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Rapidly *in situ* forming chitosan/ε-polylysine hydrogels for adhesive sealants and hemostatic materials

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ABSTRACT

A novel *in situ* forming polysaccharides/polypeptide hydrogel composed of naturally derived materials for applications as adhesive sealant and hemostatic material was developed *via* Michael addition crosslinking, taking advantage of its mild condition. Thiol-modified chitosan (CSS) was fast *in situ* crosslinked by an efficient polypeptide crosslinker (EPLM) which was prepared by introducing maleimide groups onto ε-polylysine. Gelation can happen swiftly within 15–215 s depending on the CSS concentration, the degree of substitution (DS) of maleimide groups, and the molar ratio of maleimide group to thiol group. Results indicated that storage modulus of the hydrogel increased dramatically with the increase of CSS concentration and DS of maleimide. The obtained adhesive hydrogel had an adhesion strength 4 times higher than that of the commercial fibrin glue. Notably, it is non-toxic to L929 cells and exhibits excellent prompt hemostatic property. Polysaccharides/polypeptide structure designed here facilitates to improve both the biocompatibility and the adhesive property.

1. Introduction

Adhesive biomaterials have attracted rapidly growing interests and are expected to perform various functions, such as sealing leaks, stopping bleeding, binding tissues and preferably facilitating tissue recovery (Lih, Lee, Park, & Park, 2012). Traditional fibrin glue has relatively weak mechanical strength (Mehdizadeh, Weng, Gyawali, Tang, & Yang, 2012; Spotnitz, 2001), while cyanoacrylates are cytotoxic and unsuitable for internal clinical uses (Cho, Lee, & Webb, 2012). Ideal tissue sealants should be of good biocompatibility and biodegradability with a fast curing rate and simultaneously offer adequate adhesion (Artzi et al., 2009; Lee et al., 2010). Many efforts have been done to prepare suitable alternatives of the existing adhesive materials and to overcome their drawbacks. *In situ* crosslinking hydrogels appear to be promising candidates among them. Michael addition crosslinking avoids the use of cytotoxic free-radicals, H_2O_2, photoinitiators, and UV light, which are usually involved in photocrosslinking hydrogels and enzyme-catalyzed hydrogels (Amoozgar et al., 2012; Fu & Kao, 2011; Park, Jun, Joung, Shin, & Park, 2011; Rickett et al., 2010; Sakai, Hirose, Taguchi, Ogushi, & Kawakami, 2009). The fact that Michael addition can happen by simply mixing the components under mild conditions without any byproducts makes it a better choice to form *in situ* crosslinking hydrogels which are more biocompatible (Hiemstra, Van der Aa, Zhong, Dijkstra, & Feijen, 2007a, 2007b; Li et al., 2012; Pritchard et al., 2011). However, the crosslinking rate is not quick enough to meet the criteria of tissue sealants, particularly hemostatic materials (Vanderhooft, Mann, & Prestwich, 2007). Therefore, to date, there have been few reports about using Michael addition type *in situ* forming hydrogels as tissue sealants. More recently, the Michael addition reaction between maleimide reactive groups and thiols began to gain attentions because of the fast reaction kinetics of maleimide group and its high specificity for thiols at physiological pH (Phelps et al., 2012; Vanderhooft et al., 2007). However, despite the fact that the fast reaction kinetic was verified by researchers, this reaction has not been well investigated, especially in terms of its potential applications.

Chitosan (CS) is derived from a naturally occurring polysaccharide and is well known for its superior tissue adhesive property, good biocompatibility, hemostatic activity, as well as its antimicrobial action (Muzzarelli, 2009; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012). Chitosan can be modified with thiol group to improve its solubility at physiological pH. Thiolated chitosan has shown strong mucoadhesive properties by forming disulfide bonds with cysteine-rich domains of mucus glycoproteins, leading to an improvement in mucoadhesion of up to 140-fold that of unmodified chitosan (Wang et al., 2009). ε-Polylysine (EPL) is a naturally occurring homopolyamide biodegradable and nontoxic toward human (Shi, Shen, & Van, 2006; Shukla, Singh, Pandey, & Mishra, 2012). Because of its primary amine groups, EPL is cationic

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in neutral aqueous solutions, showing potential tissue adhesive property due to the ionic attraction between itself and the target tissue. Recently, Nakajima et al. used EPL to form bioadhesive and its self-degradability, low toxicity, and stronger bonding property were proved in their report (Nakajima, Sugai, Tsutsumi, & Hyon, 2007).

