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Rapid in situ cross-linking of hydrogel adhesives based on thiol-grafted bio-inspired catechol-conjugated chitosan

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Abstract

In this paper, a novel chitosan derivative, thiol-grafting bio-inspired catechol-conjugated chitosan was synthesized. The chemical structure of the synthesized catechol-conjugated chitosan was verified by ^{1}H NMR, and its contents of thiol group and catechol group were determined by UV-vis spectrum. Four percent of catechol-conjugated chitosan aqueous solution could form hydrogels rapidly in situ in 1 min or less with the addition of sodium periodate. Rheological studies showed that the mechanical properties depend on the concentrations of catechol-conjugated chitosan and the molar ratio of sodium periodate to catechol groups. Additionally, the adhesive properties of the resulting adhesives were evaluated, and the adhesion strength of obtained adhesives was as high as 50 kPa because of the complex and multiple interactions, especially the anti-oxidation mechanism of thiol group. The in vitro cytotoxicity assays demonstrated an excellent biocompatibility of the catechol-conjugated chitosan hydrogels. Benefiting from the in situ fast cured, desired mechanical strength, biocompatibility and relatively high adhesion performance, these properties suggested that catechol-conjugated chitosan hydrogel adhesives have potential applications as tissue adhesive for soft tissues.

Keywords

Chitosan derivative, catechol, mussel adhesive proteins, hydrogel, bioadhesive

Introduction

Tissue adhesives or sealants have been shown to be a practical and effective technique for wound closure and bleeding control.[1,2] Tissue adhesives can effectively deal with the problems that the traditional mechanical wound closure devices (e.g. sutures, staples, tacks) would create, i.e. new trauma and unable to reconnect soft tissues with low cohesive properties.[3,4] Therefore, many of efforts are being invested into the development of new generation of surgical adhesives instead of existing adhesives, for example, fibrin sealants[5,6] and cyanoacrylate,[7,9] and traditional wound closure devices.[4,10]

Marine mussels secrete exceptional underwater adhesive proteins – mussel foot proteins (mfps) or mussel adhesive proteins (MAPs) that enable them to firmly adhere to various matrix surfaces tightly under wet and salty environment.[11,12] Latest studies have reported that at least six mfps (mfp-1 to mfp-6) and two collagen-like proteins (Col-D and Col-P) have been identified from the adhesive plaques of mussel.[13,14] Mfps contains a high content of unusual unique amino acid 3-(3, 4-dihydroxyphenyl)-L-alanine (DOPA) that is believed to lend outstanding adhesive performance to MAPs. Especially, the mfps-3, 5, 6 are predominantly found at plaque–substrate interface, which contributes to strong, wet adhesion of the mussels. Of the three proteins, the DOPA contents of mfps-3, 5 are 20 and 30 mole%, respectively, and the DOPA content of mfp-6 is 3 mole%, which is much less than other mfps.[15] However, mfp-6 has high cysteine content (11 mole %), which have proved to bestowed benefits on the adhesion. The high level of thiols leads to the ability to control the redox chemistry of DOPA residues present in other mfps (mfp-3, mfp-5, and so on).[16,17]

Chitosan is a partially N-deacetylated product of chitin which is the second abundant polysaccharide

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after cellulose and widely distributed in nature.[18] Chitosan has been used as biomedical material for its advantages of biodegradable, non-toxic, accelerating wound healing antibacterial, and superior tissue adhesive properties.[19] Bio-inspired catechol-conjugated chitosan (CSDS) have been studied for their excellent solubility in neutral pH and strong adhesion on tissue surfaces.[20] Furthermore, catechol-modified chitosan is biocompatible and shows enhanced mechanical properties.

Hydrogel adhesive can achieve a significant level of adhesion by covalently bonding to specific tissues. However, the adhesive strength depends on the presence of tissue surface biomolecules with specific functional groups (e.g. NH_2, SH, OH, COOH).[2,21] Catechol group of DOPA is readily oxidized to dopaquinone form with reduced adhesive strength. Yu et al.[16] reported that the thiol-rich mfp-6 restores DOPA to enhance the adhesion properties by coupling the oxidation of thiols to dopaquinone reduction. In this paper, we have synthesized a thiol-grafted bio-inspired CSDS by carbodiimide coupling method. CSDS was modified with thiol group and catechol group to improve the adhesion. The chemical structure of the synthesized CSDS was verified by ^{1}H NMR and its contents of thiol group and catechol group were determined by using UV-vis spectrum. CSDS aqueous could form hydrogels rapidly in 1 min or less with the addition of sodium periodate. Equilibrium water content (EWC) and rheological behavior were studied. Additionally, the adhesive properties and in vitro cytotoxicity of the hydrogels were evaluated.

Experimental section

Materials

Chitosan was purchased from Sigma-Aldrich, medium molecular weight, with an 85% nominal degree of deacetylation. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl) and sodium periodate were purchased from Sinopharm Chemical Reagent Co Ltd. 3, 4-Dihydroxyhydrocinnamic acid was purchased from Energy Chemical. N-acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Biosharp. Fetal bovine serum (FBS) and 0.25% Trypsin-EDTA were purchased from Gibco; Dulbecco's modified eagle medium (DMEM, high glucose) and penicillin (1000 units/ml) and streptomycin (1000 μg/ml) solution were purchased from Hyclone. Other chemical reagents were all purchased from Sinopharm Chemical Reagent Co., Ltd and were used as received.

