

Tough Polyion Complex Hydrogel Films of Natural Polysaccharides*

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Abstract The developments of tough hydrogels in recent years have greatly expanded the applications of hydrogels as structural materials. However, most of the tough hydrogels are made of synthetic polymers. To develop biopolymer-based tough hydrogels has both fundamental and practical significances. Here we report a series of polysaccharides-based tough hydrogel films prepared by polyion complexation and solvent evaporation of chondroitin sulfate (CS) and protonated chitosan (CHT) solutions with different weight ratios. The obtained CS/CHT gel films with thickness of 40–80 μm and water content of 66 wt%–81 wt% possess excellent mechanical properties, with tensile breaking stress and breaking strain being 0.4–3 MPa and 160%–320%, respectively. We found that in the mixture solutions there are large amounts of excess CHT in terms of charges; after swelling the films in water, the acetic acid, which is used to protonate the amino groups of CHT, diffuses out of the gel matrix, enhancing the intermolecular interactions between CHT molecules and thus improving the mechanical properties of gel films, besides the ionic bonds between CS and CHT. Antimicrobial tests also showed that the gel films with low weight ratio of CS to CHT, corresponding to the case with excess CHT, have evident antimicrobial effect. These CS/CHT gel films with good mechanical properties and antimicrobial effect should extend the applications of hydrogels in biomedical fields.

Keywords Polysaccharides; Polyion complexes; Tough hydrogels; Chitosan; Chondroitin sulfate

INTRODUCTION

Hydrogels have gained considerable interests due to their similarities to biological tissues, smart responses to external stimuli, and promising applications in tissue engineering, drug delivery, soft actuators, *etc*^[1–4]. Owing to the low polymer content and heterogeneous network structure, hydrogels are usually mechanically weak, which greatly limits their applications as load-bearing materials^[5]. To address this issue, scientists have developed various strategies to prepare hydrogels with significantly improved mechanical performances^[6–16]. For example, Gong *et al.* have developed double-network (DN) hydrogels with extremely high toughness (tensile breaking stress σ_b of 1–10 MPa, breaking strain ε_b of 1000%–2000%, and fracture energy T of 100–4000 J/m²)^[9]. These DN gels consist of a highly crosslinked polyelectrolyte network and a loosely crosslinked neutral polymer network; under loading, the brittle polyelectrolyte network will be sacrificially broken prior to the ductile

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polymer network and dissipate energy, thereby toughening the integrated hydrogels^[5]. Based on this principle, other tough DN hydrogels with self-recovery capacities were developed by using physically crosslinked polymers as the first network^[12–14].

Besides the hydrogels with permanent or quasi-permanent network structures, supramolecular polymer hydrogels with dynamic noncovalent bonds have advantages in adaptation, processability, *etc*^[17–25]. For example, Dai *et al.* have prepared tough physical hydrogels ($\sigma_b \sim 1.2$ MPa, $\epsilon_b \sim 1500\%$) by forming stable hydrogen bonds between glycine groups^[17]. Due to the dynamic nature of hydrogen bonds, these physical gels show thermoplastic behavior and self-healing ability. Sun *et al.*^[18] and Luo *et al.*^[19] have developed tough physical polyampholyte (PA) hydrogels ($\sigma_b \sim 2$ MPa, $\epsilon_b \sim 700\%$) and polyion complex (PIC) hydrogels ($\sigma_b \sim 4$ MPa, $\epsilon_b \sim 700\%$), in which the polyampholytes or polyelectrolytes are physically crosslinked by ionic bonds^[18, 19]. The excellent mechanical properties of these gels are rooted in the wide strength distribution of ionic bond clusters: the strong clusters serve as permanent crosslinks to maintain the network integrity, whereas the relatively weak clusters behave as reversible sacrificial bonds to dissipate the energy^[16]. Moreover, due to the electrostatic shielding effect, the strength of ionic bonds can be effectively tailored by using saline solutions, enabling processing these hydrogels into desired shapes^[26–29].

The above hydrogels exhibit remarkable mechanical performances, which should be ideal candidate materials for the design of artificial cartilages and tendons. Compared to synthetic polymers, biopolymers are sustainable and renewable materials with better biocompatibility and biodegradability^[30]. It is of both fundamental and practical significances to develop biopolymer-based tough hydrogels. In recent years, several groups have made great progress in this field^[31–35]. For example, Nakayama *et al.* have applied bacterial cellulose and gelatin to develop tough DN hydrogels; the breaking stress in tension can reach up to 3.8 MPa^[31]. Costa *et al.* have prepared tough hydrogels *via* the complexation of oppositely charged chitosan and alginate^[34]. The gels have σ_b of 0.5–1.9 MPa and ϵ_b of 30%–80%; they are also good scaffolds for cell culture. This approach should be applicable to other systems of charged biopolymers in developing tough hydrogels.

