

Article

[AMIM]Cl-Exfoliated Collagen Aggregates as Building Blocks for Structurally Defined Collagen Films

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Abstract

The exceptional mechanical strength and toughness of collagen arise from its well-defined hierarchical architecture. Conventional methods for obtaining collagen aggregates (CAs), such as direct extraction from native tissues or acid swelling followed by mechanical processing, offer limited control over dimensional uniformity and provide little insight into the underlying exfoliation mechanisms. To overcome these challenges, this study introduces a novel strategy that leverages insights into the hierarchical interactions within collagen. We employ the ionic liquid 1-allyl-3-methylimidazolium chloride ([AMIM]Cl) as an exfoliating agent to successfully isolate fibrous CAs from native bovine tendon. By precisely modulating temperature and processing time, we achieve CAs with tunable mesoscale dimensions (diameter 0.9–1.1 μm , length $> 160 \mu\text{m}$). Molecular dynamics simulations reveal that [AMIM]Cl disrupts the intramolecular hydrogen-bonding network within collagen, thereby facilitating controlled exfoliation. These exfoliated aggregates serve as fundamental building blocks for fabricating collagen films. The resulting materials exhibit robust mechanical integrity, high transparency, reversible pH-responsive behavior, and excellent biocompatibility as verified by cytotoxicity assays, which together underscore their potential as versatile biomaterial platforms. Furthermore, the integration of single-walled carbon nanotubes yields conductive composites with confirmed electrical functionality. This study thus presents an innovative pathway for the precision processing of collagen and advances the design of high-performance collagen-based biomaterials.

Keywords: collagen; aggregates; exfoliation; ionic liquid; collagen film



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1. Introduction

Collagen is the most abundant structural protein in nature, and its outstanding mechanical properties, thermal stability, and biocompatibility originate from a unique hierarchical supramolecular architecture. Type I collagen, which constitutes about 90% of the total collagen in animals, is predominantly found in skin, bone, tendon, cornea, and ligaments [1,2]. This hierarchical architecture is stabilized by various non-covalent interactions, including hydrogen bonding, van der Waals forces, hydrophobic interactions, and electrostatic forces. These interactions enable a synergistic optimization of mechanical strength and toughness at the mesoscopic scale, endowing biological tissues with exceptional performance and

thermal stability [3–6]. The natural assembly of collagen produces aggregates spanning multiple scales, such as fibrils, fibril bundles, and microfibers. Their highly ordered hierarchy, governed by dynamic non-covalent interactions, provides an ideal structural template for applications in tissue engineering, biomimetic materials, and functional medical devices, particularly in the development of advanced gel dressings for burn and diabetic wound healing where structural integrity and biocompatibility are critical [7–14].

In comparison to structures reconstituted *in vitro* from isolated collagen molecules, native CAs that retain endogenous supramolecular organization exhibit significantly superior mechanical strength and thermal stability. For instance, collagen films assembled from individual molecules show a tensile strength of about 1.49 ± 0.19 MPa [15], whereas films fabricated using CAs as building blocks achieve a tensile strength of 13.81 ± 0.39 MPa—an improvement of nearly an order of magnitude [16]. Differential scanning calorimetry (DSC) of porcine skin collagen indicates a denaturation temperature of approximately 54.9 °C for molecular collagen, compared to about 74.2 °C for its aggregated form [17].

Conventional methods for preparing CAs primarily involve direct exfoliation from native tissues or strategies combining acid swelling with mechanical processing [18–21]. Although these approaches partially retain the native assembly characteristics of collagen, they often lack precise control over aggregate dimensions and architecture, and the fundamental exfoliation mechanisms remain poorly understood. These limitations hinder parameter optimization for controllable fabrication, thereby constraining the potential application of CAs in advanced functional materials. Therefore, developing innovative and efficient strategies for the controllable production of CAs, along with systematic exploration of their functionalization pathways, represents a pivotal challenge in advancing the integration of collagen into biomedical and materials science.

Ionic liquids (ILs), a class of salts that remain liquid at relatively low temperatures, are widely regarded as green solvents due to their unique physicochemical properties. These properties include negligible volatility, high thermal stability, and tunable hydrogen-bond-disrupting capability [22–24]. Such attributes have fostered their growing application in the disintegration and reorganization of natural polymers [25–32]. For example, Ge et al. studied the dual regulatory effect of water on lignin dissolution in ionic liquids at the molecular level using molecular dynamics simulations [33]. Their results demonstrated that in highly effective solvents such as [AMIM]Cl and [EMIM]Cl, anions penetrate interchain spaces and disrupt hydrogen bonds between cellulose chains. Subsequently, imidazolium cations intercalate, increasing interchain distances and ultimately leading to dissolution. In contrast, only partial disruption occurs in moderate solvents ([BMIM]Cl, [BMIM]OAc, [EMIM]OAc), while poor solvents ([BMIM]Br, [EMIM]Br) induce no significant structural change. Compared to other ionic liquid cations such as phosphonium, ammonium, and pyridinium derivatives, imidazolium-based ILs exhibit superior hydrogen-bond disruption efficiency and better biocompatibility for collagen processing, making them particularly suitable for biomedical applications [34–36]. In another study, Jun-ichi Kadokawa and colleagues showed that the ionic liquid 1-allyl-3-methylimidazolium bromide (AMIMBr) can dissolve chitin at concentrations up to 4.8 wt% and form ionogels at higher chitin content [37]. Beyond their use as solvents, ionic liquids have found diverse applications in biomedical fields, including drug delivery, tissue engineering, and as antimicrobial agents, highlighting their versatility in biomaterial development [38].

Similarly, Hu et al. employed deep eutectic solvents (DESs)—ionic liquid analogs that are environmentally benign and recyclable—to directly pretreat natural silk fibers, efficiently downsizing them into silk nanowhiskers [39]. The resulting nanostructures exhibited diameters and contour lengths comparable to those of individual nanofibrils in native silk, and the DESs were recovered with at least 92% yield and reused over four

cycles. In general, both the “dissolution” and “exfoliation” of natural polymers mediated by ionic liquids fundamentally rely on the disruption of hydrogen-bonding networks. However, these processes differ intrinsically in objective, extent, and final morphology. Dissolution follows a “break-to-rebuild” pathway, leading to complete deconstruction and homogeneous solutions, whereas exfoliation adopts a “refining the essence” strategy, aiming for controlled disintegration to yield mesoscale structural units that retain native features. From an economic perspective, while ionic liquids have higher initial costs than conventional solvents, their recyclability and efficiency in collagen processing can lead to overall cost savings in film production when considering full lifecycle analysis. In short, a clear distinction between these two processes is crucial for designing efficient, targeted IL-based processing protocols and for advancing the development of high-performance bio-based materials.