Synthesis of thiol-grafted bio-inspired CSDS

CSDS was synthesized by carbodiimide coupling method as follows. First, 0.5 g of chitosan was dispersed in 50 ml of pH 5.0 ultrapure water under stirring for 20 min; after the solution became clear under stirring, 3, 4-dihydroxyhydrocinnamic acid (1.72 mmol) dissolved in 10 ml ultrapure water was added to the solution followed by the addition of EDC•HCl solution (5.16 mmol) (20 mL of 1:1 v/v mixture of water and ethanol). During the reaction period, the pH value of the reaction solution was maintained at 4–5 by the addition of 1 M HCl solution, and reaction was continued for 24 h at room temperature. After 24 h, the reaction solution was dialyzed in ultrapure water and lyophilized. The lyophilized product was catechol-conjugated chitosan (CS-dopa).

Second, CS-dopa (0.5 g) was dissolved in pH 5.0 ultrapure water (50 ml) under stirring. Then, NAC (5.16 mmol) was added to the clear solution under stirring for 30 h, followed by the addition of EDC•HCl solution (10.32 mmol). The reaction was continued for 12 h at room temperature and the pH of the reaction solution was maintained at pH 4–5 during the reaction. After 12 h, the reaction solution was dialyzed in ultrapure water and lyophilized. The lyophilized product was CSDS that contains thiol group and catechol group.

^{1}H NMR spectroscopy of CSDS

To confirm the successful grafting of thiol group and catechol group to chitosan, CSDS was analyzed by ^{1}H NMR spectroscopy. CSDS (0.8% (g/mL)) dissolved in deuterium oxide (D_2O) was transferred into a 5 mm NMR tube and then recorded on an Bruker 600 MHz Digital NMR spectrometer (advance 3 HD 600 MHz, Bruker Corporation) which was equipped with a MestReC processing software.

Determination of thiol group and catechol group

CSDS was modified with two functional groups, and the contents of them were measured by using UV-vis spectroscopy with standard curve. According to the described method of Waite and Benedict,[22] the catechol content was measured by constructing the standard curve at 500 nm using solutions of known catechol group concentration. Briefly, DSCS aqueous solutions were treated with nitrite reagent (0.41 M sodium molybdate dehydrate and 1.45 M sodium nitrite), subsequently, excess 1.0 M NaOH solution was added. The absorbance of the prepared solution was recorded at 500 nm, and hence the concentration of catechol

Table 1. Conditions used for the preparation of CSDS hydrogel samples.

Hydrogels	Concentration of CSDS (wt%)	Concentration of $NaIO_4$ (μmol/ml)	PI/Cate (mol/mol)[a]
CSDS-2-0.5	2	20	0.50
CSDS-2-0.75	2	20	0.75
CSDS-2-1.0	2	20	1.00
CSDS-3-0.5	3	20	0.50
CSDS-3-0.75	3	20	0.75
CSDS-3-1.0	3	20	1.00
CSDS-4-0.5	4	20	0.50
CSDS-4-0.75	4	20	0.75
CSDS-4-1.0	4	20	1.00

CSDS: catechol-conjugated chitosan; $NaIO_4$: sodium periodate.
[a]Molar ratio of sodium periodate to catechol groups (denote as PI/Cate).

group was measured. Similarly, the degree of substitution of thiol group was determined using the Ellman's test.[19] Standard curve to measure thiol group content was also constructed following the instructions given for the UV-vis spectrophotometer, model UV 1100, Techcomp Co., Ltd.

Hydrogel formation and gelation time

Hydrogel was prepared by homogeneous mixing solutions of CSDS and sodium periodate ($NaIO_4$) in phosphate buffer saline solution (PBS, pH = 7.2) in vials. For the preparation of hydrogels, predetermined concentrations of CSDS were varied from 2% to 4% and the molar ratios of $NaIO_4$ to catechol groups (denote as PI/Cate) were 0.5, 0.75, or 1.0. The gelation time was defined as the time the solutions lost fluidity upon tilting of the vial.[23] The conditions used for the preparation CSDS hydrogel samples are listed in Table 1. The gelation time measurement was performed five times on each sample.

EWC

To prepare disc-shaped hydrogels (diameter = 12 mm, measured by ruler, thickness = 2.5 mm, measured by digital caliper) for test, CSDS solution and sodium periodate solution were mixed well in 48-well cell culture plate to form hydrogels. Disc-shaped hydrogels (n = 5) for EWC testing were freeze dried to measure the initial dry hydrogel weight. Subsequently, hydrogels were submerged in 10 ml of PBS solution (pH = 7.2) for 24 h at 37°C. Then, the swelling equilibrium hydrogels were measured by using analytic balance after PBS solution on the surface of hydrogels was carefully removed. The EWC was calculated according to the following equation

$$\text{Equilibrium water content (wt\%)} = \frac{W_s - W_d}{W_s} \times 100\% \quad (1)$$

where Ws and Wd are the weights of the swelling equilibrium hydrogel and initial dry hydrogel, respectively.