The objective of the present study is to develop and characterize a novel Michael addition type *in situ* forming chitosan/ε-polylysine hydrogel with superior properties in terms of the good biocompatibility, the fast crosslinking rate, and the adequate adhesion for applications as adhesive sealant and hemostatic material. In order to accomplish this goal, maleimide groups were introduced onto EPL molecules to fabricate a peptide crosslinker for thiolated chitosan to guarantee the fast curing speed. Subsequently, a novel fast curable hydrogel crosslinked *via* Michael-type addition was prepared. We hypothesized that chitosan/polylysine could mimic the polysaccharides/polypeptide structure of natural extracellular matrices (ECMs). This feature as well as the mild condition of Michael addition makes the system more biocompatible competed with other systems (Liao, Yu, & Guan, 2009). Remarkably, high functionality and adhesion sites existing on macromolecules designed by us could offer enough adhesion force to tissues and may provide possibility for further incorporation of cells or therapeutic molecules. The gelation time, storage modulus, and adhesion strength of the hydrogel were characterized in this study, and the effects of solution parameters on gelation were investigated. *In vitro* cytotoxicity and *in vivo* hemostatic property were also evaluated.

2. Materials and methods

2.1. Material

Chitosan (deacetylation degree: 90%, $M_w = 1.5 \times 10^5$ kDa) was purchased from Aokang Biotech Ltd. (Shandong, China). *N*-acetyl-L-cysteine (NAC), 1-ethy-3-(3-dimethyl-aminopropyl-carbodiimide) hydrochloride (EDC·HCl), and 1-hydroxybenzotrizole (HOBt) were purchased from Sigma–Aldrich (St. Louis, MO, US). ε-Polylysine ($M_w = 4$ kDa) was offered by KW Chemical Ltd. (Nanjing, China). *N*-[γ-maleimidobutyryloxy] sulfosuccinimide ester (Sulfo-GMBS) was purchased from Pierce Biotechnology (Rockford, IL, US). Fibrin glue was obtained from Bioseal Biotech Co. Ltd. (Guangzhou, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Thiolation of chitosan

Thiol functionalized chitosan (CSS) was synthesized referring to a previous literature (Teng et al., 2010). The preparation schematic diagram is shown in Fig. 1A. Typically, about 500 mg chitosan powder was dispersed in 50 ml ultrapure water under stirring. Then HOBt (2.58 mmol), NAC (10.32 mmol) and EDC·HCl (20.64 mmol) were added and the pH was adjusted to 3–5. The mixture solution was then dialyzed and lyophilized. The lyophilized product was characterized on a Bruker AV300 ^{1}H NMR spectrometer.

2.3. Synthesis of maleimide group modified ε-polylysine (EPLM)

About 30 mg ε-polylysine were dissolved in pH 7.4 PBS to form a solution of 1 mmol/l. Subsequently, 14 mg or 28 mg Sulfo-GMBS was dissolved in pH 7.4 PBS to form a solution of 8 mmol/l, and then this Sulfo-GMBS solution was added to the above ε-polylysine solution (Fig. 1B). The reaction mixture was incubated for 0.5 h at room temperature under stirring and then dialyzed against ultrapure water, which was exchanged every 24 h. The success of reaction was also confirmed by ^{1}H NMR spectroscopy. The degree of substitution (DS) of maleimide groups is defined as the molar percentage of reacted α-amine groups to the primary amine groups in EPL.

2.4. Measurement of gelation time

CSS and EPLM were dissolved in pH 7.4 PBS solution, respectively. The hydrogel was synthesized by simply mixing the above CSS and EPLM solution and vibrating quickly at room temperature. Unless otherwise stated, all concentrations are expressed in wt%. In different systems, concentrations of modified chitosan were varied from 1 wt% to 4 wt% and the molar ratio of maleimide groups to thiol groups (denote as MAL/SH) was 1/1 or 2/1. The time to form a gel (denoted as gelation time) was determined using the vial tilting method (Jin, Hiemstra, Zhong, & Feijen, 2007; Tran, Joung, Lih, Park, & Park, 2010). No flow within 1 min upon inverting the vial was regarded as the gel state. The experiments were performed in triplicate.

