



Article

Impact of the Three-Dimensional Arrangements of Polyhydroxylated Crosslinkers on the Resulting Properties of Chitosan-Based Hydrogels

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Abstract: Chitosan was subjected to a crosslinking reaction with three polyhydroxylated diacids (glucaric (GlcA), mannaric (ManA), and mucic (MucA) acids) that only differ in the spatial orientation of their hydroxyl groups. This work aimed to obtain experimental evidence of the impact of the three-dimensional arrangement of the crosslinkers on the resulting properties of the products. In all the cases, the products were hydrogels, and their chemical structures were fully elucidated by FT-IR spectroscopy and conductometric titration. Thermogravimetric and morphological studies were also carried out. The specific surface area of all the products was similar and higher than that of native chitosan. Moreover, all hydrogels were characterized in terms of viscoelastic properties and long-term stability under external perturbation. Furthermore, their lead adsorption efficiency and swelling capacity were assessed. Despite the resemblant chemical structure in all the hydrogels, Ch/ManA exhibited the highest lead adsorption capacity, (Ch/ManA: 93.8 mg g⁻¹, Ch/GlcA: 82.9 mg g⁻¹, Ch/MucA: 79.2 mg g⁻¹), while Ch/GlcA exhibited a remarkably higher swelling capacity (i.e., ~30% more than Ch/MucA and ~40% more than Ch/ManA). The results obtained herein evidenced that the selection of the polyhydroxylated crosslinker with the appropriate three-dimensional structure could be crucial to finely adjust the final materials' features.

Keywords: crosslinked chitosan; polyhydroxylated crosslinkers; three-dimensional arrangement; hydrogels



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1. Introduction

Polysaccharides are outstanding materials because of a number of the following reasons: (i) natural abundance and low cost; (ii) a polyhydroxylated chemical structure that confers them versatility towards chemical modifications to impart them specific properties; (iii) environmental friendliness; and (iv) renewable origin. Therefore, in recent years, efforts have been made to take advantage of these features in multiple fields.

Among all polysaccharides, chitin ((1→4)-2-acetamino-2-deoxy-β-D-glucopyranose) is particularly attractive since it is the second most abundant polysaccharide worldwide. Chitin can be found in the shells of crustaceans, which make up a significant part of the waste generated by the fishing industry. Therefore, it is indeed noteworthy that its use would highly contribute to solving the environmental problems associated with organic waste accumulation [1,2]. Furthermore, when chitin undergoes partial deacetylation, it

results in the obtention of chitosan (Figure 1a), which has an amino group that provides it with distinctive chemical properties compared with other polysaccharides.

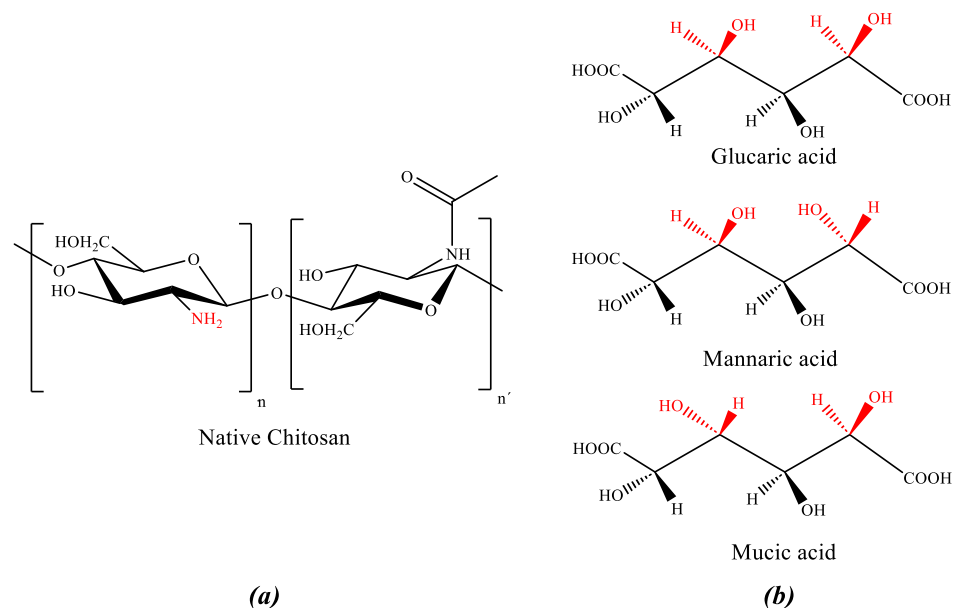


Figure 1. (a) Partially acetylated chitosan. (b) Chemical structure of polyhydroxylated diacids.

In a previous study, the authors successfully made use of chitosan's versatility for the preparation of new materials by crosslinking it with the following diacids of the same chain length: mucic acid (a polyhydroxylated acid) and adipic acid (a non-functionalized dicarboxylic acid) [3]. In such study, it was demonstrated that the presence of hydroxyl groups in a crosslinker played a role in the resulting materials' chemical structure and properties [4]. Based on those results, a new hypothesis was proposed: not only the presence but also the spatial orientation of a crosslinker's hydroxyl groups could have an impact on the chemical structure and properties of chitosan-based materials. Therefore, a polyhydroxylated crosslinker with the appropriate three-dimensional arrangement could be used to finely adjust the final materials' features.

With the aim of obtaining experimental evidence that allows for confirming or refuting the proposed hypothesis, in the present work, chitosan was subjected to crosslinking reactions with glucaric, mannaric, and mucic acids (GlcA, ManA, and MucA, respectively), three isomers that only differ in the spatial orientation of their hydroxyl groups (Figure 1b). The resulting materials' chemical structures were fully elucidated.

Furthermore, considering the increasing demand for stable hydrogels with outstanding swelling behavior in a number of industries, such as pharmaceutical, food, and even biomedical, in this work, the new products were subjected to rheological and swelling studies to assess their value for potential applications in such fields [5,6].

Hydrogels are a unique class of materials characterized by their ability to absorb and retain significant amounts of water while maintaining their structural integrity [7]. This remarkable property arises from their three-dimensional, crosslinked polymer network, which can swell significantly without dissolving [8]. The versatility and tunability of hydrogels have caught the scientific community's attention and interest in the past few decades, and they have consequently become intensely researched.

Regarding hydrogels' applications, they have been found to be of great use in a wide range of fields. For instance, in the biomedical industry, some applications include drug delivery systems, wound dressings, scaffolds in tissue engineering, and implantable bioelectronics [7–12]. Additionally, in agriculture, superabsorbent hydrogels are used as soil conditioners to retain moisture, thereby enhancing crop yield and reducing water consumption [13]. In the field of food science, hydrogels are used as carriers for flavors, nutrients, and probiotics, improving the sensory and nutritional quality of food prod-

ucts [14,15]. Moreover, in this study, their potential use for water treatment was also assessed by performing adsorption studies with lead as a model cationic pollutant.

All in all, the multifaceted applications of hydrogels indeed highlight the importance and need for ongoing research to optimize their properties for specific uses.

2. Materials and Methods

2.1. Materials

Medium molecular weight chitosan (70% of deacetylation) was purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA) and characterized in the same way as the products. Syntheses were carried out using the following reagents as purchased without further purification: mucic acid (Sigma-Aldrich), sodium hydrogen carbonate (NaHCO_3) (Sigma-Aldrich), *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (BLD pharm, No.1 Wangdong Middle Road, Building 5, Songjiang District, Shanghai 201601, China), nitric acid (Merck, 400 Summit Drive, Burlington, MA 01803, USA), hydrochloric acid (Merck), sodium hydroxide (Merck), D-mannose (Apollo Scientific, Whitefield Rd, Bredbury, Stockport, Cheshire SK6 2QR, UK), D-glucose (Sigma-Aldrich), *N*-hydroxysuccinimide (NHS) (BLD pharm), sodium nitrite (Merck), and potassium hydroxide (KOH) (Merck). All solutions were prepared with Milli-Q water (resistivity > 18 M Ω).

2.2. Preparation of Glucaric, Mannaric, and Mucic Acid Salts

2.2.1. Obtention of Glucaric Acid as Its Monopotassium Salt

D-glucaric acid was obtained as its monopotassium salt as reported by Mehlretter et al. [16]. Briefly, 1.45 mmol of sodium nitrite and 0.5 mol of D-glucose were added slowly (in a 20 min lapse) to a 128 mL nitric acid solution (65%) previously heated at 60 °C. The temperature was maintained in a 55–60 °C range, and the reaction was allowed to continue in these conditions for one hour. The mixture was then cooled to ambient temperature, and 45% potassium hydroxide solution was gradually added until a pH of at least 9 was achieved. After that, the mixture was cooled to 20 °C, and the pH was adjusted to 3.4 with 70% nitric acid. The mixture was allowed to stand overnight at room temperature, and the potassium acid saccharate was then collected by filtration. After being washed with a minimum of cold water, the product, obtained as a white powder, was dried at 50 °C. The chemical structure of the product was analyzed by NMR spectroscopy (Bruker Avance Neo-500 spectrometer, Bruker Co, Karlsruhe, Germany). The ^1H - and ^{13}C -NMR of the product agreed with those reported in the literature [17,18].

2.2.2. Obtention of Mannaric Acid as Its Sodium Salt

In the first step, mannodilactone was obtained as previously reported [19]. Briefly, 25 mL of HNO_3 solution (32.5%) was added to a flask containing 10 g of mannose (55.6 mmol). The mixture was heated at 60 °C for 2 h using magnetic stirring. Next, the temperature was set at 85 °C, and the reaction was kept at this condition for 12 h. After that, the mixture was left to cool down to ambient temperature. The solvent was evaporated, and a white solid was obtained. The crude product was recrystallized twice with ethanol and dried under vacuum.

In the second step, the mannodilactone synthesized in the previous step was transformed into sodium mannarate. Briefly, mannodilactone (1 g, 5.8 mmol) was hydrolyzed using 40 mL of an aqueous solution of NaOH (40%) and heating the mixture at 100 °C under reflux for 1 h. Then, the mixture was left to cool down to ambient temperature, and the pH was adjusted to a value between 8 and 9. After that, the mixture was filtered, and the solvent was evaporated. The resulting sodium mannarate was frozen and freeze-dried. The chemical structure of the product was analyzed by NMR spectroscopy (Bruker Avance Neo-500 spectrometer). The ^1H - and ^{13}C -NMR of the product agreed with those reported in the literature [20].