The mechanical properties of PIC hydrogels are associated with the strength of ionic bonds. Recent research effort has revealed that the strength of ionic bonds depends on the type of charges; sulfonates associate more strongly than carboxylates with positively charged groups of polymers^[36]. This principle should also be applied to the systems of charged biopolymers. Therefore, we envision to develop PIC hydrogels with improved mechanical properties by using biopolymers with sulfonate groups instead of the one with carboxylate groups.

Here we report polysaccharides-based tough hydrogel films prepared by the complexation of chitosan (CHT) and chondroitin sulfate (CS) with different weight/charge ratios. The obtained transparent hydrogels have water content of 66 wt%–81 wt% and exhibit excellent mechanical performances with σ_b of 0.4–3 MPa and ϵ_b of 160%–320%. However, the best mechanical properties of CS/CHT gels do not appear near the charge balance point, indicating that the toughening of gels is also influenced by other factors besides ionic bonding, which is rationally discussed. This work should merit the development of other tough hydrogels of biopolymers.

EXPERIMENTAL

Materials

Chondroitin 4-sulfate sodium salt (CS) was used as purchased from Macklin reagent; chitosan (CHT; medium molecular weight, deacetylation degree: 75%–85%) was used as received from Sigma-Aldrich. Acetic acid and NaCl were received from Sinopharm Chemical Reagent Co., Ltd. Millipore deionized water was used in all the experiments.

Preparation of CS/CHT Hydrogel Films

CHT and CS solutions with prescribed concentrations were prepared with the aid of dilute acetic acid. The CHT solution with a concentration of 2 mg/mL was prepared by dissolving the prescribed amount of CHT in aqueous solution of 1 wt% acetic acid. The CS solutions with concentrations of 1.4, 1.6, 1.8, 2.0 and 2.2 mg/mL were prepared in a similar way. Then, CHT and CS solutions with the equal volume (100 mL) were slowly dripped

into a beaker containing 50 mL dilute acetic acid (1 wt%) that was intensively stirred by a magnetic stirrer. The mixture of CHT and CS solutions was transferred into a petridish (diameter: 150 mm) and dried at 80 °C for 48 h, resulting in a compact film. The obtained sample was swelled in a large amount of water to remove the counterions of PIC and residual acetic acid before achieving the equilibrium state of hydrogel films.

Characterizations

Zeta (ζ)-potential measurements

Zeta (ζ)-potentials of CHT, CS, and acetic acid solutions were measured on a Malvern ZET-3000HS apparatus.

Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectra of solid CS, CHT, and dry CS/CHT films dispersed in KBr were recorded by using a BRUKER VECTOR 22 FTIR spectrometer. All the spectra were obtained at ambient temperature with a resolution of 2 cm⁻¹ in the range of 4000–400 cm⁻¹.

Scanning electron microscopy (SEM)

The microstructure of films was observed by Hitachi S4800 field emission scanning electron microscope (SEM). The hydrogel films prepared at different weight ratios of CS/CHT were freeze-dried and cryogenically fractured in liquid nitrogen. All the samples were coated with a thin layer of gold by the sputtering method before SEM characterization.

Tensile test

The tensile tests of PIC gels were performed on a commercial tensile tester equipped with a load sensor of 50 N (Instron 3343 Tester) at room temperature. The samples were cut from gel films into dumbbell-shape with initial gauge length of 12 mm and width of 2 mm. The thickness of gel films was examined by a digital stereo microscope (VH-Z100R, Keyence Corporation). The stretch velocity was 100 mm/min, and the nominal stress and strain were recorded. The elastic modulus, E , was calculated from the initial slopes (within the strain of 8%) of the stress-strain curves.

Antimicrobial test

In vitro antimicrobial tests were carried out to quantitatively evaluate the antimicrobial activity of the CS/CHT gel films. The hydrogel films were placed in 24-well plates. 1 μ L of bacterial suspension of *Staphylococcus aureus* in phosphate buffered saline (PBS) with an initial cell concentration of 1×10^7 cells/mL was pipetted onto the surface of gel films (5 mm $\times$ 5 mm). The well plates were kept in a 25 °C water bath to ensure the wet incubation atmosphere. At a predetermined time, the samples were sonicated for 1 min to separate the bacteria from the gel films. The bacterial suspension was collected by pipettes and serially diluted. 0.1 mL aliquots of the serially diluted suspension were placed onto the triplicate solid agar using the spread plate method. After incubating the plates at 37 °C for 24 h, the number of viable cells (colonies) was counted, and the bacterial survival ratio was defined as the ratio of the mean number of colonies in the experimental group to that in the control group. The killing efficiency of gel films was calculated by 100% minus the bacterial survival ratio.