Rheological experiment

The rheological measurements were carried out using a strain-controlled rheometer (ARES-RFS, TA Instruments) with a stainless steel parallel plate geometry of 25-mm diameter. The cross-linking reagent, $NaIO_4$ solution, was added to aqueous solution of CSDS, and the well-mixed solutions were loaded onto the rheometer. The samples of DSCS hydrogels were freshly prepared before rheological tests. The operation temperature was maintained at 25°C. After the samples were applied to the rheometer, the upper plate was immediately lowered to a predetermined gap size of 1 mm and the measurement was started. To ensure that the rheological measurements are within a linear viscoelastic range, the dynamic strain sweep is conducted prior to the frequency sweep, in which the frequency was set at 1.0 Hz and strain was set from 0.1 to 100%, and the strain was determined to be 5%. Storage modulus (G′) and viscous modulus (G″) were measured by performing frequency sweeps between 0.1 and 100 rad/s at a fixed strain (5%) and temperature (25°C). The contribution of a solid-like behavior (elastic modulus (G′)) and a liquid-like behavior (viscous modulus (GPrime)) was recorded.

Measurement of adhesion strength

As described in previously literature,[24,25] the measurement of adhesion strength was performed by lap shear testing. The lap shear strength was directly measured on the porcine skin which used as the adherent. Fresh porcine skin was bought from the local slaughterhouse, and the porcine skin was shed of the excessive fat beneath the dermal tissue. Then, the porcine skins were cut into rectangular sections at 10 mm × 30 mm. The CSDS solution and sodium periodate solution were applied on two pieces of porcine skin, respectively, and then these strips were immediately overlapped with an area of 10 mm × 10 mm, and hydrogel was rapidly in situ formed. Samples were incubated at 37°C for 60 min to fully cross-link, and the adhesion strength was measured by using a mechanical testing machine (HY-940FS, Shanghai

Hengyu Co., Ltd) with a crosshead speed of 5 mm per minute.

In vitro cytotoxicity test

In vitro cytotoxicity of the hydrogel adhesives was evaluated indirectly by studying the extract of the adhesives with L929 mouse fibroblast cells according to ISO 10993-5 standard test method. The prepared hydrogels were fumigated and sterilized in 75% ethanol vapor for 5 h, washed three times with sterile PBS and were transformed to a centrifuge tube containing 20 ml DMEM at a concentration of 2.0 mg/mL. The centrifuge tubes were incubated for 24 h at 37°C and at 100 r/min. The extraction of the hydrogels was obtained by filtration (filter diameter = 0.22 μm); the extract was supplemented with 10% FBS and 1% of penicillin and streptomycin (pen/strep) solution, and subsequently stored in refrigerator at −20°C. Mouse fibroblasts L929 were cultured in DMEM supplemented with 10% FBS and 1% pen/strep solution under standard culture conditions at 37°C and in 5% CO_2. L929 cells suspension was seeded into 48-well cell culture plates at a density of 10×10^4 cells per well. After 24 h incubation, the culture medium was removed and replaced with the stored extract, and incubated for another 24 h, 48 h, and 72 h at 37°C and in 5% CO_2. L929 cells were seeded to the fresh culture medium as the negative control. Twenty microliters of 5 mg/ml MTT solution and 200 μL of DMEM were added to each well at the predetermined time after the culture medium was removed, and then returned back to incubation for 4 h; the medium containing unreacted MTT was removed. Then, 200 μl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal; after incubating for 15 min at 37°C, the optical absorbance values of the solutions were measured in an ELISA reader (Multiscan GO, Thermo Scientific) at a wavelength of 570 nm to determine the number of living cells.

Cell proliferation

To assess the cell proliferation on the hydrogel adhesives, L929 mouse fibroblast cells (50,000 cells/well) were seeded into the disc-shaped hydrogel samples (diameter = 12 mm, thickness = 1 mm) in 48-well culture plate and cultured with DMEM solutions supplemented with 10% FBS and 1% of pen/strep solution at 37°C and in 5% CO_2. Culture medium was replaced every day. After five days, hydrogel samples were washed three times with sterile PBS, and then the cells were stained with the live/dead kit (Invitrogen) according to the manufacturer's instruction. The samples were observed and imaged under a fluorescence microscope (OLYMPUS-1X51, Olympus Corporation).

Statistical analysis

All date were expressed as average with standard deviations. Statistical analysis using Origin pro software 8.0 software (Origin Lab Corporation) was performed by one-way analysis of variance (ANOVA), followed by a Bonferroni test. A p-value ≤ 0.05 was considered statistically significant.

Results and discussion

Characterization of CSDS

CSDS was prepared by grafting two different functional groups, thiol group and catechol group, to chitosan through the creation of amide bonds between primary amine group in chitosan and carboxylic acid group in 3, 4-dihydroxyhydrocinnamic acid or NAC via EDC chemistry, as shown in Figure 1. EDC chemistry is widely used for the preparation of chitosan derivatives, as chitosan chain was not depolymerized by the coupling reaction conditions. Coupling reaction was carried out at pH of 4–5 to avoid chitosan precipitation; meanwhile, there are enough non-protonated amino groups for the reaction. It has been reported that non-protonated amino groups were favorable to the preparation of chitosan derivatives using EDC chemistry.[26] To confirm the obtained product, CSDS was characterized by ^{1}H NMR and UV-Vis spectroscopy.

