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Protocols

Synthesis and characterization of incorporating mussel mimetic moieties into photoactive hydrogel adhesive

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ABSTRACT

Surgical adhesive is the optimal candidate for the replacement of traditional mechanical wound closure. In our paper, mussel adhesive proteins inspired hydrogel adhesive was prepared with 3, 4-dihydroxyphyenylanine acrylamide (DOPA-AA), poly (ethylene glycol) diacrylate (PEGDAA) and thiolated chitosan (CSS) by UV irradiation. DOPA-AA, containing catechol group and vinyl group, was successful synthesized and characterized by FTIR and ^{1}HNMR. The gelation time, equilibrium water content, degradation, materials properties and adhesive strength of the hydrogels were studied. We found that the gelation time was retarded and the materials mechanical strength was decreased because of the inhibitory effect of catechol group. Equilibrium water content was slightly affect by the increasing concentration of DOPA-AA (1–5%). Nevertheless, catechol group can remarkably improve the adhesive properties because of the complex and durable interactions of the hydrogel, especially, the interaction between the thiol group of CSS and catechol group of DOPA-AA, which also greatly slowed down the degradation of the adhesive hydrogels. CSS and DOPA-AA was introduced to ensure the adhesive properties, DOPA-AA lend the adhesive nature to hydrogel and CSS can protect the catechol group from oxidation and enhance durable adhesion. Moreover, cytotoxicity of the resulting hydrogels showed that the L929 cell viability was weaken, it mostly probably induced by the catechol oxidation.

1. Introduction

Bioadhesives have increasingly gained its attraction and popularity as novel biomaterials because of their obviously advantages including rapid control bleeding and bonding, less pain, no second surgical risking and minimum infection [1,2]. Therefore, it universal utilized for surgical adhesives, hemostatic agents, wound closing, dental bonding, and so on [3]. Tissue adhesives can effectively deal with the problems that the traditional mechanical wound closure devices (such as sutures, staples, tacks) can not, for example, traditional wound closure devices would create new trauma and unable to reconnect soft tissues with low cohesive properties (e.g., dura, kidney, lung, spleen) [2,4,5]. Nevertheless, Tissue adhesives have to address the two main problems: (1) the adhesion strength is not enough strong to integrated with the local tissue and the durable adhesion is lost due to the presence of body fluids; (2) adhesives or its degradation products is not biocompatible. Existing commercial tissue adhesives, such as fibrin sealant and cyanoacrylate, are hampered by these two problems. Therefore, a biocompatible tissue adhesive with superior adhesion performance is highly desirable and urgent to develop and promote.

Marine mussels can attach to various matrix surfaces tightly under wet and salty environment by secreting exceptional underwater adhesive proteins, which was named of mussel adhesive proteins (MAPs) [6,7]. MAPs contains a high content of the unusual unique amino acid 3, 4-dihydroxyphyenylanine (DOPA), which believed to lend outstanding adhesive performance to the MAPs [8]. Waite group [9,10] identified that the catechol group contained in DOPA is mainly responsible for the high, strong moisture-resistant adhesion of MAPs. Catechol group is a reactive, sensitive DOPA residue and easily oxidized by chemical or enzymatic to form DOPA-quinone that rapidly reacts with basic amino acids or nucleophilic groups. Thus, modification of DOPA and its derivatives

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onto bioadhesive materials have a great of potential application in clinical medicine as surgical adhesive and tissue cement [2,4,11]. Several literatures suggested that un-oxidized DOPA is responsible for strong water resistant adhesion while the oxidized *o*-quinone is responsible for cross-link formation [12]. Therefore, to protect the catechol group, scientific researchers recently begin to synthesis DOPA-containing biomimetic polymers that cross-linked to form hydrogels quickly without oxidizing reagents. Some strategies are proposed to address the challenge, such as preparation of thermosensitive materials grafted with DOPA residues [13–15], incorporating DOPA or its derivatives to polymers that can cured by photopolymerization, [16,17] or metal ions [18,19], and so on. Besides, polymers containing DOPA or analogous catecholic moieties have also designed to form hydrogels via chemical cross-linking with polymers containing active groups [20].

However, the facile tendency toward auto-oxidation of DOPA-containing biomimetic polymers have to take into consideration when it was dissolved in the aqueous solution, which usually renders DOPA unreliable for adhesion. Jing Yu et al. [21] showed an interesting findings that Mussels limit DOPA oxidation by imposing an acidic reducing regime based on thiol-rich mfp-6, which restores DOPA by coupling the oxidation of thiols to dopaquinone reduction. Therefore, in this paper, in order to make use of the adhesive characteristics of DOPA and protect the catechol group from auto-oxidation, we have designed an adhesive composed of three polymers, namely, 3, 4-dihydroxyphyenylanine acrylamide (DOPA-AA), poly (ethylene glycol) –diacrylate (PEGDAA) and thiolated chitosan (CSS). DOPA-AA, containing catechol group and vinyl group, was synthesized and its chemical structure was characterized by Fourier transform infrared spectra (FTIR) and proton nuclear magnetic resonance spectroscopy (^{1}HNMR). CSS was modified with thiol group that can react with vinyl group and restore catechol group. The copolymerization of DOPA-AA, PEGDAA and CSS was initiated in aqueous solution to form hydrogel by UV irradiation. The gelation time, equilibrium water content, degradation, materials properties, adhesive strength of the hydrogel were determined. Moreover, the cytotoxicity of the resulting hydrogel was also evaluated by MTT assay.

2. Experimental section

2.1. Materials

Chitosan used was purchased from Sigma-Aldrich, medium molecular weight, with an 85% nominal degree of deacetylation. 1-Hydroxybenzotrizole Hydrate (HOBT) was purchased from Shanghai WoKai chemical Reagent co Ltd. Poly (ethylene glycol) (PEG, 2 K Da) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from sinopharm chemical reagent Co Ltd. 3,4-dihydroxyphyenylanine (L-DOPA) was purchased from Sigma-Aldrich. Acryloyl Chloride was purchased from Energy Chemical. The photoinitiator, 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, namely Irgacure 2959 (I2959) was purchased from Sigma-Aldrich. Fibrin sealant (Tisseel ®) was purchased from Baxter Healthcare Ltd, 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT) was purchased from Biosharp, Fetal bovine serum (FBS) and 0.25% Trypsin-EDTA was purchased from Gibco, Dulbecco's modified eagle medium (DMEM, high glucose) was purchased from Hyclone. Other chemical reagents were all purchased from Sinopharm Chemical Reagent Co., Ltd and were used as received.