RESULTS AND DISCUSSION

Mixture of CS and CHT Solutions for Film Fabrications

The molecular structures of CS and CHT are shown in Fig. 1(a). As shown in Fig. 1(b), the polysaccharides-based hydrogel films were fabricated by complexation of CS and CHT solutions, casting/solvent evaporation, and reswelling the films in water to achieve the equilibrium state. Mixing of bulk solutions of oppositely charged biopolymers often resulted in inhomogeneous precipitate. To improve the homogeneity of polyion complexation, the complexation rate was slowed down by simultaneously dripping the CS and CHT solutions into a beaker^[27]. The appearance of resulting mixtures varies with the weight ratio, w_r , of CS to CHT (Fig. 2). When $0.6 \leq w_r \leq 0.8$, the mixtures are translucent and stable solutions; however, when $w_r > 0.8$, some precipitates appear and form sediment during the storage. When $w_r \geq 1.2$, the complexation produces a large amount of precipitates, and the

solution phase becomes transparent. This result indicates that more charged biopolymers participate in the complexation at high w_r .

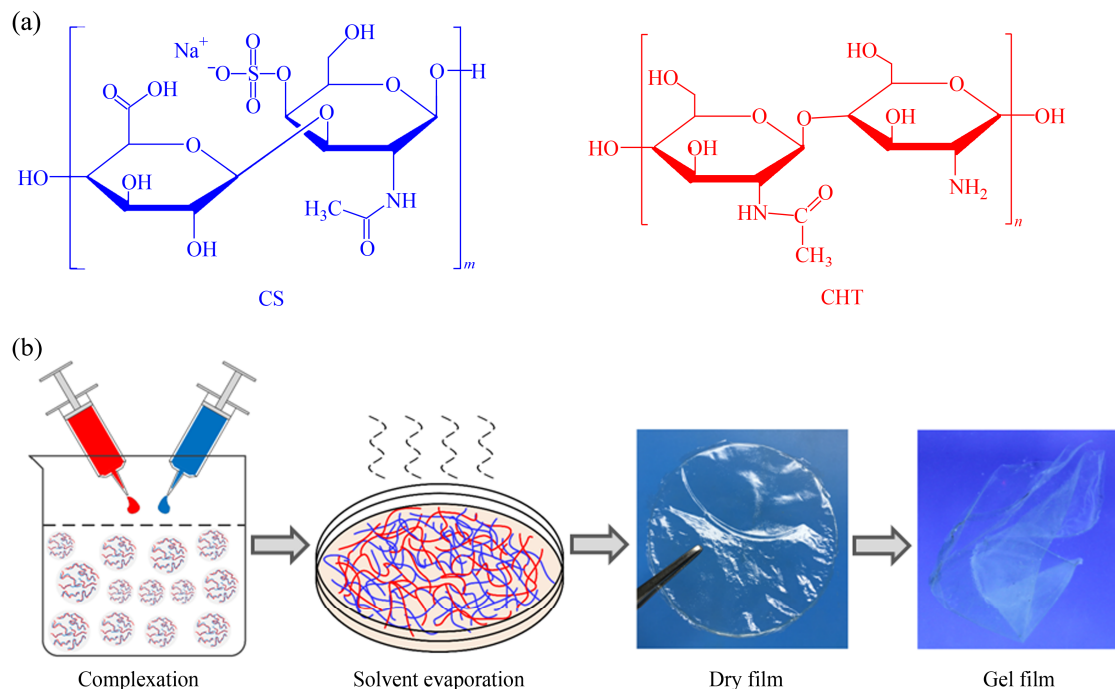


Fig. 1 (a) Chemical structures of CS and CHT; (b) Schematic for the preparation of CS/CHT hydrogel films

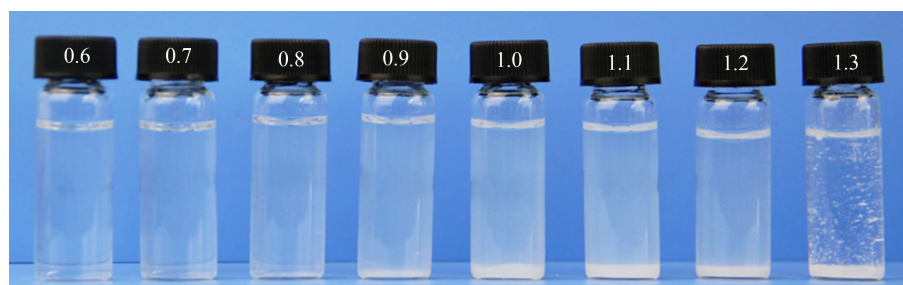


Fig. 2 Photo of the mixtures of CS and CHT solutions with different weight ratios of CS to CHT, w_r . The samples were stored for 24 h.

The different states of the mixtures should be related to the charge ratio of CS to CHT. Zeta (ζ) potentials of the solutions were measured before and after the mixing to study the complexation process. As listed in Table 1, the ζ -potential of CHT solution (2.0 mg/mL) is 70.1 mV, and the ζ -potential of CS solutions is approximate -25 mV, which only moderately changes with concentration (1.2–2.6 mg/mL). After mixing CS and CHT solutions with the equivalent volume, the ζ -potential decreases with the increase of w_r ; ζ -potentials of the mixtures with w_r of 0.6 and 1.3 are 46.3 and 14.0 mV, respectively. It should be noted that the ζ -potential gradually decreases from 46.3 mV to 29.2 mV, when w_r increases from 0.6 to 1.2, yet it dramatically decreases to 14.0 mV when w_r increases further to 1.3. The ζ -potential (14.0 mV) of the mixture with w_r of 1.3 is close to that of 1 wt% acetic acid solution, indicating that the mixture of CS and CHT is close to the charge balance point. These results are consistent with the change in the appearance of the mixtures with different w_r .

