measurement, constant amplitude sweeps were performed with constant angular frequency $\omega = 1$ Hz and oscillatory stress between 1×10^{-4} and 500 Pa to determine the linear viscoelastic region. Frequency sweeps were performed with $\tau = 20$ Pa (3.0% NFC hydrogel), $\tau = 5$ Pa (1.5% and 1.0% NFC hydrogel), $\tau = 2$ Pa (0.8% NFC hydrogel), $\tau = 1.5$ Pa (0.6% NFC hydrogel), $\tau = 0.8$ Pa (0.4% NFC hydrogel), and $\tau = 0.5$ Pa (0.125% NFC hydrogel). Data were processed with OriginPro software version 9.7.0.188 (OriginLab, Northampton, MA, USA).

2.4. Cell Culturing

PC3 (American Tissue Culture Collection, ATCC) cells were cultured in Ham's F-12K (Kaighn's) Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (*v/v*) fetal bovine serum (FBS; Merck, Darmstadt, Germany) and 1% (*v/v*) penicillin–streptomycin (P/S; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). HepG2 (ATCC) cells were cultured in DMEM with high glucose and glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (*v/v*) FBS and 1% (*v/v*) P/S. Human adipose stem/stromal cells (hASC) (Lonza, Switzerland) were cultured in MEM- α medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% of human serum (*v/v*) (Merck, Darmstadt, Germany). hASCs were used for the experiments in passage 5. All cells were cultured at +37 °C and 5% CO₂.

All three different cell types were cultured as 3D cell spheroids to study the impact of different stiffnesses of freeze-dried and reconstituted NFC hydrogels with different NFC fiber concentrations. A 1.0% (*m/V*) NFC hydrogel, 0.8% (*m/V*) NFC hydrogel, and 0.125% (*m/V*) NFC hydrogel were used for PC3, HepG2, and hASC cells, respectively. Cells were seeded with 100,000 cells/100 μ L (PC3 and hASC) or 70,000 cells/100 μ L (HepG2). Dry NFC samples with sucrose were directly reconstituted with a mixture of mQ-water, appropriate media, and cells as described above. After reconstitution, samples were homogenized by mixing with a pipette. Then, 100 μ L of the mixed NFC hydrogel with cells was pipetted on low adhesion 96-well inertGrade BRAND plates® (Merck, Darmstadt, Germany), and on top of the hydrogel was added 100 μ L of appropriate serum-supplemented cell media. Media was changed every second day by first centrifuging the 96-well plate at $150 \times g$ (PC3 and hASCs) or $200 \times g$ (HepG2) for 6 min and then replacing the old media with fresh.

Three-dimensional control spheroids were cultured on the same plate as the sample cell spheroids. To culture the control spheroids, first, the fresh 1.5% NFC hydrogel was diluted with appropriate cell media supplemented with serum to the desired NFC fiber concentration. The control cell spheroids were cultured in the same NFC hydrogel concentrations as the sample 3D cell spheroids, i.e., 1.0%, 0.8%, and 0.125% for PC3, HepG2, and hASCs, respectively. Then, the 2D cultured cells were detached, collected, and counted, and the cell density was adjusted with appropriate cell media to the cell densities described above. The NFC and cell suspension were mixed and 100 μ L of the obtained suspension was seeded on low adhesion 96-well inertGrade BRAND plates® (Merck, Darmstadt, Germany). On top of the hydrogel was pipetted 100 μ L of appropriate serum-supplemented cell media. The cell media was changed as described above.

For alamarBlue™ (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) experiments, PC3, HepG2, and hASCs were cultured in 2D on a standard 96-well plate to ensure the viability of the used cells. The cells were seeded on the same day as the 3D cell spheroids with the same cell culture media as described above. The used cell densities were 10 k cells/cm², 20 k cells/cm², and 30 k cells/cm² for PC3, HepG2, and hASCs, respectively. The media was changed on days 1, 3, 5, and 7 during the alamarBlue™ experiments.

2.5. Viability Assays

The viability of the 3D cell spheroids was evaluated by their mitochondrial activity and cell membrane integrity. Mitochondrial activity was studied with alamarBlue™ Cell

Viability Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The cell media on top of the hydrogel was replaced as described above by 100 μ L of alamarBlue™ solution diluted with culture media to a concentration of 20% (*v/v*). Cells were incubated for 4 hrs at 37 °C. After incubation, the well plates were centrifuged and 100 μ L of alamarBlue™ solution was removed to plastic tubes. A 100 μ L volume of cell media was added to replace the removed alamarBlue™ solution. Plastic tubes were spun for 15 s at 1500 $\times$ *g* to remove any NFC fibers, after which 80 μ L of alamarBlue™ solution was transferred to a black 96-well plate (Nunc® MicroWell 96 optical bottom plates; Merck, Darmstadt, Germany), and fluorescence was measured using Varioskan LUX (Thermo Scientific, Waltham, MA, USA) and SkanIt RE-program 5.0 (Thermo Scientific, Waltham, MA, USA) (excitation 560 nm, emission 590 nm). The fluorescence signal was normalized to the signal from control cells and blank control samples without cells. The 2D control samples were treated similarly, but without the centrifugation step, and 10% (*v/v*) alamarBlue™ (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) solution diluted with cell media was used.

Cell membrane integrity was studied with a Cellstain double staining kit (Merck, Darmstadt, Germany). Cells were stained with calcein-AM (1:500) and propidium iodide (PI) (1:1000) diluted in Dulbecco's phosphate-buffered saline without calcium or magnesium (DPBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) by incubating them with the staining solution for 30 min at 37 °C and 5% CO₂. Samples were imaged with Leica TCS SP5 II HCS-A confocal microscope (Leica Microsystems, Wetzlar, Germany) using argon 488 nm and DPSS 561 nm lasers.

2.6. Immunocytochemistry

Morphology and proliferation capacity of 3D cell spheroids cultured in freeze-dried and reconstituted NFC hydrogel were studied by staining their actin cytoskeleton, nuclei, and proliferation marker Ki67. The cell spheroids were cultured for 2 days (hASC) or 7 days (PC3, HepG2, and hASC) in NFC hydrogel as described above. On day 1 or 6 (one day before fixing) the NFC hydrogel was digested enzymatically with GrowDase™ enzyme (UPM Kymmene, Lappeenranta, Finland) according to the manufacturer's instructions. All the following incubations were performed in rotation until further notice.

The day after initiating enzymatic digestion of the NFC hydrogel, 3D cell spheroids were collected in low-adhesion plastic tubes and fixed with 4% paraformaldehyde for 20 min. After that, the cell spheroids were washed three times with 0.1% (*v/v*) Tween 20 (Merck, Darmstadt, Germany) in DPBS without calcium or magnesium. Cells were permeabilized with 0.1% (*v/v*) Triton X-100 (Merck, Darmstadt, Germany) in PBS for 8 min and then blocked for 1 h at RT with 3% bovine serum albumin (BSA; Merck, Darmstadt, Germany) and 0.3 M glycine (Sigma-Aldrich, Germany) in PBS-Tween 20 solution. After blocking, the cell spheroids were dried on a microscopy glass. The following steps were performed without rotation on a microscope glass. The samples were incubated overnight at 4 °C with anti-rabbit Ki67 (1:200; Abcam, Cambridge, UK) antibody and conjugated Phalloidin Alexa 488 (1:40; Thermo Fisher Scientific, Waltham, MA, USA) in 0.1% (*v/v*) Tween 20 in DPBS containing 3% (*m/V*) BSA.

On the following day, the cell spheroids were washed three times with 0.1% Tween 20 in PBS. After a careful washing step, the cell spheroids were incubated with Alexa Fluor 594 donkey anti-rabbit IgG (1:500; Life Technologies, Carlsbad, CA, USA) in 0.1% (*v/v*) Tween 20 in DPBS containing 5% (*m/V*) BSA for 1 h at room temperature. Followed by the incubation, the cell spheroids were washed carefully three times with 0.1% Tween 20 in PBS. After the last washing, all excess liquid was dried with tissue paper, and the cell spheroids were mounted with ProLong Diamond Antifade Mountant with DAPI (Life Technologies Carlsbad, CA, USA) and covered with cover glass (Menzel-Gläser, Braunschweig, Germany). Samples were imaged with a Leica TCS SP5 II HCS-A confocal microscope (Leica Microsystems, Wetzlar, Germany) using argon 488 nm and DPSS 561 nm lasers and UV diode. Images were analyzed and processed with ImageJ software.

2.7. Automatic Cell Segmentation

A novel CNN-based automatic nuclei segmentation approach was applied to three PC3 and HepG2 spheroids [35]. The approach used CNN models to create binary nuclei masks and seeds and utilized a marker-controlled watershed algorithm [36] to produce instance segmentation of the nuclei. The approach was trained and validated with data including twelve HepG2 cell spheroids introduced by Kaseva et al. (2022) [35]. The nuclei and imaging configurations of these spheroids were similar to the ones in HepG2 and PC3 spheroids, making the approach applicable to this study.

The spheroid images were preprocessed by first resizing the voxels near isotropic. To achieve this, the x–y-planes of both spheroids were resized to 512×512 voxels and the z-plane of the HepG2 spheroid was expanded by a factor of two simply by copying each x–y-plane. This procedure was not exactly the same as discussed in [35], but sufficient for a visual demonstration. Then, a $256 \times 256 \times N$ sized region of interest (ROI) was chosen from the center of each image. The height N was chosen manually to exclude x–y-planes with no visible nuclei. The segmentations of ROIs themselves were produced automatically. The computation time for each segmentation was approximately half a minute. The computed results were compared visually to the original confocal microscope images.

2.8. Statistical Analysis

Statistical significance was determined with Microsoft®Excel®software version 2211 (Build 16.0.15831.20098) (Microsoft, Redmond, WA, USA) with a two-tailed independent samples *t*-test where $p < 0.05$ was considered significant.

3. Results

3.1. Freeze-Dried NFC Hydrogel Formed a Porous Scaffold with Suitable Physicochemical Properties for 3D Cell Culturing after Reconstitution

Freeze-dried 1.5% NFC hydrogel with 300 mM sucrose formed a highly porous and regular scaffold (Figure 1A). The appearance of the freeze-drying cakes was elegant (Supplementary Figure S2). The pH and osmolality of the freeze-dried and reconstituted NFC hydrogels were evaluated to observe the suitability of the NFC hydrogels for 3D cell culturing. The volume of media was adjusted to obtain osmolality values of 300 mOsmol/kg. The osmolality of the initial formulation (1.5% NFC hydrogel with 300 mM sucrose) was 346 mOsmol/kg (342–350 mOsmol/kg). The osmolalities and pH values of fresh and freeze-dried and reconstituted NFC hydrogels are reported in Table 1.

Storage moduli (G') of the fresh and freeze-dried and reconstituted NFC hydrogels were measured to evaluate the successful reconstitution of NFC hydrogel into different fiber concentrations. Importantly, the storage moduli were higher than the loss moduli in all freeze-dried and reconstituted samples indicating the successfully recovered viscoelastic characteristics (Supplementary Figure S3). Interestingly, NFC hydrogels with sucrose, DMEM, and water had relatively higher storage moduli when compared with NFC hydrogels with sucrose and water. The storage moduli of freeze-dried and reconstituted NFC hydrogels were slightly lower than those of corresponding fresh NFC hydrogels (Figure 1B). However, when storage modulus was correlated with the fiber concentrations, we observed that the behavior of the freeze-dried and reconstituted NFC hydrogels followed the same pattern as the fresh NFC hydrogels (Figure 1B). Figure 1D shows that the storage moduli of the NFC hydrogels increased when the NFC hydrogel concentration increased. By plotting the storage moduli of NFC hydrogel with sucrose and DMEM at the angular velocity of 4.93 rad/s with different NFC concentrations on semilogarithmic coordination we obtained Equation (1):

$$\log G' = a + bc(NFC) \quad (1)$$

in which a and b are experimentally determined values and $c(NFC)$ is the concentration of NFC hydrogel as $m/V\%$. For the fresh NFC with DMEM (supplemented with 10% FBS and 1% P/S antibiotics) a is 0.599 (Standard error (SE) ± 0.173) Pa and b is 2.07 (SE ± 0.26) m^2/s^2 ($R^2 = 0.954$). For the freeze-dried and reconstituted NFC hydrogel samples with

DMEM (supplemented with 10% FBS and 1% P/S antibiotics) the value of a is 0.298 ($SE \pm 0.192$) Pa and b is 1.85 ($SE \pm 0.29$) m^2/s^2 ($R^2 = 0.931$). The correlation between the fiber concentration and measured storage modulus remained nearly unchanged after freeze-drying and reconstitution.

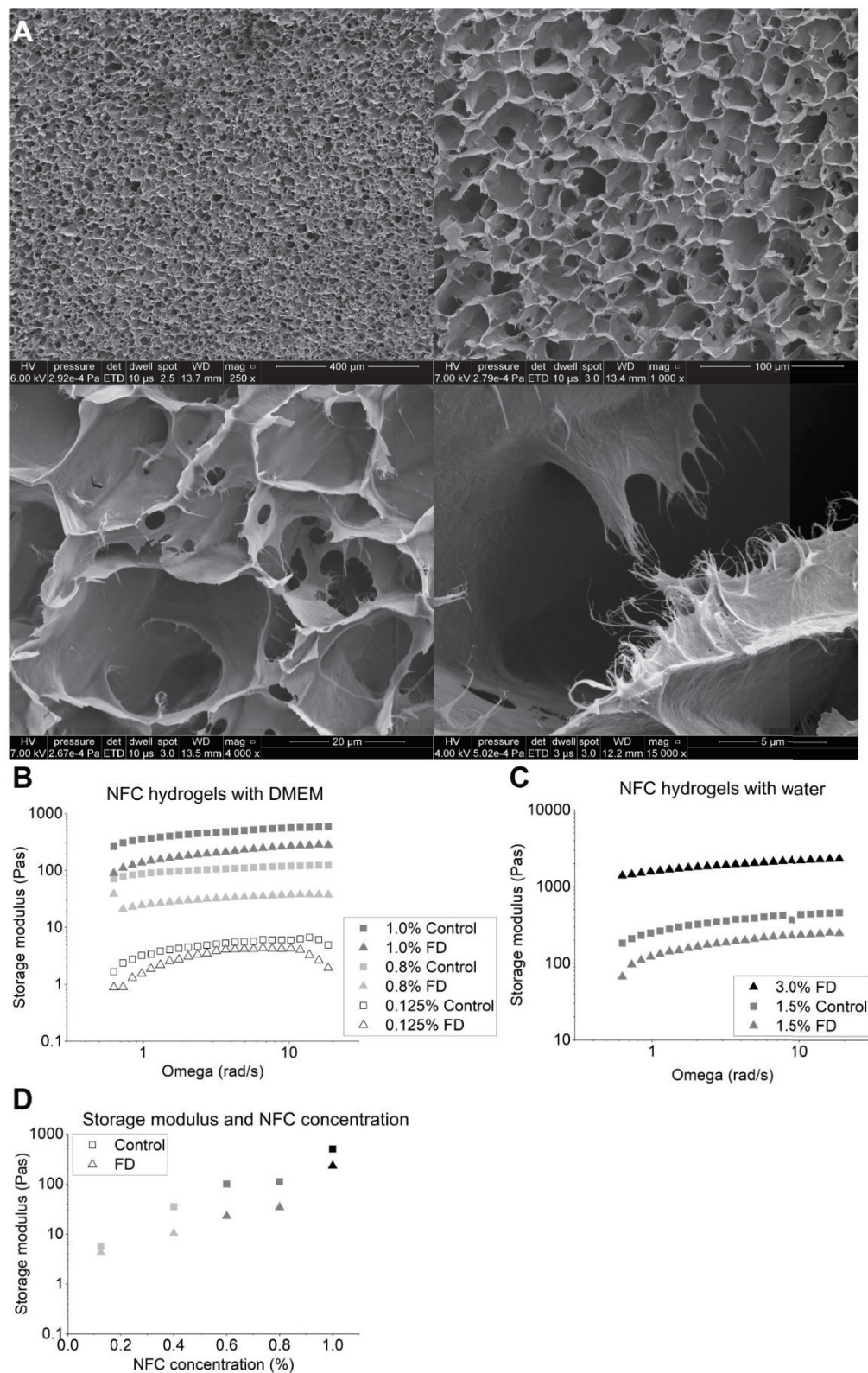


Figure 1. (A) Scanning electron microscope (SEM) micrographs of freeze-dried 1.5% nanofibrillated