2.2.3. Obtention of Disodium Galactarate

A total of 201.6 mg of mucic acid (0.96 mmol) and 161.3 mg of NaHCO_3 (1.92 mmol) were dissolved in 2 mL of H_2O , obtaining its disodium salt.

2.3. Preparation of Crosslinked Chitosans

2.3.1. General Procedure

First, 462.7 mg (1.92 mmol of deacetylated units) of native chitosan was dissolved in 19.2 mL of a HCl 0.1 M solution under mechanical stirring at 400 rpm. After that, 5 mL of water was added. Then, carboxylic acid (glucaric, mannaric, or mucic) as its corresponding salt was added according to the specific conditions detailed in Section 2.3.2. After the addition of each diacid salt, the system was cooled down to 5 °C, and 3 mL of an aqueous solution of EDC (1.83 g) and NHS (1.1 g) (9.6 mmol each) were added [3]. The reaction was kept under mechanical stirring in an ice bath for nearly 7 h until gelation was achieved. Then, stirring was suspended, and the reaction was left at 4 °C overnight. The reaction was quenched with NaOH (10%), the samples were centrifugated (5000 rpm) to remove the supernatants, and the crude product was sequentially washed with HCl 0.1 M (until pH 2), NaOH 0.1 M (until pH 10), and finally water (until neutral pH). Lastly, the samples were freeze-dried.

2.3.2. Specific Details Involved in the Reaction of the Crosslinkers with Chitosan

Chitosan/Glucaric acid derivative preparation (Ch/GlcA)

First, 239.8 mg of potassium acid glucarate (0.96 mmol) and 81.2 mg of NaHCO_3 (0.96 mmol) were dissolved in 2 mL of H_2O , and the solution was added to a flask containing the acidic chitosan solution (1.92 mmol of deacetylated units). After that, NaOH 0.1 M was added until pH 6.5 was reached. The temperature was monitored, and once it was below 5 °C, the EDC/NHS solution was added, as described in Section 2.3.1.

Chitosan/mannaric acid derivative preparation (Ch/ManA)

First, 244.8 mg of sodium mannarate (0.96 mmol) was dissolved in 2 mL of water and added to a flask containing the chitosan solution at pH 4 (1.92 mmol of deacetylated units). After that, the pH was adjusted to 6.5. The temperature was monitored, and once it was below 5 °C, the EDC/NHS solution was added, as described in Section 2.3.1.

Chitosan/mucic acid derivative preparation (Ch/MucA)

Disodium galactarate (0.96 mmol), prepared as mentioned in Section 2.2.3., was added to a flask containing the chitosan solution (1.92 mmol of deacetylated units). Then, NaOH 0.1 M was added until pH 6.5 was reached. The temperature was monitored, and once it was below 5 °C, the EDC/NHS solution was added, as described in Section 2.3.1.

2.4. Characterization Assays

2.4.1. Solubility Assays

First, 50 mg of each product in its freeze-dried form was placed in a tube, and 10 mL of water was added. The mixtures were left with continuous stirring for 2 h at room temperature. Then, the mixtures were centrifugated, and the supernatant was removed. The solids were frozen and freeze-dried. The dry samples were weighted. The same procedure was repeated using NaOH (0.05 M) and HCl (0.05 M). In all cases, the mass lost after the assays was negligible.

2.4.2. Chemical Structural Analyses

Infrared spectra (FT-IR) were acquired with a Thermo Scientific Nicolet 6700 spectrometer in transmission mode. The analyses were performed with the materials in both their basic and acidic form. Samples were prepared by mixing each material with KBr (Grade FT-IR 99+%, Thermo Spectra-Tech, Madison, WI, USA) in a 1:100 ratio and then pressing with a Hand Press accessory PIKE Technologies into a 5 mm disc. All spectra were

collected in the 400–4000 cm^{-1} wavenumber range, with a 4 cm^{-1} resolution for 32 scans. Background was measured using KBr, with the settings mentioned before.

The isoelectric point of all the products was determined by plotting Z potential as function of pH, using a Zetasizer Nano ZS coupled with MPT-2 autotitrator (Malvern Panalytical, Middle Watch Swavesey, Cambridge, CB24 4AA, United Kingdom). Each product (1 mg) was dispersed in 10 mL of HCl 0.01 M, and 1 M NaOH was gradually added to progressively increase the pH during the assay.

The free amino and carboxylic acid content of all the products was determined by conductometric titration as well as the acetylation degree of native chitosan [21]. In the case of native chitosan, titration was carried out in a homogeneous system, while in the case of the modified materials, because of their water insolubility in the whole pH range, titrations were carried out in heterogeneous systems.

For native chitosan, 300 mg of the sample was dissolved in 10 mL of HCl 0.1 M. In the case of the modified materials, 300 mg of each sample was suspended in 10 mL HCl 0.1 M solution and titrated with NaOH 0.1 M, under magnetic stirring. A Hanna Instruments HI-2300 conductometer was used to measure the conductivity values every 0.05 mL. The conductometric titration curves of Ch, Ch/GlcA, Ch/ManA, and Ch/MucA are detailed in the Supplementary Materials (Figures S1–S4), where electrical conductivity was plotted as a function of NaOH solution volume.

The percentage of the deacetylated units of native chitosan, also known as the deacetylation degree (%DD), was calculated using the equivalence points and Equation (1), where 203 and 161 are the molar masses (g mol^{-1}) of acetylated and deacetylated units, respectively, $[\text{NaOH}]$ is the sodium hydroxide concentration (mol L^{-1}), V_1 and V_2 are the volumes (L) of each equivalence point, and m is the mass of chitosan (g).

$$\%DD = \frac{203 \cdot (V_2 - V_1) \cdot [\text{NaOH}]}{m + (203 - 161) \cdot (V_2 - V_1) \cdot [\text{NaOH}]} \times 100\% \quad (1)$$

In order to determine the molar fraction of each structural unit in the products (i.e., monosubstituted (x_{COOH}), unsubstituted (x_{NH_2}), crosslinked (x_{CL}), and acetylated (x_{Ac})), it was assumed that under the reaction conditions, the acetylated units remained unchanged [22]. For the rest of the units, Equations (2)–(4) were used, where $[\text{NaOH}]$ is the sodium hydroxide concentration (mol L^{-1}), m is the mass of chitosan used (g), Mr_{COOH} , Mr_{NH_2} , Mr_{CL} , and Mr_{Ac} represent the molecular weight (g mol^{-1}) of each unit, and ΔV_1 and ΔV_2 are the volumes (L) of NaOH consumed from the first to the second equivalence point and between the second and the third equivalence point, respectively [3].

$$x_{\text{COOH}} \cdot \frac{m}{Mr} = \Delta V_1 \cdot [\text{NaOH}] \quad (2)$$

$$x_{\text{NH}_2} \cdot \frac{m}{Mr} = \Delta V_2 \cdot [\text{NaOH}] \quad (3)$$

$$\overline{Mr} = Mr_{\text{COOH}} \cdot x_{\text{COOH}} + Mr_{\text{NH}_2} \cdot x_{\text{NH}_2} + Mr_{\text{CL}} \cdot x_{\text{CL}} + Mr_{\text{Ac}} \cdot x_{\text{Ac}} \quad (4)$$

2.4.3. Morphologic Studies

Morphologic studies were carried out by scanning electron microscopy (SEM) using a Carl Zeiss Evo 10 (ZEISS, Chicago, IL, USA). Samples of each material in its freeze-dried form were suspended in water. The swollen gels were dried at 40 °C under vacuum for 120 min and then deposited on microscope glasses and coated with a gold thin layer using an ion sputter coater.

2.4.4. Determination of the Specific Surface Area

The specific surface area (SSA) of native chitosan and the new materials were determined by N_2 adsorption–desorption analysis (77 K) in a Surface Area Analyzer Beckman

Coulter 3100TM (Fullerton, CA, USA) (BET method). Before the measurement, samples of each material in its freeze-dried form were degassed at 40 °C for 30 h.

2.4.5. Thermogravimetric Analyses (TGA)

Thermogravimetric analyses were carried out in a TA Instruments Q-500 (TA Instrument Corporation, Wilmington, DE, USA) thermogravimetric analyzer. First, 2.5 mg of each freeze-dried sample was weighed in a platinum pan and then heated from 40 to 700 °C, at 10 °C min^{−1}, in a N₂ atmosphere (flow rate of 60 mL min^{−1}). At the maximum degradation rate, the temperature ($T_{\max}$) was determined from the peak of the derivative of the weight loss against temperature. The experiments were performed in triplicate.

2.4.6. Hydrogel Characterization

Oscillatory rheological experiments

Oscillatory rheology studies were carried out to assess the hydrogels' hydrodynamic viscoelastic behavior. An AR-G2 rheometer (TA Instruments, New Castle, DE, USA) with a steel parallel plate geometry, featuring a diameter of 40 mm and a gap space of 800 µm was used. The tests were performed in oscillating mode at 25 °C with 1 mL of the hydrogels in their fully swollen form. The linear viscoelastic region was explored through dynamic strain sweeps at a constant angular frequency of 1 rad s^{−1}, varying the strain from 0.01 to 100%. These amplitude sweep experiments allowed for the determination of the plateau values of the elastic or storage modulus (G') and the viscous or loss modulus (G''). A fixed strain value of 0.1% was selected to conduct dynamic frequency sweeps. G' and G'' were subsequently determined as functions of angular frequency within the range of 0.1 to 100 rad s^{−1}. The phase angle δ was obtained through the relation $\tan \delta = G''/G'$. Experiments were performed in triplicate.