2.5. Rheological measurement

A Stress Tech rheometer (Reologica Instruments Inc.) with standard steel parallel-plate geometry of 20 mm diameter was used for the rheological characterization of all hydrogel samples. The CSS and EPLM solutions were blended rapidly on the bottom plate of the rheometer and the upper plate was quickly lowered down to a gap size of 150 μm. All experiments were carried out at room temperature, and silicon oil was used to prevent evaporation during the experiment. The storage modulus (G') was measured at a stress of 1 Pa (stress control) and a frequency of 0.5 Hz.

2.6. Measurement of adhesion strength

As described in former literature, 10 wt% gelatin solution was uniformly spread on the surface of glass to simulate the living tissue (Li, Niu, Yang, Nie, & Yang, 2011; Wang, Nie, & Yang, 2012). The dimension of one piece of glass was 1 mm × 25 mm × 76 mm. The tissue adhesion strength was investigated according to the method modified from ASTM F2255-03. Briefly, 150 μl hydrogel adhesive was applied to cover an area of 25 mm × 15 mm. The adhesive joint was kept at room temperature for about 60 min in a humid environment, and then the samples were loaded to failure in shear with a crosshead speed of 5 mm/min. The maximum strength *versus* displacement was measured, and the shear stress at break (ultimate adhesion strength) was used to characterize adhesion for each sample. At least six samples were used for each measurement.

2.7. Cytotoxicity studies

Cytotoxicity of the hydrogels was assessed by contacting extracts of the hydrogels with monolayers of L929 mouse fibroblast cells according to ISO standards (ISO 10993). Firstly, the extracts were prepared by incubating the hydrogel in Modified Eagle's medium (MEM) at an extraction ratio of 15 mg/ml for 24 h at 37 °C. After removing the hydrogel, the extract was sterilized by 0.22 μm filters and supplemented with 10% fetal bovine serum (FBS). MEM medium and MEM medium containing 10% dimethylsulfoxide (DMSO) were used as negative control and positive control, respectively. Subsequently, L929 mouse fibroblast suspension (200 μl) were seeded in a 96-well culture plate at a density of 5×10^3 cells per well. The plate was incubated at 37 °C in 5% CO_2 for 24 h. After incubation, the culture media was removed and replaced with hydrogel extracts. After another 48 and 72 h, 20 μl methylthiazolydiphenyl-tetrazolium bromide (MTT) solution was added to each well and incubated for a further 4 h. Then, the culture medium and MTT were removed, after which 150 μl DMSO