cellulose (NFC) samples with sucrose demonstrating a continuous porous structure of the freeze-dried sample. **(B)** Storage moduli of NFC hydrogels with sucrose and Dulbecco's modified eagle medium (DMEM). Standard deviations (SD) of the measurements varied between 264.5–588.4 (1.0% control), 65.4–115.1 (1.0% FD), 71.6–124.2 (0.8% control), 16.5–44.6 (0.8% FD), 1.7–6.7 (0.125% Control), and 0.5–4.5 (0.125% FD) **(C)** NFC hydrogels with sucrose only. SD of the measurements varied between 256.8–462.8 (3.0% FD), 183.4–466.0 (1.5% Control), and 38.2–68.2 (1.5% FD). **(D)** Storage moduli of NFC hydrogel with sucrose and DMEM at the angular velocity of 4.93 rad/s (fresh and freeze-dried and reconstituted) with different concentrations. FD: Freeze-dried. Control: Fresh NFC formulation diluted from 1.5% NFC with 300 mM sucrose.

Table 1. The osmolalities in the form of average (range) and pH of fresh and freeze-dried and reconstituted nanofibrillated cellulose (NFC) hydrogels. FD: Freeze-dried. DMEM: Dulbecco's modified eagle medium.

Formulation	Osmolality (mOsmol/kg) (<i>n</i> = 3)	pH
Fresh 1.5% NFC, sucrose	346 (342–350)	7
Fresh 1.0% NFC, DMEM, sucrose	352 (368–379)	7
Fresh 0.8% NFC, DMEM, sucrose	352 (344–365)	7
Fresh 0.125% NFC, DMEM, sucrose	343 (341–345)	7
FD and reconstituted 1.5% NFC, sucrose	315 (302–338)	7
FD and reconstituted 3.0% NFC, sucrose	562 (538–586)	7
FD and reconstituted 1.0% NFC, DMEM, sucrose	314 (292–328)	7
FD and reconstituted 0.8% NFC, DMEM, sucrose	328 (319–335)	7
FD and reconstituted 0.125% NFC, DMEM, sucrose	340 (337–345)	7

3.2. Cell Spheroids Cultured in NFC Maintained their Viability at Least for 7 Days and Formed Cell-Type Typical Spheroids

PC3, HepG2, and hASC cells were cultured in 1.0%, 0.8%, and 0.125% NFC hydrogels, respectively, and in both fresh and freeze-dried and reconstituted NFC hydrogels to study any differences in the viability of the spheroids. Metabolic activity of the PC3, HepG2, and hASC cells cultured in freeze-dried and reconstituted NFC hydrogel was 102%, 104%, and 88%, respectively, on day 7, when normalized to the metabolic activity of the control cells on the same day (Figure 2). When normalized to the metabolic activity of day 1 cells of the same culture, i.e., sample normalized to sample day 1, and control normalized to control day 1, metabolic activities of 127% (control) and 156% (sample) were obtained for PC3, 145% (control) and 181% (sample) for HepG2, and 255% (control) and 206% (sample) for hASCs on day 7. This indicates that for the cancer cell lines PC3 and HepG2, the metabolic activity of the cells increased relatively more in the freeze-dried and reconstituted samples than in the control.

Statistically significant differences were observed between the 3D cell spheroids cultured in fresh and freeze-dried and reconstituted NFC on days 3 and 5, and on days 3, 5, and 7, for PC3 and hASC spheroids, respectively. However, when evaluated with live/dead staining using confocal microscopy, both cell lines showed equal viability and individual spheroids had approximately 100% viability in all cell lines (Figure 2). The 3D cultured PC3 cells grew with loose morphology, HepG2 spheroids with tight morphology, and hASC spheroids with tight and round morphology.

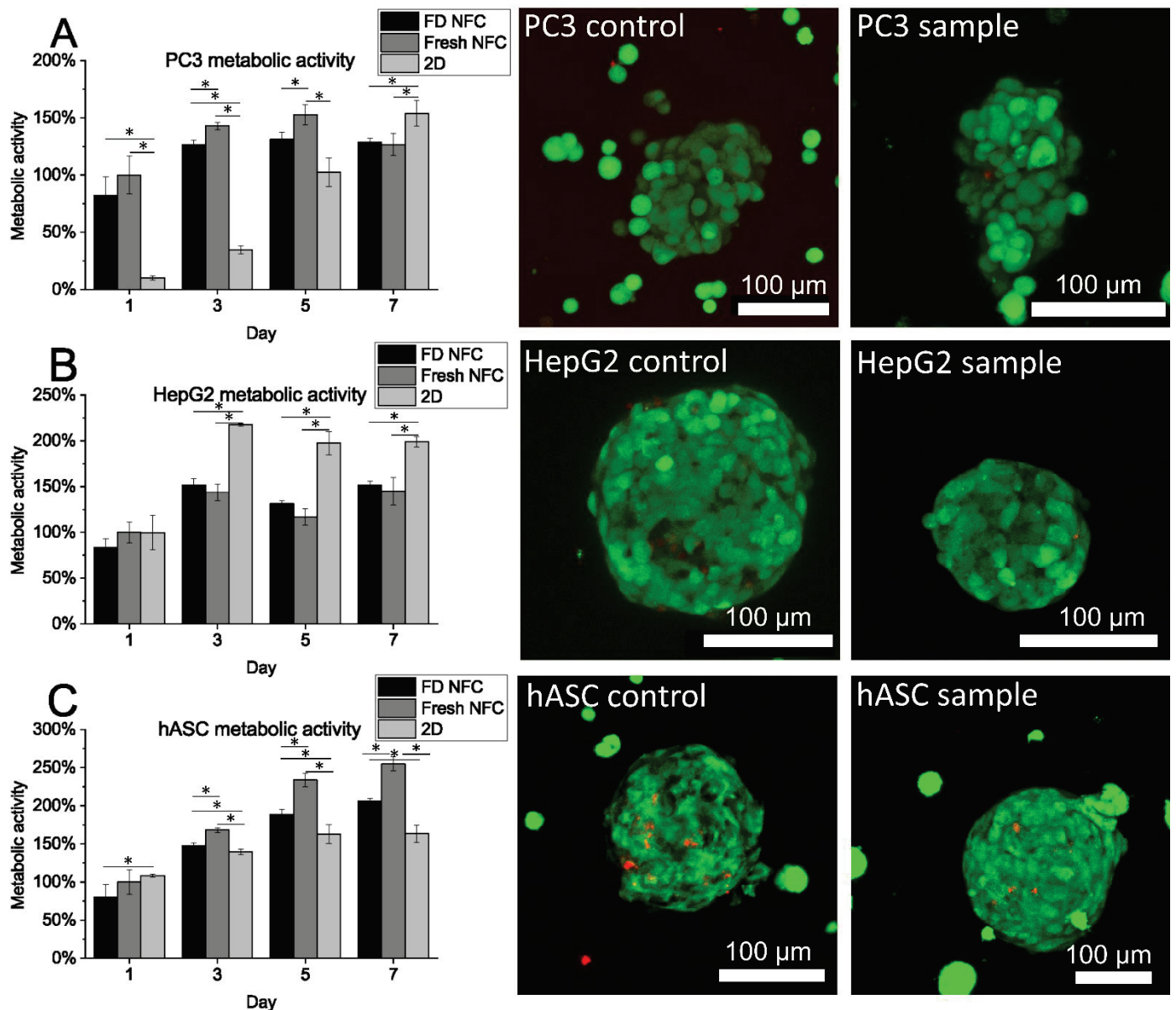


Figure 2. (A) Metabolic activity and viability of prostate cancer (PC3) cells cultured in freeze-dried and reconstituted 1.0% nanofibrillated cellulose (NFC) hydrogel, fresh 1.0% NFC hydrogel, and in 2D format. (B) Metabolic activity and viability of hepatocellular carcinoma (HepG2) cells cultured in freeze-dried and reconstituted 0.8% NFC hydrogel, fresh 0.8% NFC hydrogel, and 2D format. (C) Metabolic activity and viability of human adipose stromal/stem cells (hASCs) cultured in freeze-dried and reconstituted 0.125% NFC hydrogel, fresh 0.125% NFC hydrogel, and 2D format. Live/dead staining was performed on day 7 for all cell lines. Green: viable cells. Red: Dead cells. FD: Freeze-dried. Columns represent average values normalized to the metabolic activity of the specimen of day 1 fresh NFC. Error bars: standard deviation. Asterix: Statistically significant difference (two-tailed independent samples t-test, $p < 0.05$ considered significant).

Immunocytochemistry staining was performed to obtain sophisticated images of the morphology and proliferation capacity of 3D cell spheroids cultured in hydrogels with different stiffnesses and to compare morphology between 3D cell spheroids cultured in fresh and freeze-dried and reconstituted NFC hydrogel.

PC3 cells grew with grape-like morphology in 1% NFC hydrogel. Grape-like morphology was observed using actin and nuclei staining (Figure 3) [37]. Actin cytoskeletons of

individual cells could be recognized, and the cells were not tightly bound. In addition, cell nuclei were disorganized. No difference was observed in the morphology or number of proliferating PC3 cells cultured in fresh or freeze-dried and reconstituted NFC hydrogel.

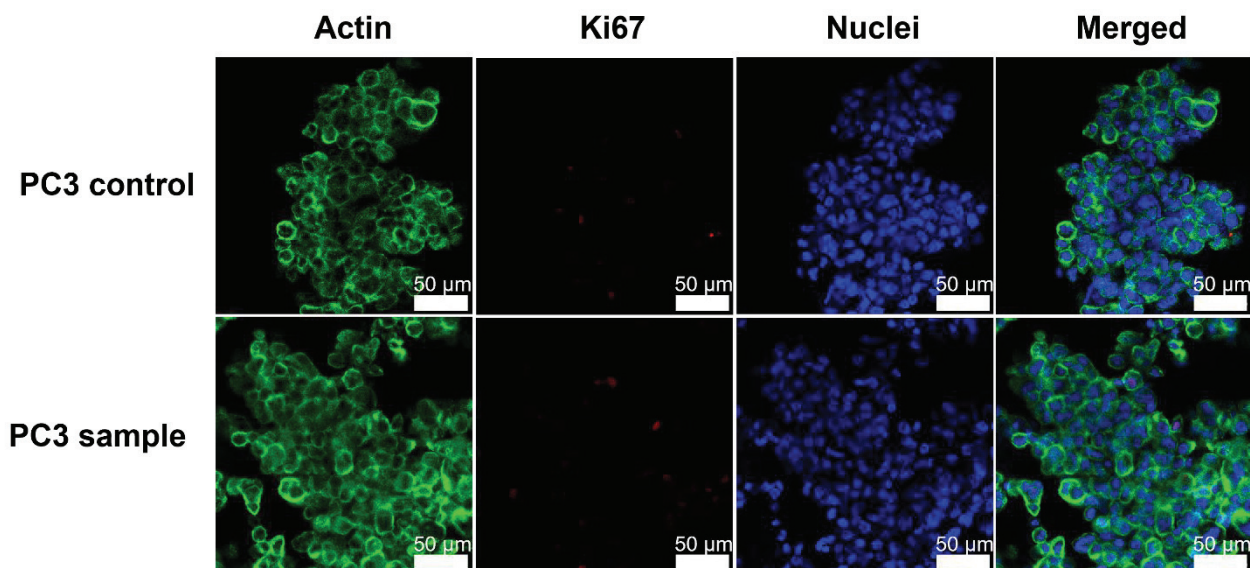


Figure 3. Immunocytochemical staining showing loose actin (green), proliferating cells (red), and nuclei (blue) of the 3D cultured prostate cancer (PC3) cell spheroids cultured either in 1.0% NFC hydrogel (control) or in freeze-dried and reconstituted 1.0% NFC hydrogel (sample). Green: Phalloidin conjugated Alexa Fluor 488. Red: Alexa Fluor 594. Blue: DAPI.

HepG2 cell spheroids grew with mass spheroid morphology in the 0.8% NFC hydrogel (Figure 4). Mass-like morphology could be observed by a tighter actin cytoskeleton network throughout the cell spheroid and with the higher organization of the cells observed by the location of the nuclei [37]. However, some disorganization was still observed, which indicated the mass-like morphology. Again, no difference was observed between HepG2 cell spheroids cultured in the fresh or freeze-dried and reconstituted NFC hydrogel.

The hASC spheroids grew with an organized structure indicating round morphology [37] in 0.125% NFC hydrogel (Figure 5). Already on day 2, hASCs had formed 3D cell spheroids. Polarization of hASCs on the perimeter of the 3D cell spheroid and their adjustment as spheroids could be observed already on day 2 from the appearance of the actin cytoskeleton and nuclei. The actin cytoskeleton formed round structures around the 3D spheroid. On day 7 the spheroids were extensive in terms of their diameter. The morphology of the 3D cell spheroids was round also on day 7 despite some loosely attached cells observed on the perimeter of the 3D cell, i.e., the structure of hASC spheroids appeared tighter on day 2 than on day 7. In addition, the number of spheroids was lower on day 7, and the size of individual cell spheroids was larger. Proliferating cells were observed only sparsely, on both days 2 and 7. In general, more proliferating cells were observed in cancer cell spheroids than in primary adipose stem cell spheroids.