Swelling studies

To determine swelling capacity, 10 cm × 10 cm filter papers were folded in a cone shape and placed in centrifuge tubes [23]. Inside each one, 25 mg of each freeze-dried polymer were placed. The tubes were filled with water, ensuring the totality of the polymer was immersed. After 10 min, each cone containing a swollen polymer was removed from the water-filled tube and placed in an empty tube with a hole underneath and left to dry out. After 10 min of drying, each paper cone containing the polymer was weighted, and swelling was calculated using Equation (5) [23]. The procedure was repeated 20 times in order to estimate the swelling capacity of each material, where m_s is the mass of the swollen gel and m_d is the mass of the dried material in the paper.

$$Swelling\% = \frac{m_s - m_d}{m_d} \times 100\% \quad (5)$$

To calculate m_s , in each case, the mass of the wet paper was discounted to the total mass of the filter paper cone containing the swollen gel.

Differential scanning calorimetry (DSC) and fusion endotherms of water in hydrogels

To determine the form of water in the fully swollen hydrogels, DSC was used (TA Instruments Q200, TA Instrument Corporation, Wilmington, DE, USA). First, 9 mg of each sample was placed into Tzero[®] aluminium pans, which were sealed with hermetic lids. The samples were initially equilibrated at 20 °C for 1 min and then cooled to −80 °C at a rate of 5 °C min^{−1}. After an additional equilibration step at −80 °C for 1 min, thermograms were obtained in the range of −80 °C to 40 °C, with the temperature increasing at a rate of 5 °C min^{−1}. The melting enthalpy of the frozen water of the hydrogel was determined by analyzing the thermograms using OriginPro 2023b (10.0.5.157 (Academic), OriginLab Corporation, Northampton, MA, USA). All experiments were performed in duplicates.

The percentage of non-freezing water $W_{nf}(\%)$ in the swollen hydrogels was determined from the melting enthalpies using Equation (6) [24]:

$$W_{nf}(\%) = W(\%) - W_f(\%) = W(\%) - (Q_{endo}/Q_f) \times 100 \quad (6)$$

where $W(\%)$ is the percentage of the total water in the hydrogel system calculated from the swelling experiments and $W_f(\%)$ is the percentage of freezing water in the hydrogel. Q_{endo} and Q_f are the heat of fusion of freezing water in the hydrogel and the heat of fusion of pure water, respectively [25].

2.5. Adsorption Studies

Pb (II) aqueous solutions were prepared using solid $\text{Pb}(\text{NO}_3)_2$ p.a. (Merck). Lead concentrations were determined on an air-acetylene flame-type atomic absorption spectrometer (FAAS) (Model iCE 3000, Thermo Scientific, Cambridge, UK). For all measurements, standard solutions of Pb (II) were prepared from a 1000 mg L^{-1} Pb (II) standard solution (Merck). All studies were conducted in duplicate.

2.5.1. Influence of pH

First, 25 mg of each product in its freeze-dried form was added to 25 mL of Pb (II) solution (150 mg L^{-1}), and the pH was adjusted at specific values (2–6) using HCl 0.01 M. The mixtures were shaken at 150 rpm in a water bath at 20°C for 24 h. After that, the supernatants were filtered for lead concentration determination by FAAS.

2.5.2. Influence of Adsorbent Dosage

Chitosan or its derivatives in their freeze-dried form (12.5, 25 or 50 mg) were added to 25 mL of Pb (II) solution (150 mg L^{-1}) at pH 6.5. The mixtures were shaken at 150 rpm in a water bath at 20°C for 24 h. After that, the supernatants were filtered for lead concentration determination by FAAS.

2.5.3. Kinetic Studies

First, 25 mg of chitosan or the derivatives prepared in their freeze-dried form was added to 50 mL of Pb (II) solution (100 mg L^{-1}) at pH 6.5. Then, the mixtures were shaken at 150 rpm in a water bath at 20°C . Aliquots were taken at specific times (5, 10, 15, 20, 25, 30, 45, 60, 120, and 180 min and 24 h), filtered, and the Pb (II) concentrations of the supernatants were determined by FAAS.

2.5.4. Adsorption Isotherms

First, 25 mg of chitosan or the new products in their freeze-dried form was added to 25 mL of Pb (II) solutions with specific concentrations (25, 50, 75, 100, 125, and 150 mg L^{-1}) at pH 6.5. Then, the mixtures were shaken at 150 rpm in a water bath at 20°C for 24 h. After that, the supernatants were filtered for lead concentration determination by FAAS.

2.5.5. Regeneration and Reusability Studies

First, 25 mg of each product in its freeze-dried form was suspended in 25 mL of a 150 mg L^{-1} Pb (II) solution and left for 2 h under shaking at 150 rpm. After that, the supernatants were filtered, and their lead concentrations were determined by FAAS. Regeneration of the adsorbents was carried out by suspending the remaining gels in 25 mL of HCl 6 mM and shaking the suspensions at 150 rpm for 2 h. Next, the supernatants were filtered, and their lead concentrations were determined by FAAS.

The reusability of the materials was assessed by subjecting the regenerated gels to a new adsorption–desorption cycle. For this, the gels were conditioned by adding NaOH 10 mM, centrifugating at 5000 rpm, washing with water until neutral pH, and then freeze-drying.

The reused materials were regenerated, conditioned as described before, and analyzed by FT-IR spectroscopy.

3. Results

3.1. Characterization of the Crosslinked Chitosans

The products (Ch/GlcA, Ch/ManA, and Ch/MucA) were characterized in terms of their chemical structure by FT-IR spectroscopy and conductometric titration. The FT-IR spectra of native chitosan and its derivatives were acquired in both basic and acidic forms (Figures 2a and 2b, respectively). The complete spectra are shown in the Supplementary Materials (Figure S5a,b).

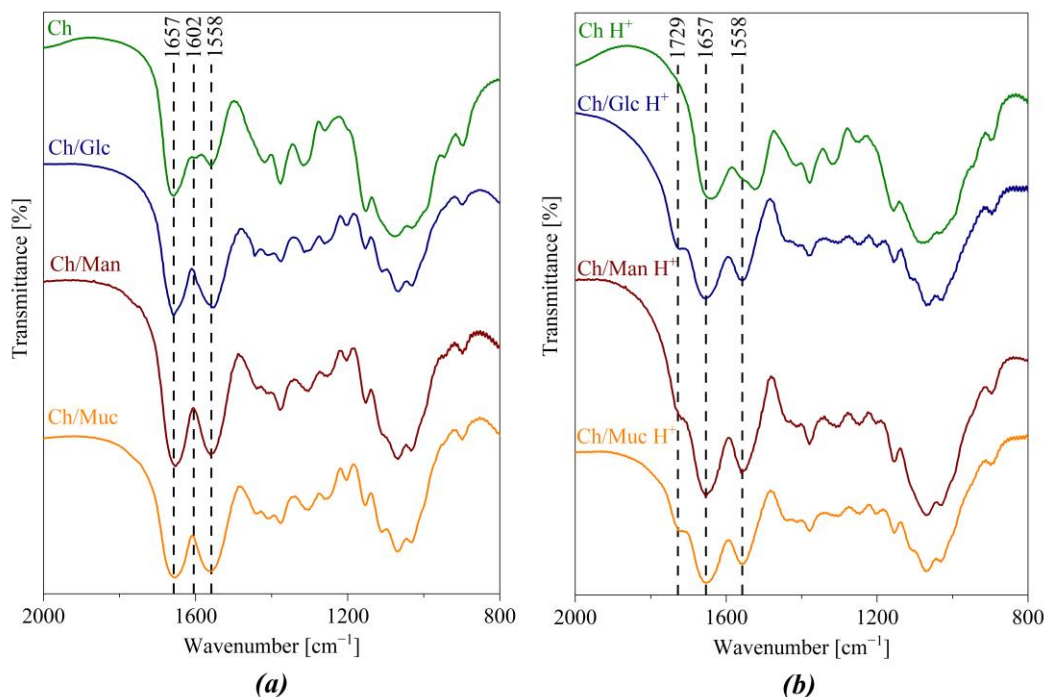


Figure 2. FT-IR spectra of chitosan and its derivatives acquired in their (a) basic and (b) acidic forms.

The reactions of the diacids with the free amino groups of chitosan to form covalent amide bonds can involve either both of their carboxylic groups or just one of them, leading to crosslinked or monosubstituted units, respectively.

Conclusive evidence of the covalent attachment of the crosslinkers to the chitosan chains through amide bonds obtained from the FT-IR spectra in basic form is described as follows: (i) the enhanced intensity of the signals associated with the presence of amide groups (1558 and 1657 cm^{-1} , corresponding to the N-H bending and the C=O stretching of the amide group, respectively [26]) and (ii) the diminished N-H scissor bending (1602 cm^{-1}) to non-observable levels with respect to those observed in the spectrum of native chitosan (Figure 2a).

The spectra acquired in the basic form did not provide information on whether monosubstituted units with free carboxylic groups were present or not in the products since the signal corresponding to the carboxylate ions' C=O stretching overlapped with the amide groups' N-H bending (1558 cm^{-1}). Therefore, the spectra of the products in their acidic form were also acquired. At low pH, the carboxylate groups were protonated, and the signal corresponding to the C=O stretching shifted to a higher wavenumber. The observation of a new signal at 1729 cm^{-1} in these spectra, together with the decrease in the intensity of the signal at 1558 cm^{-1} , revealed the presence of monosubstituted units in all products (Figure 2b).

Furthermore, the presence of monosubstituted units with free carboxylic groups in all the products was also evidenced from the isoelectric points (all in the 4–5 range) calculated from Z potential vs. pH curves (Figure 3).