Table 1 Zeta potentials of CS and CHT solutions and their mixtures with different concentrations

Sample	Zeta potential (mV)
1 wt% acetic acid solution	19.7 ± 1.9
2 mg/mL CHT solution	70.1 ± 2.2
1.2 mg/mL CS solution	-26.5 ± 1.3
1.4 mg/mL CS solution	-25.6 ± 1.2
1.6 mg/mL CS solution	-23.7 ± 1.2
1.8 mg/mL CS solution	-23.8 ± 1.5
2.0 mg/mL CS solution	-24.3 ± 1.2
2.2 mg/mL CS solution	-23.2 ± 1.5
2.4 mg/mL CS solution	-27.2 ± 0.7
2.6 mg/mL CS solution	-27.1 ± 0.7
Mixture of 1.2 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 0.6$)	46.3 ± 0.1
Mixture of 1.4 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 0.7$)	41.6 ± 1.2
Mixture of 1.6 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 0.8$)	40.4 ± 0.9
Mixture of 1.8 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 0.9$)	36.4 ± 1.7
Mixture of 2.0 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 1.0$)	34.2 ± 1.0
Mixture of 2.2 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 1.1$)	33.0 ± 1.6
Mixture of 2.4 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 1.2$)	29.2 ± 0.6
Mixture of 2.6 mg/mL CS and 2 mg/mL CHT solutions ($w_r = 1.3$)	14.0 ± 1.5

Structural Features of CS/CHT Hydrogel Films

The mixture of CS and CHT solutions was used for the preparation of hydrogel films, according to the procedure in Fig. 1(b). The obtained gel films were transparent with the thickness of 40–80 μm and water content of 66 wt%–81 wt% (Fig. 3). The formation of these robust hydrogels should be associated with the noncovalent interactions between CS and CHT through the functional groups, which were characterized by FTIR spectroscopy (Fig. 4). In the spectrum of CS, the peak at 1253 cm^{-1} is the characteristic band of $\text{S}=\text{O}$ (SO_4) bond; the peaks at 1414 and 1382 cm^{-1} are ascribed to the coupling of $\text{C}-\text{O}$ stretch vibration and $\text{O}-\text{H}$ variable-angle vibration, indicating the existence of carboxyl group. In the spectrum of CHT, the peaks at 1654 and 1592 cm^{-1} correspond to amide I (CONH_2) and amide II (NH_2 group), respectively^[37]. In comparison, in the spectrum of CS/CHT, the peak at 1654 cm^{-1} (CONH_2) in the spectrum of CHT shifts to 1639 cm^{-1} , and the peak at 1240 cm^{-1} ($\text{S}=\text{O}$) in the spectrum of CS shifts to 1223 cm^{-1} ; the peaks of coupled $\text{C}-\text{O}$ stretch vibration and $\text{O}-\text{H}$ variable-angle vibration of carboxyl group of CS are evidently weakened. These variations indicate the status of ionic bonding between sulfate and carboxylate groups of CS and protonated amino groups of CHT^[38, 39], which accounts for the formation of polyion complexes and precipitates after mixing the solutions of CS and CHT.

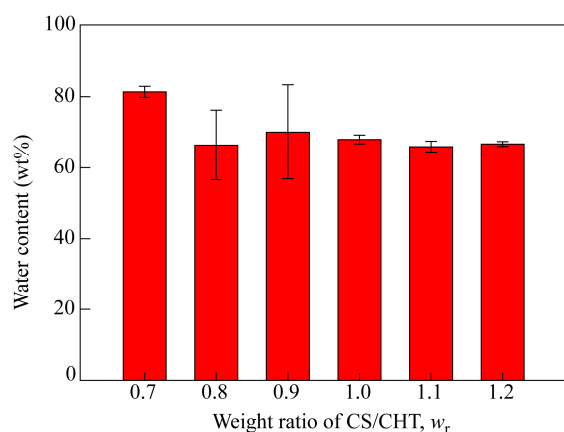


Fig. 3 Water content of CS/CHT hydrogel films with different weight ratios of CS to CHT, w_r . Error bars represent the standard deviation of the mean.

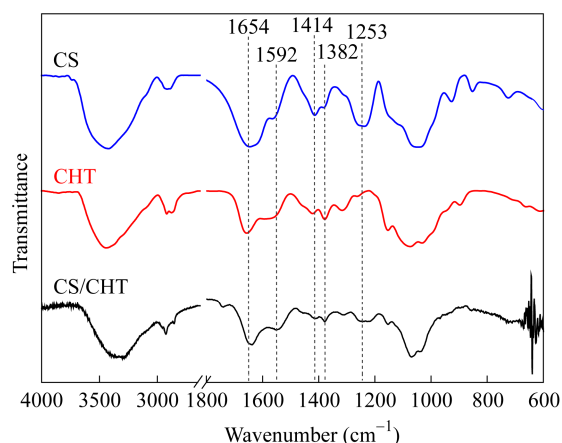


Fig. 4 FTIR spectra of CS, CHT, and CS/CHT films (w_r value of the films is 1.0.)