2.2. Synthesis of L-DOPA (DOPA-AA)

The reaction media was prepared by dissolving 2.288 g (0.006 mmol) of sodium borate and 1.272 g (0.012 mmol) of sodium bicarbonate in 50 mL of distilled water, the resulting aqueous solution was degassed with N_2 for 30 min to remove the air from the solution to protect the catechol group. After 1.183 g (0.006 mmol) of L-DOPA was added and stirred for 20 min, the solution was cooled to 0 °C, 0.652 g (0.072 mmol) of acryloyl chloride in 5 mL of dichloromethane was prepared and added dropwise slowly into the aqueous solution with stirring. The reaction mixture solution was stirred for 24 h at room temperature with nitrogen bubbling meanwhile the pH of the reaction solution was maintained above 9.0 with 1 M of NaOH. The resulting reaction solution was acidified to pH 2 with concentrated HCl and then extracted with ethyl acetate for 3 times. The extracted solution was dried over anhydrous MgSO4, filtered and concentrated, then added to 200 mL hexane with vigorous stirring to precipitate a brownish solid. The formed suspension was refrigerated to maximize crystal formation size. After filtration, to purify, the obtained crude product was dissolved in ethyl acetate and precipitated in hexane again. After dried in a vaccum drying oven, the samples were dissolved in ultra-pure water, then lyophilized to afford the final powder product.

2.3. Synthesis of CSS and PEGDAA

Thiolated chitosan (CSS) and poly (ethylene glycol) –diacrylate (PEGDAA) were synthesized as the literature description [22]. Briefly, 0.5 g of chitosan powder was dispersed in 50 mL ultrapure water on a flask under stirring for 20 min. The solution became clear under stirring after HOBT (2.58 mmol) was added, and then NAC (5.16 mmol) was added to the mixture solution followed by the addition of a solution of EDC·HCl (10.32 mmol). The reaction was proceeded for 5 h at room temperature. The pH value of the solution was keeping at 4–5 by the addition of 1 M HCl solution during the reaction. After 5 h, the reaction solution was dialyzed and lyophilized. The lyophilized product was CSS, and content of thiol group was determined by Ellman's test. (^{1}HNMR Data was shown in Fig. S1).

8 wt% of PEG was dissolved in toluene on a flask then the moisture contained in the PEG was removed by azeotropic distillation using a Dean-Stark trap. After cooling to room temperature, the PEG solution was degased with N_2 for 30 min. Triethylamine (TEA) dissolved in dichloromethane (volume ratio = 1:9) was added to the PEG solution with stirring. The reaction begun with the acryloyl chloride added into the solution dropwise and proceeded for 24 h under N_2 atmosphere. The reaction solution was filtered and concentrated. Then PEGDAA was precipitated from the concentrated solution by addition of cooled diethyl ether. The precipitate was washed with diethyl ether for three times and then dried in vacuum drying oven at room temperature. The react molar ratio of the PEG: TEA: acryloyl chloride was 1:2:2. The substitution of the vinyl group was determined using the ^{1}HNMR spectrum. (^{1}HNMR Data was shown in Fig. S2).

2.4. Characterization of DOPA-AA monomer

2.4.1. FTIR spectra measurement

The resultant DOPA-AA was analyzed by FTIR spectra which recorded on Nicolet 6700 instrument (Thermo Fisher Company, USA) equipped with an attenuated Total Reflection (ATR) (Thermo Fisher Company, USA) attachment. The measurement was carried out fast at wavenumber from 4000 to 600 cm^{-1} with resolution of 4.0 cm^{-1} so that DOPA-AA was not oxidized to form DOPA-quinone. Besides, the FTIR spectroscopy of L-DOPA was also collected.

2.4.2. ^{1}HNMR spectroscopy of DOPA-AA

To confirm the successful grafting of acrylamide group to L-DOPA, DOPA-AA was also analyzed by ^{1}HNMR spectroscopy. 1.5% (g/mL) of DOPA-AA solution in deuterium oxide (D_2O) was trans-

ferred into a 5 mm NMR tube and recorded on an advance 400 (400 MHz) Bruker NMR spectrometer which equipped with a MestReC processing software.

2.5. Preparation of hydrogels

30% of (g/mL) PEGDAA, 2% of (g/mL) CSS and 0.1% (g/mL) of photoinitiator I2959 were dissolved in phosphate buffer saline solution (PBS, pH = 7.2), then 1%, 2%, 3%, 4%, 5% of DOPA-AA relative to the PBS solution (wt%) was added, respectively. Hydrogel adhesive formulations were abbreviated as CPD-X, where X is the wt.-% of the DOPA-AA (as shown in Table 1). The precursor solution was transferred into 48-well cell culture plate and gelled by UV irradiation at the wavelength 365 nm, the disc-shaped hydrogels with various proportions of DOPA-AA were obtained.

2.6. Measurement of gelation time

200 μL of each gel precursor solution was placed on a 2-mL centrifuge tube and illuminated with UV lamp (250 W) at a distance of 25 cm. After UV irradiation for 30, 60, 90 or 120 s, or more, the samples were observed the fluidity when the tube was tilted (Fig. S3). If the precursor solution of the centrifuge tube could not flow, then it was formed a hydrogel. So the gelation time was defined as the irradiation time for the hydrogel forming. The measurement of gelation time was repeated for five times and took the average.

2.7. Equilibrium water content and degradation

Disc-shaped hydrogels (diameter = 12 mm, thickness = 2.5 mm, n = 3) were submerged in 10 mL PBS solution (pH = 7.2) for 24 h at 37 °C, the mass of swelling equilibrium hydrogels (Ms) was measured by the analytic balance after the liquid on the surface of hydrogels was wiped off. Then hydrogels were dried in a vacuum drying oven at room temperature for at least 48 h and the mass of hydrogels at dried state (Md) was measured. The equilibrium water content (EWC) was calculated as follows:

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

In vitro degradation properties of the materials were investigated by weighing the mass loss of the prepared hydrogels (n = 3) which was submerged in 10 mL PBS solution (pH = 7.2) and placed into an incubator set at 37 °C. The time for the onset of the hydrogels degradation is taken at the point when the weight of hydrogels immerging into the PBS solution reached the maximum value (Ws), which was not changed obviously. The degraded hydrogels were weighed at various time intervals after removal the PBS solution, the obtained data were set as Wt, which express as the weight of degraded hydrogels at time "t". The overall weight ratio was determined by the following equation:

$$\text{Weight ratio (wt\%)} = \frac{\text{Wt}}{\text{Ws}} \times 100\% \quad (2)$$

2.8. Compression testing

Materials testing system (HY-940FS, Shanghai Hengyu Co., Ltd) was used for uniaxial compression testing to study the mechanical properties of the prepared hydrogel discs. Hydrogel discs (n = 3) were compressed at a rate of 5 mm per minute until the sample fractured. Before testing, the diameter and thickness of the samples were precisely measured using a ruler and a digital caliber, respectively. Stress and strain were recorded with the configuration software of the materials testing system when the compression testing was performing. The compressive elastic modulus (E) was taken from the slope of the stress-strain curve at a strain between 0.1 and 0.3 while the toughness was determined from the area underneath the stress-strain curve.