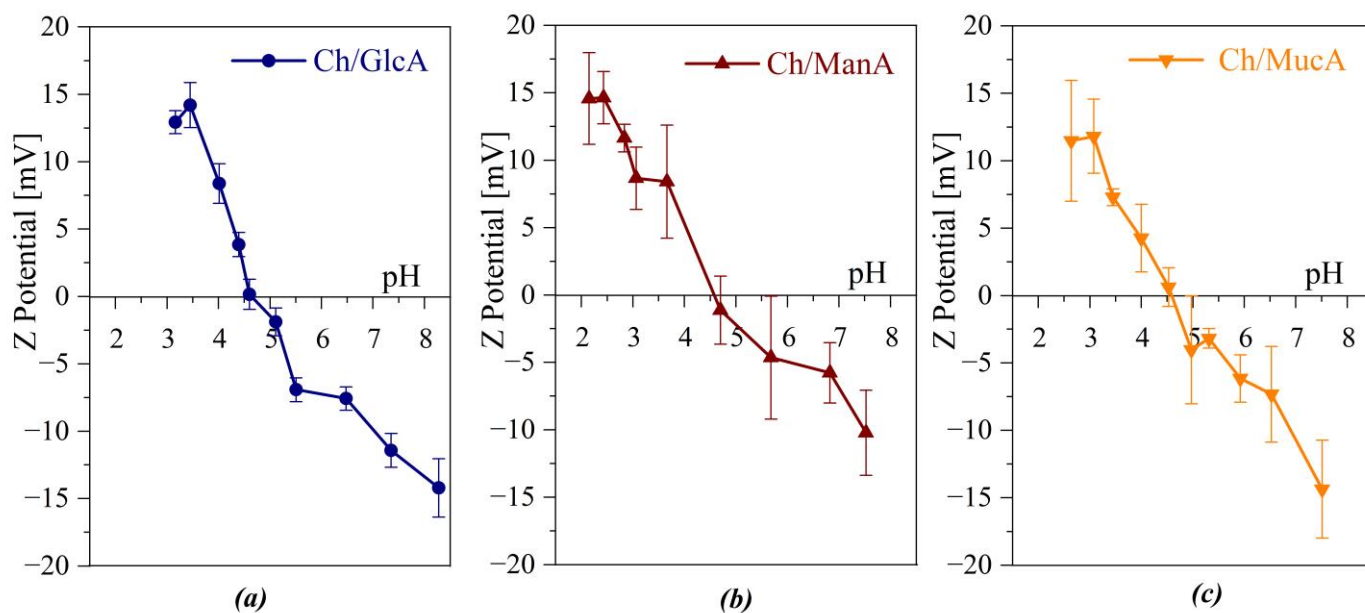


Figure 3. Measurements of Z potential against pH for the chitosan derivatives: (a) Ch/GlcA; (b) Ch/ManA; (c) Ch/MucA.

Figure 4 shows the structural units that could be present in the products according to the previous discussion.

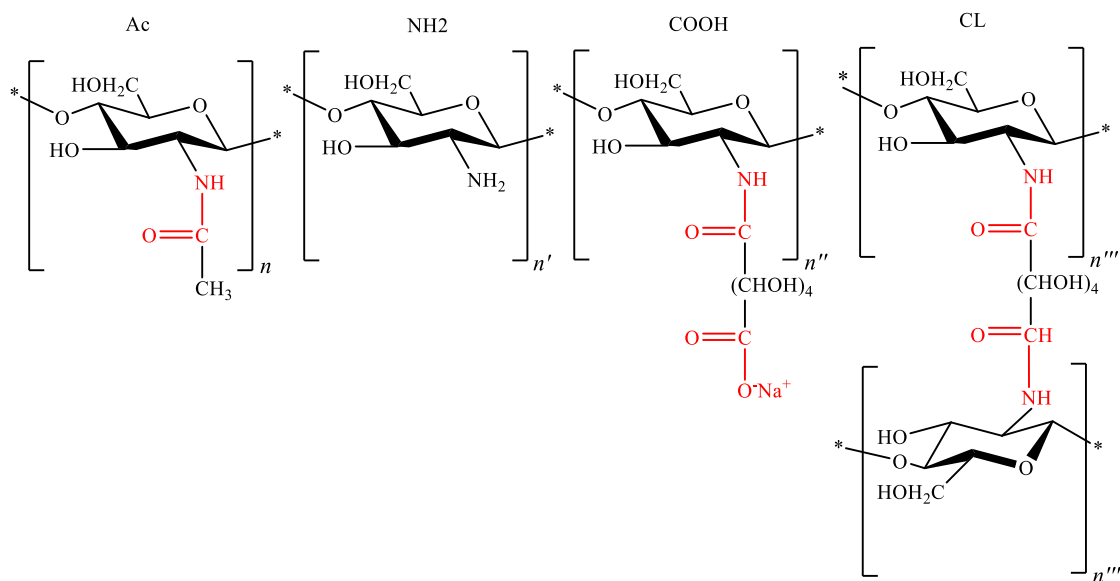


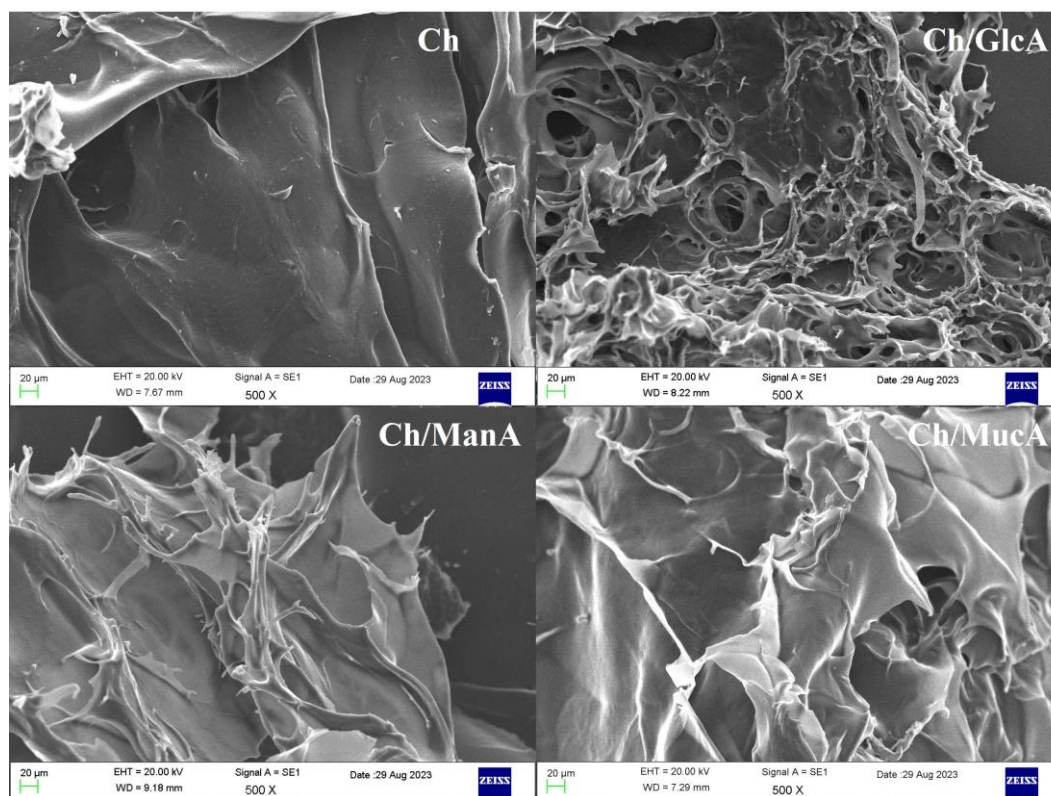
Figure 4. Structural units present in chitosan-based materials.

Once the structural units that could be present in the new materials were identified, the next step involved carrying out titration studies in order to determine the proportion of each one in the samples. The results achieved from titration curves (shown in the Supplementary Materials, Figures S1–S4), using the equations detailed in the Materials and Methods Section (Section 2.4.2), are summarized in Table 1. No significant differences were observed in the proportion of the different structural units between Ch/GlcA and Ch/ManA or between Ch/GlcA and Ch/MucA. However, in the case of Ch/MucA, the total degree of substitution (monosubstitution plus crosslinking) was slightly higher with respect to that achieved in Ch/ManA.

Table 1. Structural units' percentages of native chitosan and its derivatives.

[%] $\pm$ SD	x_{Ac}	x_{NH_2}	x_{COOH}	x_{CL}
Ch	30.0 ± 1.3	70.0 ± 1.3	-	-
Ch/GlcA	30.0 ± 1.3	21.2 ± 4.9	31.8 ± 3.1	17.1 ± 4.8
Ch/ManA	30.0 ± 1.3	24.7 ± 3.9	28.1 ± 3.8	17.3 ± 3.6
Ch/MucA	30.0 ± 1.3	17.7 ± 3.7	36.2 ± 3.5	16.1 ± 4.1

The SEM micrographs from the morphological studies are shown in Figure 5. In the chitosan micrograph, a smooth surface is observed, while in the case of the modified materials, the roughness observed can be attributed to the loss of crystallinity due to the introduction of substituents that disrupt the hydrogen bond interaction in the chitosan chains [27], as previously reported [3]. This disruption also impacted the specific surface area of the new materials since all of them (Ch/GlcA, Ch/ManA, and Ch/MucA) exhibited higher values than that of native chitosan because of their less compact structure (Table 2) [28].

**Figure 5.** SEM micrographs of Ch, Ch/GlcA, Ch/ManA, and Ch/MucA.**Table 2.** BET surface area.

	BET Surface Area [m ² g ⁻¹]
Ch	1.25 ± 0.03
Ch/GlcA	1.35 ± 0.03
Ch/ManA	1.33 ± 0.03
Ch/MucA	1.35 ± 0.03

The thermal stability of the products and native chitosan was also studied (Figure 6). For all samples, below 150 °C, a weight loss of about 7% was observed, which was attributed to the loss of the water that remained in the powders after the freeze-drying process [29].

The greatest weight loss and maximum degradation rate of all samples occurred in the range of 150 °C–500 °C, which was related to the degradation of the chitosan chains [30,31].