In this work, we employed 1-allyl-3-methylimidazolium chloride ([AMIM]Cl) as an exfoliation medium to efficiently delaminate native collagen, thereby generating and extracting collagen microstructures with preserved structural integrity. By precisely regulating processing parameters such as temperature and duration, we successfully produced gradient CAs spanning micrometer to sub-micrometer scales. In addition, molecular dynamics simulations uncovered the dynamic interaction mechanism between the ionic liquid and collagen. The imidazolium ring of [AMIM]Cl inserts into the collagen triple-helix interface via hydrophobic interactions, while the chloride anion weakens the hydrogen-bonding network through charge shielding. Together, these actions facilitate the ordered exfoliation of the collagen hierarchy.

2. Materials and Methods

2.1. Liquid-Phase Exfoliation of Bovine Tendon Collagen by Ionic Liquids

Commercially sourced bovine Achilles tendon was subjected to a series of pretreatment steps. First, surface-adherent fat was removed under frozen conditions, and the material was sectioned into cubic particles with an edge length of approximately 0.5 cm. These particles were then immersed in a 0.2 wt% sodium hydroxide (Aladdin, Shanghai, China) solution and stirred at room temperature for about 12 h to facilitate sufficient swelling. The swollen collagen was subsequently rinsed repeatedly with deionized water until a neutral pH was attained. The resulting material was transferred to an ultra-centrifugal mill (ZM200, Retsch, Haan, Germany), mixed with a suitable amount of deionized water, and homogenized. The homogenized slurry was freeze-dried to yield pretreated bovine tendon collagen fiber bundles. These bundles, which retained their fibrous aggregates structure, were then mixed with the ionic liquid [AMIM]Cl (Sigma-Aldrich, Shanghai, China) at a collagen-to-[AMIM]Cl mass ratio of 1:30. The mixture was heated and stirred at 60 °C for 0.5, 1, 2, and 3 h, respectively. After each treatment, the product was exhaustively washed with deionized water until the conductivity of the supernatant, monitored using a conductivity meter (S230, Mettler Toledo, Shanghai, China), approached that of pure deionized water. The washed mixture was subsequently subjected to high-speed centrifugation (TGL-20B, Anting, Shanghai, China). The resulting pellet, enriched in CAs with a refined fibrillar architecture, was isolated as the final product. The obtained samples were designated as S-0.5 h, S-1 h, S-2 h, and S-3 h, corresponding to the duration of [AMIM]Cl treatment (Figure 1a).

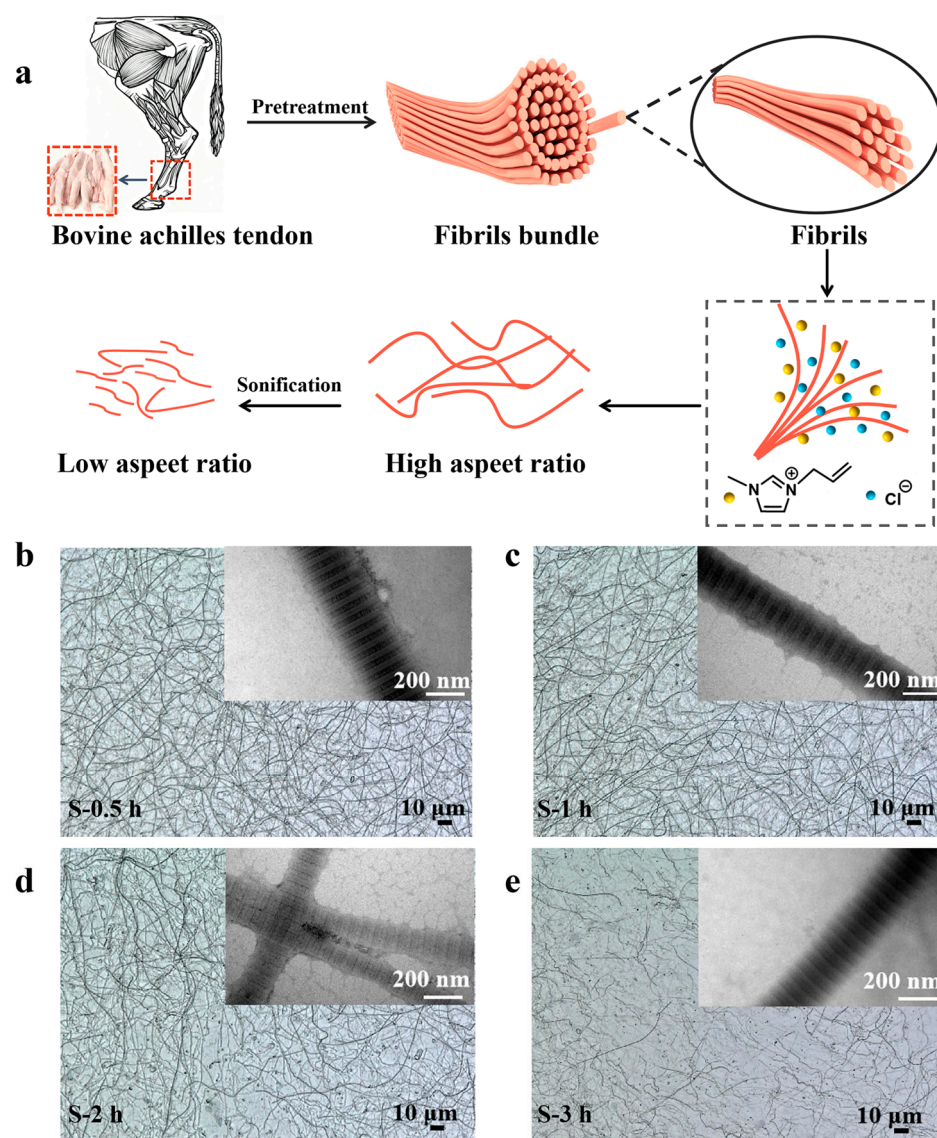


Figure 1. (a) Schematic and morphologies of the exfoliation process of bovine tendon collagen using [AMIM]Cl to obtain CAs structural units; (b–e) super-depth-of-field microscope images and corresponding transmission electron microscopy (TEM) insets of CAs obtained after [AMIM]Cl treatment for different durations: (b) 0.5 h, (c) 1 h, (d) 2 h, and (e) 3 h.