The structure of CSDS was confirmed by ^{1}H NMR spectra in D_2O (Figure 2). The specific peaks of CSDS appeared between 6.63 and 7.22 ppm and were contributed to the Ph-H of catechol group. Peaks at about 1.91 ppm were assigned to the N-acetyl methyl proton which was present in both chitosan and CSDS; however, new resonance peaks at 3.05 ppm were detected in CSDS, and it was believed to correspond to the side chain methylene of CH_2SH and $CH_2C=O$. Furthermore, peaks at 0.78 and 1.23 were assigned to the methyl proton of the backbone of 3, 4-dihydroxyhydrocinnamic acid. According to Waite and Benedict' method, the content of catechol groups on CSDS was 75.21 ± 5.2 μmol/g (Figure S1). In addition, the content of thiol groups on CSDS was 215.34 μmoL/g by Ellman's test (Figure S2).

Preparation and gelation of CSDS hydrogel adhesives

Mussel-inspired catechol-conjugated polymer derivatives are usually cross-linked to form hydrogels by the addition of metal ions, oxidized reagents or enzymes.[15,27] For the preparation of hydrogels, predetermined amounts of CSDS and $NaIO_4$ in PBS solution (pH = 7.4) were simplified and mixed to form hydrogels

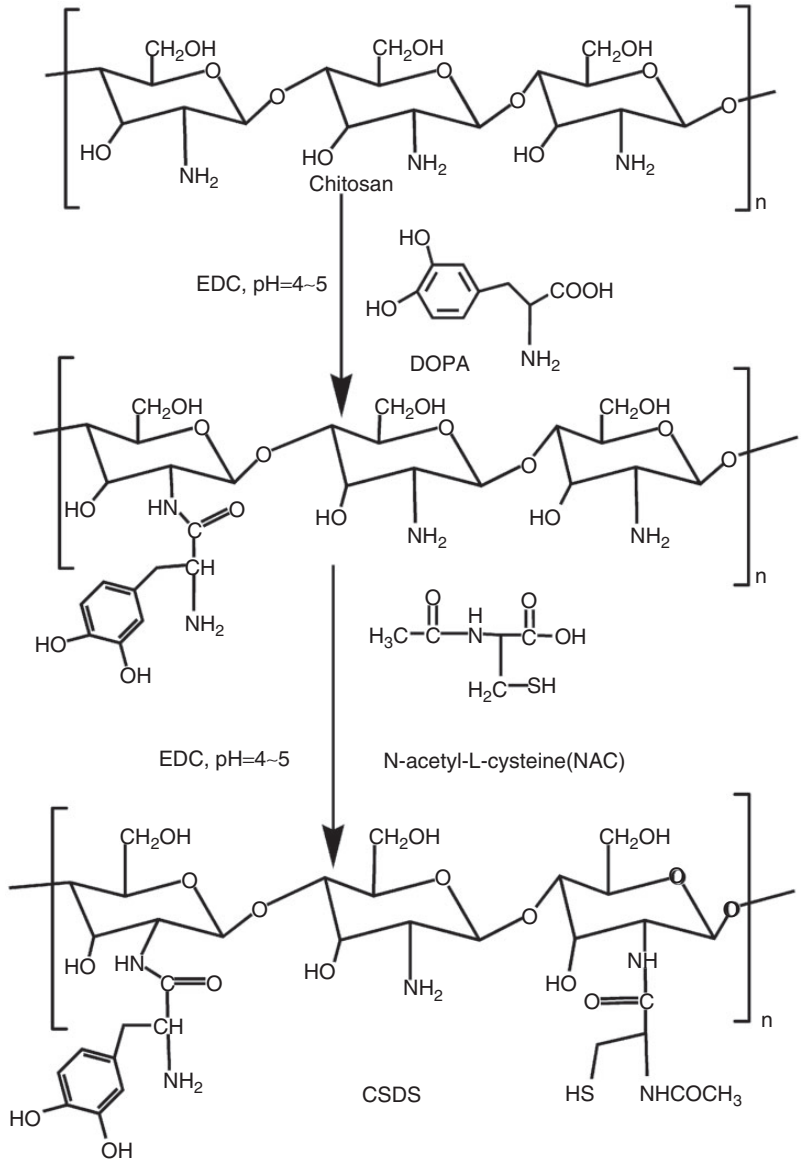

Figure 1. Synthesis of CSDS through carbodiimide chemistry.

rapidly. Gelation time of the hydrogels with different concentrations and molar ratios of PI/Cate were measured (Figure 3). As can be seen, hydrogels can be formed rapidly within 1 min as the concentration of CSDS was 4% and gelation time can be modulated from about 5.5 min to 30 s by altering the CSDS concentration and molar ratio of PI/Cate. Gelation time was decreased as the concentration of CSDS and the molar ratio of PI/Cate increased. Catechol group was oxidized to form hydrogels in this study, and hence the reaction between catechol group and $NaIO_4$ was the key to the gelation times of CSDS hydrogel adhesives. It is well known that catechol groups easily formed semiquinone and quinone by one-electron and two-electron oxidation, respectively. Quinone is a highly reactive compound that can participate in intermolecular covalent forming, resulting in the cross-linking of Dopa-containing hydrogel adhesives.