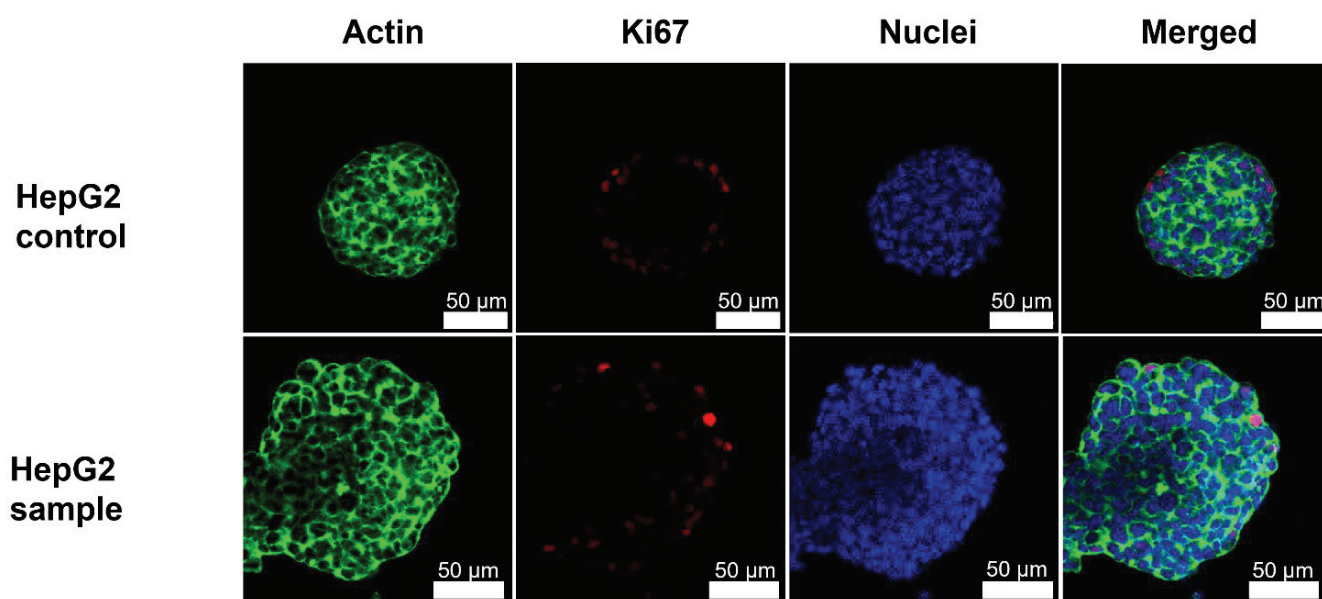


Figure 4. Immunocytochemical staining shows robust cell adhesion by actin (green), some proliferating cells (red) on the outer sphere of the cell spheroid, and disorganized nuclei (blue) locations of HepG2 cell spheroids cultured in fresh 0.8% NFC hydrogel (control) and freeze-dried and reconstituted 0.8% NFC hydrogel (sample). Green: Phalloidin conjugated Alexa Fluor 488. Red: Alexa Fluor 594. Blue: DAPI.

3.3. Automatic Segmentation of Different Cell Types

The quality of the segmentation of 3D PC3 and HepG2 cell spheroids was visually assessed (Figure 6). The ground truths for PC3 spheroids were not available nor created and quantitative evaluation scores could not be computed. However, the qualitative visual assessment of the segmentation results was satisfactory. The segmentation itself was fully automatic and non-subjective. Visual evaluation was performed to verify that the results of the automatic segmentation were acceptable. While some segmentation mistakes were present, most of the segmented cells were visually reasonable. The results emphasized that the automatic segmentation of 3D cell cultures discussed in this work can be viable. In order to exploit this approach on hASC spheroids as well, the training and evaluation set of the approach should be extended to include similar nuclei as in these spheroids. Automatic segmentation indicated clearly the different morphologies of the 3D cell spheroids. The grape-like morphology and looser nuclei structures of PC3 spheroids and the mass spheroid morphology and denser packing of the nuclei of HepG2 spheroids were easily observed from the automatic segmentation.

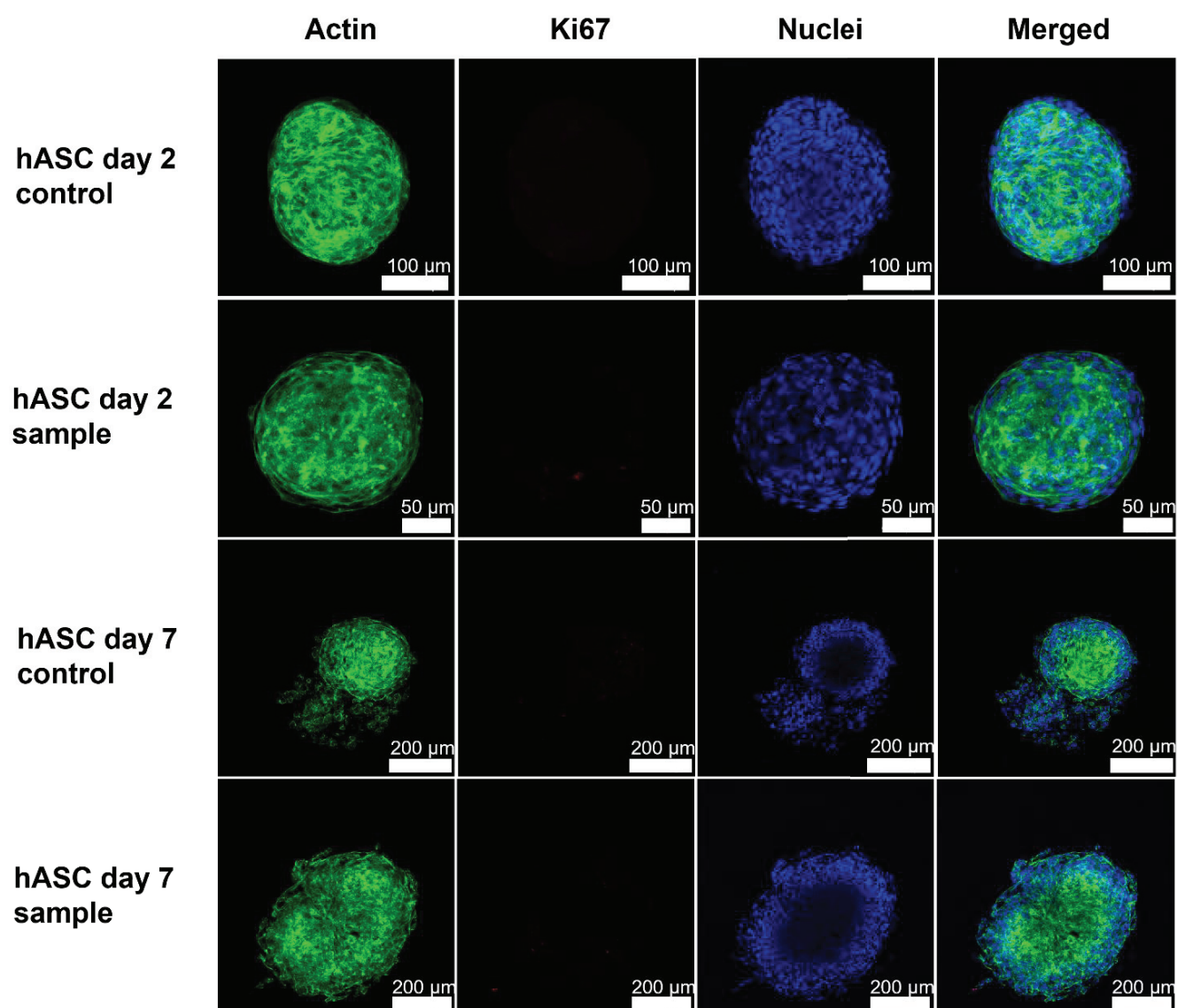


Figure 5. Immunocytochemical staining showing an organized structure of actin (green) and polarization of the cell observed as the shape of the nuclei (blue) of human adipose stromal/stem cells (hASCs) in fresh 0.125% NFC hydrogel (control) and freeze-dried and reconstituted 0.125% NFC hydrogel (sample). Proliferating cells (red) were observed only sparsely on both days 2 and 7. Green: Phalloidin conjugated Alexa Fluor 488. Red: Alexa Fluor 594. Blue: DAPI.

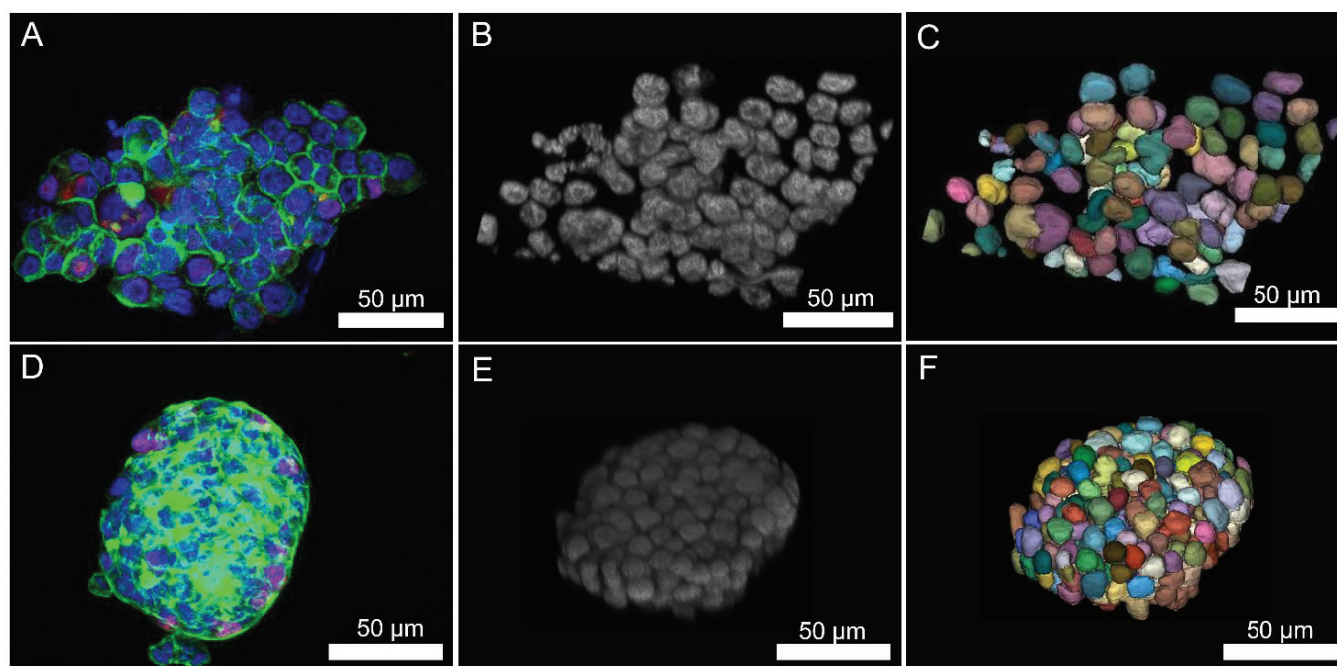


Figure 6. (A) PC3 cell spheroid cultured in 1.0% nanofibrillated cellulose (NFC) hydrogel for 7 days. Nuclei were stained with DAPI (blue), Ki67 proliferation marker with Alexa Fluor 594 (red), and actin cytoskeleton with Phalloidin conjugated Alexa Fluor 488 (green). (B) A grayscale figure of nuclei of PC3 spheroid for automatic segmentation. (C) Automatically segmented nuclei of the multicellular 3D PC3 cell spheroid. (D) HepG2 cell spheroid cultured in 0.8% NFC hydrogel for 7 days. Nuclei were stained with DAPI (blue), Ki67 proliferation marker with Alexa Fluor 594 (red), and actin cytoskeleton with Phalloidin conjugated Alexa Fluor 488 (green). (E) A grayscale figure of nuclei of HepG2 cell spheroid for automatic segmentation. (F) Automatically segmented nuclei of the multicellular 3D HepG2 cell spheroid.

4. Discussion

Most drugs that fail in the clinical phase lack efficacy or their pharmacokinetic profile is unsuitable. More precise screening of these properties in early drug discovery would reduce the need for animal testing and the costs of drug development. Novel approaches to reduce the gap between *in vitro* and *in vivo* experiments in drug discovery and research are required to address these issues. In this study, we showed the suitability of freeze-dried wood-derived nanofibrillated cellulose hydrogel for 3D cell culturing with a rapid one-step cell-seeding process. In addition, we demonstrated the possibility to reconstitute NFC hydrogel into different fiber concentrations and the possibility to estimate the stiffness of the hydrogel with a certain fiber concentration. Lastly, we showed the suitability of CNN-based models to automatically segment individual cells of multicellular HepG2 and PC3 spheroids.

Stiffness and porosity are recognized as important parameters of the biomaterials used for cell culturing. The stiffness of the material affects proliferation, growth, cell migration, differentiation, and organoid formation [23,38]. The storage moduli (describing the stiffness) of the NFC hydrogel samples with the cell media were relatively higher than the storage moduli of the samples with only water. This was observed with both fresh and freeze-dried and reconstituted samples. Interestingly, the opposite has been observed in crosslinked biomaterials. Hastruk et al. (2020) observed that higher ionic strength resulted in lower storage moduli [39]. However, NFC hydrogel is not cross-linked, which can explain the observed difference. The relatively higher storage moduli in our study can result from interactions between the fibers when the media is present. The slight differences observed with the fresh and freeze-dried and reconstituted hydrogels

with the same fiber concentration could result from the orientation of the NFC polymers after freeze-drying and reconstitution. A lesser decrease in the stiffness of the freeze-dried hydrogel is observed if the polymers have an anisotropically extruded orientation [40]. In our study the freezing was isotropic, and the NFC polymers were randomly aligned after freeze-drying and especially after reconstitution. This can explain the observed decrease in the storage moduli of the hydrogels. Furthermore, minor inaccuracy in the weighing of the sample and subsequent reconstitution also affects the observed stiffness. Nevertheless, despite the differences in the storage moduli, no difference was observed in the viability or morphology of the 3D cell spheroids cultured in the fresh NFC hydrogel and in the freeze-dried and reconstituted NFC hydrogel.

As stated above, porosity is another important material attribute when considering freeze-dried biomaterial scaffolds [29]. Wu et al. (2010) showed in their study how the porosity of the scaffolds could be controlled by changing the gelatin concentration and crosslinking before unidirectional freeze-drying [41]. In addition, by changing the process parameters of freeze-drying, especially by changing the freezing step, different porosities of the material can be obtained [42]. We have previously characterized the porosity of the freeze-dried NFC formulations throughout and similar results were observed in this study evaluated from the SEM micrographs [32,33,43]. The similar porosities observed in the SEM micrographs of this study and our previous studies underline the reproducibility of the freeze-drying cycle and sample preparation. Porosity directly affects the cellularization of the cell scaffolds, for example, cell attachment and viability [44,45]. However, our results indicate that the pore size is unspecific for cell types, yet the porosity can be considered vital to facilitate mass transport in the scaffold. We believe that in our study the pore size played a less significant role than in the other studies of dry scaffolds because we cultured the 3D cell spheroids eventually in the hydrogel despite starting from the dry NFC scaffold. Parisi et al. (2021) discussed in their recent review article how different material manufacturing methods and pore size affect cellularization by cell colonization or encapsulation [46]. They concluded that the cellularization is impaired below a certain threshold of porosity and cytocompatibility of the used method. In this study, we showed that the porosity of the freeze-dried NFC hydrogel was suitable for all three different types of cells despite the diverse tissue origins of the used cells.