2.2. Preparation of CAs Films

A CAs suspension (0.4 wt%) was prepared by dispersing the aggregates in a 0.02% (*v/v*) acetic acid solution (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). A specified volume of the suspension was cast onto a polycarbonate film (pore size 0.22 μm , diameter 47 mm) placed in a vacuum filtration apparatus (Sigma-Aldrich, Shanghai, China). After filtration, the film was dried at 37 $^{\circ}\text{C}$, carefully peeled off from the substrate, and yielded a CAs film with an average thickness of 0.06 mm. The resulting films were designated as F-0.5 h, F-1 h, F-2 h, and F-3 h, corresponding to the exfoliation times of the CAs used for their preparation.

2.3. Structural Characterization of CAs

The structural characteristics of the CAs, which retained a distinct fibrous morphology despite processing, were comprehensively analyzed using a suite of complementary techniques. Morphological examination was first conducted using a super-depth-of-field microscope (VHX-6000, Keyence, Osaka, Japan). A droplet of the suspension was deposited

on a clean glass slide and air-dried. Three-dimensional morphologies at different focal lengths were then acquired by combining optical magnification with image overlay technology, clearly revealing the persistent fibrous architecture of the CAs. The microstructure was further investigated by transmission electron microscopy (TEM, Tecnai G220, FEI, Hillsboro, OR, USA). A droplet of the suspension was applied to a copper grid, allowed to dry naturally, and negatively stained with phosphotungstic acid. Images were acquired at an accelerating voltage of 8 kV, confirming the presence of fine, fibril-like assemblies within the CAs. The disassembly behavior of collagen fiber bundles in the presence of [AMIM]Cl was monitored using polarized light microscopy (BX51, Olympus, Tokyo, Japan). Pretreated collagen fiber bundles were placed on a hot stage, treated with [AMIM]Cl, and their morphological evolution—including progressive loss of birefringence and fibrous integrity—was recorded in the temperature range of 25–70 °C at a heating rate of 3 °C/min. For comparison, control experiments were performed using untreated samples and those treated with deionized water. At the molecular level, the chemical structure of the freeze-dried CAs was analyzed by Fourier-transform infrared (FTIR) spectroscopy (N601-P, Bruker, Billerica, MA, USA) in attenuated total reflection (ATR) mode. Spectra were collected from 800 to 2000 cm^{-1} at a resolution of 4 cm^{-1} , with a gas-phase background scan, providing insights into secondary structural changes associated with the preserved fibrous conformation. Finally, the crystalline properties were examined by X-ray diffraction (XRD, D8 ADVANCE, Bruker, Billerica, MA, USA). Freeze-dried samples were ground into a fine powder, and XRD patterns were recorded over a 2θ range of 5–50° at a scan speed of 10°/min to determine the lattice structural parameters, which reflected the semi-crystalline nature of the fibrous CAs.

2.4. Molecular Dynamics Simulations

Molecular dynamics (MD) simulations were conducted to elucidate the interactions between [AMIM]Cl and collagen at the molecular level and their influence on system properties. All-atom simulations were performed using GROMACS 2021.2. The initial structure of the collagen molecule was obtained from the RCSB Protein Data Bank (PDB ID: 2MQS) [40]. The structure of the 1-allyl-3-methylimidazolium cation (AMIM⁺) was constructed in Visual Molecular Dynamics (VMD) and parameterized with the Generalized Amber Force Field (GAFF) using the acpype tool [41,42]. To mimic the experimental environment, the simulation system was constructed containing one collagen molecule and 1764 ion pairs of AMIM⁺ and Cl[−]. The system was energy-minimized until the maximum force fell below 1000 $\text{kJ}\cdot\text{mol}^{-1}\cdot\text{nm}^{-1}$ to eliminate unfavorable atomic contacts. Simulations were carried out using the amber99sb-ildn force field. After energy minimization, the system was equilibrated sequentially in the NVT (constant number of particles, volume, and temperature; 333 K, V-rescale thermostat) and NPT (constant number of particles, pressure, and temperature; 1 bar, Parrinello–Rahman barostat) ensembles. Production MD was then run for 150 ns with a 2 fs integration time step. Trajectory analysis and visualization were performed using VMD to extract key interaction parameters and generate representative snapshots.

2.5. Mechanical Testing of CAs Films

The mechanical properties of the CAs films (designated F-0.5 h, F-1 h, F-2 h, and F-3 h) were evaluated using a dynamic mechanical analyzer (DMA Q800, TA Instruments, New Castle, DE, USA). The films were cut into rectangular strips (15 mm × 8 mm) and fully hydrated by immersion in deionized water for 1 h prior to testing. Tensile tests were performed at room temperature with a 5 kg load cell and a crosshead speed of 0.1 mm/s. Complementary texture analysis (TA.HD.plus, Waters, Milford, MA, USA) was further

employed to compare the tensile behavior of the films in both dry and hydrated states. Representative stress–strain curves, processed and selected using the instrument’s native software, are presented to illustrate the mechanical performance.

2.6. Transparency and pH-Responsive Behavior of CAs Films

The optical transparency and pH responsiveness of the CAs films were evaluated using the F-0.5 h sample as a representative. The film was immersed in aqueous solutions at different pH levels (adjusted with HCl and NaOH (Aladdin, Shanghai, China)). The transmittance of the hydrated film was measured using a haze meter (WGT-S, Labthink Instruments Co., Ltd., Jinan, China) across a pH range of 1, 5, 7, 9, and 12. To assess the reversibility of its optical response, the same film was subjected to cyclic alternation between pH 1 and pH 12 solutions. The transmittance was recorded after each transfer to quantify the stability and reproducibility of the pH-dependent switching behavior.