2.9. Lap shear adhesion strength measurement

Gelatin coated glasses and SD rat skin tissue were used as adherent models for lap shear test. 20 wt% of gelatin solution was coated on the surface of rectangular glass slides (10 mm × 30 mm) then a thin gel film was formed after the solvent removed (thickness of the gelatin was about 0.1 mm). The prepared precursor solution, namely CPD-X solution, was dropped and spread uniformly on the gelatin coated glass and immediately overlapped with another gelatin coated glass and fixed with clips tightly. The adhesion area was 10 mm × 10 mm and irradiated for 10 or 15 min by the UV lamp (250 W) at a distance of 25 cm. The photocured samples were incubated for different time at 37 °C and then measured the lap shear strength using the mechanical testing machine (HY- 940 FS, Shanghai Hengyu Co., Ltd) with a crosshead speed of 5 mm per min at room temperature.

The lap shear strength was also directly measured on the tissue model that SD rat skin used as the adherent. Fresh rat skin tissue obtained from the SD rats was shaved with a razor and shed of the excessive fat beneath the dermal tissue (thickness of the skin was about 0.12 mm). The rat skins were cut into rectangular sections at 10 mm × 30 mm and placed in PBS solution for immediately use so that the skins were moist. After the prepared solutions were applied onto the rat skins and overlapped with the area of 10 mm × 10 mm, then the samples were clamped and irradiated for 15 min by the UV lamp (250 W) at a distance of 25 cm. The photocured samples were incubated for 3 h at 37 °C and measured the adhesion strength using the mechanical testing machine (HY- 940FS, Shanghai Hengyu Co., Ltd) with a crosshead speed of 5 mm per minute at room temperature.

2.10. Cytotoxicity assays

Cytotoxicity of the samples was evaluated by an indirect contact method according to ISO 10993 standard test. The hydrogel extraction was prepared to study the viability of L929 mouse fibroblast which was measured by MTT cell viability assay. Hydrogels (n = 4) were incubated in PBS solution (pH = 7.2) for 24 h, then sterilized in 75% ethanol vapor for 5 h. The sterilized samples were incubated in DMEM supplemented with 10.0% FBS and 1.0% penicillin-streptomycin solution at 37 °C for 24 h under orbital stirring at 100 rpm and the extraction ratio was 25 mg/mL. After incubation, the hydrogel extraction was achieved by filtering the medium "conditioned" by the hydrogel (filter diameter = 0.22 μm). L929 cells were seeded into 48-well cell culture plate at a density of 10×10^3 cells per well and incubated at 37 °C in 5% CO_2 for 24 h. Then the culture media was replaced with the hydrogel extraction solutions supplemented with 10.0% FBS and 1.0% penicillin-streptomycin solution and incubated for another 24 h, 48 h and 72 h. Then 20 μg of 5 mg/mL MTT assay solution in PBS was added to each well. After incubating the cells for 4 h, the medium was removed and 200 μL of dimethyl sulfoxide was used to dissolve the formazan crystal which was generated when MTT reagents incubated with the alive cell, additionally, DMEM with 10.0% FBS and 1.0% penicillin-streptomycin solution was used as negative control. The dissolved solution was swirled for 15 min in an incubator. MTT assay was performed to detected cytotoxicity using an ELISA reader (Multiscan GO, Thermo Science) by analyzing the absorbance at 570 nm and 630 nm of the dissolved solution. The cell viability was calculated as the following equation:

$$\text{Cell viability} = \frac{A_{\text{sample}}570 - A_{\text{sample}}630}{A_{\text{control}}570 - A_{\text{control}}630} \times 100\% \quad (3)$$

Table 1
Ratio of components for the CPD-X hydrogels cured by UV irradiation.

hydrogels	Concentration of CSS (wt%)	Concentration of PEGDAA (wt%)	Concentration of DOPA-AA (wt%)	Molar ratio of thiol group: vinyl group: catechol group (mol: mol: mol)
CPD-0	2%	30%	0	1:34:0
CPD-1	2%	30%	1%	1:34:5
CPD-2	2%	30%	2%	1:34:10
CPD-3	2%	30%	3%	1:34:15
CPD-4	2%	30%	4%	1:34:20
CPD-5	2%	30%	5%	1:34:25

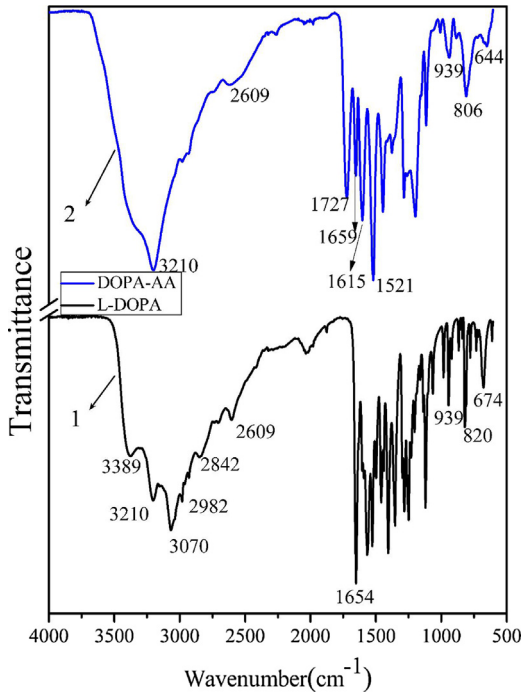


Fig. 1. FTIR spectra of L-DOPA (1, —— black) and DOPA-AA (2, — — blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.11. Statistical analysis

All data are expressed as the mean ± S.E.M. Statistical analysis was performed using the Origin Pro software 8.0 software (Origin Lab Corporation). One-way analysis of variance (ANOVA) followed by a Bonferroni test was conducted. *P*-Value less than 0.05 were considered statistically significant.