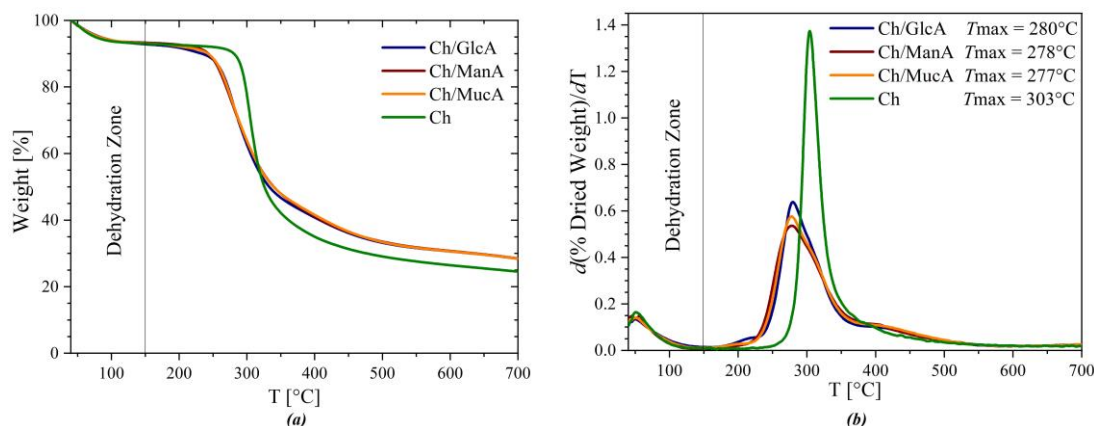


Figure 6. Thermogravimetric analyses of freeze-dried samples. (a) Weight loss as a function of temperature. (b) Derivative of weight loss with respect to temperature, as a function of temperature. The temperatures at the maximum degradation rate ($T_{\max}$) are displayed.

On the other hand, the onset temperature of all new products was approximately 25 °C lower than that of the native polysaccharide (Figure 6a). This reduction in thermal stability is consistent with the disruption of interchain hydrogen bonds and the loss of crystallinity in native chitosan due to the chemical modifications discussed above. However, native chitosan exhibited a higher $T_{\max}$ and the narrowest peak (Figure 6b), indicating a more homogeneous decomposition event that is consistent with a more regular and stable structure. Among the products, no significant differences in thermal stability were observed (Figure 6), in agreement with their similar crosslinking degree (Table 1).

3.2. Hydrogel Characterization

All the chitosan derivatives were insoluble in water in the whole pH range, and when immersed in water, they readily exhibited a hydrogel-like appearance (Figure 7).

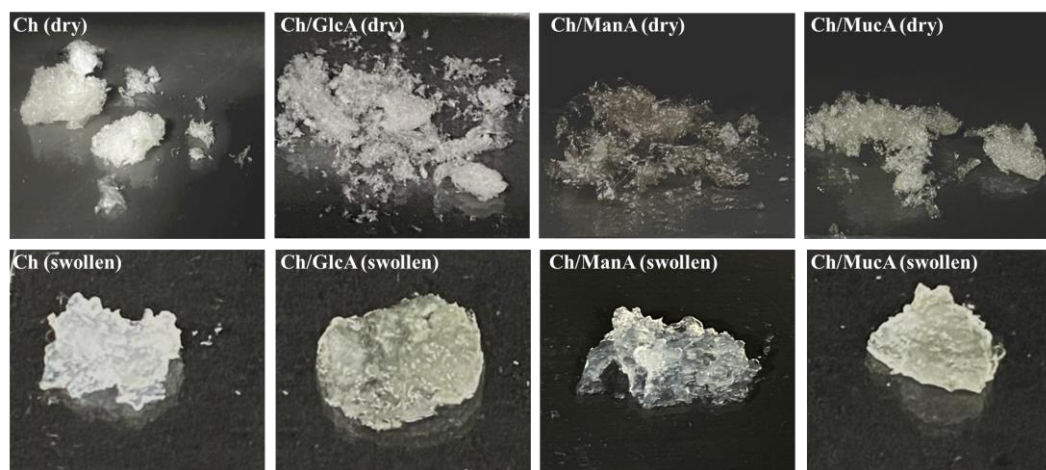


Figure 7. Dry and swollen samples of Ch, Ch/GlcA, Ch/ManA, and Ch/MucA.

The viscoelastic properties of the materials were assessed by oscillatory rheological experiments (Figure 8). Initially, the variation in the elastic storage modulus (G') and the viscous loss modulus (G'') was studied as a function of the shear strain at a constant angular frequency of 1 rad s^{−1} (Figure 8a,b). All samples exhibited a linear viscoelastic region in the strain range of 0.02% to 0.5%, in which both G' and G'' exhibited constant values (Figure 8a). G' was greater than G'' for all samples in this viscoelastic region, which

is consistent with a predominantly elastic nature. Therefore, the chitosan derivatives can be considered viscoelastic solids, displaying a gel-like structure [32,33]. Furthermore, in the case of Ch/GlcA, the flattening of the δ vs. strain curve (Figure 8b) was slightly extended to higher strain values with respect to the other products, suggesting that the hydrogel formed by Ch/GlcA was more stable under strain perturbations than the others.

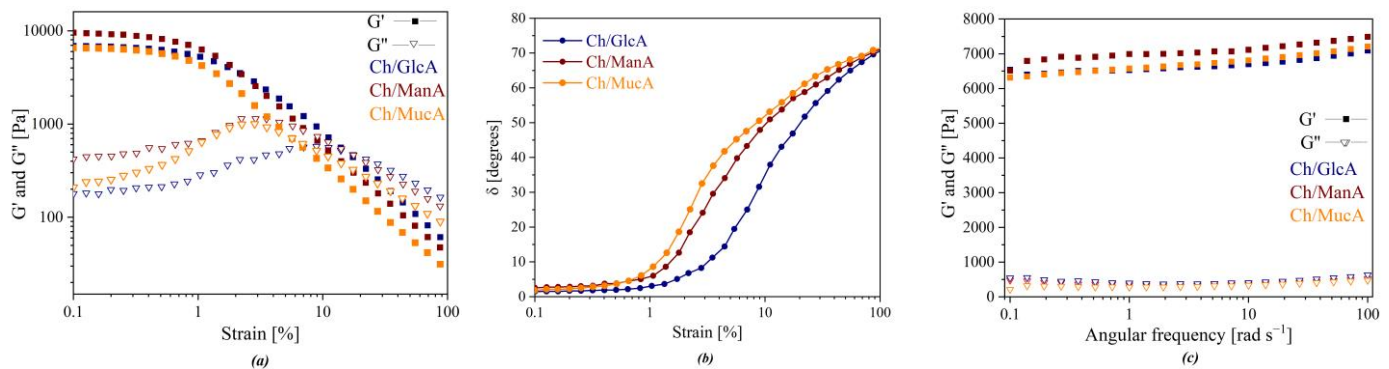


Figure 8. Rheological properties of the materials at constant frequency (a,b) and strain (c).

The elastic solid behavior of all products was also assessed by studying the variation in the elastic and viscous modulus (G' and G'' , respectively) against the angular shear frequency in the viscoelastic region at a constant strain amplitude of 0.1% (Figure 8c). The strain amplitude was chosen to simulate slow motion on long timescales or at rest. The greater values of G' with respect to G'' throughout the entire frequency sweep were in accordance with the predominantly elastic solid behavior described above [12,34].

Furthermore, as shown in Figure 8c, both G' and G'' remained nearly constant for all angular frequencies, which indicates the stability of all the hydrogels under external perturbations. These results are consistent with the covalently crosslinked structure of these materials (Figure 3).

Regarding the swelling studies (Figure 9), the enhancement in the swelling capacity of the modified materials with respect to native chitosan could be attributed to the following: (i) the disruption of the hydrogen bond interaction in the chitosan chains due to the chemical modification; (ii) the introduction of ionic groups, which contribute to improving the interaction between the adsorbents and water; and (iii) the feasibility of interaction between water and the hydroxyl groups of the crosslinking agents [35–38]. The remarkably higher swelling capacity of Ch/GlcA is noteworthy.

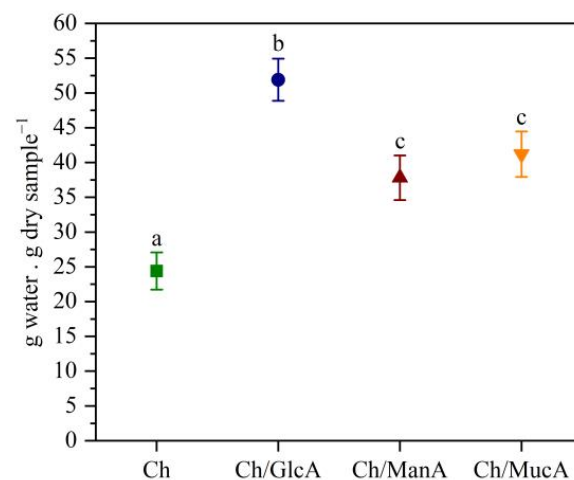


Figure 9. Swelling studies of Ch, Ch/GlcA, Ch/ManA, and Ch/MucA. Values marked with different letters indicate statistically significant differences ($p \leq 0.01$) using the Tukey HSD post hoc test.

The total water content of the hydrogels W (grams of water/100 g of swollen sample) calculated from the swelling studies can be divided into freezable and non-freezable water. Non-freezable water represents the fraction tightly bound to the polymer chains, not exhibiting a phase transition observable by DSC. In contrast, freezable water is less associated with polymers, behaving more similarly to free water and displaying phase transitions during DSC analyses [24,25].

In this context, DSC studies of swollen hydrogels were performed (Figure 10). The percentages of freezing water (W_f) were determined as the ratio between the endothermic peak area of the water-swollen hydrogel (Q_{endo}) and the melting endothermic peak of pure water (Figure 10, $Q_{\text{ice}} = 334 \pm 3 \text{ J g}^{-1}$) [25]. Non-freezing water (W_{nf}) was calculated as the difference between W and W_f (Table 3).