2.7. Cytotoxicity Assay of CAs Films

The cytotoxicity of the collagen films (F-pre, F-0.5 h, F-1 h, F-2 h, F-3 h) was evaluated in vitro against L929 mouse fibroblast cells, using established commercial dressing controls (Commercial Gelatin Sponge Dressing (CGS), Commercial Chitosan Dressing (CCD), and Commercial Collagen Sponge Dressing (CCS)) as reference materials. Sample extracts were prepared by agitating the materials in MEM culture medium containing 10% fetal bovine serum (concentration: 5.0 mg/mL) at 37 °C for 24 h. L929 cells were maintained in complete MEM medium (supplemented with 10% FBS and 1% penicillin-streptomycin) at 37 °C in a 5% CO₂ atmosphere. For the assay, cells were harvested, resuspended, and seeded into 96-well plates at a density of 1×10^5 cells/mL. After 24 h of incubation to allow cell attachment, the culture medium was replaced with 100 µL of the sample extracts, blank control (medium alone), negative control (polyethylene extract), or positive control (phenol solution). Following another 24 h of incubation, cell morphology was observed microscopically. Subsequently, the MTT assay was performed: 50 µL of MTT solution (1 mg/mL) was added to each well, and the plates were incubated for 2 h. The formazan crystals formed were then dissolved by adding 100 µL of isopropanol, and the absorbance was measured at 570 nm using a microplate reader (T6, Beijing Puxi General Instrument Co., Ltd., Beijing, China). Cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{100 \times \text{OD}_{570\text{e}}}{\text{OD}_{570\text{b}}}$$

where OD_{570e} is the average optical density value of the 100% extract of the test sample and OD_{570b} is the average optical density value of the blank control.

2.8. Conductive SWNTs/CAs Paper

Electrically conductive collagen paper was prepared by incorporating single-walled carbon nanotubes (SWNTs, (Sigma-Aldrich, Shanghai, China), carbon content > 85%, diameter 1.3–2.3 nm) into the CAs suspension. The SWNTs were dispersed by sonication in an ice bath for 30 min using a probe sonicator (Branson, Danbury, CT, USA; 450 W, 30% amplitude). The resulting homogeneous dispersion was subsequently processed into free-standing SWNT/collagen composite paper through vacuum filtration. The electromechanical response of the conductive paper was characterized by monitoring the change in electrical resistance under mechanical deformation. Using a digital multimeter (UT33C, UNI-T, Dongguan, China), the resistance was recorded in situ during repeated compression–tension cycles to evaluate the correlation between strain and electrical response.

3. Results

3.1. Structure of CAs

The morphological and structural evolution of CAs during [AMIM]Cl-mediated liquid-phase exfoliation was systematically characterized using a multi-scale approach. Super-depth-of-field microscopy images (Figure 1b) revealed that untreated collagen exhibited a characteristic fibrous architecture, with aggregates appearing randomly interwoven. With increasing exfoliation time, the CAs underwent progressive refinement, showing a progressive decrease in average diameter from 1.07 μm to 0.90 μm , while retaining lengths exceeding 116 μm across all samples (Table 1). This tunable dimensional reduction can be attributed to the selective disruption of the hydrogen-bonding network within collagen by [AMIM]Cl.

Table 1. Geometrical dimensions of CAs after [AMIM]Cl treatment for different exfoliation durations.

Samples	Diameter (μm)	Length (μm)	Aspect Ratio
S-0.5 h	1.1 ± 0.2	>200	>160
S-1 h	1.0 ± 0.1	>200	>160
S-2 h	0.9 ± 0.1	165 ± 44	116~251
S-3 h	0.9 ± 0.1	160 ± 35	121~253

Controlled disassembly of collagen fiber bundles was achieved through targeted disruption of the inter-microfibrillar non-covalent interactions. The observed size heterogeneity primarily stems from the intrinsic size gradient inherent in natural collagen fibers [43], a phenomenon also reported in studies using acid swelling combined with mechanical processing [44]. To further characterize the microstructure of the exfoliated aggregates, high-resolution transmission electron microscopy (TEM) was conducted on stained specimens (Figure 1b). All treated samples displayed distinct periodic banding patterns aligned along the fibril axis, with a measured spacing of approximately 64 nm. This value corresponds precisely to the characteristic D-periodicity of native collagen fibrils [45], which originates from the staggered arrangement of collagen triple helices. The preservation of this periodic structure confirms that the [AMIM]Cl-mediated exfoliation process maintains the intrinsic supramolecular architecture of collagen without significant disruption. Together, these multi-scale characterization results demonstrate that the [AMIM]Cl-based liquid-phase exfoliation not only enables tunable sizing of CAs but also effectively preserves their essential structural integrity, thus providing a robust foundation for their subsequent functional deployment.

3.2. Mechanistic Analysis of Bovine Tendon Collagen Exfoliation

The exfoliation mechanism of bovine tendon collagen by [AMIM]Cl was investigated using polarized optical microscopy (POM) to monitor collagen fiber bundles in situ under heating in air, water, and [AMIM]Cl (Figure 2a). The pretreated collagen fiber bundles initially exhibited well-defined birefringent regions, corresponding to their native D-periodic (~64 nm) superhelical structure. When heated to 70 °C in air or water, the fibrous architecture remained largely intact, with only moderate swelling observed in the aqueous medium due to water uptake and local reorganization of hydrogen bonds. In contrast, collagen displayed a distinctly different thermal behavior in [AMIM]Cl. At 60 °C, the birefringent regions in POM images began to diminish, and by 70 °C, they were nearly absent, indicating effective disruption of the periodic superstructure. The underlying mechanism involves three synergistic effects. First, [AMIM]Cl penetrates the fiber interior through hydrophobic interactions and electrostatic shielding, which weakens non-covalent cross-links between microfibrils. Second, cations and anions competitively interfere with

the hydrogen-bonding network between collagen molecules. Third, progressive decoupling of the D-periodic arrangement occurs as these non-covalent interactions are disrupted. Based on this mechanistic insight, 60 °C was identified as the optimal processing temperature, enabling [AMIM]Cl to achieve controlled exfoliation of collagen fiber bundles while largely preserving their native structural conformation. These findings establish a thermodynamic basis for the precise regulation of collagen micro- and nano-structures and further demonstrate the potential of ionic liquids in the deconstruction and functionalization of biomacromolecules.