Swelling properties

Hydrogels are highly hydrated three-dimensional networks similar to the nature extracellular matrix (ECM) that can provide excellent conditions for cell growth. Swollen properties of hydrogels were indirectly used to indicate the potential as scaffolds for cell infiltration and growth. Figure 4 shows the EWC of CSDS hydrogel adhesives. As the concentration of CSDS increased from 2% to 4%, the water content of the hydrogels was also increased. Nevertheless, the increasing tendency of EWC was dropped. Furthermore, the influence of the molar ratio of PI/Cate is shown in Figure 4. Water

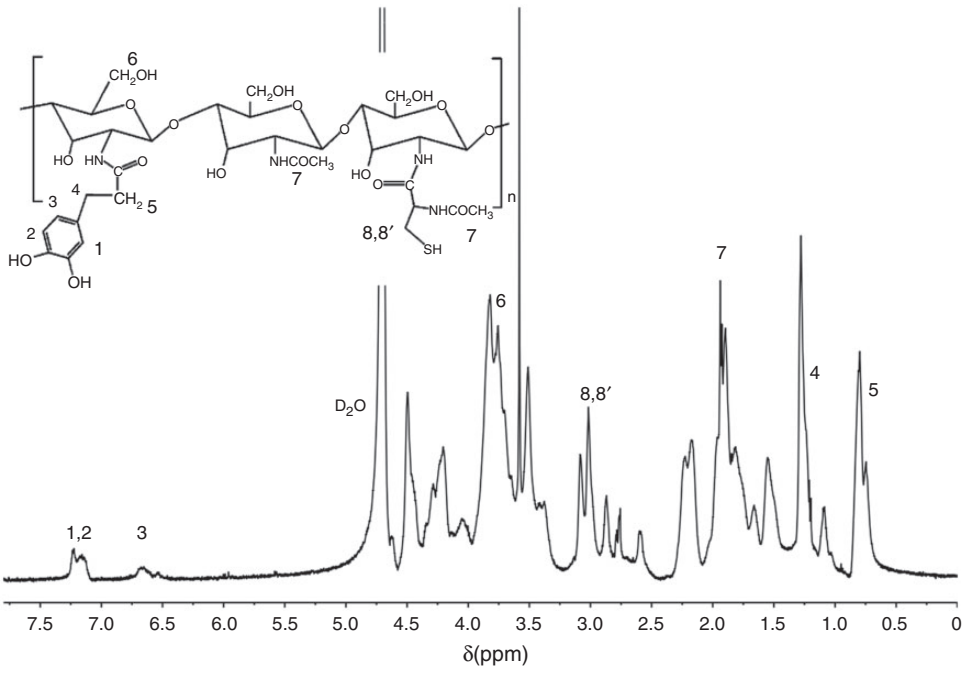

Figure 2. ^{1}H NMR spectra of CSDS in D2O.

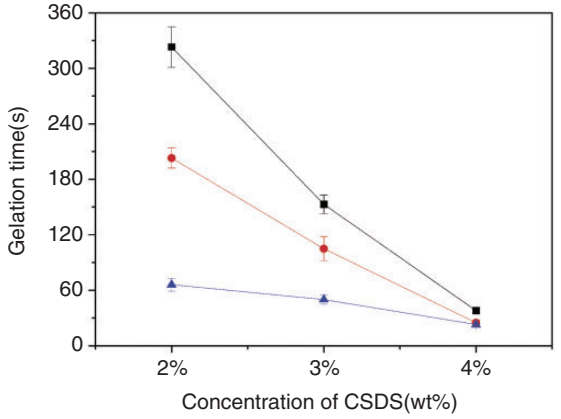

Figure 3. Gelation times determined by the vial tilting method when mixing solution of $NaIO_4$ and CSDS in PBS solution (pH = 7.4) at molar ratio of PI/Cate was 0.5 (-■-); 0.75 (-●-); and 1.0 (-▲-).

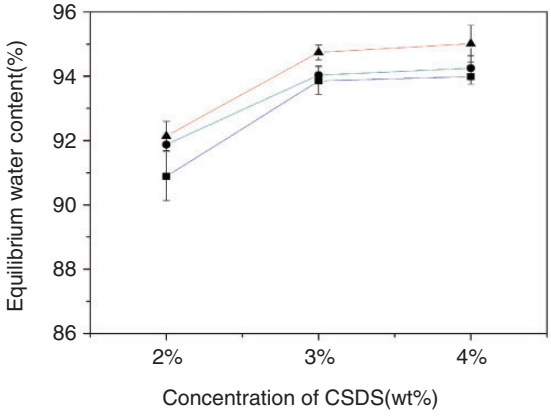

Figure 4. Equilibrium water contents of the prepared hydrogel adhesives in PBS solution (pH = 7.4) at molar ratio of PI/Cate was 0.5 (-■-); 0.75 (-●-); and 1.0 (-▲-).

contents of hydrogels were increased with the increase in molar ratio of PI/Cate. Thus, EWC of sample CSDS-4-1.0 was the largest of all the samples. The results indicated that EWC of the CSDS hydrogel adhesives was controlled by the intermolecular network structure, which was influenced by the contents of catechol group and thiol group. Catechol group was oxidized to form cross-linking point and thiol group can form disulfide linkage between the molecules. CSDS-4-1.0 hydrogels contained much more catechol group and thiol group to generate intermolecular cross-linking point so that the cross-linking density of the hydrogels was bigger. Therefore, the CSDS-4-1.0 hydrogels can keep in amounts of water to measure by weight method when reached in swelling equilibrium.