3. Results

3.1. Synthesis and characterization of DOPA-AA

Synthesized sample of DOPA-AA was analyzed using FTIR spectra and ^{1}H NMR spectroscopy. As shown in Fig. 1, compared with the spectrum of L-DOPA, the successful formation of an amide covalent bond between L-DOPA and acryloyl chloride (Scheme 1a), was confirmed by the new FTIR signal of C=O of amide bond, which appeared at a wavenumber of 1727 cm^{-1}. Otherwise, the wavenumber of 1615 cm^{-1} and 806 cm^{-1} was contributed to the C=C stretching and bending vibration, respectively. Similar to what was reported in a previous publication, signals from the catechol group were visible at the wavenumber of 3210 cm^{-1}, however, DOPA-AA showed a broad and smooth peak of 3210 cm^{-1} because of the N—H stretching vibration of –CO—NH— and O—H stretching vibration. The other signals such as 2609 cm^{-1} was assigned to the Ph-H stretching vibration, and 1659 cm^{-1} and 1521 cm^{-1} was contributed to the C=O stretching vibration of —COOH and N—H bending vibration, respectively.

The ^{1}H NMR spectrum of DOPA-AA was showed in Fig. 2. It showed that the protons belonging to the benzene ring (Ph-H) were clearly shown at 6.55–6.75 ppm (a, b, a'). Also, protons from the vinyl groups (—CH=CH$_2$) were apparent with signals at 5.40 ppm (g), 5.27 ppm (h) and 4.55–4.42 ppm (f), respectively. Since the synthesized DOPA-AA is soluble in deuterium oxide (D_2O), its solvent peak was observable as a strong signal at 4.65 ppm. The multiple resonance peaks at 3.09–2.95 ppm (c') and 2.86–2.72 ppm (c) was assigned to Ph—CH$_2$—, the protons of CH—COOH showed a single peak at 2.57 ppm (d). The results of FTIR and ^{1}HNMR were demonstrated that DOPA-AA containing catechol group and vinyl group was synthesized by the reaction between L-DOPA and acryloyl chloride which formed amide covalent bond.

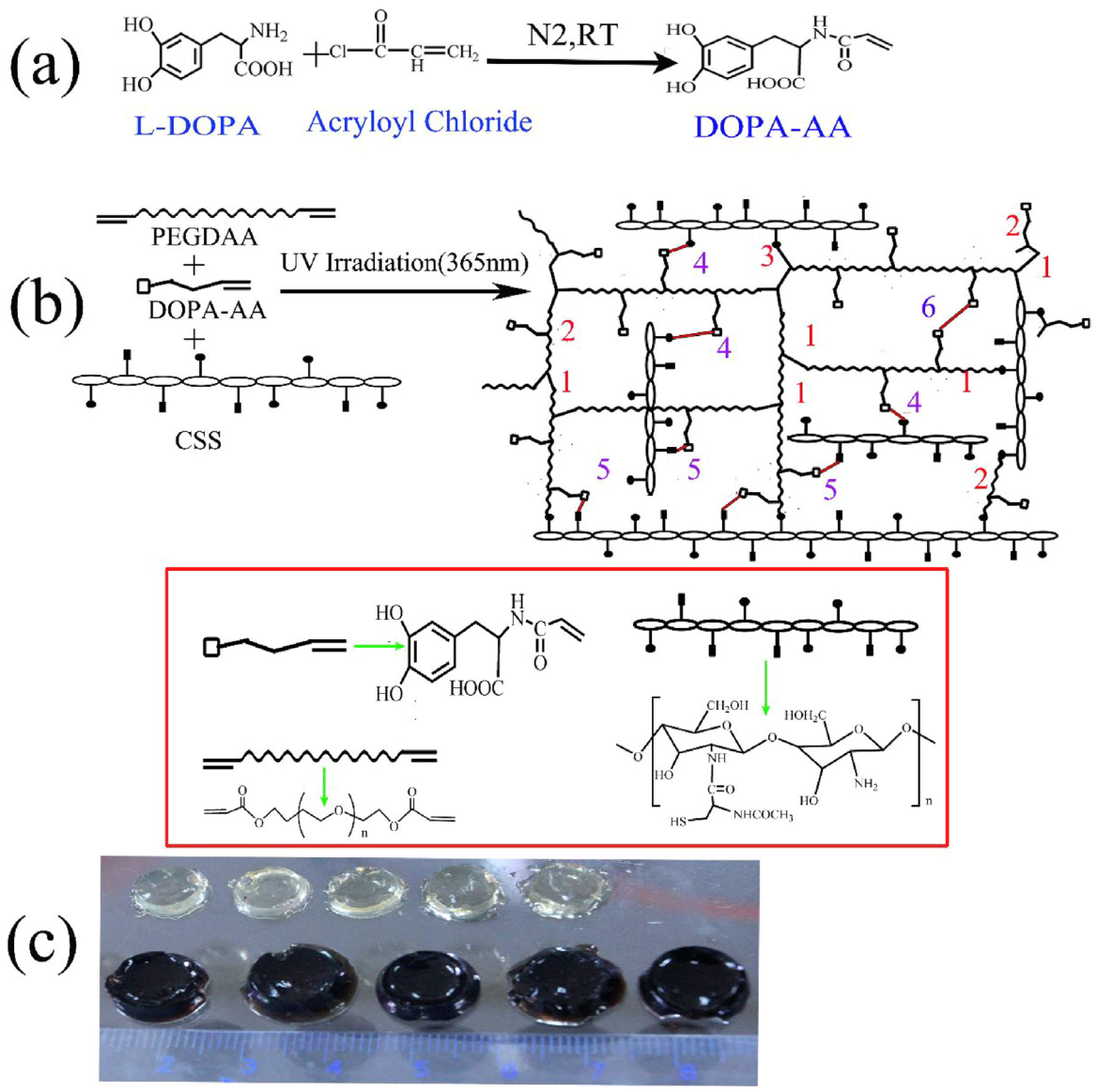
3.2. Preparation and gelation time of hydrogels

The substitution of the vinyl group of PEGDAA was 85%, and the extent of thiol group for the CSS was 350 μmol/g, which measured by the Ellman's method. The molar ratio of components for the CPD hydrogels were shown in Table 1. The copolymerization of DOPA-AA with PEGDAA and CSS was initiated in aqueous solution to form hydrogel by UV irradiation (Scheme 1b). The gelation time of CPD-X hydrogels was influenced significantly by the concentration of DOPA-AA in the precursor solution. As shown in Fig. 3, the gelation time was increased when the content of DOPA-AA increasing, for example, the CPD-0 without DOPA-AA photocured in 40 ± 6.1 s while CPD-3 containing 3% of DOPA-AA formed hydrogel by illustration for 257 ± 12.5 s. Thus the CPD-5 took much more time than others to cross-link and its cured time was as long as 586 ± 8.9 s. The results showed that the gelation time of hydrogel adhesives were increased with the DOPA-AA concentration increasing for the decelerating or inhibitory effect of catechol group.