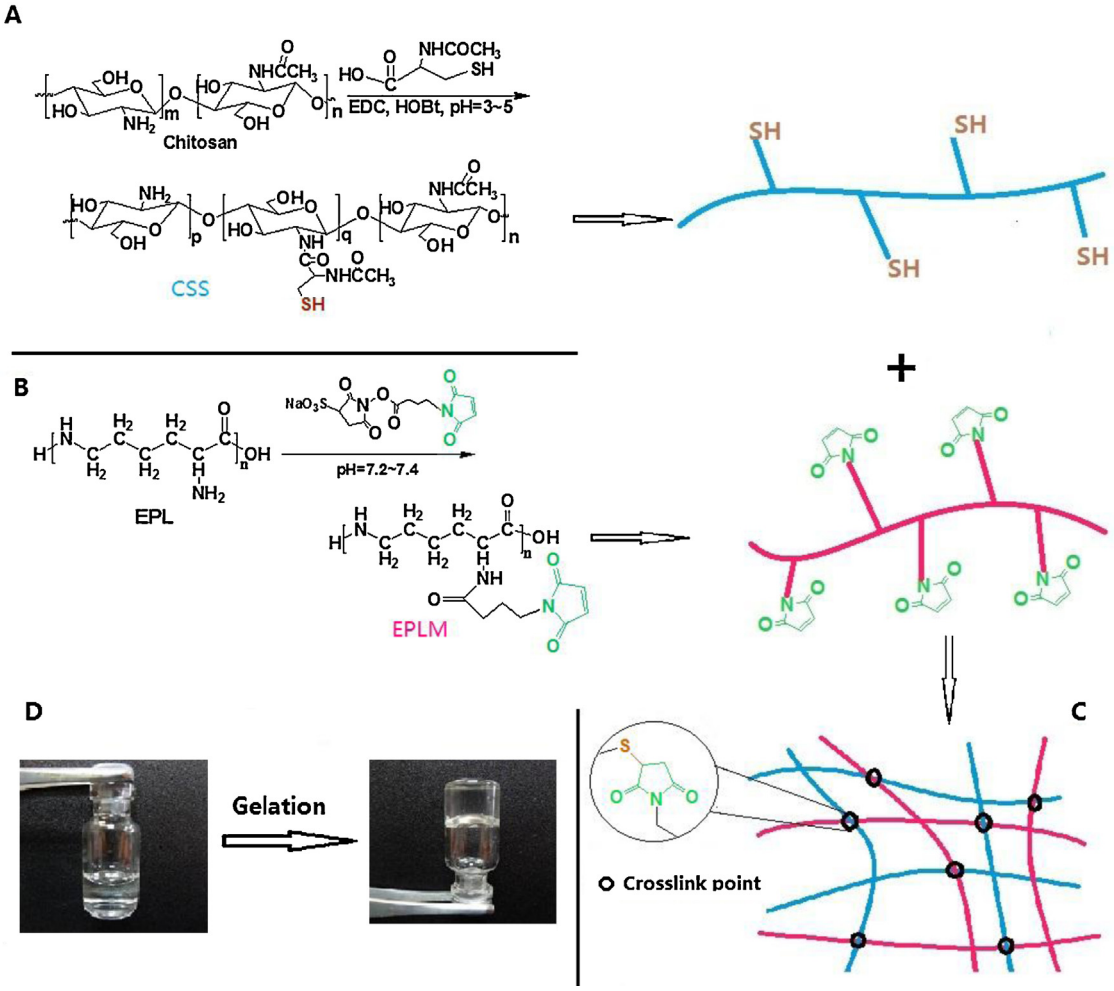

Fig. 1. Schematic representation of the synthesis of CSS (A); and the synthesis of EPLM (B); demonstration of the *in situ* hydrogel formation (C); photographs of hydrogel formation (D).

was added to each well. The absorbance was measured at 570 nm and 630 nm. Cell viability was defined as the following equation:

$$\text{Cell viability}(\%) = \frac{A_{\text{sample }570} - A_{\text{sample }630}}{A_{\text{control }570} - A_{\text{control }630}} 100\% \quad (1)$$

all reported values were the means of six measurements.

2.8. In vivo hemostatic ability test

All animal studies were performed in compliance with guidelines set by national regulations and were approved by the local animal experiments ethical committee. In brief, a rat (normal SD rat, 200–230 g, 6w, M) was paralyzed using ketamine and immobilized on a surgical corkboard. The liver of the rat was exposed by abdominal incision, and serous fluid around the liver was carefully removed to prevent inaccuracies in the estimation of the blood weight obtained by the filter paper. A pre-weighed filter paper was placed beneath the liver. Bleeding from the liver was induced using 18 G needle with the corkboard tilted at about 30° and 100 μl of hydrogel was immediately applied to the bleeding site using the dual syringe kit filled with solutions. After 3 min, the weight of the filter paper with absorbed blood was measured and compared with a control group (no treatment after pricking the liver). Six rats were used for each experimental group.

3. Results and discussion

3.1. Synthesis and characterization of CSS and EPLM

CSS was synthesized by the covalent attachment of NAC to the amine groups of chitosan. The structure of CSS was confirmed by ^{1}H NMR. From Fig. 2A, the peak at 1.87 ppm of N-acetyl methyl proton of chitosan was seen both in modified CS and unmodified CS whereas a new resonance peak at 2.78 ppm was only detected in CSS, which was believed to correspond to the side-chain methylene of CSS (2H, CH_2SH, CSS). According to Ellman's method (Krauland, Hoffer, & Bernkop-Schnürch, 2005), the content of thiol groups on CSS was 151.8 μmol/g.