The metabolic activity of 3D cultured cells was lower than 2D cultured cells and the number of proliferating cells in the 3D cell spheroids was low. Yet, the viability of the cells in the spheroid observed from the live/dead staining was nearly 100%. We think that this indicates the closer relevance of 3D cell tumors to *in vivo* than unlimitedly proliferating 2D cell cultures [47]. For example, if the studied cells have artificially too high metabolic activity, drug candidates affecting them may show falsely positive effects resulting later in a failure in clinical trials. The statistical significance observed in the metabolic activities can be also explained by the different cell densities used in the 2D and 3D cell cultures. Subia et al. (2021) concluded in their review considering breast tumor-on-a-chip applications that tumor-on-chip models can compensate for 2D and 3D models [5]. Combining the fluidic system into the well plate with 3D culture spheroids would be a viable option to obtain an even more relevant human-on-a-plate approach suitable for HTS. Furthermore, combining the automatic segmentation with the HTS screening would hasten the analysis of HTS results.

In this study, segmentation results on one HepG2 spheroid and one PC3 spheroid were illustrated. The results were produced by manually choosing representative 3D ROIs from both spheroids and applying the 3D nuclei segmentation system introduced by Kaseva et al. [35] to these ROIs. The system operates automatically. At this stage, no ground truths have been generated for the spheroids and visual inspections of the results were performed. The inspections revealed that the system could, despite being trained with a small-sized dataset, obtain reasonable results. This is a promising outcome since it outlined that the automatic segmentation of HepG2 and PC3 spheroids could be viable in the future.

To summarize, according to our results, freeze-drying of NFC hydrogel results in suitable porosity for cell colonization and facilitates subsequent reconstitution and adjustment of the stiffness of the hydrogel to promote 3D cell culturing of different cancer cell lines and primary cells. In addition, we showed the suitability of the material for microscopy imaging and subsequent CNN-based automatic cell segmentation. These properties are important for the materials used in in vitro experiments for drug discovery in the near future. Material sciences promote drug delivery applications, for example, with vaccines and contraception [48]. We believe that in addition to the applications reviewed by Sadeghi et al. (2021), material sciences will become increasingly important in drug discovery [48].

5. Conclusions

Freeze-dried NFC hydrogel can be reconstituted to different stiffnesses relative to the fiber concentration. The freeze-dried and reconstituted NFC hydrogel can be used for 3D cell culturing of different human-origin cell types, such as primary hASCs, PC3 cells, and HepG2 cells, of which PC3 and HepG2 cell spheroids were suitable for automatic cell segmentation. The porosity of the material was sufficient to facilitate mass transfer and colonization of the material by the seeded cells. The 3D cultured cell spheroids showed cell-typical morphology, and first increasing and then stable metabolic activity with nearly 100% viability. Taken together, freeze-dried NFC hydrogel offers a versatile 3D cell culturing platform for cells of different origins.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym14245530/s1>, Figure S1: Freeze-drying cycle used in this study. Figure S2. Freeze-dried 1.5% nanofibrillated cellulose with 300 mM sucrose and reconstituted 1.5% NFC hydrogel with 300 mM sucrose. Figure S3. Storage (G') and loss (G'') moduli of A) 3.0%, B) 1.5%, C) 1.0%, D) 0.8%, and E) 0.125% NFC hydrogel.

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Article

Determining the Relationship between Delivery Parameters and Ablation Distribution for Novel Gel Ethanol Percutaneous Therapy in Ex Vivo Swine Liver

Erika Chelales ^{1,*}, Katriana von Windheim ¹, Arshbir Singh Banipal ¹, Elizabeth Siebeneck ¹, Claire Benham ¹, Corrine A. Nief ¹, Brian Crouch ¹, Jeffrey I. Everitt ², Alan Alper Sag ³, David F. Katz ¹ and Nirmala Ramanujam ¹

¹ Department of Biomedical Engineering, Duke University, Durham, NC 27708, USA; katriana.von.windheim@duke.edu (K.v.W.); arshbir.banipal@duke.edu (A.S.B.); cnief@stanford.edu (C.A.N.)

² Department of Pathology, Duke University Medical Center, Durham, NC 27710, USA; jeffrey.everitt@duke.edu

³ Department of Radiology, Division of Vascular and Interventional Radiology, Duke University Medical Center, Durham, NC 27710, USA

* Correspondence: erika.chelales@duke.edu

Abstract: Ethyl cellulose–ethanol (ECE) is emerging as a promising formulation for ablative injections, with more controllable injection distributions than those from traditional liquid ethanol. This study evaluates the influence of salient injection parameters on forces needed for infusion, depot volume, retention, and shape in a large animal model relevant to human applications. Experiments were conducted to investigate how infusion volume (0.5 mL to 2.5 mL), ECE concentration (6% or 12%), needle gauge (22 G or 27 G), and infusion rate (10 mL/h) impacted the force of infusion into air using a load cell. These parameters, with the addition of manual infusion, were investigated to elucidate their influence on depot volume, retention, and shape (aspect ratio), measured using CT imaging, in an ex vivo swine liver model. Force during injection increased significantly for 12% compared to 6% ECE and for 27 G needles compared to 22 G. Force variability increased with higher ECE concentration and smaller needle diameter. As infusion volume increased, 12% ECE achieved superior depot volume compared to 6% ECE. For all infusion volumes, 12% ECE achieved superior retention compared to 6% ECE. Needle gauge and infusion rate had little influence on the observed depot volume or retention; however, the smaller needles resulted in higher variability in depot shape for 12% ECE. These results help us understand the multivariate nature of injection performance, informing injection protocol designs for ablations using gel ethanol and infusion, with volumes relevant to human applications.

Keywords: ethyl cellulose; ethanol; ablation; low resource; injectable therapy; computed tomography; infusion force

1. Introduction

Tissue ablation is an inexpensive [1,2] and minimally invasive [3–5] alternative to surgical tissue removal for many therapeutic and prophylactic interventions. Ablation typically requires less procedure time [6,7] and shorter hospital stays [7,8] than the surgical approaches. Thermal ablation (e.g., radiofrequency ablation, RFA; microwave ablation, MW; or cryoablation) is common in high-income countries (HICs). Thermal ablation can apply heat or cold: a heating probe can emit high-frequency alternating current (radio frequency ablation, RFA), causing ionic agitation and heat to denature proteins and induce tumor cell necrosis [9,10], or a cryoprobe can induce cold temperatures in tissue which induce intracellular ice crystals that destroy the tumor cells [10–12]. However, access to electricity and the cost of supplies significantly limit thermal ablation use in many low- and middle-income countries (LMICs) [13].

Ethanol ablation is a chemical ablation method involving direct injection of ethanol into a lesion to induce coagulative necrosis through protein denaturation [14] and cytoplasmic dehydration [15]. Ethanol ablation is a potentially more suitable global treatment for some cancers, especially superficial solid tumors accessible to injections, since it can be performed by minimally trained personnel in the absence of electricity and/or surgical infrastructure. However, although inexpensive, ethanol is not widely used for tumor ablation. Historically, thermal ablation has produced more predictable and effective outcomes [9]. It has achieved higher rates of complete necrosis and survival for hepatocellular carcinoma treatment as compared to ethanol ablation [16–18]. Currently, ethanol ablation often requires multiple injections or treatment sessions [14,19–29], which are not feasible in LMICs, where patients are often lost to follow-up [30]. Moreover, traditional liquid ethanol ablation tends to cause non-target leakage, resulting in irregularly shaped injectate distributions in tumors that typically do not span the entire tumor and may extend to normal cells at the tumor margins [31]. Ethanol volumes that are significantly larger than the corresponding tumor volumes are often needed to achieve efficacy [28]. This exacerbates ethanol leakage into vessels or bile ducts [32], which can result in acute and delayed vascular injuries [33]. Overall, current ethanol ablation methodology results in relatively ineffective treatment, in part due to high interstitial pressure in tumors [34].

Our group previously developed a simple method for tumor ablation by mixing ethyl cellulose (EC), an inexpensive, natural polymer and derivative of cellulose, with ethanol prior to injection. EC increases ethanol viscosity [35] and leverages the endogenous polarity difference between ethanol and water to induce a phase change from liquid to fibrous gel (gelation) upon injection into tissue [36]. This mechanism of *in situ* gelation sequesters cytotoxic ethanol in the target region, increasing on-target coverage and reducing off-target leakage [37,38].

The physical characteristics and performance of EC depend on its degree of substitution and its molecular weight. EC is obtained through the etherification of cellulose as hydroxyl groups are replaced with ethoxyl groups. At low degrees of substitution, solubility in water can be maintained; however, at higher degrees of substitution EC is only soluble in organic non-polar solvents [39]. When mixed with ethanol, a homogenous sol/gel is formed. This is a slow process that takes between three to twelve hours depending on the concentration of the polymer. The polymer first undergoes a swelling phase that must be continuously mixed to ensure complete dissolution. The viscosity of the solution is dependent on the amount of EC added and has been investigated in previous work [35]. Upon introduction to water, the solution undergoes a phase transition as the ethanol disperses and the EC contacts the poor solvent environment (tissue). This *in situ* phase change creates a gradient between insoluble EC–fibrous gel–sol/gel as the ethanol diffuses over time, increasing on-target coverage and reducing off-target leakage [37,38]. The speed and degree to which the phase change occur depend on the initial concentration of EC [35]. Higher ECE concentrations provide better retainment of ethanol as they generate more of a gel, but the higher viscosity creates a tradeoff between increasing efficacy and decreasing injectability. EC has been highlighted as a promising cellulose derivative for modified drug delivery due to these properties and has been implemented in pharmaceutical research [40].

We previously showed that ECE ablation induced complete regression of squamous cell carcinomas in a hamster cheek pouch model, while conventional ethanol ablation did not induce regression [35]. Injected ECE also exhibited decreased localized adverse events and increased overall survival in a syngeneic model of breast cancer, demonstrating the increased safety and efficacy of ECE in small animal models compared to ethanol alone [41]. Finally, injected ECE reduced tumor volume and was demonstrated as a feasible treatment for feline squamous cell carcinoma [42]. Overall, injected ECE ablation has the potential to address the need for a low-cost ablative therapeutic for the treatment of cancer in LMICs. Further, HICs could benefit from non-thermal ablation methods to treat tumors in locations not amenable to thermal ablation [43], or due to patient exclusion criteria, preference, and/or cost.

Ideally, any clinically suitable ablation technique should achieve predictable and repeatable injection distribution volumes in humans. Those volumes should span and ablate the target tumor volume, while minimizing off-target toxicity. In RFA, the temperature decreases with distance from the source; only the area within a lethal temperature threshold is destroyed [15,19,21]. Clinical data for thermal ablations have been used to generate charts that show an effective ablation diameter, height, or width vs. various procedure settings (i.e., temperature, ablation time, number of probes). These charts guide clinical practice. Analogously, an understanding of the influence of key injection parameters on ECE ablation will be important for its clinical translation.

The goal of the present study was to help inform an analogous approach to optimizing ECE injections, as has been created for thermal ablations. We specifically focused upon the formulation of the therapeutic ECE (viz. ethanol concentration) and key injection parameters in relation to the resulting force and distribution volumes for clinically relevant (less than 5 cm in diameter as is typical for ablative therapies) tumor treatment [2]. This study followed up our previous work, which developed methodologies to relate key injection parameters to injected ECE injection volumes (50–200 μ L) in ex vivo swine and rat liver [37,38]. We also investigated the force required for infusion to inform the impact that the high viscosity of ECE will have on the safety and efficacy of these injections. The force during injection has previously been tied to pressure during injection. However, the literature is limited on substances with viscosities as high as ECE's [44]. Higher forces during injection correlate with higher pressures in the tissue, leading to tissue cracking, leakage, and lower retainment in the zone of ablation [37]. Typically, clinical thermal ablation devices, such as RFA, treat lesions that are 1 to 5 cm in diameter, corresponding to a spherical volume ranging from approximately 0.52 mL to 65.45 mL [14,16–18,21,23–25,27]. Our prior work focused upon smaller volumes (50–200 μ L). Scaling up injection volume is a complex biophysical process, where injection variables (e.g., volume, rate, needle gauge, EC concentration) are likely to be salient and do not scale up obviously. The work here addressed this problem.

Our previous preclinical studies with small injection volumes (50–200 μ L) considered swine liver, as well as ex vivo and in vivo rat liver tissue models [37,38]. In these studies, we found that for small infusion volumes (50–200 μ L), 6% ECE significantly increased depot volume compared to pure ethanol and 3% ECE at infusion rates of 10 mL/h in ex vivo swine liver [37]. We also found in ex vivo rat liver that 12% ECE, vs. lower ECE concentrations and pure ethanol, significantly increased ethanol spatial distribution and that there was a strong radiologic–pathologic correlation between ethanol distribution volumes observed on CT imaging and resultant necrosis [38]. We then followed this work up with an in vivo rat liver model and demonstrated superior ethanol spatial distribution of 12% ECE compared to pure ethanol in vivo, thus confirming the ex vivo results [38]. The present study builds upon the foundational knowledge of our previous preclinical work, to investigate injection volumes relevant to human applications. In so doing, we also began a more in-depth analysis of biophysical factors governing the injection process, focusing upon the force required to push the formulation through the needle.

2. Materials and Methods

2.1. Formulation of ECE

For this study, the EC obtained from Sigma-Aldrich (St. Louis, MO, USA) had a viscosity of 100 cP and a 48% ethoxyl content. This corresponds to a degree of substitution of 2.35–2.62 [45]. The molecular weight of 100 cP EC is characterized as 80.8 ± 24 kDa [46]. Ethanol 200 proof (anhydrous ethanol, Koptec, King of Prussia, PA, USA) was mixed with EC within 24 h of the experiment.

2.2. Assessment of Distribution Volume of ECE In Ex Vivo and In Vivo Swine Liver

Previous work in our lab used ex vivo swine liver, as well as ex vivo and in vivo rat liver tissue models [37,38]. However, the infusion volume in all of these studies was

50–200 μL , which is quite small compared to lesion sizes that are typically treated with ablation in humans. The swine liver is an appropriate model choice for ablation assessment. While the swine liver differs from the human liver in external morphology, it has a similar size, segmental anatomy, vascularity, and biliary tree structure [47]. The efficacy of ablative therapies is commonly examined and in healthy liver tissue [48–56] specifically, traditional ethanol ablation [32,48,57] (PEI), radiofrequency ablation [54,58] (RFA), microwave ablation [55,59–61] (MWA), drug-eluting bead embolization [62], and high-intensity focused ultrasound [63] (HIFU) are used. These relatively homogeneous tissues provide a consistent environment for such studies. Further, ex vivo liver is commonly used by medical device companies to create guidelines for ablation parameter choices to achieve ablation zones of varying sizes. Therefore, porcine liver was an appropriate choice here for assessment of ECE ablation distribution for clinically relevant volumes.