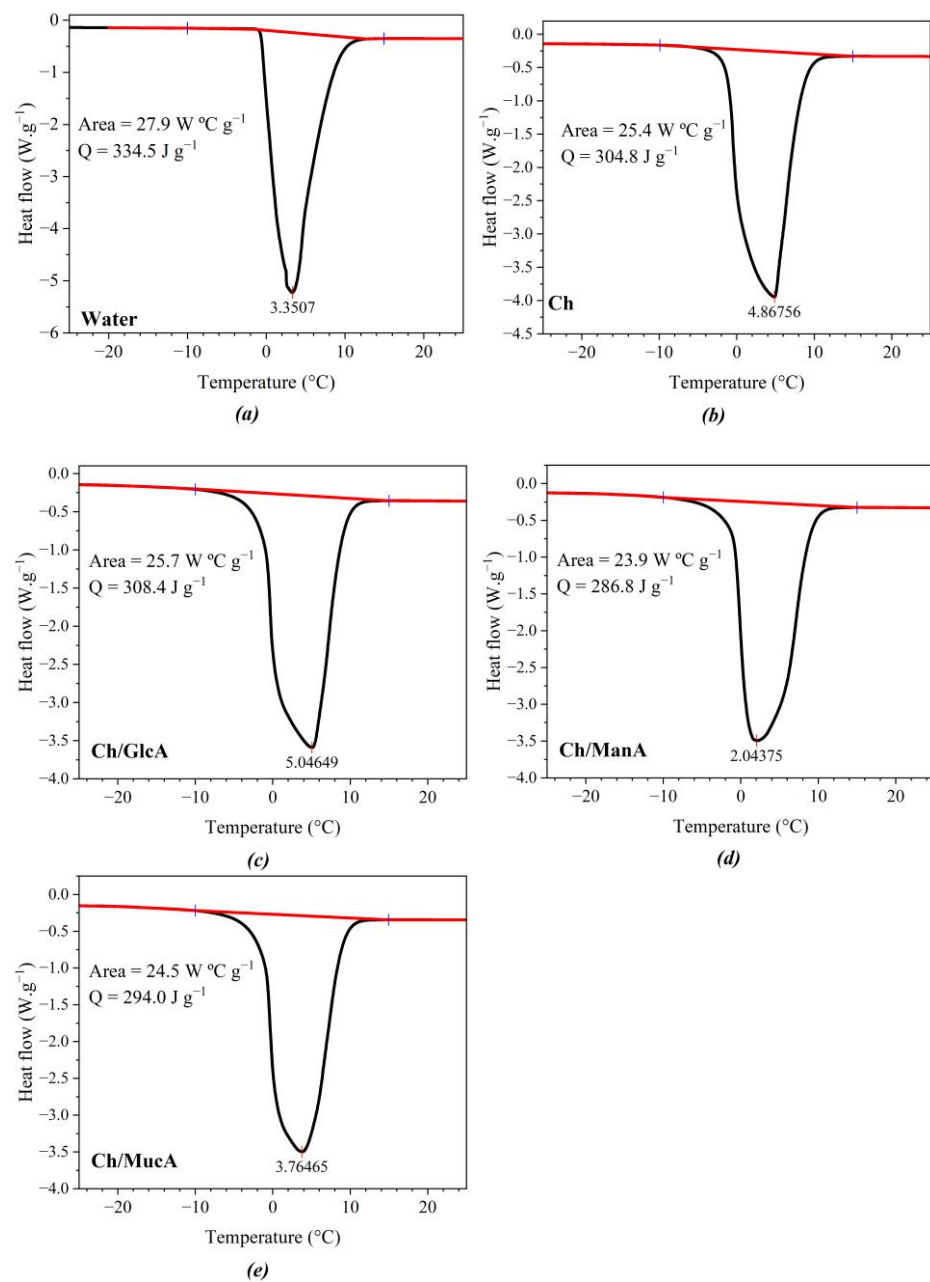


Figure 10. DSC thermograms of swollen hydrogels. The heat of the fusion of freezing water in the hydrogel (Q_{endo} (J/g)) for each sample is shown: (a) water; (b) Ch; (c) Ch/GlcA; (d) Ch/ManA; (e) Ch/Muc/A.

Table 3. Results of swelling experiments and DSC studies. W : total water content (%); Q_{endo} : heat of fusion of freezing water in the hydrogel (J/g); Q_{ice} : melting endothermic peak of pure water; W_{nf} : percentage of non-freezing water (%); W_{f} : percentage of freezing water (%).

Samples	Swelling [g H ₂ O g ⁻¹ Dry Sample]	W [%]	Q_{endo} (J/g)	$Q_{\text{endo}}/Q_{\text{ice}}$	Non-Freezable Water		Freezable Water	
					W_{nf} (%)	g H ₂ O g ⁻¹ Dry Sample	W_{f} (%)	g H ₂ O g ⁻¹ Dry Sample
Ch/GlcA	52 ± 3	98.1 ± 0.1	308 ± 3	0.92 ± 0.01	6 ± 1	3 ± 1	92 ± 1	49 ± 1
Ch/ManA	38 ± 3	97.4 ± 0.2	287 ± 3	0.86 ± 0.01	11 ± 1	4 ± 1	86 ± 1	33 ± 1
Ch/MucA	41 ± 3	97.6 ± 0.2	294 ± 3	0.88 ± 0.01	10 ± 1	4 ± 1	88 ± 1	37 ± 1
Ch	24 ± 3	96.1 ± 0.4	305 ± 3	0.91 ± 0.01	5 ± 1	1 ± 1	91 ± 1	23 ± 1

Native chitosan had the lowest value of non-freezable water, which is consistent with its well-known highly compact structure related to the hydrogen bonding interchain interaction, which does not favor its interaction with water [28].

3.3. Adsorption Studies

The results obtained for the adsorption capacity assessment of the materials against Pb (II) are presented below.

3.3.1. Effect of pH

Figure 11 shows the strong dependence of the lead adsorption capacity of all the modified materials with pH. The results are in agreement with their chemical structures. In accordance with the Z-potential measurements, at low pH values, both amino and carboxyl groups are protonated; therefore, the modified materials are positively charged, which prevents the retention of lead cations because of electrostatic repulsion. As the pH increases, the deprotonation of amino and carboxyl groups occurs, resulting in the presence of negative charges in the modified materials, which enhances lead adsorption through ionic interactions. The maximum pH tested was 6.5 because, above this value, a water-insoluble lead hydroxide precipitate appears. As expected, the maximum adsorption capacity in all cases was achieved at the maximum pH assayed (6.5), as can be seen in Figure 11.

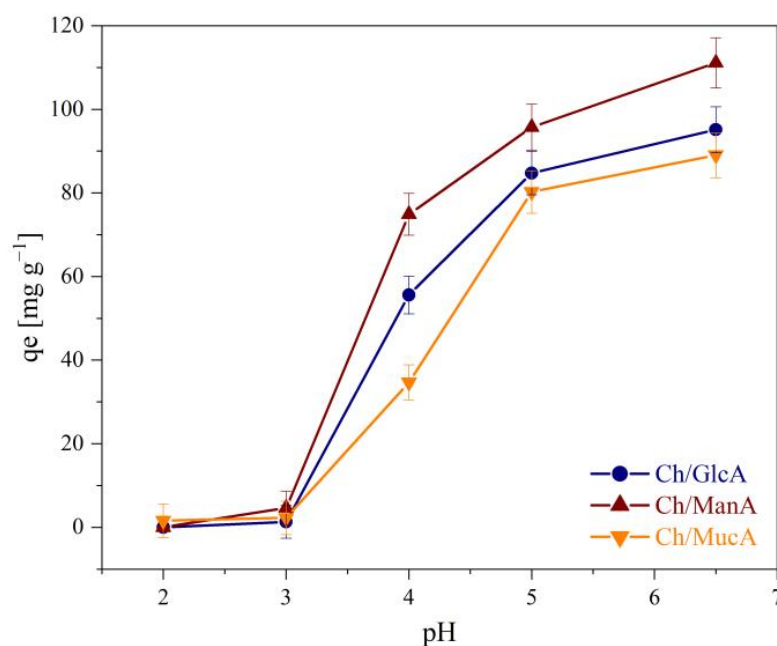


Figure 11. Effect of pH on lead adsorption capacity.

The effect of pH was not studied for native chitosan because of its solubilization at acidic conditions [39,40].

3.3.2. Effect of Adsorbent Dosage

The dependence of adsorbent dose on Pb (II) adsorption was studied by varying the amount of native chitosan or its derivatives from 0.5 to 2 mg mL⁻¹ at pH 6 for 24 h. The results in Figure 12a show that the adsorption capacity of the derivatives presents no significant difference at a dosage in the 0.5–1 mg mL⁻¹ range. When higher adsorbent doses are employed (2 mg mL⁻¹), 100% lead removal was achieved without reaching saturation of the adsorbent (Figure 12b).

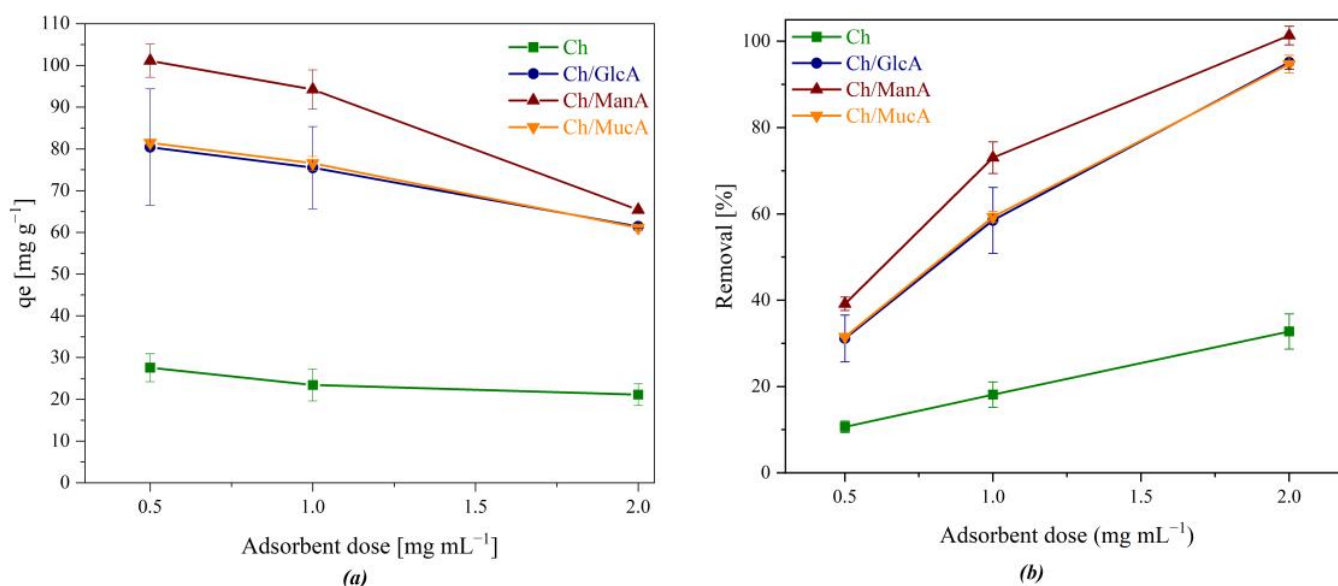


Figure 12. Effect of adsorbent dosage on (a) lead adsorption capacity and (b) lead removal.