EPLM were achieved through reaction between EPL and water-solubale Sulfo-GMBS, Sulfo-GMBS is heterobifunctional cross-linker that contains both NHS ester and maleimide group. In the present study, NHS ester reacted with primary amines of EPL in PBS solution. Maleimide groups were thus successfully introduced onto EPL. Structure of EPL and EPLM was confirmed by ^{1}H NMR spectra in D_2O (Fig. 2B). The specific peaks of EPL appeared between 1.2 and 4.0 ppm. Peaks at about 3.8 ppm are assigned to the methine groups. In the spectrum of EPLM, new signal at 6.6 ppm (the double bond protons) was clearly observed. The peak of the methine groups of EPL shifted partially from 3.8 to 4.0 ppm when reaction occurred in the amine groups, indicating the presence of major functional groups linked to EPL. The DS of maleimide groups can

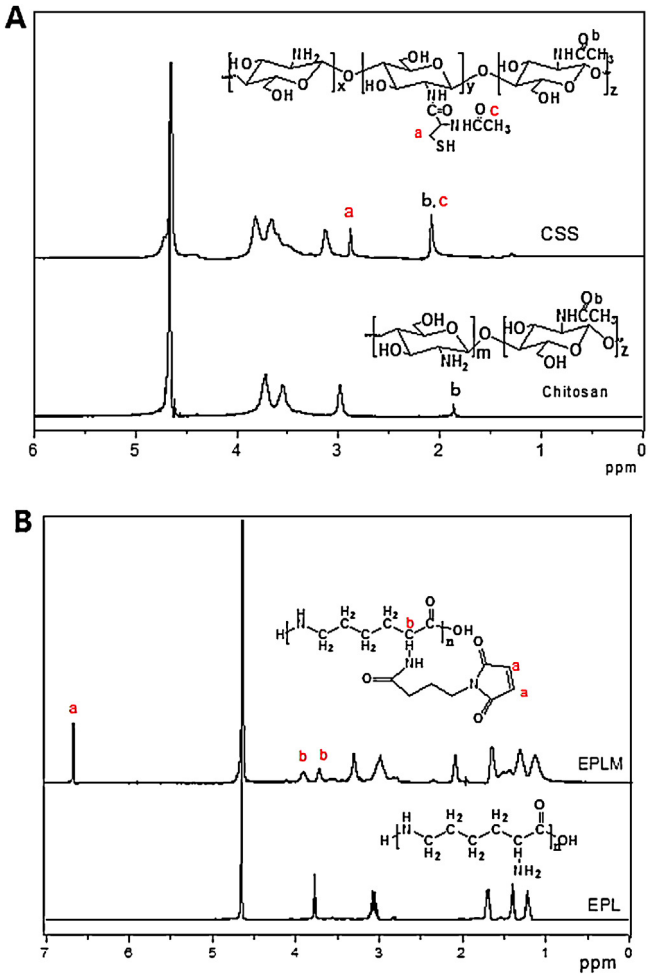

Fig. 2. ^{1}H NMR spectra of CSS (A) and EPLM (B).

be calculated as the area ratio of proton on the double bond (δ 6.6) to the total methine protons (δ 3.8 and 4.0) (Yu, Huang, & Huang, 2009). Two different molar feed ratios of Sulfo-GMBS to EPL were used in our study, which were 5/1 and 10/1, respectively. The DS of maleimide was found to increase with the increase of the feed ratio. As a result, two kinds of EPLM, the DS of maleimide were 5% and 30% respectively, were prepared, meaning 5% and 30% of amine groups in EPL were reacted with Sulfo-GMBS.

3.2. Gelation time

Fast crosslinking of the gel is preferred, especially for the adhesive sealant application where the gel must set up quickly and not flow from the administration site or be diluted with fluid. On the other hand, when using combined with cells or therapeutic molecules, slow gelation may lead to the leaking of bioactive molecules to surrounding areas or the heterogeneous incorporation of cells due to gravity. Most of the published articles report gelation times on the order of 10–60 min for Michael-type addition crosslinking (Phelps et al., 2012). This time scale is too long to meet the requirements of tissue sealants and hemostatic materials. It is urgent to improve the reaction kinetics to make hydrogels crosslinked *in situ* through Michael-addition possible to be used as tissue sealants.

In the present study, hydrogels were formed *via* Michael addition between maleimide groups on EPLM and thiols on CSS (Fig. 1C). It is well known that very rapid gelation may result in insufficient mechanical properties and adhesiveness because of the formation