Mechanical testing of hydrogels

Strain-controlled rheometer was used to determine the mechanical properties of the hydrogels. Shear strain was performed in a dynamic strain sweep model which was conducted to determine the linear

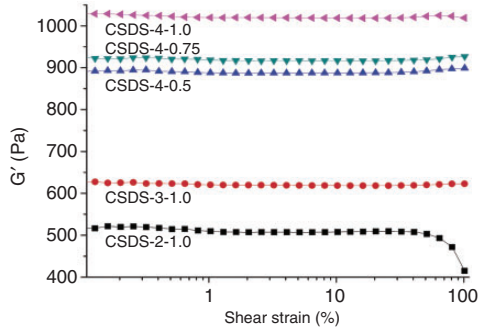

Figure 5. Shear strain was performed in a dynamic strain sweep model to measure the storage modules (G′) of hydrogel adhesives: CSDS-2-1.0, CSDS-3-1.0, CSDS-4-0.5, CSDS-4-0.75, and CSDS-2-4.0.

viscoelastic region and storage modulus (G′) of CSDS hydrogel adhesives as a function of the concentration of CSDS and molar ratio of PI/Cate (Figure 5). As can be seen, with increasing cross-linking density of the hydrogels, the storage modulus of CSDS hydrogels was increased. Undoubtedly, more catechol groups and molar ratio of PI/Cate resulted in higher cross-linking density. Therefore, the G′ of CSDS-4-1.0 was the highest of all the testing samples, which was as high as 1030 Pa. Additionally, compared with other CSDS hydrogels in Figure 5, CSDS-2-1.0 hydrogel samples showed decreased values of G′ at high shear strain, which indicated a structural breakdown. On the basis of these data, a strain of 5% was chosen for comparison of various hydrogels in dynamic frequency sweep model of the strain-controlled rheometer.

Storage modulus (G′) and viscous modulus (G″) were measured by performing dynamic frequency sweep between 0.1 and 100 rad/s at 25°C. Storage modulus (G′) and viscous modulus (G″) of the testing hydrogels are recorded and shown in Figure 6. The effects of molar ratio of PI/Cate to the mechanical properties of CSDS hydrogels (4% of CSDS) are studied from Figure 6(a) and (b), and the concentrations of CSDS to the mechanical properties are investigated as shown in Figure 6(c) and (d). Obviously, with the molar ratio of PI/Cate and concentration of CSDS increasing, storage modulus increased while the viscous modulus decreased. And when the samples were compressed with dynamic frequency (0.1–100 rad/s), the values of G′ were slightly increased in Figure 6(a) and (c); meanwhile, the values of G″ was decreased and then increased as shown in Figure 6(b) and (d). As we know, hydrogels are full of water in their three-dimensional network structure, resulting in showing both liquid properties (G″) and solid behaviors (G′). The samples of DSCS hydrogels were freshly prepared before rheological tests, and the higher concentration of CSDS solution indicates the cross-linking of more catechol group and thiol group and lesser water content in the prepared hydrogels. Therefore, the higher the concentration of CSDS solution and molar ratio of PI/Cate, the higher the values of G′; on the contrary, the values of G″ are low. It is noteworthy that the values of G″ were decreased and then increased with the frequency from 0.1 to 100 rad/s, and it is believed that at lower frequencies, the imposed mechanical motion to the hydrogels is hindered by the cross-link network; hence the longer chain needs more relaxation time, and it is dispelled as the frequencies increase; however, at higher frequencies, the hydrogels become stiffened up because the long chain in the network cannot rearrange themselves in the time scale of the imposed motion. Polymer network is the key to the viscoelastic response of hydrogels which is controlled primarily by the nature of imposed mechanical motion and the flexibility of polymer chains (length, numbers and distribution).

Evaluation of adhesion strength

Porcine skin was chosen as adherent to mimetic human tissue under clinical conditions. The adhesion strength of DSCS hydrogel adhesives was investigated by lap shear testing. The preparation of samples for adhesion tests was much simple and short in operation time without compression. Figure 7 shows the lap shear strength of samples cross-linked under different conditions. From Figure 7(a), lap shear strength of the prepared hydrogel adhesives, as shown in Table 1, that incubated for 60 min at 37°C was investigated. Of all the samples, the largest adhesion strength was 38.3 kPa, belonging to CSDS-4-0.75. The adhesive values are higher than the Mussel-inspired hyperbranched poly(amino ester) polymer reported by Zhang et al.[25] The values of the lap shear strength was increased with the increasing concentration of CSDS. However, samples with 0.75 of molar ratio of PI/Cate showed to be more adhesive than the samples with other molar ratios of PI/Cate. Lap strength of CSDS-4-0.75 hydrogel adhesives as a function of incubated time was also studied, as shown in Figure 7(b), and adhesion strength was increased as the incubated time increased.