The microstructure of CS/CHT gel films was investigated by SEM. As shown in Fig. 5, the microstructure of PIC gel films changes with w_r . At low w_r ($w_r = 0.7$), the film shows a condensed microstructure (Fig. 5a). As w_r increases ($w_r = 0.8, 0.9$), the samples show a typical microporous structure (Figs. 5b and 5c). When w_r increases further to 1.0 and 1.1, the films exhibit compact microstructure again; nanometer-sized grains are nested in the network, and the size and density increase with w_r (Figs. 5d and 5e). The structural variation of CS/CHT gel films is associated with the formation of aggregates during the complexation process. At low w_r , the major component of the film is CHT and the complexation between CS and CHT is incomplete due to the severe excess of positive charges. At high w_r , the complexation is well proceeded, which produces compact aggregates. The nanometer-sized aggregates are incorporated into the gel matrix. Since the charge balance point appears at $w_r = 1.3$, the amount of CHT in the mixture solutions is relatively excess in terms of charges. These uncomplexed CHT molecules will bind each other due to the strong hydrogen bonding after dialyzing out the acetic acid which is used to protonate the amine groups of CHT^[40]. At relatively high w_r , more CHT molecules form PIC with CS and the amount of uncomplexed CHT decreases; therefore, the effect of hydrogen bonding between CHT molecules is weakened and that of electrostatic interaction between CS and CHT molecules is enhanced, leading to the formation of compact PIC aggregates maintained in the gel matrix.

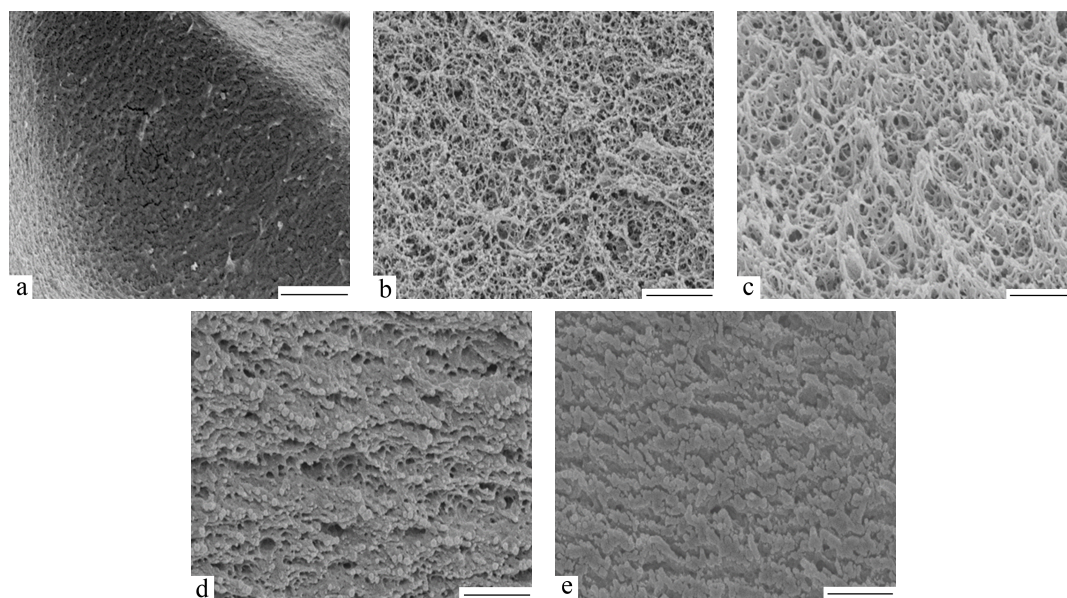


Fig. 5 SEM images of the cross-section of hydrogel films with different weight ratios of CS to CHT, w_r : (a) $w_r = 0.7$; (b) $w_r = 0.8$; (c) $w_r = 0.9$; (d) $w_r = 1.0$; (e) $w_r = 1.1$ (Scale bar: 1 μm ; accelerating voltage: 3 kV.)