3.3.3. Kinetic Studies

Lead adsorption on native chitosan, Ch/GlcA, Ch/ManA, and Ch/MucA was studied against time at 25 °C and 100 mg L⁻¹. As depicted in Figure 13, the adsorption kinetics were fast for all materials, reaching equilibrium in less than 20 min. A decline in the capture rate was noted as the active sites of the chitosan-based materials became occupied and, upon reaching saturation of the adsorbent, the curves reached a plateau.

The controlling mechanism of the absorption process was inferred by fitting the experimental data with pseudo-first- and pseudo-second-order models (Equations (7) and (8), respectively) [41], where k_1 (min⁻¹) and k_2 (g mg⁻¹ min⁻¹) are the kinetic constants, q_e (mg g⁻¹) is the adsorption capacity, and q_t (mg g⁻¹) is the adsorption uptake at time t (min).

$$q_t = q_{\max} \cdot (1 - \exp(-k_1 \cdot t)) \quad (7)$$

$$q_t = \frac{q_{\max}^2 \cdot k_2 \cdot t}{1 + q_{\max} \cdot k_2 \cdot t} \quad (8)$$

The mentioned parameters were obtained for both models, which are shown in Table 4 along with the adjusted determination coefficient (R_{adj}^2), the root-mean-square error (RMSE), and Akaike's information criterion (AIC).

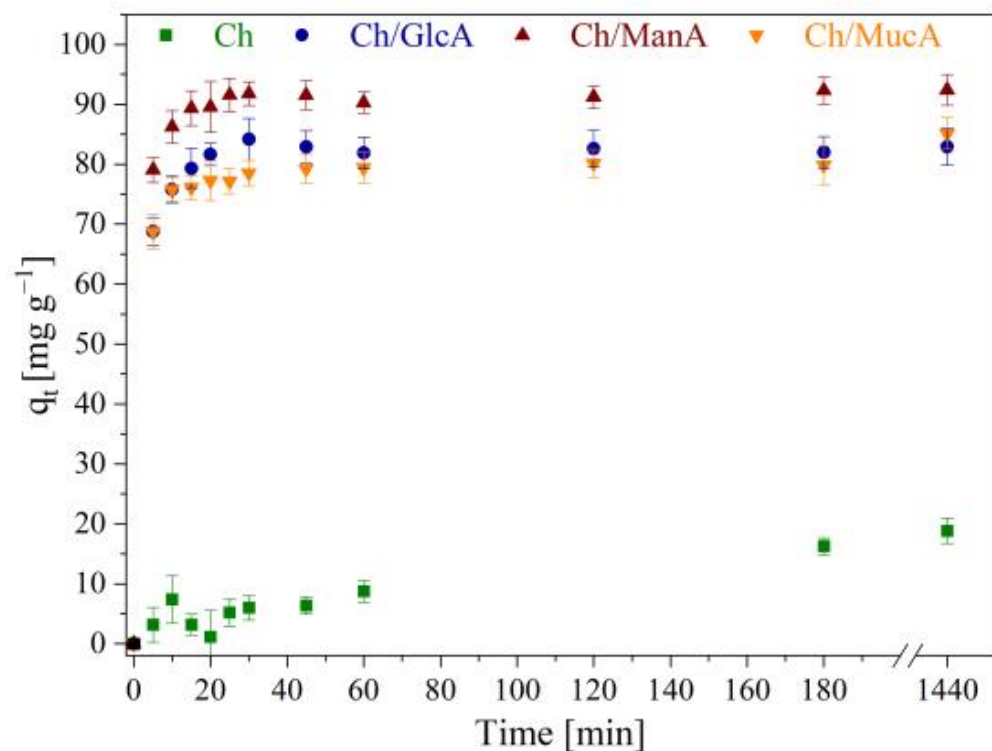


Figure 13. Amount of Pb^{+2} absorbed on the chitosan-based materials at different times.

Table 4. Adsorption kinetic models (mean $\pm$ standard deviation).

Model	Parameters	Ch	Ch/GlcA	Ch/ManA	Ch/MucA
Pseudo-first-order model	k_1 [min^{-1}]	0.011 ± 0.003	0.343 ± 0.023	0.393 ± 0.022	0.395 ± 0.045
	q_e [mg g^{-1}]	18.7 ± 2.0	82.1 ± 0.5	90.9 ± 0.4	79.1 ± 0.8
	R_{adj}^2	0.8632	0.9951	0.9970	0.9891
	RMSE	42.7	1.6	1.4	2.4
	AIC	24.3	18.9	14.3	27.6
Pseudo-second-order model	k_2 [$\text{g mg}^{-1} \text{min}^{-1}$]	$6.6 \cdot 10^{-4} \pm 2.5 \cdot 10^{-4}$	0.012 ± 0.001	0.013 ± 0.002	0.013 ± 0.002
	q_e [mg g^{-1}]	20.4 ± 2.3	83.9 ± 0.6	93.0 ± 0.4	81.3 ± 0.6
	R_{adj}^2	0.8594	0.9966	0.9986	0.9960
	RMSE	43.9	1.4	1.4	1.4
	AIC	24.6	14.3	6.7	15.6

The native chitosan experimental data were best fitted by the first-order model, which is consistent with the fact that the adsorption mechanism is kinetically mainly controlled by the diffusion process. On the other hand, the best fit for the chitosan-based materials was attained using the pseudo-second-order kinetic model, indicating that the adsorbent–adsorbate interactions mainly control the kinetics for the adsorption mechanism. This result is in agreement with the fact that the strong electrostatic interactions established between lead cations and the free carboxylate groups govern the adsorption mechanism [3,42–45].

3.3.4. Adsorption Isotherm

Adsorption isotherms were obtained for native chitosan, Ch/GlcA, Ch/ManA, and Ch/MucA by determining the amount of lead retained on the adsorbents against the supernatant's concentration under equilibrium at room temperature.

In particular, the adsorbents were contacted with lead solutions of concentrations in the 25–150 mg g^{-1} range, and the variables (i.e., dosage, time, pH) were chosen based

on the results shown above (Sections 3.3.1–3.3.3). The experimental data were modeled using well-known equations of adsorption isotherms (Langmuir, Freundlich, Dubinin–Radushkevich, and Frumkin) by nonlinear fit using OriginPro 2023b software (10.0.5.157 (Academic) [41]. The parameters of the models as well as the adjusted determination coefficient (R_{adj}^2), the root-mean-square error (RMSE), and Akaike’s information criterion (AIC) are shown in Table 5.

As shown in Table 5, the best fit to the data is given by the Langmuir equation, and Figure 14 shows the experimental results against this model. The separation factor (R_L) is always between 0 and 1 for any initial concentration (C_0). Taking into account that R_L indicates whether the type of the isotherm is unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$), or irreversible ($R_L = 0$), the values obtained herein are consistent with a favorable lead adsorption process onto the chitosan-based materials [3,45–48].

In all cases, a remarkable enhancement in the adsorption capacity of the derivatives with respect to native chitosan was observed. This could be attributed to (i) ionic interactions between the carboxylate groups (present in the monosubstituted units) and the lead cations; (ii) the disruption of chitosan’s interchain hydrogen bonds that upturned the exposure of chitosan’s hydroxyl groups to the lead cation, promoting their interaction via chelation; and (iii) the three-dimensional arrangement of the crosslinkers which can either facilitate or hinder the approach of lead cations to the hydroxyl groups.

Table 5. Comparison of the adsorption isotherm models (mean $\pm$ standard deviation).

Adsorption Isotherm Model		Adsorbent			
Equation	Parameters	Ch	Ch/GlcA	Ch/ManA	Ch/MucA
Langmuir $q_e = \frac{q_{max} \cdot k_L \cdot C_e}{1 + k_L \cdot C_e}$ $R_L = \frac{1}{1 + k_L \cdot C_0}$	q_{max} [mg g ^{−1}]	18.2 $\pm$ 5.3	82.9 $\pm$ 0.9	93.8 $\pm$ 1.3	79.2 $\pm$ 1.4
	k_L [L mg ^{−1}]	0.659 $\pm$ 2.438	18.0 $\pm$ 1.14	7.08 $\pm$ 0.49	37.72 $\pm$ 3.80
	R_{adj}^2	0.2929	0.9975	0.9966	0.9941
	RMSE	8.6	1.7	2.1	2.4
	AIC	41.8	18.7	22.1	23.9
Freundlich $q_e = k_F \cdot C_e^{1/n}$	k_{Fre} [mg g ^{−1} (mg L ^{−1}) ^{−1/n}]	15 $\pm$ 12	60.2 $\pm$ 5.0	63.7 $\pm$ 5.8	60.4 $\pm$ 5.0
	n	0.036 $\pm$ 0.211	0.102 $\pm$ 0.026	0.128 $\pm$ 0.032	0.084 $\pm$ 0.024
	R_{adj}^2	0.2794	0.8890	0.8767	0.8692
	RMSE	8.7	11.0	12.8	11.3
	AIC	41.9	45.2	47.4	45.6
Dubinin–Radushkevich $q_e = q_{D-R} \cdot e^{-k_{D-R} \cdot \epsilon^2}$ $\epsilon = R \cdot T \cdot \ln(1 + C_e^{-1})$ $E = \frac{1}{\sqrt{2 \cdot k_{D-R}}}$	q_{D-R} [mg g ^{−1}]	18.4 $\pm$ 4.2	83.0 $\pm$ 1.2	91.6 $\pm$ 1.7	80.0 $\pm$ 1.8
	k_{D-R} [mol ² J ^{−2}]	0.009 $\pm$ 0.017	3.4 $\cdot 10^{-5} \pm 1.9 \cdot 10^{-6}$	6.3 $\cdot 10^{-5} \pm 4.3 \cdot 10^{-6}$	2.1 $\cdot 10^{-5} \pm 1.9 \cdot 10^{-6}$
	E [J mol ^{−1}]	7.5	121.3	89.1	154.3
	R_{adj}^2	0.4399	0.9954	0.9933	0.9890
	RMSE	8.4	2.2	3.0	3.3
	AIC	41.4	22.8	27.0	28.3
Frumkin $\theta = \frac{q_e}{q_{max}}$ $\frac{\theta}{1-\theta} \cdot e^{-2 \cdot a \cdot \theta} = K_{Fru} \cdot C_e$	a [g mg ^{−1} min ^{−1}]	4.06 $\pm$ 0.71	3.3 $\pm$ 9.4	2.8 $\pm$ 4.4	2.9 $\pm$ 5.0
	K_{Fru} [mg g ^{−1}]	0.001 $\pm$ 0.79	0.038 $\pm$ 0.70	0.068 $\pm$ 0.56	0.041 $\pm$ 0.40
	R_{adj}^2	−0.2108	−0.2709	0.6569	0.5857
	RMSE	44.9	22.8	8.9	14.3
	AIC	64.9	55.4	42.2	48.9