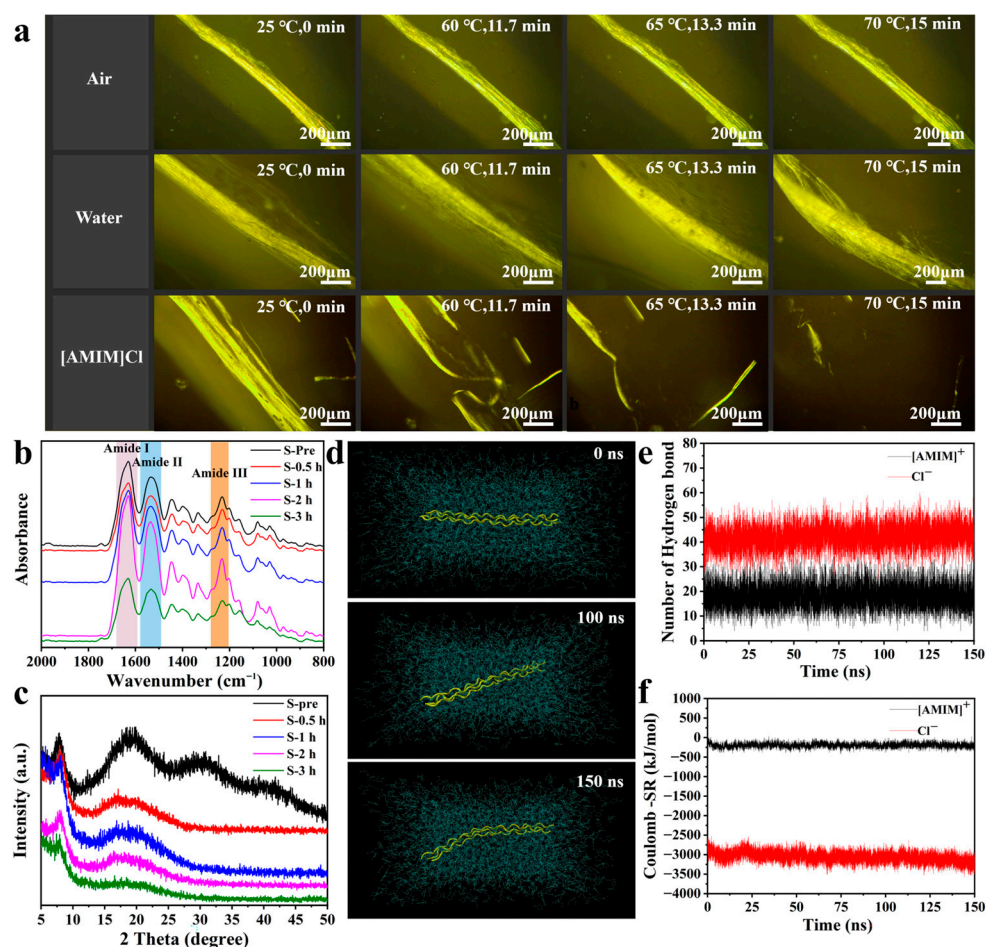


Figure 2. (a) Polarized optical micrographs of pretreated bovine tendon collagen fiber bundles during in situ heating in air, water, and [AMIM]Cl, respectively; (b) Fourier-transform infrared (FTIR) spectra and (c) X-ray diffraction (XRD) patterns of bovine tendon collagen before and after [AMIM]Cl treatment. (S-Pre denotes the pretreated bovine tendon collagen sample, and the others represent samples obtained after different treatment durations by [AMIM]Cl); (d) visual representation from molecular dynamics simulation of a collagen macromolecule in [AMIM]Cl at 60 °C; yellow, collagen polypeptide chains; cyan, [AMIM]Cl molecules; (e) variation in the number of hydrogen bonds between the collagen macromolecule and [AMIM]Cl; (f) variation in Coulombic interaction energy between the collagen macromolecule and [AMIM]Cl.

To further elucidate the structural evolution of bovine tendon collagen upon [AMIM]Cl treatment, FTIR and XRD were performed (Figure 2b). The FTIR spectra display characteristic amide bands: amide I at ~1645 cm⁻¹ (C=O stretching), amide II at ~1558 cm⁻¹ (N-H bending coupled with C-N stretching), and amide III at ~1240 cm⁻¹ (C-N stretching and N-H bending) [46–48]. These features are indicative of the collagen-specific secondary structure. The persistence of these bands in all treated samples confirms that the fundamental molecular conformation is preserved after [AMIM]Cl exfoliation. The XRD patterns

of the mesoscale CAs are presented in Figure 2c. The sharp diffraction peak near 8° corresponds to the interchain spacing, while the broad feature centered at $\sim 22^\circ$ originates from diffuse scattering between collagen molecules [49]. A distinct peak near 30° , attributed to the axial height of the triple-helical unit [50], is evident in the untreated sample. Notably, this peak disappears after [AMIM]Cl treatment, suggesting a partial disruption of the triple-helical packing arrangement.

Molecular dynamics (MD) simulations were conducted to elucidate the molecular-level interactions between collagen and [AMIM]Cl. As depicted in Figure 2d, collagen adopts a triple-helical structure composed of three polypeptide chains, surrounded by uniformly distributed [AMIM]Cl molecules. Hydrogen bonding and electrostatic interactions between collagen and the ionic liquid species were statistically analyzed at 150 ns (Figure 2e,f). The results show that the number of hydrogen bonds formed between the 1-allyl-3-methylimidazolium cation (AMIM^+) and collagen fluctuates around 20, whereas chloride anions (Cl^-) form approximately 40 hydrogen bonds with collagen. This indicates that both ions disrupt the intrinsic hydrogen-bonding network of collagen by establishing competitive hydrogen bonds, with Cl^- exhibiting a greater disruptive capacity due to its higher electronegativity. Analysis of electrostatic interactions reveals a near-zero Coulombic interaction energy between AMIM^+ and collagen, but a significantly negative value of approximately -3000 kJ/mol for Cl^- -collagen interactions. This suggests the absence of substantial electrostatic attraction between AMIM^+ and collagen, in contrast to the strong attractive interaction mediated by Cl^- . In summary, Cl^- disrupts both hydrogen bonding and electrostatic interactions within collagen, while AMIM^+ primarily interferes with hydrogen bonding. These complementary interactions at the molecular level provide a mechanistic basis for the exfoliation of higher-order CAs.

3.3. Mechanical Properties of Collagen Films

The collagen films fabricated via vacuum filtration (Figure 3a) were freestanding, uniform, transparent, and elastic. As shown in Figure 3b,c, the water-insoluble CAs served as fundamental building blocks, self-assembling into a densely interconnected network during filtration, which conferred excellent structural stability and flexibility to the resulting films. The mechanical properties of the films were evaluated in both dry and hydrated states. For dry films (Figure 3d), tensile strength and elongation at break gradually decreased with prolonged [AMIM]Cl treatment time, accompanied by a transition to brittle fracture behavior. Specifically, the film prepared from aggregates treated for 0.5 h exhibited a tensile strength of 44.44 MPa and an elongation at break of 12.95%. The decline in mechanical performance is attributed to the reduced dimensions and intrinsic mechanical integrity of the CAs, as well as the weakened physical entanglement between them after extended ionic liquid treatment.