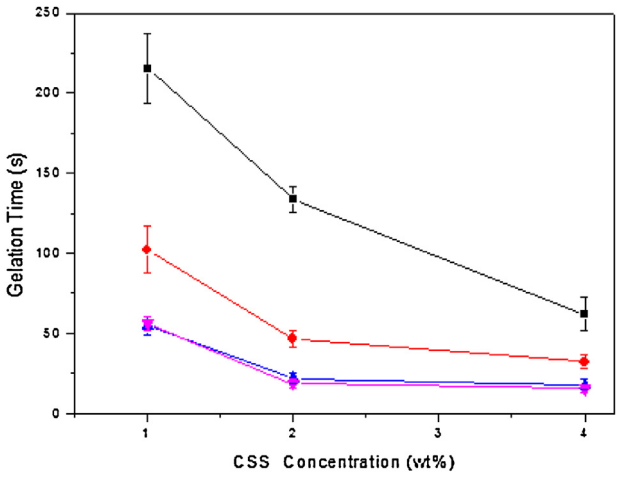

Fig. 3. Gelation times determined by the vial tilting method when mixing solution of CSS and EPLM in PBS (pH 7.4): (—■—) DS of maleimide is 5%, MAL/SH (mol/mol) is 1/1; (—●—) DS of maleimide is 5%, MAL/SH (mol/mol) is 2/1; (— ▲ —) DS of maleimide is 30%, MAL/SH (mol/mol) is 1/1; (— ▼ —) DS of maleimide is 30%, MAL/SH (mol/mol) is 2/1.

of non-homogeneous hydrogels (Lih et al., 2012). Obviously, transparent and homogeneous hydrogels were observed and no phase separation was detected under all conditions studied in our work despite the very short gelation time (Fig. 1D). DS of maleimide groups, concentration of CSS solution, and molar ratio of maleimide group to thiol group appeared to influence the gelation time. From Fig. 3, it can be seen that DS of maleimide groups significantly affected the rate of gelation. The hydrogel crosslinked by EPLM with maleimide DS of 30% showed shorter gelation times compared with that by EPLM with maleimide DS of 5%. It was worthy to notice that in spite of very low CSS concentration, a relatively fast gelation of CSS/EPLM hydrogels was still observed, which was attributed to the higher reactivity of maleimide groups compared with other reacting groups such as vinylsulfone and acrylate groups (Phelps et al., 2012). Higher contents of maleimide groups greatly improved the crosslinking kinetics. Furthermore, when the DS of maleimide was fixed at 5%, the gelation time was found to decrease obviously with the increase of the molar ratio of maleimide groups to thiol groups (MAL/SH). In terms of hydrogels crosslinked by EPLM with maleimide DS of 30%, however, the effect of MAL/SH on gelation time became negligible. This suggested that the kinetics of maleimide groups' reaction might approach saturation when DS of maleimide was as high as 30% (Phelps et al., 2012). In addition, the gelation time decreased with increasing CSS concentration. Higher CSS concentration increases the probability of chemical interaction and the network is expected to form more efficiently. When the concentration reached 4 wt%, the gelation time reduced to the range from 62 ± 10 s to 15 ± 3 s, which is appropriate for the *in situ* clinical use. Some of them are even quick enough for the use of adhesive sealants.

3.3. Rheological analysis

The mechanical properties of the CSS/EPLM hydrogels were studied by oscillatory rheology. PBS solutions of CSS and EPLM were mixed using a double syringe and quickly applied to the rheometer. The conditions used in Michael addition reaction for the formation of CSS/EPLM hydrogels are listed in Table 1. The storage modulus G' gradually increased after mixing solutions of CSS and EPLM due to the Michael addition reaction, until reaching its plateau values, indicating the completion of the crosslinking (Fig. 4). When the DS of maleimide increased from 5% to 30%, the G' plateau values of hydrogels increased dramatically. Take CSS/EPLM-1 and

Table 1
Conditions used in Michael addition reaction for the formation of CSS/EPLM hydrogel.

Hydrogel	DS of maleimide (%)	Concentration of CSS (wt%)	MAL/SH (mol/mol)[a]
CSS/EPLM-1	5	2	1/1
CSS/EPLM-2	30	1	1/1
CSS/EPLM-3	30	1	2/1
CSS/EPLM -4	30	2	1/1
CSS/EPLM -5	30	2	2/1
CSS/EPLM -6	30	4	1/1
CSS/EPLM -7	30	4	2/1

[a] Molar ratio of maleimide group to thiol group (denote as MAL/SH).

CSS/EPLM-4 for example, G' plateau values increased about 60 folds when DS of maleimide reached 30%. This suggested that DS of maleimide has the crucial impact on the modulus of CSS/EPLM hydrogel, for more maleimide groups can effectively increase the intermolecular crosslinks to form a stronger network.