It is well known that the adhesive property of catechol highly relies on its oxidation state, and the unoxidized catechol is more adhesive than the oxidized catechol,[28] and thus, the more the catechol group, the higher the adhesive strength. However, the largest adhesion strength was shown at 0.75 of the molar ratio of PI/Cate, not at 0.5. Reasons are that the thiol group of the CSDS provides an anti-oxidation mechanism that likely reacts with some $NaIO_4$ and protects the reduced form of catechol and restores catechol by coupling the oxidation of thiols to dopaquinone reduction. Furthermore, abundant contents of thiol groups

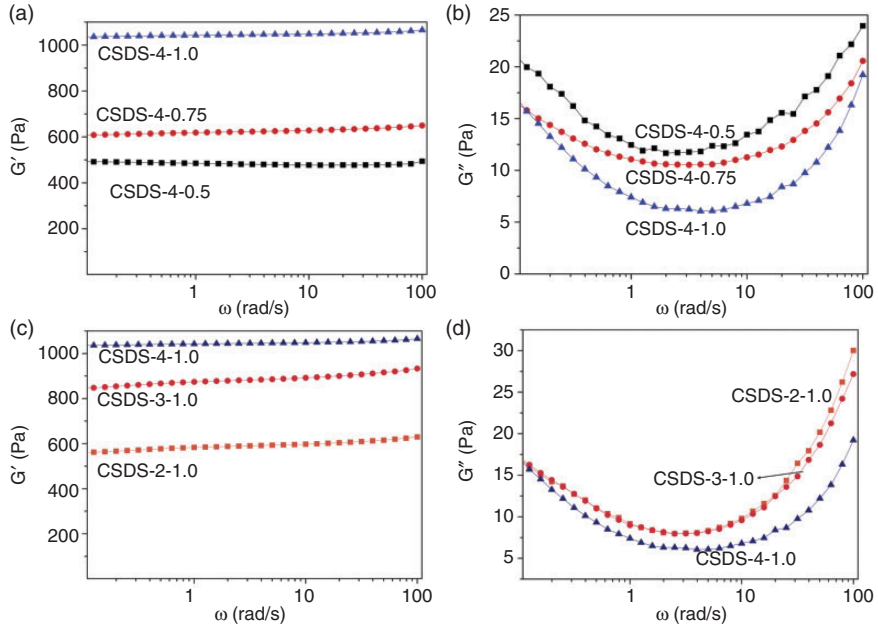

Figure 6. Storage modulus (G′) and viscous modulus (G″) was measured by performing dynamic frequency sweep between 0.1 and 100 rad/s at 25°C. (a) Storage modulus (G′) of CSDS-4-0.5, 0.75, 1.0 hydrogels; (b) viscous modulus (G″) of CSDS-4-0.5, 0.75, 1.0 hydrogels; (c) storage modulus (G′) of CSDS-2, 3, 4- 1.0 hydrogels; (d) viscous modulus (G″) of CSDS-2, 3, 4- 1.0 hydrogels.

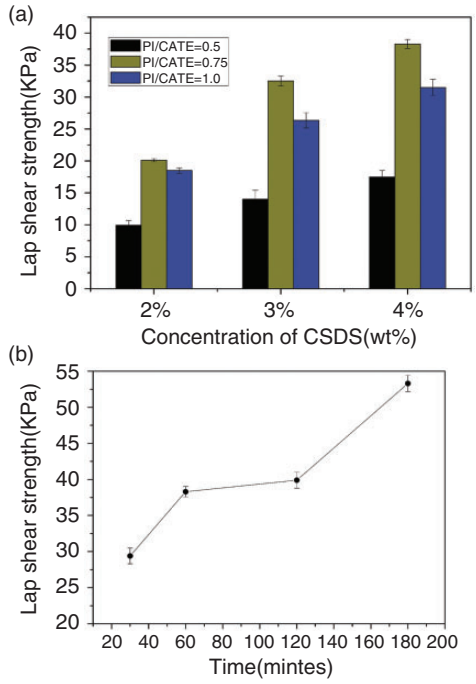

Figure 7. Adhesion strength of CSDS hydrogel adhesives. (a) Lap shear strength of the prepared hydrogel adhesives as shown in Table 1 that incubated for 60 min at 37°C; (b) lap shear strength of CSDS-4-0.75 hydrogel adhesives as a function of incubated time.

can form disulfide bond or generate Michael Addition Reaction to enhance the adhesion strength, which was characterized by a sharp increase in lap shear strength from 120 min to 180 min.