In contrast, the hydrated film (Figure 3e) demonstrated a tensile strength of 1.49 MPa and an elongation at break of 74.94%. Its stress-strain curve exhibited a characteristic “J”-shaped profile, typical of soft biological tissues [51–53]. The incorporation of water molecules into the inter-aggregate spaces induced swelling, loosened the physical connections, and enhanced structural flexibility [54], thereby reducing mechanical strength compared to the dry state. As illustrated in Figure 3f, the tensile behavior of the wet film can be described in three stages. In Region I, CAs gradually align along the tensile direction under low stress. In Region II, the aligned aggregates undergo slippage and deformation, leading to progressive elongation. In Region III, further stretching induces extensive deformation of the highly oriented structure, resulting in a rapid increase in stress until failure.

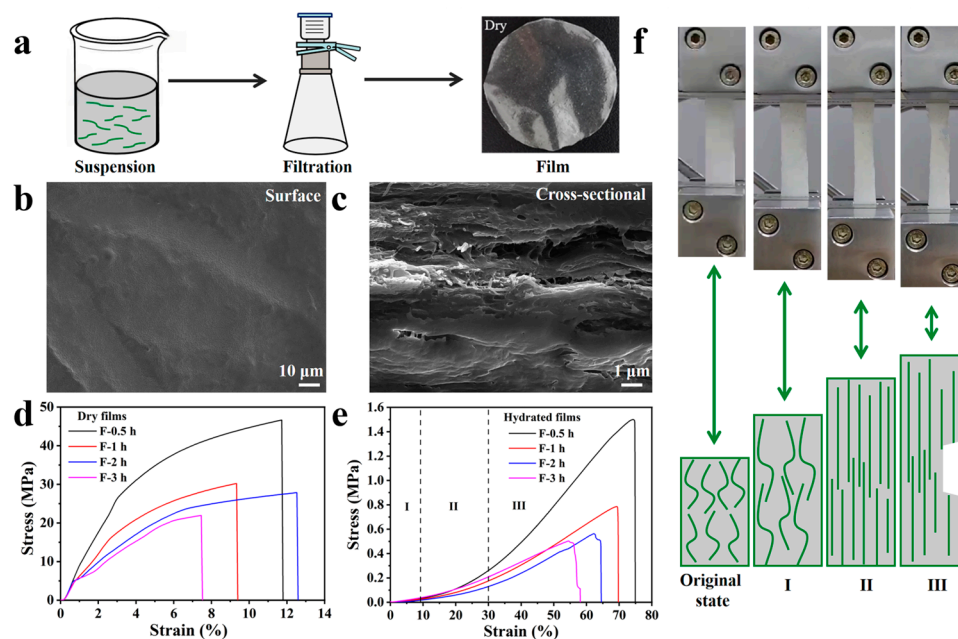


Figure 3. (a) Schematic illustration of the preparation process and photographs showing the appearance of the collagen film (F-0.5 h); scanning electron microscopy (SEM) image showing (b) the surface and (c) cross-sectional morphology of the collagen film; typical stress–strain curves of the (d) dry-state and (e) hydrated-state collagen films; (f) typical stress–strain curves of hydrated collagen films, with an illustration showing collagen fibril arrangements in the film under force.

Statistical analysis confirmed that exfoliation time significantly influenced the mechanical properties (Table S1, Figures S1 and S2). F-0.5 h exhibited the highest tensile strength in both dry (49.05 ± 6.36 MPa) and hydrated states (1.56 ± 0.08 MPa), with prolonged treatment leading to significantly lower values (F-3 h dry: 22.36 ± 3.56 MPa, $p < 0.001$; hydrated: 0.46 ± 0.04 MPa, $p < 0.001$). Processing temperature also played a critical role (Figures S3 and S4); films processed at 60 °C showed significantly higher dry tensile strength (49.05 ± 6.36 MPa) compared to other temperatures ($p < 0.05$ to $p < 0.001$), confirming it as the optimal condition. As shown in Table S2, the F-0.5 h film exhibits superior mechanical properties compared to many reported collagen materials [55–60], attributed to the preservation of native aggregated architecture.

3.4. Optical Transmittance and pH Responsiveness of Collagen Films

As illustrated in Figure 4a, the collagen film displays distinct optical transmittance under different pH conditions. It exhibits high transparency in strongly acidic (pH 1) and alkaline (pH 12) environments, whereas significant turbidity is observed in neutral solution (pH 7). These macroscopic observations are corroborated by quantitative measurements of transmittance and haze (Figure S5). The film also maintains structural stability in organic solvents such as ethanol and methanol (Figure S6). Systematic evaluation across a broader pH range (pH 1, 5, 7, 9, and 12) consistently confirmed the pH-dependent transparency (Figure 4b). To assess reversibility, cyclic immersion tests were conducted between pH 1 and 7, and pH 12 and 7. The results reveal fully reversible transmittance changes in response to pH variations (Figure 4c,d). After multiple cycles, the film repeatedly regained high transparency at pH 1 and 12, while reverting to low transparency at pH 7 (Figure 4e).