2.3. Experimental Design

We scaled up previous work using small volumes in ex vivo and in vivo rat livers to investigate infusion parameters for ECE ablation to achieve distribution volumes of a clinically relevant size. Since percutaneous ablation is most often used for, and most successful in, the treatment of smaller tumors [15,19,21,22,25], we investigated parameters to achieve ablation volumes in the range of 0–1.5 mL, since a sphere with a radius of 1 cm corresponds to an approximately 0.5 mL volume. Using the methods established in prior work [38], we conducted injections of ECE while varying the parameters of formulation (6, 12% ECE), infusion volume (0.5, 1, 1.5, 2, 2.5 mL), needle gauge (22, 27 G), and infusion rate (10 mL/h, manual infusion). Amounts of 6% and 12% were the most commonly used EC concentrations in our previous studies [37,38]. Parameters were systemically varied in a series of three experiments, selectively reducing the parameter space from a full factorial design. The completed experiments are detailed in Table 1. Experiment (Exp.) 1 was designed to establish an optimal ECE concentration, with the goal of maximizing distribution volume in the tissue to reduce the amount and cost of the formulation. Thereafter, we kept the ECE concentrations constant. Exp. 2 was designed to examine the effects of using a larger needle, which would result in reduced pressure during injection and is closer to commonly used clinical needle and probe sizes for current ablation methods [9,10,14,27,28,64–66]. Finally, Exp. 3 focused upon the effect of infusion rate on resultant distribution volume. We included manual infusions, which would enable easier clinical translation, greater physician flexibility, and reduced need for costly injection equipment. For Exp. 3, smaller infusion volumes were investigated, since they achieved superior ratios of distribution volumes to infusion volumes in previous experiments. Both needle gauges were studied. Notably, the smaller needle would enhance patient comfort [38].

Table 1. Summary of experiments and key parameters varied in each experiment.

Experiment	Parameters Assessed ($n > 6$ for Each Group)				
	Infusion Volume (mL)	EC Concentration (%)	Needle Gauge (G)	Needle Length * (inches)	Infusion Rate (mL/h)
1	0.5, 1, 1.5, 2, 2.5	6 12	27	0.5	10
2	0.5, 1, 1.5, 2, 2.5	6 12	22	0.75	10
3	0.5, 1	12	27 22	0.5 0.75	Manual Infusions

* Needle length varied due to manufacturer availability; however, all needles were inserted 0.5 inches into the tissue for all injections.

2.4. Experimental Procedure

Ablations in ex vivo swine livers, acquired from Hatley Farms or Animal Biotech Industries, were completed using the respective conditions detailed in Table 1. Upon delivery, the swine livers were immediately preserved and stored in Krebs–Ringer bicarbonate buffer (Sigma-Aldrich, K4002) on ice until injection (within 1–2 h). For injections, the livers were submerged in the Krebs–Ringer bicarbonate buffer in a gallon plastic zip bag which contained no air bubbles to promote higher contrast in imaging. ECE was infused from a 3 mL syringe (BD Medical, Columbus, NE, USA) through 10 cm of rubber tubing (1/4 in. inner diameter; McMaster-Carr, Douglasville, GA, USA), using a syringe pump (NE-1000; New Era, Farmingdale, NY, USA). The needle was inserted directly through the plastic zip bag and into the liver. In all experiments, needles were inserted to a depth 0.5 inches below the surface of the liver.

Pre- and post-ablation computed tomography (CT) images were acquired using a GE Discovery 690 PET/CT Scanner (General Electric, Waukesha, WI, USA) and segmented using 3D Slicer software [67] version 4.11.20210226 r29738/7a593c8 (minimum of $n = 6$ per group). In each imaging session, ethanol–water solutions with 0%, 25%, 50%, 75%, and 100% ethanol concentrations were prepared to assess ethanol CT contrast and to create the calibration equation (established in our prior studies [38]). This enabled us to quantify a 20% ethanol concentration cutoff value for ablation segmentation. Radiodensity values corresponding to at least 20% ethanol concentration [68] were included in the segmentation of ECE. To help elucidate the effect of infusion parameters (infusion volume, EC concentration, needle gauge, and infusion rate) on the resultant ablation, we quantified the ablation metrics: the depot volume (distribution volume of the injectate without inclusion of non-depot injection spread into nearby collapsed vasculature), percent of infusion volume retained in the depot, and aspect ratio of the depot.

2.5. Computed Tomography Image Acquisition

All CT images were acquired on a GE Discovery 690 PET/CT, obtained using a helical scan with a 1 sec rotation time, full rotation length, 20 mm detector coverage, and 0.625 mm helical thickness. The pitch and speed (mm/rot) were 0.969:1 and 19.37, respectively. A medium body single field of view (SFOV) was used with 120 kV and auto/smart mA. For reconstruction, a 36 cm field of view (FOV) was used with Standard Plus reconstruction and no adaptive statistical iterative reconstruction (ASiR) Setup.

2.6. Image Segmentation in 3D Slicer

Images were processed in 3D Slicer [67]. The radio threshold for ethanol segmentation was determined using the radiodensities of ethanol–water vials. The average radiodensity of in vitro ethanol–water samples was computed by segmentation of a sphere within each vial. Specifically, spheres with diameters of 30 mm were created in the Segment Editor module and centered in each vial. Smaller spheres were used if the 30 mm diameter sphere was larger than the liquid volume in the vial. The Segment Statistics module was used to calculate the mean radiodensity in each vial.

Ex vivo liver images were segmented by selecting the tissue surrounding the injected ethanol distribution without including surrounding buffer. Ethanol was segmented using the 20% cutoff value determined from standard vials imaged at each imaging session, as detailed in prior work [38]. Specifically, segmentations were made in the Segment Editor modules by using a sphere brush to paint around the injection site through all image slices, excluding areas outside the liver. This segment was named the “true volume” of the ethanol distribution and is depicted in Figure S1A, for 1 mL infusions of both 12% ECE and 6% ECE, using 27 G and 22 G needles. The collapsed nature of the vasculature in the ex vivo swine liver leads to a low-resistance path for flow of the injectate. The true volume includes non-depot injection spread into nearby collapsed vasculature. This is not representative of the ablation zone, since in an in vivo system, such a spread would be cleared away from the target by the vascular system. The true volume segmentation

was duplicated into a new segmentation and any portions of the segmentation not in the depot (the ablation region at the target) were removed. The new segment was named the “depot volume” and is depicted in Figure S1B as the “segmented depot”. The depot volume does not include any non-depot injection spread into nearby collapsed vasculature. The Segment Statistics module was used to calculate the volume of both the true volume and depot volume distributions in the tissue. The Screen Capture module was used to export the segmentation volumes as videos and images. Segmentation and cross-sectional images were generated directly from 3D Slicer; analysis of segmentations and generation of histograms was performed in MATLAB R2022a. Statistical analysis was completed in JMP Software (JMP®, Version 17, 1989–2023; SAS Institute Inc., Cary, NC, USA).

2.7. Force Measurements

All force measurements were conducted with a calibrated load cell (Loadstar Sensors, Fremont, CA, USA) connected to a DI-1000 interface obtained through McMaster-Carr (Elmhurst, IL, USA). All injections were conducted into air. The configuration for these injections was the same as for the ex vivo swine liver injections, except that the tubing between the syringe and needle was omitted. Table 1 gives the parameter values tested, except manual measurements that were also performed. The load cell was placed at the back of the syringe plunger to record the force required to expel the ECE. Force was recorded every 1 s using LoadVUE Pro software v2021.8.20.1 (Loadstar Sensors, Fremont, CA, USA). The load cell was zeroed before each measurement, and the syringe pump base was placed a few millimeters away from the syringe plunger for each injection to capture the break loose force. When the desired injection volume was reached, the movement in the syringe pump was halted, and the pressure was allowed to drop off for a few seconds before the force-recording software was stopped. Force was measured every second, and thus, the number of data points varied with the infusion volume ($n = 180$ for 0.5 mL, $n = 900$ for 2.5 mL). For data analysis, the injection force profiles were summarized by averaging two time points, an initial one at 100 s and a final one at 50 s, prior to the end of the injection.

2.8. Selection of Injection Parameters

Previous studies in our group investigated different combinations of ECE concentrations, needle gauges, and volumes. These selections were motivated by experimental questions and model limitations (i.e., tumor size limiting volume delivered). While these studies were critical for establishing the potential efficacy of the treatment, the variety of parameters chosen did not lead to an algorithm for determining the best injection parameters. The work here contributes to the development of this algorithm, by combining information on injection parameters gained in our previous studies with new knowledge about how parameter choice impacts the amount of ECE that is maintained in the depot. The goal was to maximize the amount of ECE that is retained while operating within clinical guidelines. Except for volume, which was increased to clinically relevant volumes, all injection parameters were selected from our previous studies. The needle gauges 22 and 27 were also what we previously studied.

2.9. Statistical Analyses

All statistical analysis was conducted in JMP. The force data were tested for unequal variances (Levene’s test) and analyzed with a general linear models methodology to assess the impact of ECE concentration, needle gauge, and volume on the in vitro injection force. In vivo experiments were analyzed with multi-factor ANOVA to assess the impact of ECE concentration, needle gauge, and volume on depot size, percent retained, and aspect ratio. A significance level of $p = 0.05$ was applied throughout.

3. Results

3.1. Force of Injection Increases with Higher ECE Concentration and Smaller Needle Gauge

We investigated the force required for the injection of 6% and 12% ECE through 22 and 27 G needles at a 10 mL/h flow rate into air (called the “infusion force”). The volumes used were matched to the swine liver experiments detailed in Table 1. The flow rate was held constant for all injections; thus, infusion time varied depending on injection volume.

Figures 1A and 2A show box plots for the average force of injection for each ECE concentration and needle gauge combination. In Figure 1B–F, the force profiles vs. time are shown for all injections ($n = 3$) through 22 G needles. Figure 2B–F show the force profiles vs. time for all injections ($n = 3$) through 27 G needles. Approximately 90 s lapsed before the force plateau during an injection.

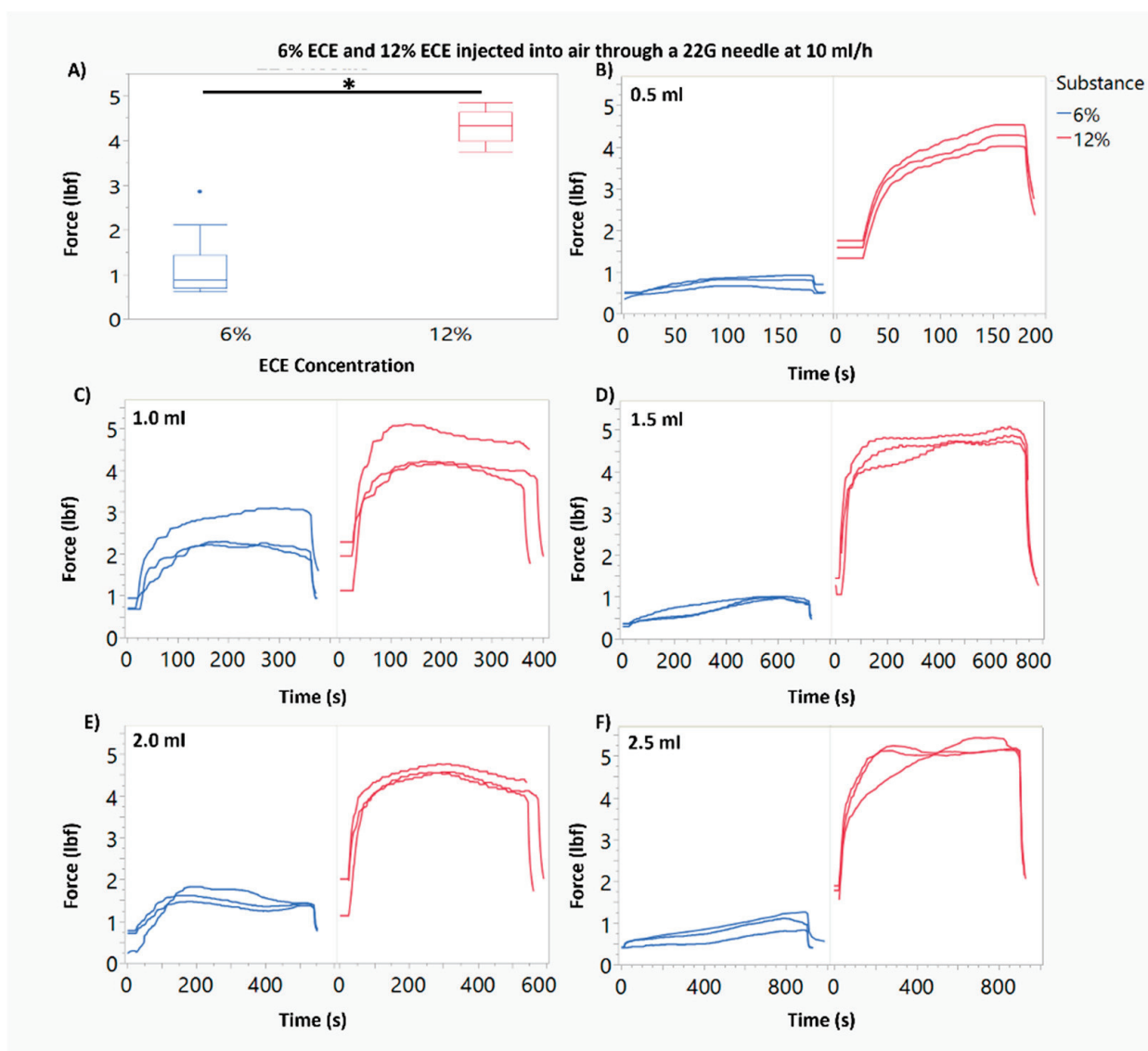


Figure 1. Comparison of force during injection for 6% and 12% ECE through 22 G needles. (A) Force measurements for 6% ECE and 12% ECE through a 22 G needle at 10 mL/h. The force required for 12% ECE was significantly larger than the force for 6% (* $p < 0.05$). (B–F) Plots of the force measured over time during an infusion for 0.5–2.5 mL infusions through a 22 G needle at 10 mL/h.