The observed differences in the maximum adsorption capacity values between the products could not be attributed to either ionic interactions (because of the similar carboxylate content) or non-specific adsorption since the surface area measurements were comparable. Therefore, the major lead adsorption capacity of Ch/ManA (Table 4) with respect to the other derivatives could mainly be attributed to a higher efficiency in the

interaction of the lead cation with the hydroxyl groups of Ch/ManA via chelation. This could be explained in terms of the different spatial orientation of the hydroxyl of C-2 of mannaric acid (with respect to glucaric and mucic acids), which could enhance the interaction between Pb (II) and the hydroxyl groups of the adsorbent via chelation.

These results, as well as those obtained from the swelling analyses, evidenced the impact of the three-dimensional arrangement of the crosslinkers on some relevant properties of the products.

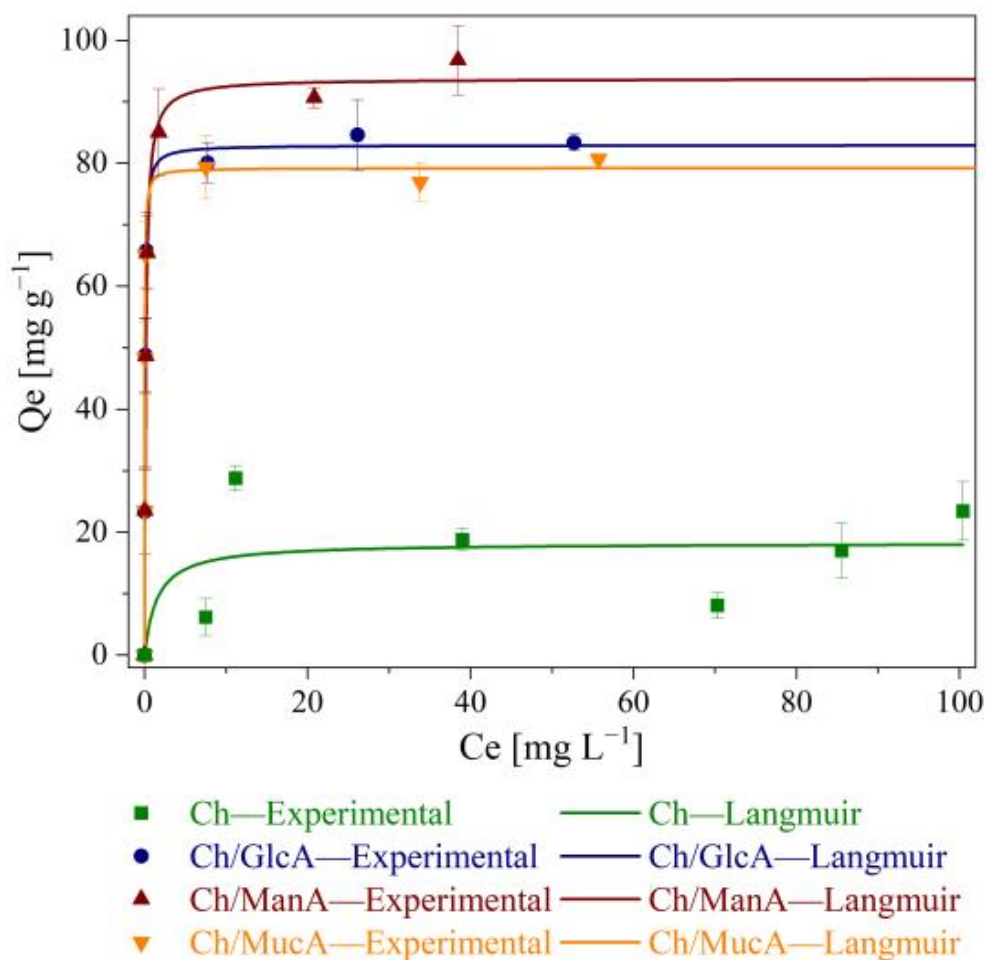


Figure 14. Lead adsorption isotherm on native chitosan and its derivatives and comparison with the Langmuir model.

3.3.5. Regeneration and Reusability Studies

Since the economic feasibility of an industrial-scale application of the adsorbents requires their reusability, regeneration and reuse studies were carried out, and the results are shown in Figure 15.

As can be seen in Figure 15, all desorption processes were successful, releasing practically the totality of the lead originally adsorbed. In addition, no significant changes in the adsorption capacity were observed in the reused materials (Figure 15).

The preservation of the chemical structure of the reused and regenerated materials was corroborated by FT-IR spectroscopy since their spectra did not show significant changes with respect to the original ones (Figure S6).

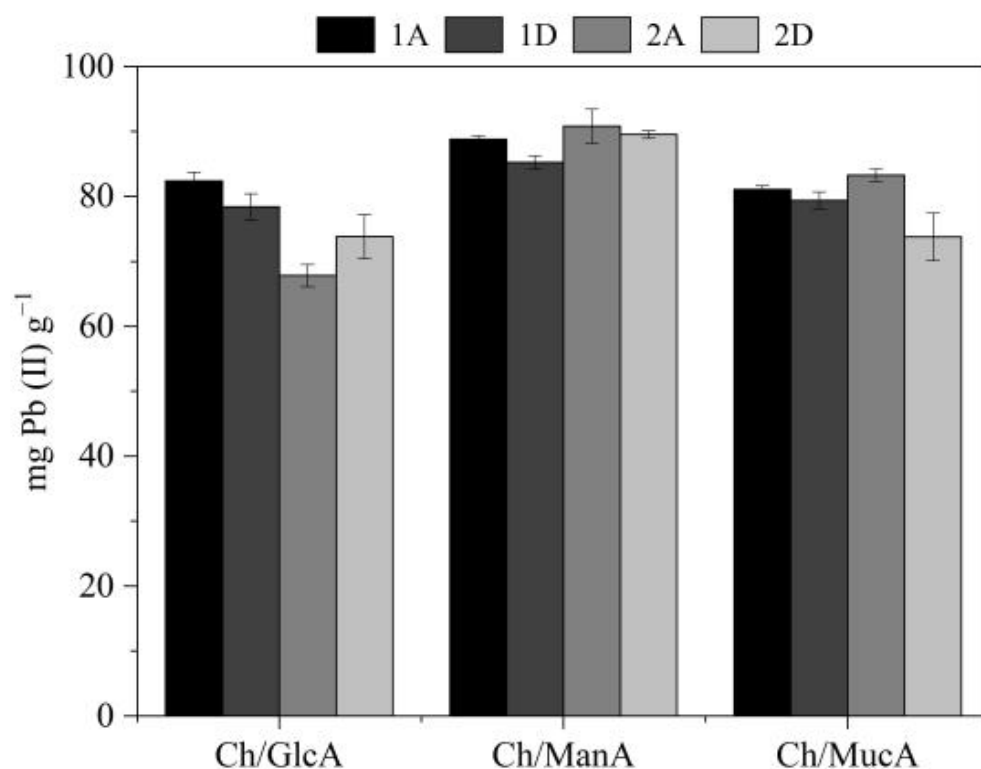


Figure 15. Regeneration and reusability study of the chitosan-based materials (A: adsorption; D: desorption).

4. Conclusions

Herein, in an effort to move forward to the rational design of materials, chitosan (Ch) was subjected to crosslinking reactions with three polyhydroxylated diacid isomers that only differ in the spatial orientation of their hydroxyl groups. We aimed to correlate the three-dimensional arrangement of the crosslinkers with the chemical structures and physicochemical properties of the new materials, which, in all cases, were stable hydrogels.

Given that the structure of all the products was very similar, the differences observed in their properties can be attributed solely to the variations in the spatial arrangement of the hydroxyl groups of the crosslinkers. Particularly, the product crosslinked with glucaric acid exhibited a remarkably higher swelling capacity (i.e., ~30% more than Ch/MucA and ~40% more than Ch/ManA) and the highest elastic nature, while the product crosslinked with mannaric acid exhibited a higher lead adsorption capacity (Ch: 18.2 mg g⁻¹, Ch/ManA: 93.8 mg g⁻¹, Ch/GlcA: 82.9 mg g⁻¹, Ch/MucA: 79.2 mg g⁻¹).

These results contribute to the rational design of new materials, highlighting the impact of the spatial arrangement of the crosslinkers on some relevant properties of the grafted polymers. These confirm the proposed hypothesis that the polyhydroxylated crosslinker with the appropriate three-dimensional structure could be used to finely adjust the final materials' features. For instance, highly swollen hydrogels like Ch/GlcA are sought for numerous applications in multiple fields such as the pharmaceutical, biomedical, and food industries. Overall, when seeking specific properties in a material, stereoisomery cannot be left aside.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/polysaccharides5030023/s1>, Figure S1: Ch conductometric titration curve; Figure S2: Ch/GlcA conductometric titration curve; Figure S3: Ch/ManA conductometric titration curve; Figure S4: Ch/MucA conductometric titration curve; Figure S5: FT-IR spectra of chitosan and its derivatives in their (a) basic and (b) acidic forms; Figure S6: FT-IR spectra of the

adsorbents (Ch/GlcA, Ch/ManA and Ch/MucA) with their respective regenerated forms (Ch/GlcA R, Ch/ManA R and Ch/MucA R).

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