3.3. EWC and degradation properties

Equilibrium water content (EWC) of the prepared hydrogels was shown in Fig. 4A. The EWC of the testing samples were above 90%, and values were not significantly changed with the content of DOPA-AA increased in the hydrogels. ANOVA followed by a Bonferroni test was performed for comparing the CPD-0 to evaluated the influence of the DOPA-AA to the EWC, and results showed that with (1–5%) or without DOPA-AA, the EWC of hydrogels composed by CSS and PEGDMA was not statistically significant differences ($p \geq 5\%$).

To show the data clarify, Fig. 4B showed the degradation properties of three hydrogels containing DOPA-AA (CPD-1, CPD-3 and CPD-5). The degradation curve was increased slightly after about 15 days, and then went down slightly. However, it was increased again and finally went down as the time past. It was noteworthy that CPD-5 hydrogels showed a decreasing tendency during the beginning five days. After 75 days, the weight ratio of CPD-5 was



Scheme 1. (a) Schematic of synthetic rout for DOPA-AA; (b) Preparation of adhesive hydrogels containing CSS, PEGDAA and DOPA-AA; and schematic of the structure and reaction: "1" was the reaction between PEGDAA and PEGDAA, "2": reaction between PEGDAA and DOPA-AA, "3": Micheal addition reaction between vinyl group and thiol group(●), "4", "5" and "6": catechol group (□) coupled with thiol group(●), amine group(■) of CSS and itself, respectively.

63.7% while CPD-1 and CPD-3 was 60.0% and 42.3%, respectively. CPD-5 degraded more slowly than CPD-1 and CPD-3.

3.4. The influence of DOPA-AA to the mechanical properties

Compression testing was carried out to study the mechanical properties of the prepared hydrogel discs (n = 3) directly. The compression testing results including the maximum stress and strain, compressive elastic module (*E*) and toughness were showed in Table 2. As seen from Table 2, the measured values of CPD–1–5 hydrogels for materials properties was decreased with increasing DOPA-AA contents. For example, with the DOPA-AA content increased from 1% to 5%, the maximum stress was decreased from 338.9 ± 26 to 120.2 ± 16.4 KPa, and the toughness values decreased from 50.456 ± 8.184 to 19.027 ± 3.057 KJ/m^3. At the highest DOPA-AA concentration tested (5%, CPD-5), the maximum stress and strain, compressive elastic module (*E*) and toughness values were the smallest of all samples tested.

3.5. Evaluation of adhesion strength

Adhesive performance of the CPD hydrogel adhesives was measured by lap shear test. To evaluated fully the adhesion properties of the CPD–0–5 adhesive hydrogels, gelatin coated glass and SD rat skin were used as adherents, and results were showed in Fig. 5. Moreover, the adhesion strength of the hydrogels incubated for 2 h, 3 h, 24 h at 37 °C were also measured for testing the durable adhesion.

Fig. 5A showed the adhesive strength of adhesive hydrogels on gelatin coated glass (cured for 10 min and incubated for 3 h) was increased from 41.7 ± 2.34 KPa to 163.93 ± 4.95 KPa with the DOPA-AA concentration increasing from 0% (CPD-0) to 4% (CPD-4), however the values was decreased when DOPA-AA concentration was 5% (CPD-5). For comparing, adhesive strength of fibrin sealant on the gelatin coated glass incubated for 3 h was also tested and the value was 49.53 ± 2.12 KPa that was less than the CPD hydrogels. The results indicated that the adhesive strength was enhanced by the addition of DOPA-AA, and hydrogels containing DOPA-AA were showed higher adhesive than fibrin sealant. However, as the

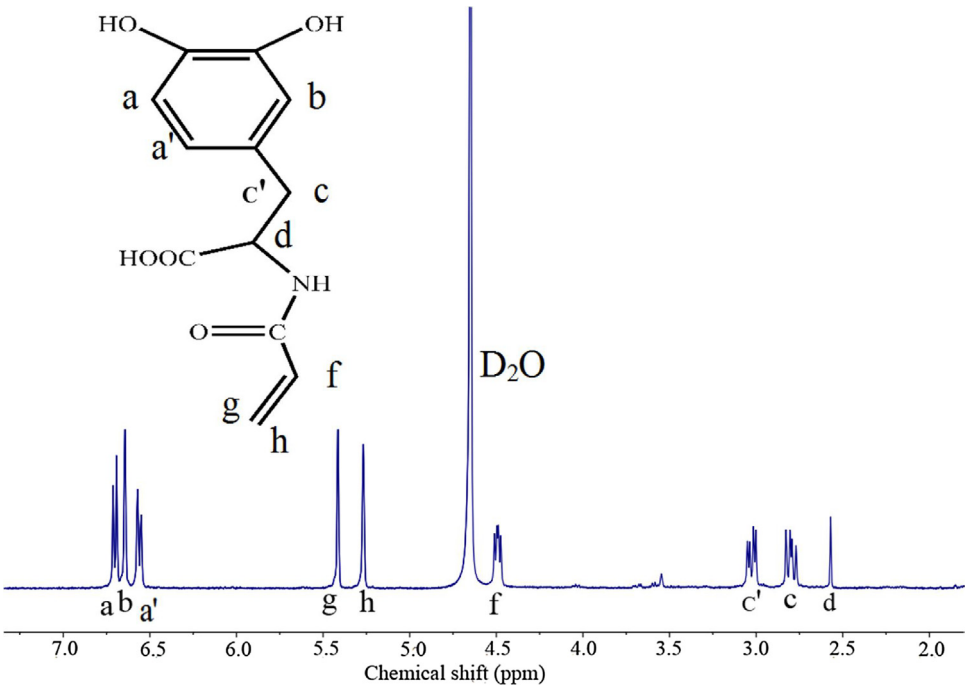


Fig. 2. ^{1}HNMR spectrum of DOPA-AA which was dissolved in D_2O.