By applying the Hagen–Poiseuille equation (Hagen–Poiseuille, 1846) to our injections, volume should have had no impact on force for injections to air. Therefore, using multiple

volumes was not theoretically critical. However, by using multiple volumes, we were able to further ensure that the force measurement methodology was working properly. Volume was found to only be significant ($p < 0.05$) when the 0.5 mL volume was included as a group. This is theorized to be due to the long rise in force required for the ECE injections to reach the plateau. The 0.5 mL infusion is small enough that the time spent at plateau is very short, putting it on the lower end of force measurements. With this in mind, volume was collapsed as a factor to elucidate the statistical power of the remaining factors, ECE concentration and needle gauge. The injection force ranged from an average (over replicates) of 1.19 lbf for 6% ECE through 22 G needles, to an average of 12.2 lbf for 12% ECE through 27 G needles (Figures 1A and 2A).

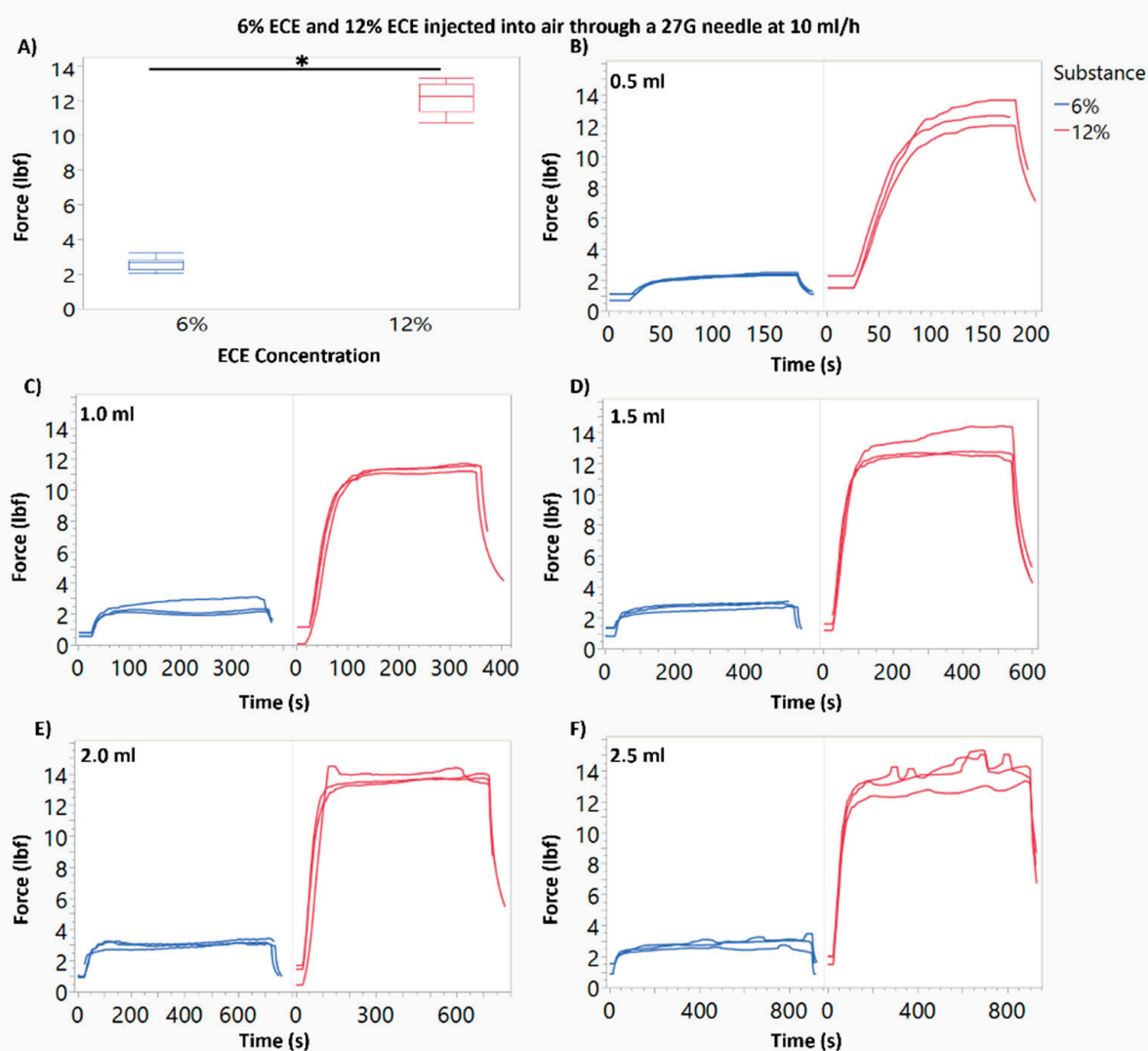


Figure 2. Comparison of force during injection for 6% and 12% ECE through 27 G needles. (A) Force measurements for 6% ECE and 12% ECE through a 27 G needle at 10 mL/h. The force required for 12% ECE was significantly higher than 6% ECE (* $p < 0.05$). (B–F) Plots of the force measured over time during an infusion for 0.5–2.5 mL infusions through a 27 G needle at 10 mL/h.

For each ECE concentration (6% and 12% ECE), needle gauge had a significant impact on the force required for infusion. The 27 G needle required approximately a three-fold increased infusion force compared to the 22 G needle, per ECE concentration. ECE concentration also had a significant impact on force. For the 27 G needle, 12% ECE required

approximately a three-fold increased force compared to 6% ECE. For the 22 G needle, 12% ECE required approximately a five-fold higher force compared to 6% ECE.

Additionally, we observed increased variability in the required force for infusion when using a higher ECE concentration and smaller needle gauge. This can be noted visibly in Figure 2F. Statistically, Levene's test confirmed that variances were significantly different between both ECE concentrations and needle gauges, but not across the volumes.

3.2. Increasing Infusion Volume of 12% ECE Achieves Significantly Greater Treatment Volumes, While Increasing the Infusion Volume 6% ECE Does Not Yield a Significant Increase in Treatment Volume, for Both 22 G and 27 G Needles

As detailed in Table 1, Exp. 1 aimed to examine the influence of EC concentration on the resultant distribution volume and distribution shape for clinically relevant ablation volumes (0.5–2.5 mL) using a 27 G needle. The relevant treatment volume is referred to as the “depot volume”, which is defined as the distribution of the injectate within the tissue, excluding any leakage of the injectate into nearby collapsed vasculature as a result of the ex vivo model (Figure S1). All infusions were performed using 27 G needles and an infusion rate of 10 mL/h, as was used in previous preclinical studies in mice [41,69,70].

Figure 3A,B show representative images of the depot volumes for 0.5 mL, 1 mL, 1.5 mL, 2 mL, and 2.5 mL infusion volumes for both 6% ECE (A) and 12% ECE (B). The quantified depot volume is printed in white in the top right of each image. Figure S2 shows the corresponding representative images of the non-depot volumes prior to segmentation for 0.5 mL, 1 mL, 1.5 mL, 2 mL, and 2.5 mL infusion volumes for both 6% ECE (Figure S2A) and 12% ECE (Figure S2B).

Figure 3C,D show box plots for the depot volume achieved at various infusion volumes, for concentrations of 6% ECE (Figure 3C) and 12% ECE (Figure 3D). No significant differences in depot volume were observed between any of the infusion volumes (0.5–2.5 mL) for 6% ECE. However, for 12% ECE, both 2 mL and 2.5 mL infusion volumes achieved significantly larger depot volumes than the 0.5 mL infusion volume ($p < 0.05$). Increasing the infusion volume significantly increases the treatment volume when 12% ECE is used, but not 6% ECE.

Figure 3E,F show box plots for the percent infusion volume retained in the depot for various infusion volumes for 6% ECE (Figure 3E) and 12% ECE (Figure 3F). Significant differences were not observed for either 6% ECE or 12% ECE, indicating that the infusion volume did not significantly influence the percent of the infusion volume retained in the depot for either polymer concentration. Figure 3G,H show box plots for the depot aspect ratio achieved at various infusion volumes for 6% ECE and 12% ECE, respectively. Again, no significant differences were observed for either 6% ECE or 12% ECE, indicating that the infusion volume does not significantly influence the shape of the distribution in tissue. However, it was observed that there was a wider range of aspect ratios for 12% ECE compared to 6% ECE. That is, the lower polymer concentration was more effective at achieving a predictable distribution shape. It is possible that this was due to the larger forces generated when using 12% ECE compared to 6% ECE (Figures 1 and 2). The mechanics of tissue deformation, and possible localized rupture due to these increased forces, may have produced more irregularly shaped distributions in the tissue. Interestingly, after segmenting leakage into nearby vessels for both polymer concentrations, the median aspect ratio was near 1 for all infusion volumes. That is, the shape of the depot volume (without any leakage included) of these large volume infusions (0.5–2.5 mL) was relatively spherical. Spherical ablation zones allow clinicians improved prediction of treatment effect effects; they are common for existing clinically used modalities such as microwave or radiofrequency ablation and cryoablation.

We then examined how the same injection parameters' (infusion volume, EC concentration) influence on the depot volume, injection volume retention, and depot shape using a more clinically relevant needle size, the 22-gauge needle (Exp. 2). This size is appropriate for human applications, but too large for humane use in small animals. The results of Exp. 2 are shown in box plots in Figure 4. Figure 4A,B show representative images of the depot

volumes for various infusion volumes for both 6% ECE and 12% ECE, respectively. The quantified depot volume is printed in white in the top right of each image. Figure S2 shows corresponding representative images of the non-depot volumes prior to segmentation for both 6% ECE (C) and 12% ECE (D) for the 22 G needle.

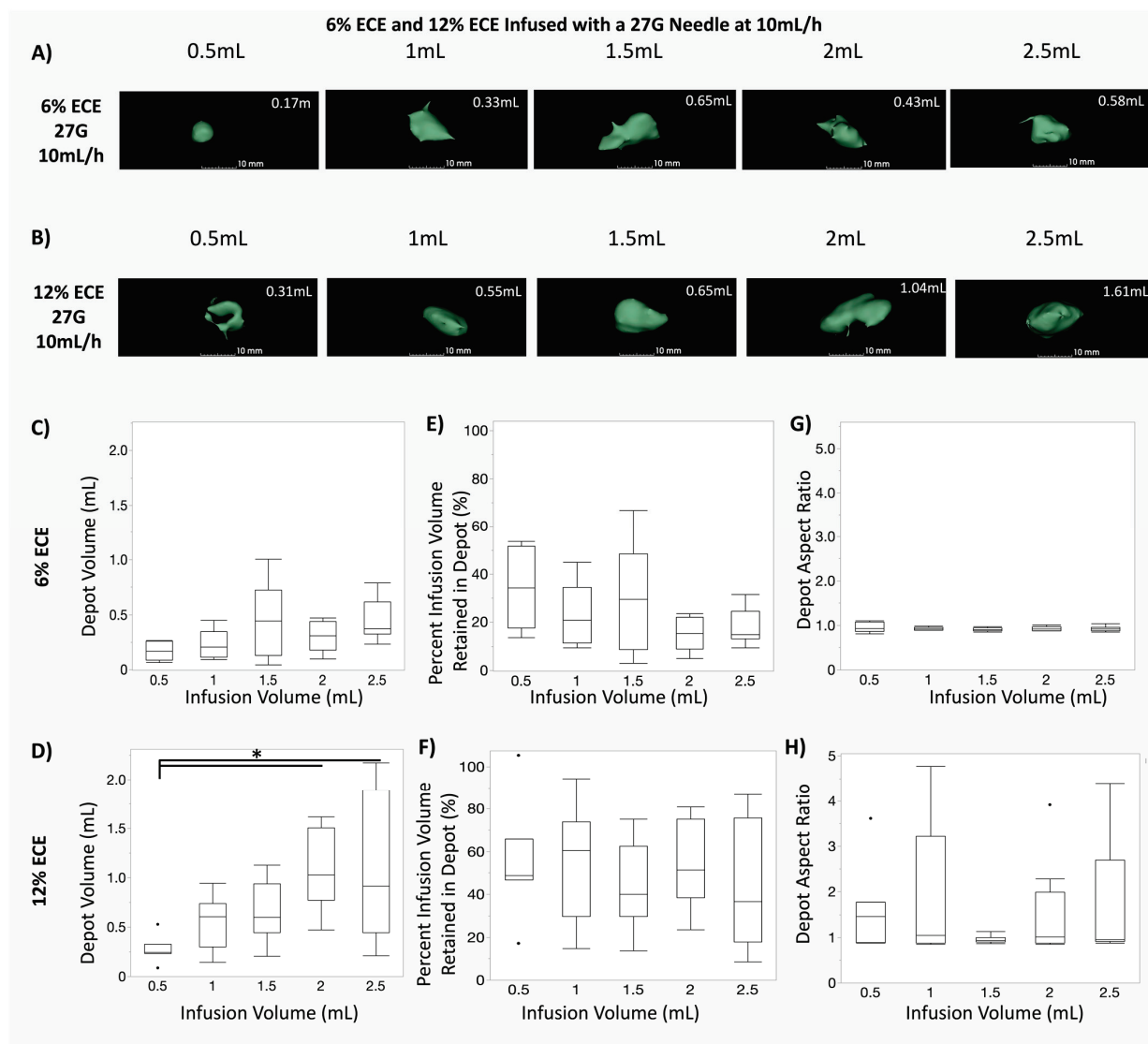


Figure 3. Comparison of infusions of 6% and 12% ECE through a 27 G needle. (A,B) Representative 3D segmentations of the depot volume for 0.5 mL, 1 mL, 1.5 mL, 2 mL, and 2.5 mL infusion volumes for 6% ECE (A) and 12% ECE (B) using 27 G needles and a 10 mL/h infusion rate. (C,D) Box plots showing the resultant depot volume achieved at various (0.5–2.5 mL) infusion volumes of 6% ECE (C) and 12% ECE (D) using 27 G needles and a 10 mL/h infusion rate (* $p < 0.05$). No significant differences were observed between any of the infusion volumes (0.5–2.5 mL) for 6% ECE. However, both 2 mL and 2.5 mL infusion volumes achieved significantly larger distribution volumes than the 0.5 mL infusion volume for 12% ECE. (E,F) Box plots showing the percent infusion volume retained in the depot for various infusion volumes (0.5–2.5 mL) of 6% ECE (E) and 12% ECE (F) using 27 G needles and a 10 mL/h infusion rate. Significant differences were not observed for either 12% ECE or 6% ECE. (G,H) Box plots of the aspect ratio achieved at various infusion volumes (0.5–2.5 mL) for 6% ECE (G) and 12% ECE (H) using 27 G needles and a 10 mL/h infusion rate. While no significant differences in the aspect ratio were found, there was a larger range of aspect ratios for 12% ECE compared to 6% ECE.