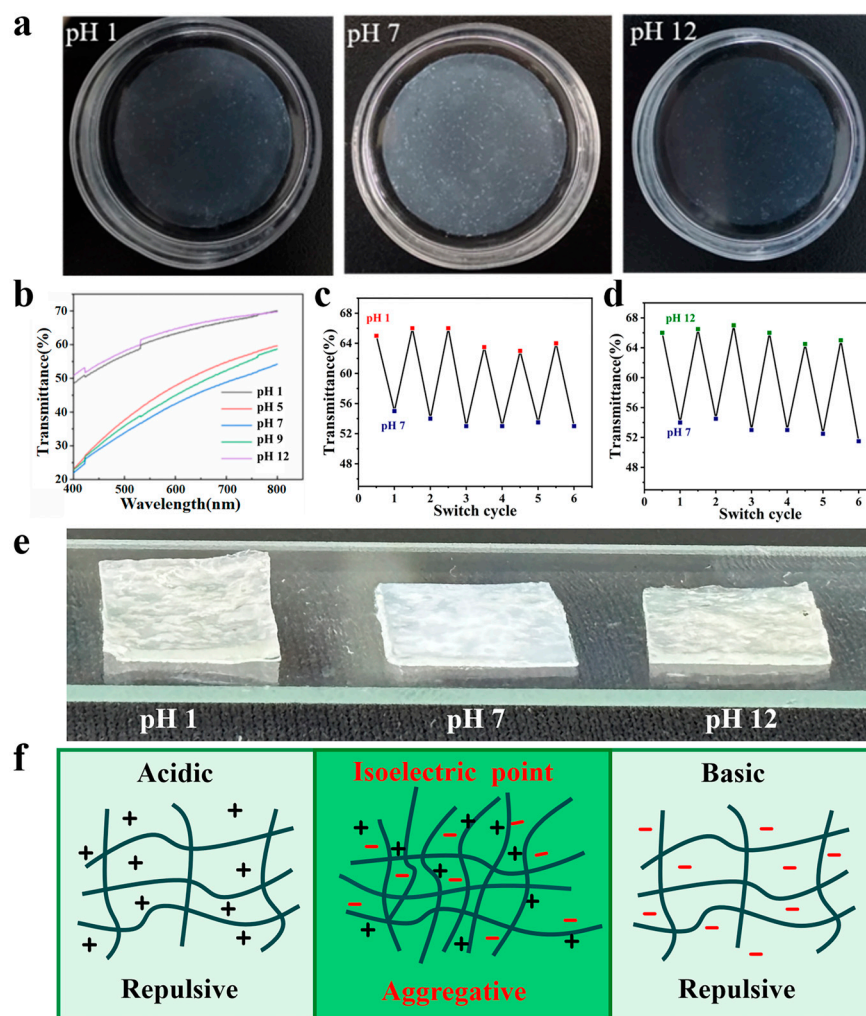


Figure 4. (a) Digital photographs of the collagen films (F-0.5 h) after immersion in solutions of various pH values for 12 h under dry-state conditions (pH 1, pH 7, pH 12); (b) the transmission of collagen films (F-0.5 h) after immersion in different pH solutions; transmission changes with collagen films (F-0.5 h) switching in solutions of (c) pH 1 and 7 (d) and pH 12 and 7 for different cycles; (e) cross-sectional images of collagen films (F-0.5 h) after immersion in aqueous solutions of pH 1, 7, and 12 for 12 h; (f) schematic showing the arrangements of collagen mesostructures in acidic, basic, and near-isoelectric point pH solutions (where the '+' and '-' signs represent cations and anions, respectively).

This optical switching behavior is governed by the ampholytic nature of collagen. Based on our previous report [43], collagen exhibits an isoelectric point (pI) near pH 6–8. Zeta potential measurements of the F-0.5 h confirm electrical neutrality at pH 6.5 (Figure S7). Near the pI, the loss of electrostatic repulsion promotes aggregation of collagen micro/nanofibers, forming a denser network that scatters light and reduces transmittance. In acidic or alkaline media (away from pI), the fibers acquire net positive or negative charges, respectively. Electrostatic repulsion expands the fibrous network, creating a more open structure that facilitates light transmission. Swelling further reduces optical scattering, thereby enhancing transparency. Consequently, film transparency is governed by both solution pH and the resultant structural state of the collagen network.

Based on these findings, we propose a mechanism for the pH-responsive optical behavior (Figure 4f). When the environmental pH deviates from the pI, like-charged collagen fibers repel each other, inducing swelling and increasing light transmission pathways. Near the pI, charge neutralization promotes fiber aggregation and network contraction, enhanc-

ing light scattering and reducing transmittance. Conversely, as the environmental pH approaches the pI, the collagen micro/nanofibers approach electrical neutrality, promoting their aggregation and entanglement. This results in reduced film swelling, fewer effective optical transmission pathways, and consequently, decreased light transmittance. The collagen micro/nanofibers, which preserve the native aggregated architecture of collagen, act as the fundamental structural units of the film. This structural integrity enables the film to retain excellent shape stability during cyclic soaking in acidic and alkaline solutions while supporting fully reversible optical switching. Therefore, the optical properties of the collagen film are dynamically regulated by the ambient pH, which governs the electrostatic state and spatial organization of the constituent collagen fibers.

3.5. Cytotoxicity Analysis

Excellent biosafety and cellular compatibility are essential prerequisites for biomaterials intended for direct contact with tissues, particularly in applications such as hemostatic dressings. To evaluate the biocompatibility of the collagen-based composite films, their effects on cellular behavior were examined after 24 h of co-culture (Figure 5). Cell morphology and viability were assessed via live/dead staining. As shown in Figure 5a, cells maintained a characteristic spindle-shaped morphology and were evenly distributed across all experimental groups, indicating normal cellular attachment and growth. Quantitative analysis via MTT assay (Figure 5b) confirmed the excellent biocompatibility of all collagen films, with cell viability exceeding 80% in all groups. Importantly, cells cultured with F-0.5 h and F-1 h films exhibited viability above 95%. Statistical analysis revealed that F-3 h ($p < 0.001$), F-2 h ($p < 0.05$), and F-0.5 h (though not significant numerically, showed a positive trend) all supported cell viability significantly better than the negative control. Furthermore, both commercial gelatin sponge (CGS, $p < 0.01$) and commercial chitosan dressings (CCD, $p < 0.05$) resulted in significantly lower cell viability compared to F-0.5 h. These results demonstrate that our collagen films are not only non-cytotoxic but also actively promote a more favorable cellular response than some commercial alternatives, supporting their potential for wound-healing applications.

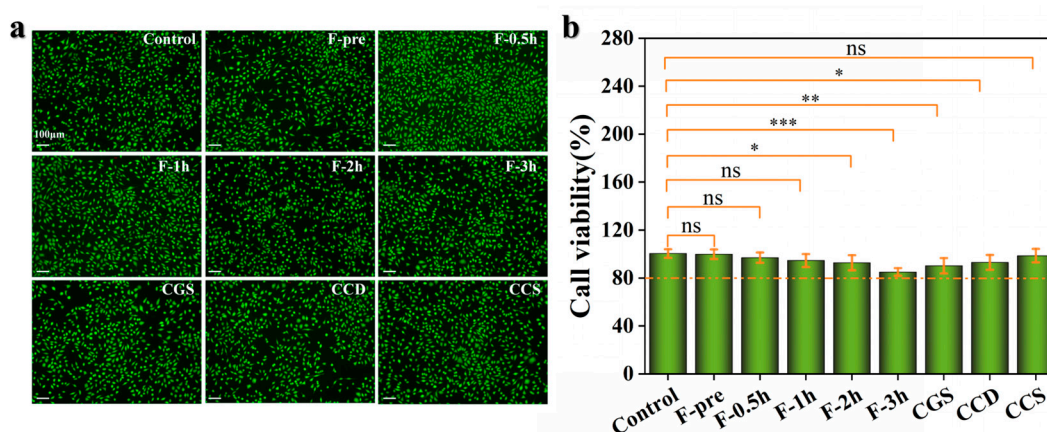


Figure 5. (a) Live/dead staining images of mouse fibroblasts (L-929) cultured for 24 h with different dressing materials; (b) cell survival rates of different materials. Data are presented as mean $\pm$ SD ($n = 6$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's test (ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control group).