Table 2
Results of uniaxial compression testing on CPD–1 ~ 5 hydrogels.

Samples	Maximum stress (KPa)	Maximum strain (100%)	Elastic module (*E*, KPa)	Toughness (KJ/m^3)
CPD-1	338.9 ± 26	63.6 ± 0.57	177.2 ± 3.9	50.456 ± 8.184
CPD-2	223.8 ± 22.1	62.34 ± 0.18	143.5 ± 7.3	35.98 ± 5.488
CPD-3	222.3 ± 30.4	56.87 ± 5.29	133.1 ± 1.8	34.763 ± 2.271
CPD-4	136.9 ± 8.0	54.51 ± 2.66	130.2 ± 3.3	21.700 ± 2.333
CPD-5	120.2 ± 16.4	52.01 ± 3.13	101.8 ± 10.9	19.027 ± 3.057

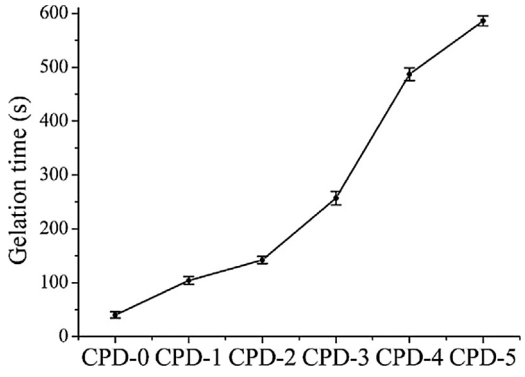


Fig. 3. Gelation time of the prepared CPD-X hydrogels (X = 1 ~ 5) with variety contents of DOPA-AA.

concentration of DOPA-AA in CPD-5 hydrogel was too much, the inhibitory effect of catechol led the adhesive strength to decrease. Adhesive strength of CPD–1–5 hydrogels on gelatin coated glass cured for 15 min and incubated for difference time was also measured (Fig. 5B). The lap shear strength of the CPD–1–5 hydrogels were increased with the incubated time increasing. After 24 h, the highest value of the adhesive strength was reached to about 1000 KPa (CPD-5), it was much higher than the adhesive strength of CSS/PEGDMA adhesive [22]. As shown in Fig. 5C, the adhesive strength of CPD hydrogel adhesives were measured by using a SD rat skin model as the living tissue adherent. Samples for lap shear test were irradiated by UV for 15 min and incubated for 3 h at 37 °C. Compared with the adhesive strength of CPD-0 hydrogel (131.9 ± 3.39 KPa), the adhesive strength of other samples were higher. CPD-5 hydrogel showed the highest adhesive strength of 251.54 ± 10.57 KPa.

3.6. Cytotoxicity assay

Biocompatible is one of the most important properties for the tissue adhesive. MTT assay was used to determine the effect of the CPD–1–5 hydrogels with different DOPA-AA concentration and incubation time on the viability of L929 fibroblast cells. The L929 cell were incubated with the hydrogels extraction solutions (25 mg/mL) supplemented with 10.0% FBS and 1.0% penicillin-streptomycin solution, then the cells viability was test and showed in Fig. 6. As seen in Fig. 6A, after incubation with the CPD–1–5 hydrogels extraction solutions for 48 h, L929 cell viability was slightly decreased but they were all above 80%, and the cell viability of CPD-1 hydrogel extraction solution was as high as 98.79 ± 1.23%. To investigate the cell viability of L929 cell in different incubation time, the L929 cell incubation in CPD-1, 3, 5 hydrogel extraction solutions for 24 h, 48 h, and 72 h was also studied, and the result was showed in Fig. 6B. The L929 cell viability incubated in CPD-1 hydrogel extraction solution was increased from 94.98 ± 1.33% to 99.14 ± 0.73% with the time from 24 h to 72 h. However, The L929 cell viability incubated in CPD-3, 5 hydrogel extraction solutions were decreased with the time from 24 h to 72 h, especially the L929 cell viability incubated in CPD-5 hydrogel extraction solution, was 64.79 ± 4.63%. The results showed that incorporation DOPA-AA into the PEGDAA/CSS hydrogels would decrease the compatibility of the

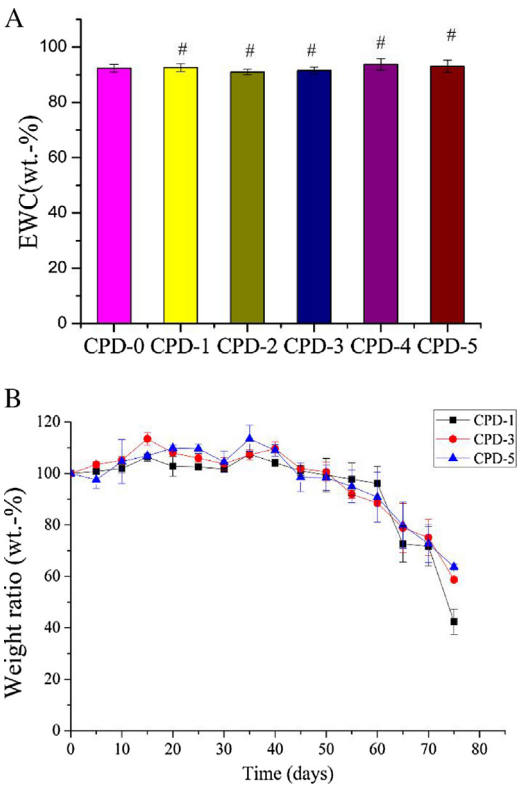


Fig. 4. (A) EWC and (B) degradation properties of the prepared adhesive hydrogels in PBS solutions (pH = 7.2) at 37 °C. "#" denote as $p \geq 5\%$, to show that data is not statistically significant differences.

hydrogels, reasons maybe were that catechol group of DOPA-AA oxidized to quinone that react with the proteins of the cell membrane so that the cells were hurt or even killed. Additionally, the catechol oxidation would generate O^{2-} and H_2O_2 that harm to the cells.