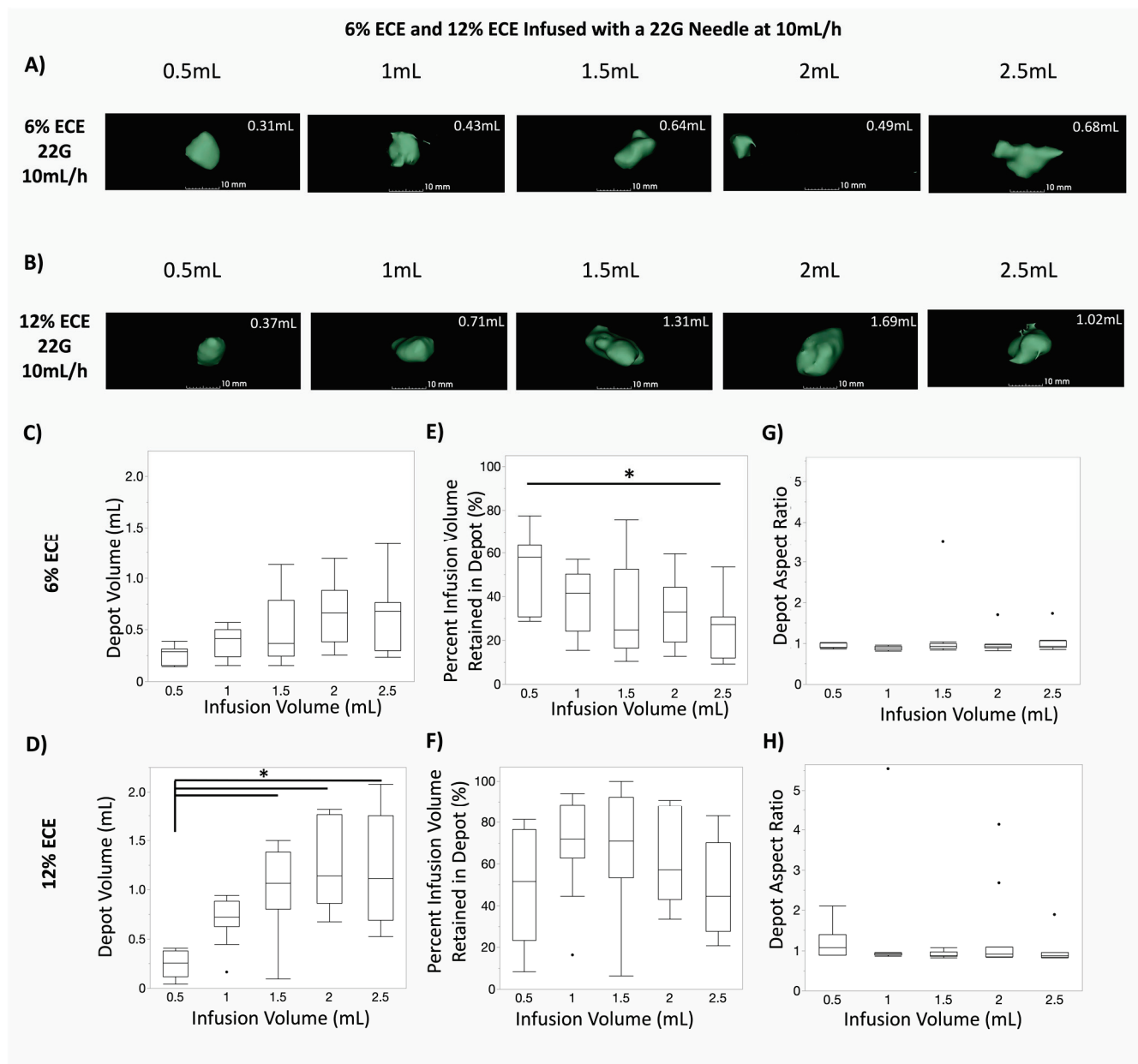


Figure 4. Comparison of infusions of 6% and 12% ECE through a 22 G needle. **(A,B)** Representative 3D segmentations of the depot volume for 0.5 mL, 1 mL, 1.5 mL, 2 mL, and 2.5 mL infusion volumes for 6% ECE **(A)** and 12% ECE **(B)** using 22 G needles and a 10 mL/h infusion rate. **(C,D)** Box plots showing the resultant depot volume achieved at various (0.5–2.5 mL) infusion volumes of 6% ECE **(C)** and 12% ECE **(D)** using 22 G needles and a 10 mL/h infusion rate (* $p < 0.05$). No significant differences were observed between any of the infusion volumes (0.5–2.5 mL) for 6% ECE. However, 1.5 mL, 2 mL, and 2.5 mL infusion volumes achieved significantly larger distribution volumes than the 0.5 mL infusion volume for 12% ECE. **(E,F)** Box plots showing the percent infusion volume retained in the depot for various infusion volumes (0.5–2.5 mL) of 6% ECE **(E)** and 12% ECE **(F)** using 22 G needles and a 10 mL/h infusion rate (* $p < 0.05$). A significantly lower percent of the infusion volume was retained in the depot for 2.5 mL compared to 0.5 mL infusions for 6% ECE. Significant differences were not observed for 12% ECE. **(G,H)** Box plots of the aspect ratio achieved at various infusion volumes (0.5–2.5 mL) for 6% ECE **(G)** and 12% ECE **(H)** using 22 G needles and a 10 mL/h infusion rate. No significant differences in aspect ratio were found for 12% ECE or 6% ECE.

Figure 4C,D show box plots for the resultant depot volume at various infusion volumes, for ECE concentrations of 6% ECE (Figure 4C) and 12% ECE (Figure 4D). No significant differences in depot volume were found between any of the infusion volumes for 6% ECE using a 22 G needle, consistent with results for 6% ECE using a 27 G needle (Figure 3C). However, 1.5 mL, 2 mL, and 2.5 mL infusion volumes achieved significantly larger depot volumes than the 0.5 mL infusion volume for 12% ECE ($p < 0.05$), consistent with the depot volume results for 12% ECE using a 27 G needle (Figure 3D). Thus, increasing the infusion volume significantly increased the treatment volume when 12% ECE was used for both 27 G and 22 G needles, but not 6% EC. Taken together, the results in Figures 3 and 4 suggest that the higher polymer concentration could be more effective at maximizing the depot volume, regardless of the needle size.

Figure 4E,F show box plots for the percent infusion volume retained in the depot in the 22 G needle for 6% ECE and 12% ECE, respectively. The 6% ECE showed a significant decrease in percent retained for 2.5 mL infusions compared to 0.5 mL infusions ($p < 0.05$), indicating that increasing the infusion volume of 6% ECE with a 22 G impairs improved injection coverage. No significant differences were observed for 12% ECE, indicating that injection coverage was relatively stable across volumes. Finally, Figure 4G,H show box plots for the depot aspect ratio achieved at various infusion volumes for 6% ECE and 12% ECE, respectively. No significant differences were observed for either 6% ECE or 12% ECE, indicating that the infusion volume did not have a significant influence over the shape of the distribution in tissue. Interestingly, the larger range of aspect ratios for 12% ECE and the 27 G needle (Figure 3H) was not observed for the 12% ECE with the 22 G needle. These results indicate that the combination of the small 27 G needle gauge and a higher polymer concentration may be less effective at achieving reliable and predictable distribution shapes. Finally, for all experimental groups, the median aspect ratio was near 1 when using the 22 G needle, indicating a relatively spherical ablation shape, consistent with results from Exp. 1 using the 27 G needle (Figure 3).

3.3. At Smaller Infusion Volumes, 12% ECE and 6% ECE Achieve Similar Treatment Volumes, but 12% Achieves Superior Treatment Volumes and Higher Percent Retained at the Target Site as Infusion Volume Increases Compared to 6% ECE, Regardless of Infusion Volume or Needle Gauge

Next, we sought to understand relationship trends amongst infusion volume, depot volume, and percent retained for the various injection parameters explored. While Figures 3 and 4 allow us to examine the impact of increasing infusion volume at set intervals, they do not allow us to easily compare the trends in the relationships of our independent and dependent variables or investigate the influence of a needle gauge within a set EC concentration. Figure 5 shows line plots and confidence intervals between the infusion volume vs. resultant depot volume, and the percent infusion volume retained in the depot, respectively, for both 6% ECE (blue) and 12% ECE (red). These plots are not meant to be used for predictive modeling, but rather to illuminate trends in the overall relationships of these injection parameters. Figure 5A,B show a positive, significant (albeit weak) correlation between infusion and depot volume, regardless of polymer concentration ($p < 0.05$ for both 6% and 12% ECE), and for both the 22 G and 27 G needles. Regardless of the needle gauge, 12% ECE trends toward a higher positive slope (22 G: 0.5709; 27 G: 0.4527) than 6% ECE (22 G: 0.1856; 27 G: 0.1260) and a higher, albeit low, r^2 value (22 G: 0.40 vs. 0.20; 27 G 0.40 vs. 0.14). These trends cannot be accurately used for predictive modeling but are insightful to the overall relationships between these parameters. Slopes for the 22 G and 27 G needle were similar, regardless of EC concentration, indicating that when the polymer concentration was constant, the influence of the needle gauge on the resultant depot volume was negligible. The stronger correlation between the infusion volume and resultant depot volume for 12% ECE indicates that it is easier to maximize the depot volume by increasing the infusion volume for 12% ECE compared to 6% ECE, regardless of the needle gauge used for infusion. There is significant overlap in the 6% ECE and 12% ECE curves for 0.5 mL and greater separation between the curves as infusion volume increased for both needle gauges investigated. Thus, at the smaller infusion volumes, the

impact of EC concentration on depot size was minimal. For smaller target volumes, the tradeoff in EC concentration is negligible, and resources could be conserved by using lower concentrations.

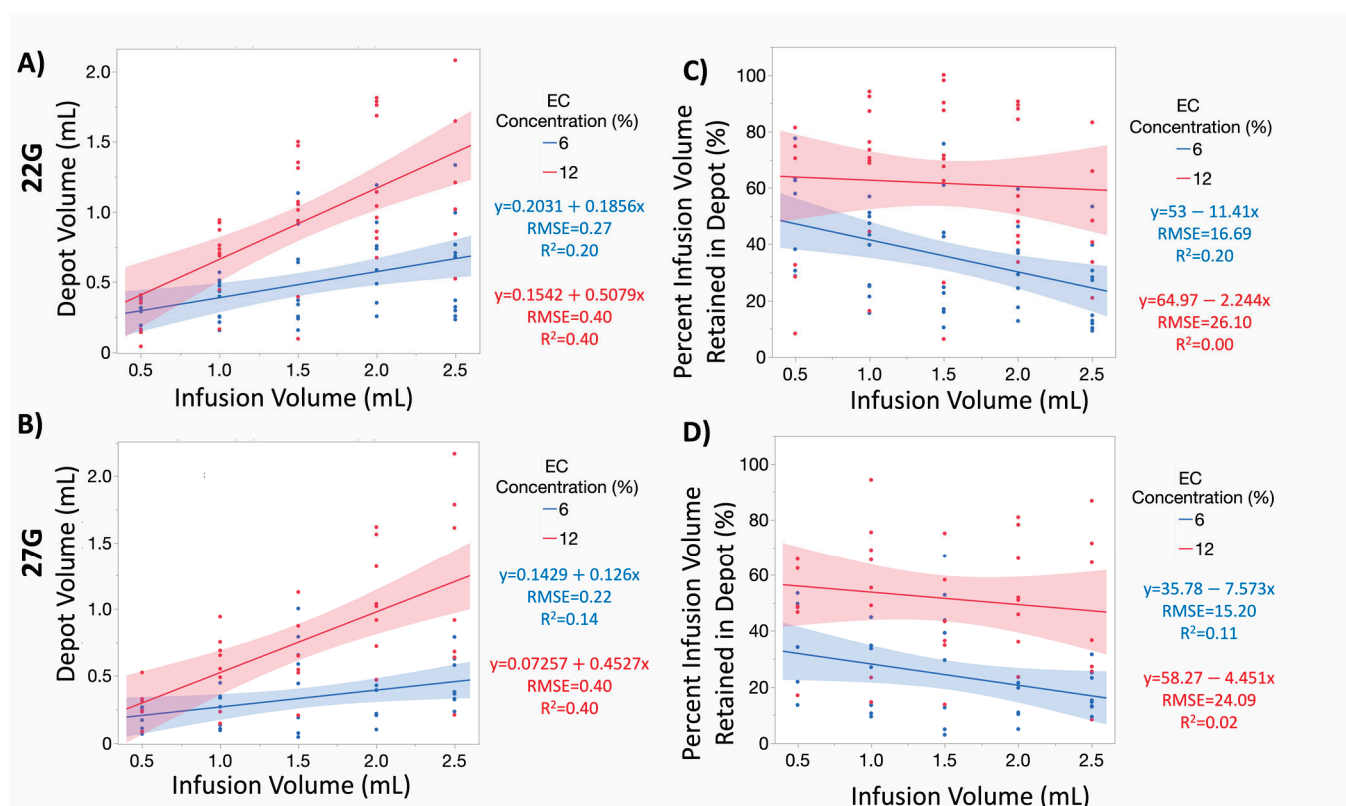


Figure 5. Trends in the relationship between depot volume and percent infusion volume retained in the depot to the infusion volume. (A,B) Line plots of the relationship between the depot volume and infusion volume for infusions performed with 22 G (A) and 27 G (B) needles. For the 22 G needle (A), the 6% ECE ($p < 0.05$, $r^2 = 0.24$) and 12% ECE ($p < 0.05$, $r^2 = 0.40$) both show positive relationships between depot volume and infusion volume, with the 12% ECE achieving a stronger correlation between depot volume and infusion volume. Similar to the 22 G, for the 27 G needle (B), the 6% ECE ($p < 0.05$, $r^2 = 0.14$) and 12% ECE ($p < 0.05$, $r^2 = 0.40$) both show positive relationships between depot volume and infusion volume, with the 12% ECE achieving a stronger correlation between depot volume and infusion volume. (C,D) Line plots of the relationship between the percent of the infusion volume retained in the depot and infusion volume for infusions performed with 22 G (A) and 27 G (B) needles. For the 22 G needle (C), the 6% ECE ($p < 0.05$, $r^2 = 0.20$) shows a negative relationship between the percent retained and the infusion volume. The 12% ECE ($p < 0.05$, $r^2 = 0.00$) shows a more stable percent retained as infusion volume increases. Similar to the 22 G, for the 27 G needle (D), the 6% ECE ($p < 0.05$, $r^2 = 0.11$) shows a slightly negative relationship between the percent retained and the infusion volume. The 12% ECE ($p < 0.05$, $r^2 = 0.02$) shows a similar slight negative relationship between the percent retained and the infusion volume, but with greater retention at all volumes compared to the 6% ECE.