Mechanical Properties of CS/CHT Gel Films

The obtained CS/CHT hydrogel films with thickness of 40–80 μm are mechanically robust except the cases with w_r of 0.6 and 1.3, which are too weak to be tested. As shown in Fig. 6(a), the gels ($0.7 \leq w_r \leq 1.2$) show excellent mechanical performances; the tensile breaking stress σ_b , breaking strain ϵ_b , and Young's modulus E are 0.4–3.0 MPa, 160%–310%, and 0.06–0.25 MPa, respectively (Fig. 6b). As expected, w_r remarkably influenced the mechanical properties of gel films. The gel with w_r of 0.8 has the maximum σ_b and E , which decrease with further increase in w_r . However, the maximum ϵ_b appears in the gel with w_r of 1.1, and the deviation of this value leads to a decrease of ϵ_b . We should note that maximum σ_b and E of the gels do not appear near the charge balance ($w_r \sim 1.2$ –1.3), and the gels with large deviations from the charge balance still exhibit good mechanical properties, different from other PIC hydrogels^[19, 20]. This special behavior originates from the multiple intermolecular interactions and heterogeneous microstructure of the gels. Ionic bonds between CS and CHT contribute to the extensibility, whereas the strong hydrogen bonds between CHT molecules account for high

strength and modulus. As a result, the gel with proper excess of CHT (corresponding to w_r of 0.8) has balanced ionic and hydrogen bonds and thus shows the highest σ_b and E . The gel with more ionic bonds (w_r of 1.1) has the highest ϵ_b . The homogeneity of the microstructure should also influence the mechanical properties of the gel films; the existence of large compact grains in gel matrix generally leads to the decrease in strength.

Cyclic tensile tests were also performed to investigate the self-recovery behavior of the gel films^[16, 18]. As shown in Fig. 6(c), only moderate hysteresis and residual strain were observed in the first loading-unloading cycle with a maximum strain of 120%, indicating the good resilience of CS/CHT hydrogels. Subsequent loading-unloading path (without waiting time between consecutive cycles) produces a loop approaching the first cycle, indicating the quick self-recovery behavior of the gel; the corresponding hysteresis ratio, calculated by the areal ratio of the second hysteresis loop to the first, is 67% (Fig. 6d). With increased waiting time after the first loading-unloading, the hysteresis loop gradually overlaps with the first one. The hysteresis ratio increases to 95% after a waiting time of 30 min, indicating the good self-recovery ability of these hydrogels, which is important for the application of the material under dynamic cyclic loading.

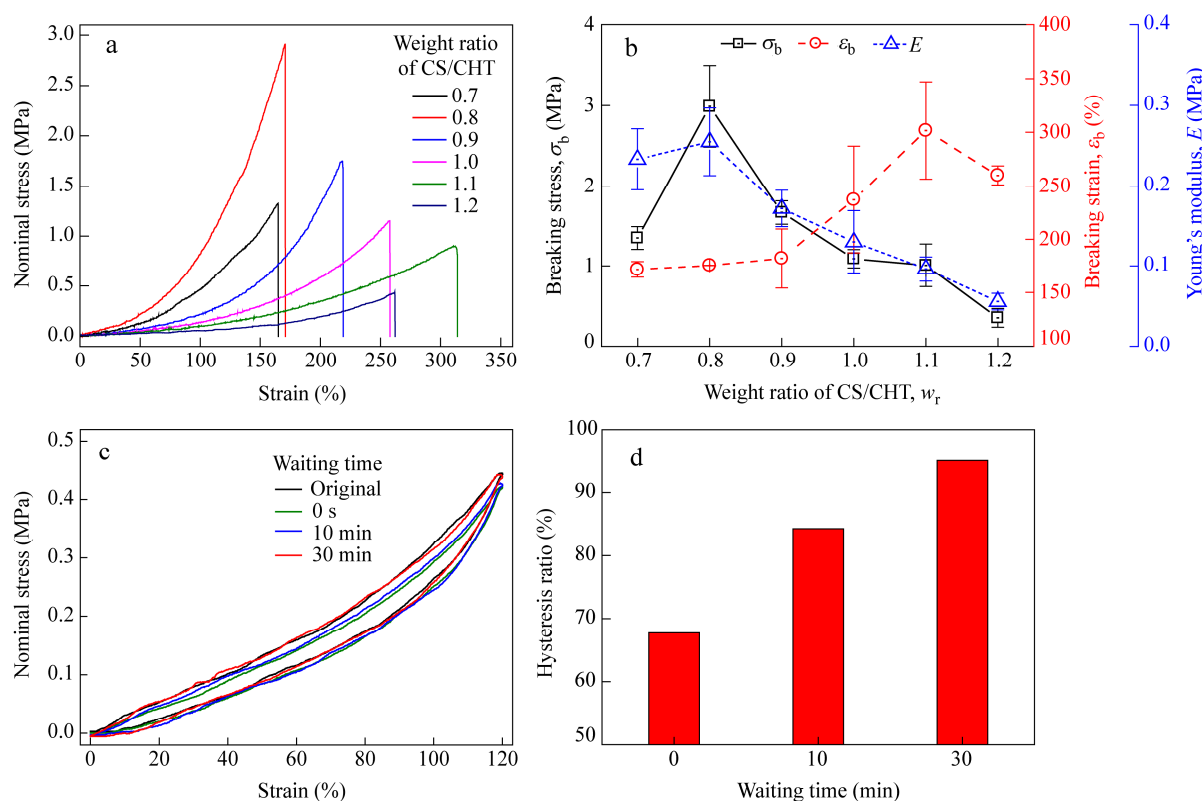


Fig. 6 (a) Tensile stress-strain curves and (b) measured mechanical properties of hydrogel films with different w_r (Error bars represent the standard deviation of the mean.); (c) Cyclic tensile stress-strain curves and (d) hysteresis ratio of the gel ($w_r = 0.9$) after different waiting time