4. Discussion

Catechol chemistry is responsible for the strong adhesive strength, rapidly solidification, and mechanical enhancement of mussel adhesive proteins. However it is hard to understand the adhesive mechanism. In this paper, DOPA-AA, containing catechol group and vinyl group, was successful synthesized through the reaction between L-DOPA and acryloyl chloride (Scheme 1a), and a marine mussel inspired photo-cured hydrogels containing CSS, PEGADAA and DOPA-AA was designed to prepare and study (Scheme 1b). As shown in Table 1, the molar ratio of three groups (thiol group, vinyl group, catechol group) contained in the polymers (CSS, PEGDAA and DOPA-AA) was calculated, and the molar ratio of catechol group of the precursor solution was increased from 0 (CPD-0) to ~41.7% (CPD-5). The extent of CSS and PEGDAA in the precursor solution was keep unchanged when DOPA-AA was added into the precursor solution, but the molar ratio of thiol and vinyl group were relatively dropped, and the molar of vinyl group was still the most of the three groups. Therefore, the hydrogels was formed mainly by the free radical polymerization of the vinyl group. In addition, the Michael addition reaction between vinyl group and thiol group was possible take part in the cross-linking reaction even if the thiol group was relatively a little and coupled with the catechol group. Because the catechol group of DOPA-AA provided inhibitory effect on free radical polymerization, the gelation time of adhesive hydrogels was decreased as the molar ratio of catechol group increased (Fig. 3).

A hypothesis was provided for study the network structure and interactions between the components of the hydrogels. As shown in Scheme 1b, Firstly, the hydrogels was formed by the CSS, PEGADAA and DOPA-AA, and PEGDAA chains was act as the cross-linker and the "backbone" of the network structure of hydrogels. Secondly, when the precursor solution was irradiating by the UV light, the main reaction was free radical polymer reaction, including the reaction between PEGDAA and PEGDAA (Scheme 1b "1"), PEGDAA and DOPA-AA (Scheme 1b "2"). Other reactions or interactions were possible: such as Michael addition reaction between vinyl group and thiol group (Scheme 1b "3"), and catechol group coupled with thiol group (Scheme 1b "4"), amine group of CSS (Scheme 1b "5") or itself (Scheme 1b "6"). Thirdly, because of the thiol group of CSS, catechol group may be efficiently restored and unoxidized, in other words, CSS may be an efficiently antioxidant at first and then as a cross-linking partner for the adhesive hydrogels. In addition, after the hydrogels formed, the catechol group is easily oxidized or auto-oxidized to form semiquinone or quinone that is highly reactive and can participate in intermolecular covalent bond generated.

This hypothesis was useful to explain the adhesive hydrogels properties. The EWC of the CPD–0–5 hydrogels was slightly changed with the concentration of DOPA-AA increased, because DOPA-AA (0–5%) have little influence to the segment spacing of the network structure. During the degradation of the sample hydrogels (Fig. 4), CPD-5 hydrogels have 41.6% (molar ratio) of catechol group, CPD-5 hydrogels showed a decreasing tendency during the beginning five days as the un-react prepolymer was leaked from the hydrogels. In addition, the compression test of the CPD–0–5 hydrogels was also indicated that the hydrogels were not cross-linked well with the content of catechol group increasing. When CPD-1, 3, 5 hydrogels was incubating in the PBS solution, the catechol group would oxidized to form semiquinone or quinone that can react with various groups (amine group, imidazole group, thiol group, catechol group) as previous reported, and then complex and various interactions, especially the intermolecular covalent cross-linked, would generate in the testing hydrogels, therefore the degradation curve was fluctuate up and down (Fig. 4). In addition, during the degradation proceed, the CPD-1, 3, 5 hydrogels were show different color with time, the color would change from light brown to yellowish-red, finally to black (Scheme 1c and Fig. S4). It was believed that DOPA was oxidized to dopaquinone, so the color is change from light brown to yellowish-red, then the high reactive dopaquinone would react with various groups (amine group, imidazole group, thiol group, catechol group), the yellowish-red color then changed to black. This phenomenon proved that complex and various interactions was occurred during the degradation proceed.

Catechol group is believed to fulfill the dual role of interfacial binding and the solidification of the adhesive proteins secreted by the marine mussel. To measure the adhesive strength of the CPD–0–5 hydrogels, the gelatin coated glass and SD rat skin were used as adherents (Fig. 5) and the adhesive hydrogels were photocured for different time (10 min, 15 min) and stored for 2 h, 3 h and 24 h to study the durable adhesion. When the samples were photocured for 15 min, the lap shear strength was increased with the extent of DOPA-AA increased, whatever the adherents is gelatin coated glass or SD rat skin. However, when the adherent was gelatin coated glass, samples were photocured for 10 min and stored for 3 h, the adhesive strength of CPD-5 was decreased in Fig. 5A, it was because that the high concentration of catechol group inhibited the solidification of the precursor solution to from hydrogels. As shown in Fig. 3, the gelation time of CPD-5 was more than 10 min, therefore, to the adhesive strength of CPD-5, samples photocured for 10 min and stored for 3 h was lower than the samples photocured for 15 min and stored for 3 h. Obviously, when samples were photocured for 15 min and stored for 3 h, the adhesive strength of the samples was determined by the UV light transmission of the