It was also found from Fig. 4 that when DS of maleimide was fixed at 30%, the G' plateau values of the hydrogels ranged from *ca.* 16 Pa to *ca.* 380 Pa with the increase of CSS concentration from 1 wt% to 4 wt%. Higher CSS concentration enhances material stiffness as it may increase the chain entanglements and the probability of chemical interactions. Another reason may be lie in more disulfide formed *via* the air oxidation of thiols under ambient conditions during the process of gel formation (Wu et al., 2009), for there are more thiols in higher concentration solutions. Additionally, changes in MAL/SH showed slightly effects on G' when CSS concentration reached 4 wt% (CSS/EPLM-6 and CSS/EPLM-7). This suggests that the extent of network formation approaches saturation at critical concentrations of CSS. It became more difficult for the excess maleimide groups to participate gel formation because of the viscosity of higher concentration. In this case, excess EPLM appeared more likely to arouse a dilution effect, which was also found in some other two components *in situ* crosslinking hydrogel systems (Artzi et al., 2009). As MAL/SH showed very slight influence on gelation time and gel modulus, it was fixed at 1/1 in the subsequent study to avoid additional biocompatible issue caused by the excess of carbon-carbon double bonds.

3.4. Evaluation of adhesion strength

Considering the comparatively appropriate gelation time and modulus of CSS/EPLM hydrogel, DS of maleimide was fixed at 30%

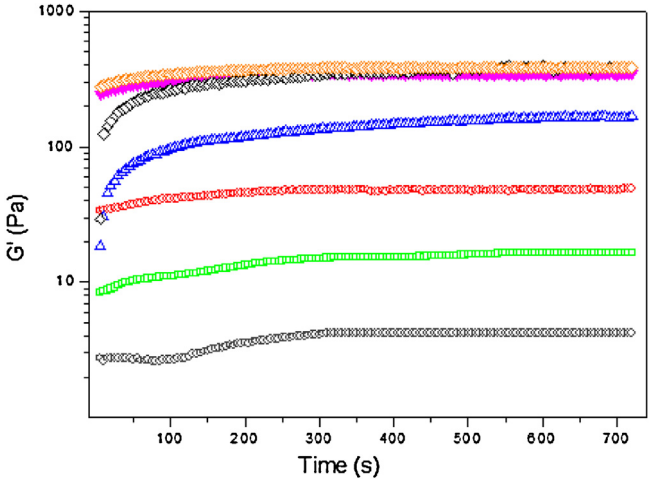

Fig. 4. Rheological analysis of the obtained hydrogels at a stress of 1 Pa and a frequency of 0.5 Hz: (—○—) CSS/EPLM-1; (—□—) CSS/EPLM-2; (—○—) CSS/EPLM-3; (—△—) CSS/EPLM-4; (—◇—) CSS/EPLM-5; (— ▼ —) CSS/EPLM-6; (—◇—) CSS/EPLM-7.

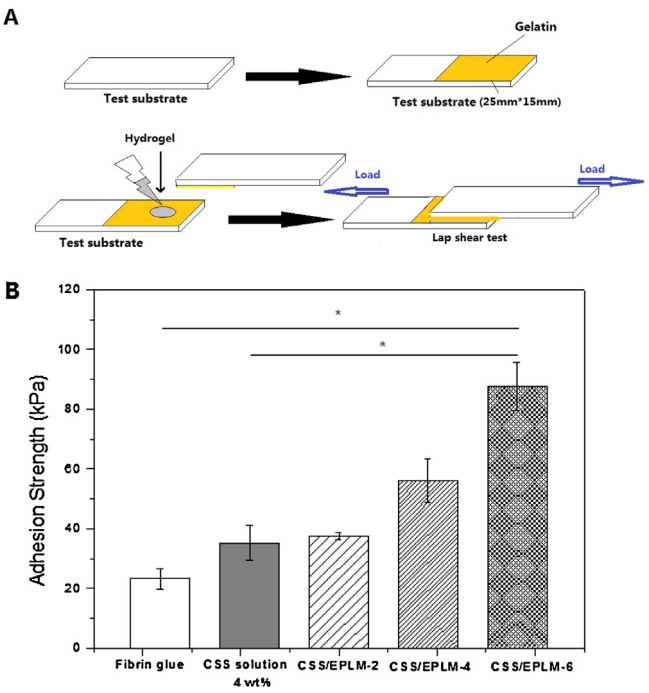

Fig. 5. Adhesion strength test: demonstration of the adhesion strength measurement (A); adhesion strength ($^*p < 0.01$) of the obtained CSS/EPLM hydrogels in comparison with fibrin glue and pure CSS solution (4 wt%) (B).

when adhesion test was conducted, that is, CSS/EPLM-2, CSS/EPLM-4, and CSS/EPLM-6 were chosen to further evaluate the adhesive performance of the present hydrogels.