As we stated before, the high toughness of CS/CHT hydrogels is ascribed to the synergism of the ionic bonds between oppositely charged biopolymers and the hydrogen bonds between CHT molecules. Therefore, tuning the strength of these noncovalent bonds should result in a variation of the mechanical properties of CS/CHT gels. As shown in Figs. 7(a) and 7(b), swelling of the gels in saline solution leads to decrease in σ_b , yet ϵ_b and E remain unaffected. This is different from other synthetic PIC hydrogels^[18, 19]. On the other hand, the gels swelled in aqueous solutions of acetic acid exhibit slight expansion in volume and dramatic decrease in σ_b and E (Figs. 7c and 7d). This is because of the protonation of amino groups of CHT in acidic solution, which

weakens the hydrogen bonding between CHT molecules. In the presence of both acetic acid and concentrated NaCl, the gels still maintain considerable toughness, much better than those gels in acetic acid solutions. These results further confirm the existence of multiple noncovalent interactions between the biopolymers, which accounts for the high toughness of CS/CHT hydrogels though the essential synergism is not yet clear.

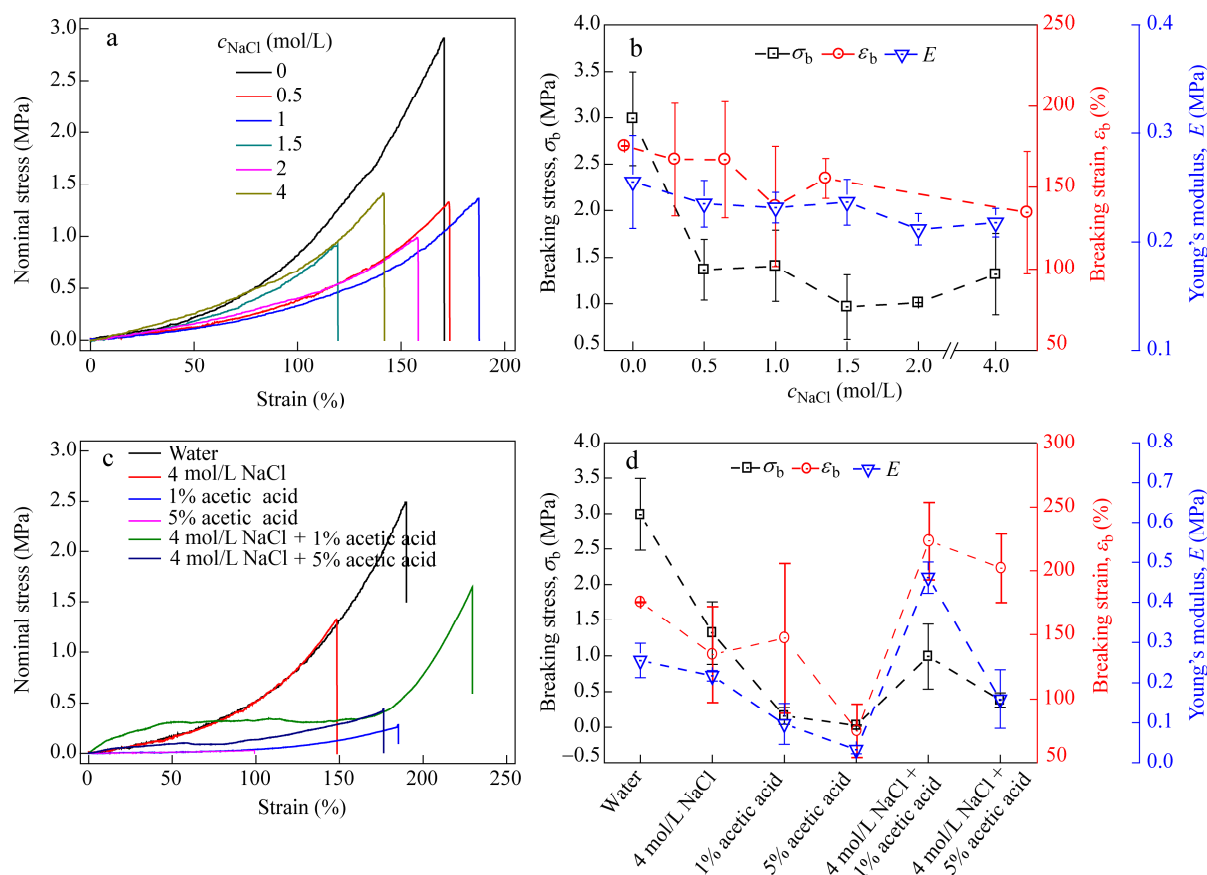


Fig. 7 (a) Tensile stress-strain curves and (b) corresponding mechanical properties of hydrogel films ($w_r = 0.9$) swelled in the saline solutions with different concentrations of NaCl, c_{NaCl} ; (c) Tensile stress-strain curves and (d) corresponding mechanical properties of the hydrogel films ($w_r = 0.9$) swelled in saline solution ($c_{\text{NaCl}} = 4$ mol/L), acetic acid solution (1 wt% or 5 wt%) or their combinations (Error bars represent the standard deviation of the mean.)