3.6. Conductive Properties of SWNTs/CAs Paper

SWNTs were homogeneously dispersed into a collagen aggregate suspension and fabricated into freestanding composite paper through vacuum filtration. The resulting paper displayed a uniform black appearance, high mechanical flexibility, and notable

structural stability in aqueous environments (Figure 6a–d). Electrical characterization at room temperature confirmed that the composites exhibit tunable conductivity, which can be precisely modulated by varying the SWNT content (Figures 6e and S8). To evaluate the electromechanical response, the resistance of the composite paper was monitored during repeated folding–unfolding cycles (Figure 6f). Interestingly, the electrical resistance did not increase upon mechanical deformation over 500 consecutive cycles. Instead, it progressively decreased with cycling. This behavior can be attributed to a dynamic self-optimization process induced by folding. Under cyclic mechanical stress, initially loose or suboptimal conductive pathways are progressively reorganized, slid, and compacted, leading to the formation of more efficient electron transport networks and thus improved conductivity.

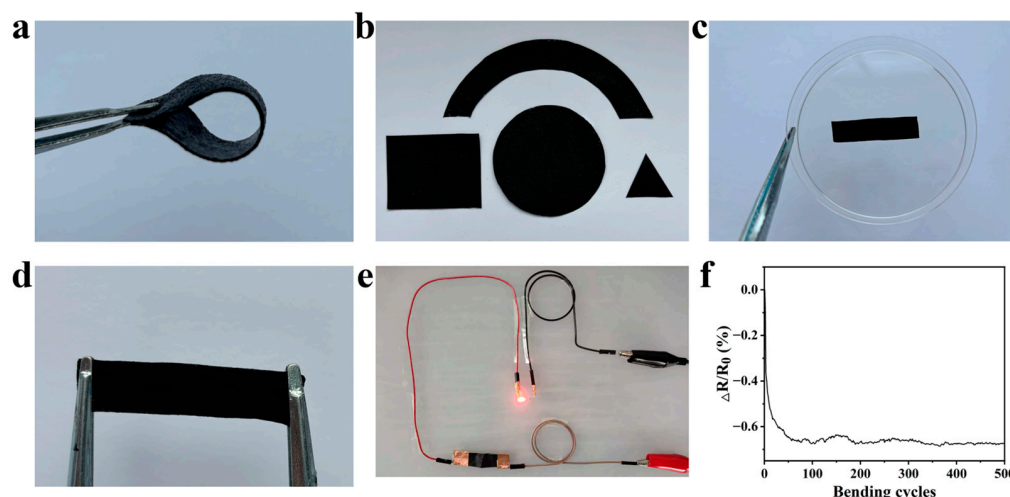


Figure 6. (a) Digital photographs of the SWNT/collagen paper; (b) SWNT/collagen paper of different shapes; (c) SWNT/collagen paper after soaking in water for 2 h; (d) SWNT/collagen paper after 500 cycles of folding–unfolding treatment; (e) digital photographs of the conductivity experiment of the SWNT/collagen paper; (f) electrical-resistance variation in the SWNT/collagen paper about cyclic (500 cycles) folding–unfolding.

Notably, the composite films retained stable electrical performance and high recovery over 500 mechanical cycles, which can be attributed to the inherent elasticity and resilience of the collagen fiber network. This network effectively accommodates mechanical strain without structural degradation, enabling consistent electromechanical response under repeated deformation. The combination of environmental stability, mechanical durability, and a sustainable biopolymer matrix underscores the potential of SWNT-reinforced collagen films as promising material candidates for next-generation flexible and wearable electronics.

4. Conclusions

In summary, this study presents a controlled exfoliation strategy for natural bovine tendon collagen, guided by a mechanistic understanding of the non-covalent interactions within its hierarchical architecture. By employing the ionic liquid [AMIM]Cl at 60 °C, we produced fibrous collagen aggregates with tunable mesoscale dimensions while largely preserving their native supramolecular order. Molecular dynamics simulations elucidated the synergistic exfoliation mechanism, wherein AMIM⁺ cations disrupt hydrogen bonds and Cl[−] anions interfere with both hydrogen bonding and electrostatic interactions. Collagen films fabricated from these optimized building blocks (F-0.5 h, 60 °C) exhibited a remarkable combination of properties, including robust mechanical strength (44.44 MPa dry, $p < 0.01$; 1.49 MPa hydrated, $p < 0.001$), high optical transparency, reversible pH-responsive behavior, and excellent biocompatibility. Furthermore, the integration of

single-walled carbon nanotubes yielded conductive composites with stable electromechanical performance. This study not only provides an innovative and efficient pathway for the precision processing of collagen but also advances the fundamental design principles for high-performance, biomimetic materials by establishing clear structure–property–function relationships, thereby offering new avenues for advanced biomedical and flexible electronic applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polym18050595/s1>, Figure S1: Mechanical properties of dry-state structural units of CAs prepared by treating bovine tendon collagen with [AMIM]Cl for varying durations at a constant temperature; Figure S2: Mechanical properties of hydrated-state structural units of CAs prepared by treating bovine tendon collagen with [AMIM]Cl for varying durations at a constant temperature; Figure S3: Mechanical properties of dry-state structural units of CAs fabricated from bovine tendon collagen treated with [AMIM]Cl at varying temperatures under a fixed processing time; Figure S4: Mechanical properties of hydrated-state structural units of CAs fabricated from bovine tendon collagen treated with [AMIM]Cl at varying temperatures under a fixed processing time; Figure S5: Transmission and haze changes with collagen film (F-0.5 h) switching in solutions of pH 1 and 7 and 12; Figure S6: Digital photographs of collagen films (F-0.5 h) soaked in different organic solvents for 12 h; Figure S7: Zeta potentials of the collagen film (F-0.5 h) at different pH levels; Figure S8: Photos of conductivity of SWNT/collagen films with different proportions of SWNT; Table S1: The mechanical properties of dry and hydrated collagen films; Table S2: Mechanical properties compared with other collagen materials.

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