Figure 5C,D show trends for negative correlations between infusion and percent infusion volume retained in the depot, regardless of polymer concentration ($p < 0.05$ for both 6% and 12% ECE) for both the 22 G (Figure 5C) and 27 G (Figure 5D) needles. This negative correlation is likely due to the leakage of the ECE into nearby vessels (the non-depot volume), due to the high prevalence of collapsed vasculature in the ex vivo swine liver. For larger injections, there is a larger potential for leakage as the probability of the depot expanding to hit nearby vasculature is increased. Regardless of the needle gauge, the 12% ECE trended toward a more positive slope (22 G: -2.244 ; 27 G: -4.451) than 6% ECE

(22 G: -11.41 ; 27 G: -7.573), indicating that retention of the injection at the target site was more consistent regardless of the volume infused for 12% ECE. The percent retained for the smallest infusion volume (0.5 mL) for the 22 G needle infusions was more similar for the 6% ECE and 12% ECE, than when a 27 G needle was used. We hypothesize that this difference is due to the reduced pressure when a 22 G needle is used (Figure 1), which may reduce crack formation. However, when infusion volume is increased, this increases the overall pressure (Figure 1), enough to result in leakage and reduced retention.

Notably, the slopes for the 22 G and 27 G needles appeared comparable when the EC concentration was constant, indicating negligible influence of the needle gauge. However, while retention remained consistent regardless of infusion volume for 12% ECE, retention decreased with increasing infusion volume for 6% ECE. To improve retention at the injection site, a higher EC concentration or reduced infusion volume may be necessary, but may not always be practical due to injection pressure or resource limitations.

3.4. The Impact of the Infusion Rate Is Not Significant for Smaller (0.5–1 mL) Infusion Volumes of 12% ECE for Both 22 G and 27 G Needles

Finally, Exp. 3 aimed to examine the influence of a manual infusion rate for the smaller infusion volumes (0.5 mL and 1 mL). Figure 6A shows representative images of the 12% ECE ablations performed using 27 G and 22 G with either manual (red outline) or 10 mL/h (blue outline) infusion rates. Figure S3 shows the corresponding representative images of the non-depot volumes prior to segmentation for 0.5 mL and 1 mL infusion volumes for both 6% ECE and 12% ECE and both 22 G and 27 G needles.

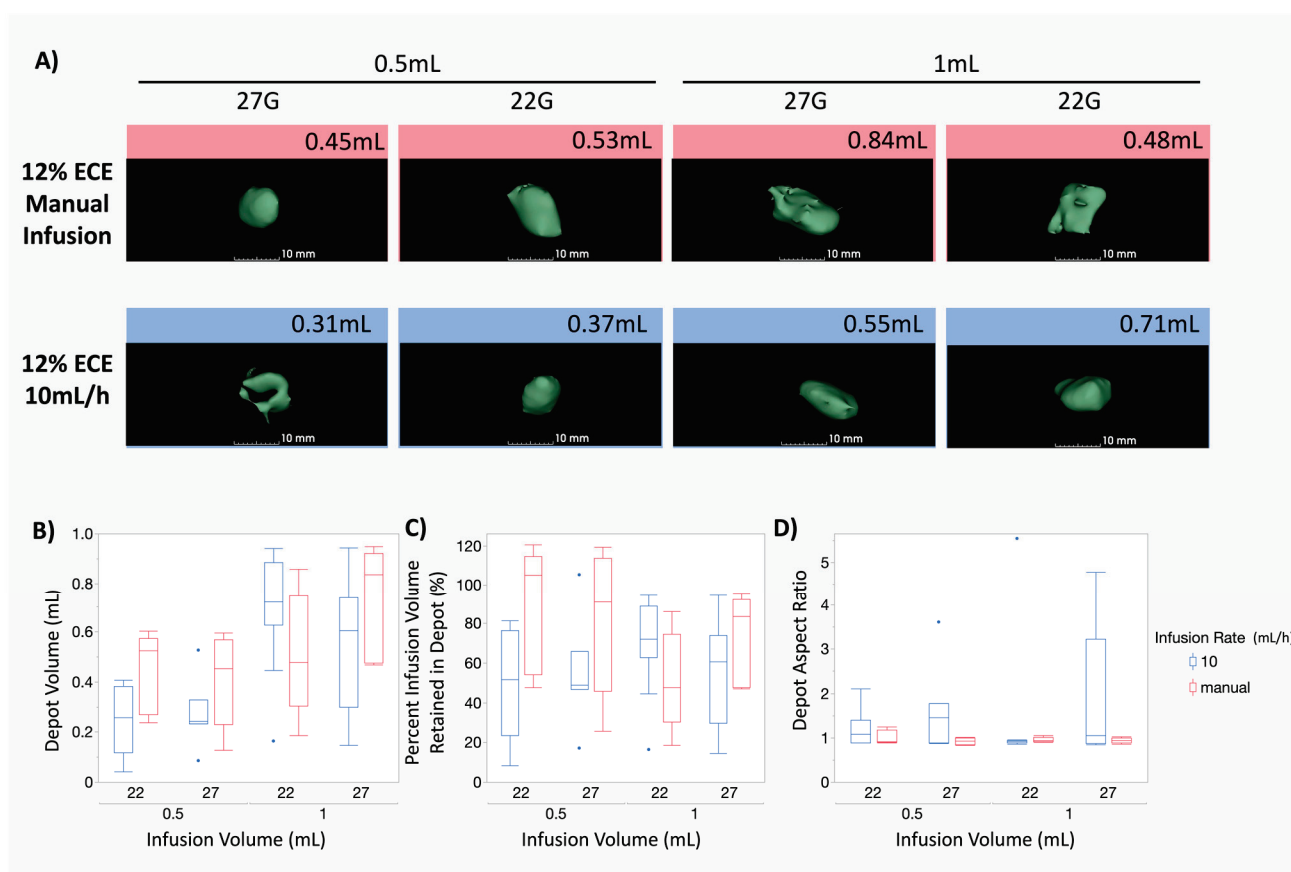


Figure 6. Comparison of manual infusion and 10 mL/h infusion rates for 0.5 mL and 1 mL infusions of 12% ECE. (A) Representative 3D segmentations of the depot volume for 0.5 mL and 1 mL infusion volumes for 12% ECE using both 22 G and 27 G needles and 10 mL/h and manual infusion rates.

(B) Box plots showing the resultant depot volumes achieved at small infusion volumes (0.5–1 mL) of 12% ECE for both 22 G and 27 G needles at both 10 mL/h (blue) and manual (red) infusion rates. No significant differences were observed. (C) Box plots showing the percent infusion volume retained in the depot for at small infusion volumes (0.5–1 mL) of 12% ECE for both 22 G and 27 G needles at both 10 mL/h (blue) and manual (red) infusion rates. No significant differences were observed. (D) Box plots showing the aspect ratio at small infusion volumes (0.5–1 mL) of 12% ECE for both 22 G and 27 G needles at both 10 mL/h (blue) and manual (red) infusion rates. No significant differences were observed.

Figure 6B compares the depot volume achieved using a 10 mL/h infusion rate (blue) and manual infusion (red). Interestingly, no significant differences were observed between the depot volume achieved, regardless of infusion volume or needle gauge, consistent with the results of Figure 5; needle gauge does not significantly influence ablation depot volume or retention. Further, these results indicate that for smaller ablations (0.5–1 mL), the infusion rate has no significant influence on the resultant depot volume.

Figure 6C shows box plots for the percent infusion volume retained in the depot for the same ablations depicted in Figure 6B. No significant differences in retention were observed, indicating that the infusion rate did not significantly influence retention, regardless of the needle gauge for small infusion volumes. Figure 6D shows box plots for the aspect ratios achieved for the same dataset. Consistent with the results of Exp. 1 and Exp. 2, no significant differences were observed for either a 10 mL/h infusion rate or manual infusion, regardless of the needle gauge, indicating that the needle gauge did not have a significant influence over the distribution shape. There is a larger range of aspect ratios (n.s.) for the 10 mL/h ablations, indicating that manual infusion may achieve more reliable and predictable distribution shapes.

4. Discussion

ECE ablation has the potential to be a low-cost alternative to thermal ablation for settings that lack access to thermal ablation and for tumors located in areas not amenable to thermal ablation. For ECE ablation to be translated to a clinical setting a full understanding of the multivariate influence of injection parameters on the resultant ablation volume and shape is necessary. This study examined the influence of multiple injection parameters (EC concentration, infusion volume, needle gauge, and infusion rate) on the resultant force, distribution volume, and shape. The experiments detailed here were not aimed at finding the optimal conditions for ablation. Rather, we focused upon the biophysical relationship between ECE injection parameters and the resultant distribution of ECE in tissue, as visualized with CT imaging.

The force studies, while limited in their conclusions due to only being collected into air, showed the high variability in force during an ECE injection, especially at higher ECE concentrations and using smaller needle diameters. ECE concentration was the largest contributor, as determined by the sum of squares, compared to needle gauge. The 12% ECE injections required force nearly four times higher than 6% ECE injected with the same needle gauge and a 27 G needle required force about three times as high as a 22 G when injecting the same ECE concentration. Combinations of high ECE concentrations and small needle gauges might not be feasible as manual injections due to the infusion force required.

Some volumes did vary significantly from each other; however, this is likely due to the higher variability seen in the longer injection times. The high variability in force seen in these injections could help explain the variability in injection distribution. We previously found that keeping injection pressures below a critical pressure helped reduced cracking in tissue [44]. The force measurement setup used is accurate for such a viscous substance but will struggle in cases where the force plateau is not reached due to a short injection time. Future studies will need to be conducted in tissue to investigate how these high forces impact the back pressure of different tissue types.

The 12% ECE achieved a superior distribution volume and retention compared to 6% ECE and exhibited a wider range of aspect ratio values across distribution volumes.

We found that increasing the infusion volume had a greater impact at the higher ECE concentration. Further, 12% ECE had a stronger positive correlation with depot volume and infusion volume than did 6% ECE, but they produced similar results at smaller volumes. When selecting a polymer concentration for treatment, lower concentrations like 6% ECE may achieve more predictable ablation shapes and more comparable distribution volumes to 12% ECE for smaller infusion volumes, like 0.5 mL. However, if larger infusion volumes are needed, higher concentrations, like 12% ECE, may be preferable to achieve better retention and coverage. Studies of traditional ethanol ablation found similar positive correlation and variability in its resultant distribution, as measured by the radius of necrosis measured from cross sections of excised tissue [48], which may be a less accurate representation of ablation volume compared to CT, which gives a full three-dimensional rendering.

Interestingly, neither needle gauge nor infusion rate showed a significant influence on depot volume or retention. Thus, for our ablative therapy, the choice in needle may be left up to the physician or availability of supplies. A wider range in aspect ratio values was observed for the smaller needle (27 G) with the 12% ECE, likely resulting from the higher pressures needed to push a more viscous fluid through the smaller needle. Few studies have examined the effect of needle gauge on ablation distribution, but several studies evaluated the effect of needle gauge on the spread of analgesic blockades with mixed results: some studies reported that larger needles resulted in a higher level of spread [71] while others found that needle gauge did not influence spread [72]. It is likely that the results of ECE injections, including their variability, are dependent not only on the injectate's physical properties, e.g., rheology, but also the anatomical location and biophysical properties of the tissues being injected. Clearly, follow-up examination of these additional parameters is necessary in various tissue types to better elucidate best practices for infusions depending on the treatment goals.

Lastly, infusion rate did not have a significant influence on depot volume or retention at smaller infusion volumes, suggesting that physicians could opt to use a controlled rate or manual infusion. Interestingly, this contrasts with results from studies of traditional ethanol ablation, which found that slower flow rates allowed for more spherical distribution of ethanol [64]. It is likely that our results differ from the conclusions of that study because of the EC polymer, which increases the injectate viscosity and undergoes a phase change upon injection into tissue. Ethanol alone has a viscosity close to water (1.2 cP), while the lowest ECE concentration used here had a viscosity of nearly 1000 cP before it underwent phase transition [35]. The EC polymer used has a strong impact on the conclusions drawn about the causative impacts of multiple injection parameters. Higher variability in distribution shape was observed for the 10 mL/h rate, likely due to dissipation of pressure during a manual injection, which is not possible when using an infusion pump.

We recognize several limitations with this work. First, we used *ex vivo* swine liver, which is highly vascular and impacts injection distributions *in vivo* and *ex vivo*. *In vivo*, vascular blood flow acts to carry away injection molecules that enter blood vessels. *Ex vivo*, the vasculature is collapsed, so fluid within it is essentially stationary. When the injectate material spreads into a vessel, the vessel provides a path with reduced resistance relative to the tissue, causing ready spread of the injectate through the vessels. While we accounted for this factor by analyzing the depot volume and using large sample sizes, it may not be appropriate to extrapolate these results to other tissues without further investigation. Second, high variability was observed for all depot volume and retention results. Again, due to the high vascularity of the liver, it was difficult to ensure consistent distance from vessels during needle placement. Future *in vivo* studies should use ultrasound or CT to guide needle placement and avoid large vasculature, as this would likely reduce the variance in the depot volume and retention. Finally, force studies conducted only allowed for the force of ECE through a syringe and needle to be characterized. Future studies will need to repeat measurements in tissue to understand its contribution. Conducting the studies into tissue could help to connect the variability seen in force to the variability seen in injection distribution.

Although the liver is commonly used for the assessment of the distribution and efficacy of ablative therapies [48–56], we plan to expand this work in the future to include other healthy tissues as well as cancerous lesions. Future studies should elucidate the effect of tissue biophysical differences (i.e., vascularity, stiffness, density). The liver is extremely vascular [73] and has low stiffness [74], therefore comparison to other tissues of lower vascularity and higher stiffness, more similar to tumors, is necessary to inform parameter selection for the treatment of larger lesions or tumors.

5. Conclusions

These experiments investigated the influence of various parameters (infusion force, infusion volume, EC concentration, needle gauge, and infusion rate) to determine relationships between the force required for infusion, resultant depot volume, retention, and shape. This study can serve as the basis for the future creation of a predictive computational model that uses tumor biophysical properties as inputs to determine optimal injection parameters (infusion volume, infusion rate, and needle gauge). This would not only improve the overall injection optimization process, but also objectively account for the interacting roles of the many formulation and injection characteristics that govern injection outcomes. Thus, there are tradeoffs when making decisions for ECE delivery: for smaller infusion volumes, the ECE concentration has little impact, while for larger infusion volumes, higher EC concentrations are necessary. While needle gauge has little impact on depot volume or retention, the use of a smaller needle with higher EC concentrations could reduce the predictability of the depot shape. Due to the high forces observed for 12% ECE, feasible flow rates for certain injection parameter combinations could be limited. Therefore, it is important to consider all parameters, the available supplies, as well as the tumor's anatomical position and shape, to select delivery parameters to meet the ablation goals.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym16070997/s1>, Figure S1: Representative 3D segmentations of the depot volume with and without non-depot injection spread; Figure S2: Representative 3D segmentations including non-depot injection spread for infusions using a 10 mL/h infusion rate; Figure S3: Representative 3D segmentations including non-depot injection spread for infusions performed manually.

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Conflicts of Interest: N.R. and D.F.K. have a patent application under Duke University of the tumor ablation process (ECE) utilized in this report. (Morhard, Robert, Nirmala Ramanujam, and David Katz. "Methods for the treatment of cancer and benign lesions by ablation". U.S. Patent Application No. 439 16/490,227.).

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