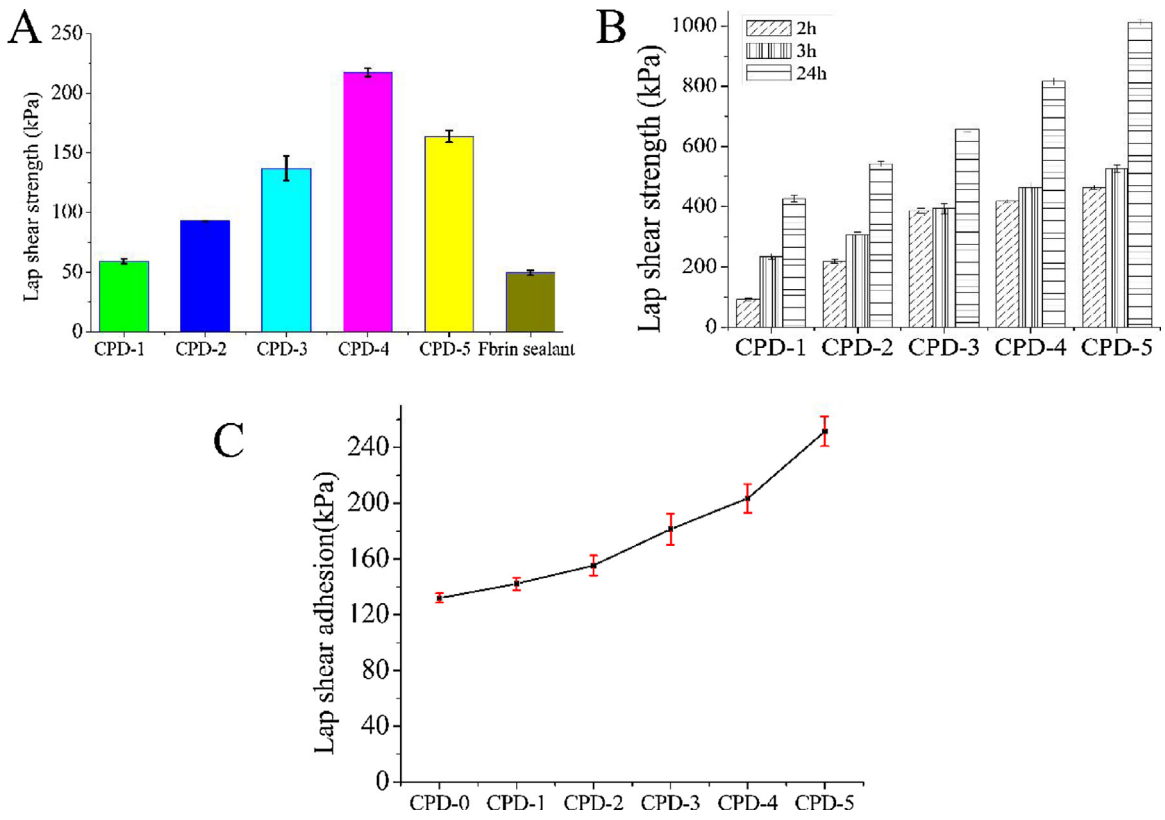


Fig. 5. (A) Adhesive strength of hydrogels on gelatin coated glass photocured for 10 min and incubated for 3 h; (B) Adhesive strength of hydrogels on gelatin coated glass photocured for 15 min and incubated for 2, 3, 24 h; (C) Adhesive strength of hydrogels on SD rat skin photocured for 15 min and incubated for 3 h.

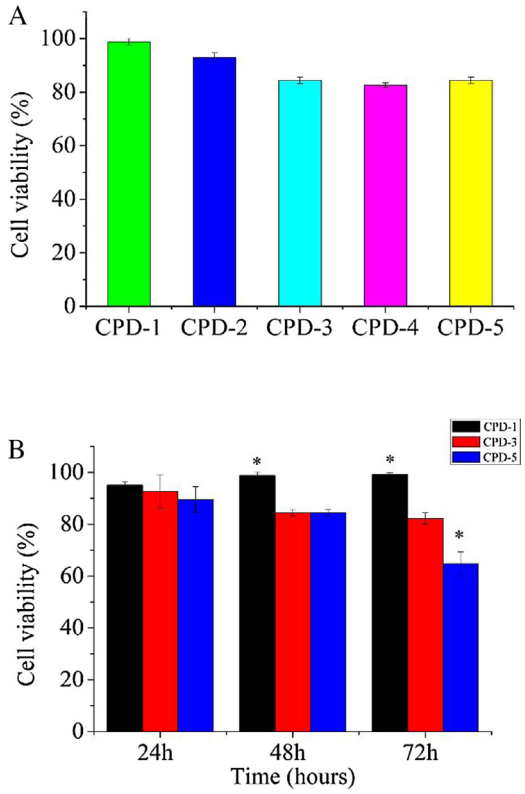


Fig. 6. (A) L929 cell viability analysis in the presence of the CPD–1 ~ 5 hydrogels extraction solutions after 48 h and (B) CPD-1, 3, 5 hydrogels extraction solutions after 24 h, 48 h, and 72 h. "*": $p < 0.05$, data is considered statistically significant differences.

adherents. Thus, the adhesive strength on SD rat skin was lower than the adhesive strength on gelatin coated glass. Nevertheless, catechol group of DOPA-AA were easier diffused into the interface of the gelatin or SD rat skins to bond the adherents tightly through chemical bond. As the hypothesis says, catechol group was oxidized to quinone that can react with amine group, imidazole group and thiol group, and these nucleophilic functional groups were found on biological substrates. However, in other side, during catechol oxidation, the highly reactive quinone was generated to bond with interfacial of the cell through various reaction, meanwhile the reactive oxygen species such as super oxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are generated, these are doing harm the cell viability. Thus the catechol group reduced the cell viability when it was oxidized.

Furthermore, Jing Yu [21] et al. found that Mussels foot proteins-6 (Mfp-6) is rich in cysteine residues (thiol group) which restores DOPA by coupling the oxidation of thiols to dopaquinone reduction. The mechanism of anti-oxidant action by Mfp-6 was also adapt to the CSS that rich in thiol group. As shown in Fig. 5B, to the samples photocured for 15 min and stored for 2 h, 3 h and 24 h, the adhesive strength of the hydrogels was increased with the time. Especially the adhesive strength of CPD-5 (molar ratio of catechol group is 41.6%) is increased to about 1.0 MPa (1000 KPa) after stored for 24 h, and the adhesion was keeping increased stored for 48 h or more (data not shown here), the results proved that the thiol group of CSS can protect the reduced form of catechol for enhanced adhesion.

6. Conclusions

The adhesive mechanism of marine mussels and the catechol chemistry have been extensively studied for many years, but it need more devotion to understand the strategies and overcome the challenge. In this study, DOPA-AA, containing catechol group and

vinyl group, was synthesized. Mussel adhesive proteins inspired adhesive hydrogel was prepared with DOPA-AA, PEGDAA and CSS by UV irradiation. The chemical structure of DOPA-AA was characterized and conformed using FTIR and ^{1}HNMR. The gelation time, equilibrium water content, degradation, materials properties and adhesive strength of the hydrogels were studied. Gelation time of the hydrogels was retarded and the materials mechanical strength was decreased because of the inhibitory effect of catechol group. Equilibrium water content was slightly affect by the increasing concentration of DOPA-AA (1–5%). Nevertheless, catechol group can remarkably improve the adhesive properties because of the complex and durable interactions of the hydrogel, especially, the interaction between the thiol group of CSS and catechol group of DOPA-AA, which was also greatly slowed down the degradation of the adhesive hydrogels. CSS and DOPA-AA was introduced to ensure the adhesive properties, DOPA-AA lend the adhesive nature to adhesive and CSS can protect the catechol group from oxidation and enhance durable adhesion. Cytotoxicity of the resulting hydrogels showed that the L929 cell viability was weaken, it mostly probably induced by the catechol oxidation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.colsurfb.2017.10.041.

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