Biocompatibility of cross-linked hydrogel adhesive

The biocompatibility of CSDS hydrogels were indirectly evaluated in vitro on L929 fibroblast cells by the quantitative MTT cytotoxicity assay, and the cell proliferation on the disc-shaped hydrogels was observed directly by staining with the live/dead kit. Considering the high adhesion strength, we have chosen CSDS-2, 3, 4-0.75 samples for the investigation. From Figure 8(a), the average optical absorbance values of the testing samples ($n = 4$) increased as a function of culture time. However, when compared with the negative control in same culture time, the values of hydrogel extractions were slightly lower, but statistically significant differences ($p \leq 0.05$) were not seen. Furthermore, L929 cells cultured in CSDS-3-0.75 showed highest values of the three samples. It has been reported that during catechol oxidation, the reactive oxygen species (ROS) such as super oxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are generated,[29,30] and the $NaIO_4$ was reduced to $NaIO_3$; meanwhile, the highly reactive quinone was formed to bond with interfacial of the cell

through various reactions; all of these reduced the cell viability. Nevertheless, the cells were appeared to be recovered after 72 h, according to the statistically significant difference analysis. The anti-oxidation of thiol group mostly counteracts the effects of ROS in some ways.

Additionally, the L929 cell proliferation on the disc-shaped hydrogels (CSDS-2, 3, 4-0.75 samples) was evaluated by live/dead assay. As shown in Figure 9, L929 cells were viable in live/dead stained images of all samples after five days of culture. CSDS-3-0.75 showed excellent cell spreading when compared to CSDS-2-0.75 or CSDS-4-0.75. The pore diameter of the hydrogels was bigger than the cells so that cells can easily spread into the inner hydrogels. Results demonstrated excellent biocompatibility of the CSDS hydrogels.

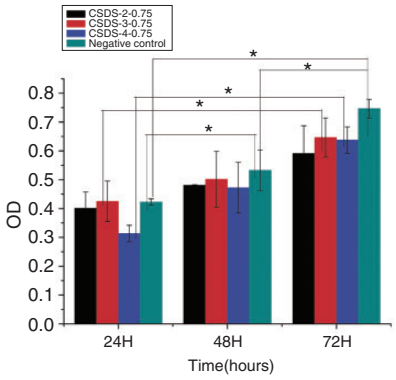

Figure 8. Cytotoxicity of CSDS-2, 3, 4-0.75 hydrogel adhesives extract was measured on the L929 cells.

Conclusion

Thiol-grafting bio-inspired CSDS was synthesized. CSDS, a novel chitosan derivative, was prepared with thiol group and catechol group to improve the adhesion. The chemical structure of the synthesized CSDS was verified by using ^{1}H NMR, and its contents of thiol group ($75.21 \pm 5.2\,\mu mol/g$) and catechol group ($215.34\,\mu mol/g$) were determined by using UV-vis spectrum. Gelation time, EWC, mechanical strength, the anti-oxidation mechanism of thiol group and adhesion performance of the hydrogels can be modulated by the concentration of CSDS and the molar ratio of PI/Cate. Four percent of CSDS aqueous solution could form hydrogels rapidly in situ in 1 min or less with the addition of $NaIO_4$. EWC of the CSDS hydrogel adhesives was above 90%. Rheological studies showed that the mechanical properties depend on the concentrations of CSDS and the molar ratio of PI/Cate. Additionally, the adhesive properties of the resulting adhesives were evaluated, and the obtained adhesion strength was as high as 50 kPa because of the complex and multiple interactions, especially the anti-oxidation mechanism of thiol group. The in vitro cytotoxicity assays demonstrated excellent biocompatibility of the CSDS hydrogels. Benefiting from the in situ fast cured, desired mechanical strength, biocompatibility and relatively high adhesion performance, these properties suggested that CSDS hydrogel adhesives have potential applications as tissue adhesive for soft tissues.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

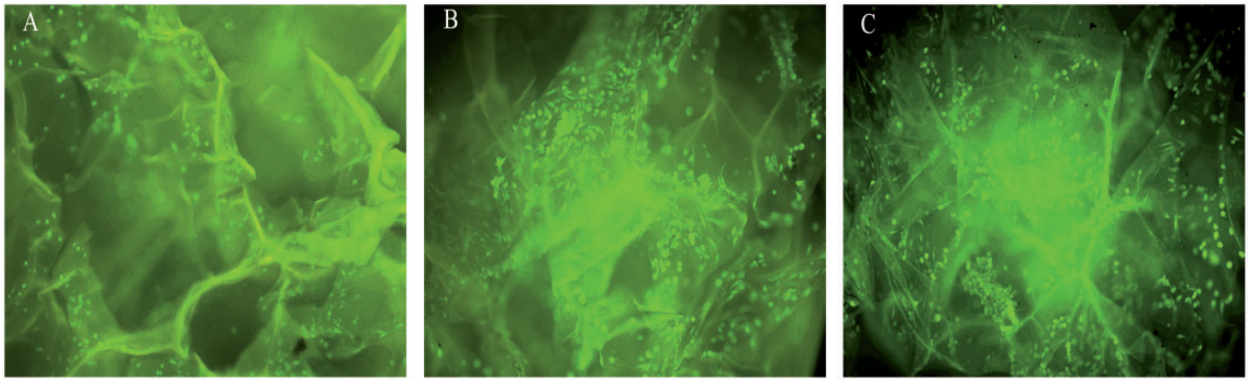

Figure 9. Live/dead staining images of L929 cells cultured in (a) CSDS-2-0.75; (b) CSDS-3-0.75; and (c) CSDS-4-0.75 hydrogels for five days.

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