Antimicrobial Properties of CS/CHT Hydrogels

It is well known that CHT has excellent antimicrobial activity because of the amine groups with the ability to contact with negatively charged bacteria^[41]. The CS/CHT hydrogel films with considerable amounts of CHT should have antimicrobial properties as well, which are important for the practical applications of hydrogel films such as wound dressings. As expected, the gel films with w_r of 0.8 and 0.9 exhibit pronounced antimicrobial effect on the Gram-positive bacteria, *Staphylococcus aureus* (Fig. 8); the killing efficiencies of gel films, as calculated by 100% minus bacterial survival ratio, are 97% and 90%, respectively. However, the gel film with w_r of 1.0 only shows weak antimicrobial effect with the killing efficiency being 5%. The gels with $w_r \geq 1.1$ have no apparent antimicrobial effect. These results indicate that the formation of ionic bonds between CS and CHT decreases the antimicrobial effect of CHT; only the gels with $w_r < 1.0$ have sufficient excess of CHT, as confirmed by the ζ -potential measurements, and thus have an evident antimicrobial effect. However, in practical applications, other antimicrobial agents together with more functional materials can be easily incorporated into these gel films during the fabrication process.

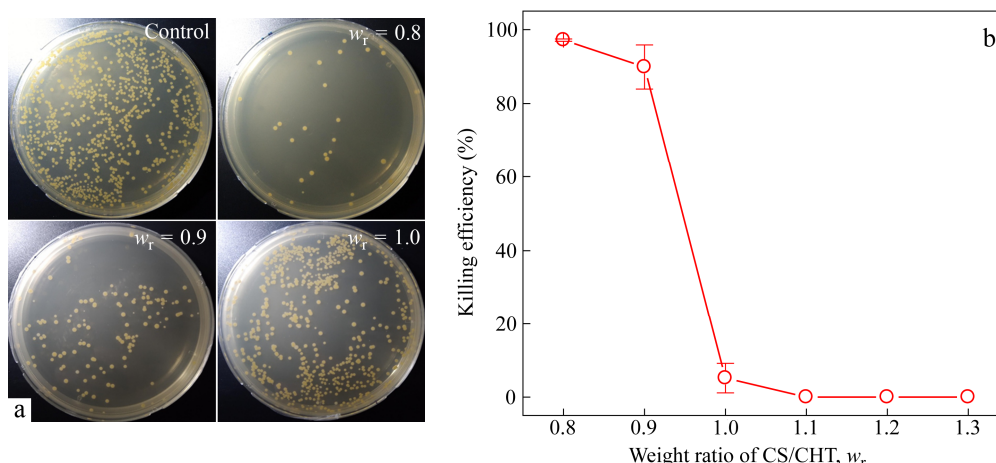


Fig. 8 (a) Photos of colony-forming units on Luria-Bertani agar plate of *Staphylococcus aureus* suspension incubated on gel films with different w_r and (b) the corresponding killing efficiency (Error bars represent the standard deviation of the mean.)

CONCLUSIONS

We have developed a facile approach to prepare tough and resilient hydrogel films by complexation and solvent evaporation of chondroitin sulfate (CS) and protonated chitosan (CHT) solutions. The transparent CS/CHT gel films with water content of 66 wt%–81 wt% and thickness of 40–80 μm possess excellent mechanical properties with the tensile breaking stress σ_b of 0.4–3 MPa and breaking strain ε_b of 160%–320%, superior to most existing biopolymer-based hydrogels. The mechanical properties and microstructure of the gel films change with the weight ratio (w_r) of CS/CHT, yet the highest strength and modulus of the gels do not appear near the charge balance point. When $0.8 \leq w_r \leq 1.2$, there are excess CHT molecules that do not form polyion complex with CS; after swelling the films in water, the acetic acid diffuses out of the gel matrix, resulting in the suppression of protonation in amino groups of CHT and the enhancement of hydrogen bonds between CHT molecules, which improve the resultant mechanical properties of gel films, besides the ionic bonds between CS and CHT. Due to the multiple noncovalent interactions between CS and CHT molecules, the mechanical properties of these gels are insensitive to concentrated saline solutions, compared with other polyion complex hydrogels. Antimicrobial tests also show that the gel films with low w_r , corresponding to the sufficient excess of CHT, have pronounced antimicrobial effect. These CS/CHT gel films with good mechanical properties and antimicrobial effect should find applications in biomedical fields such as wound dressings. The present approach should be applicable to other systems of biopolymers for the design of more sophisticated soft materials.

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