Because of the far more chemical and physical similarities that gelatin shares with collagen, the most widespread component in most soft tissues as skin (Bellucci, Sola, Gentile, Ciardelli, & Cannillo, 2012), gelatin matrices have long been used as suitable alternatives to collagen-rich tissues that require reduced stiffness (Grover, Cameron, & Best, 2012). Furthermore, gelatin has also been acknowledged to ensure conformation to tissue surfaces (Emilia et al., 2011). Thus, Yang and Nie have recently established a method to employ gelatin matrix which was coated on glass as a model of soft tissue for adhesion strength test (Li et al., 2011; Wang et al., 2012). By referring to their method, we have the adhesion strength of our hydrogels measured (Fig. 5A). Commercially available fibrin glue was used as a control. As shown in Fig. 5, the adhesive strength of all the CSS/EPLM hydrogels was higher than that of the fibrin glue.

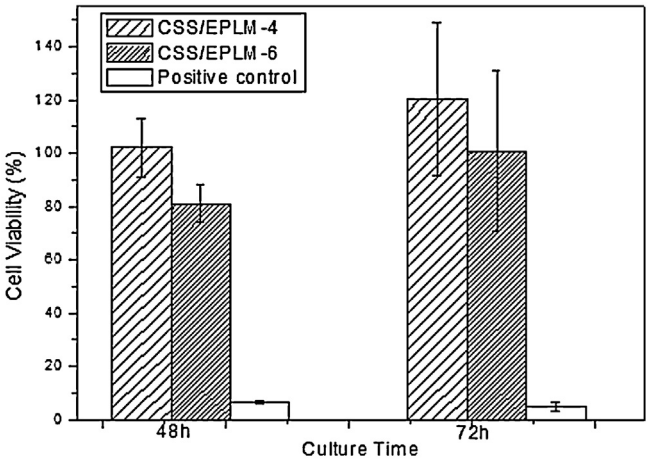

Fig. 6. Cytotoxicity test of the obtained hydrogels with a positive control.

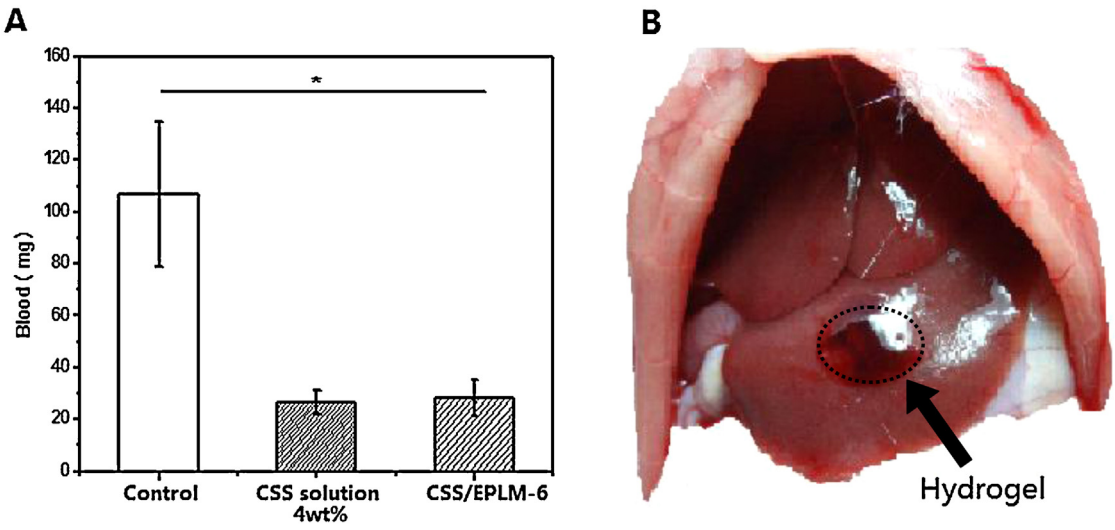

Fig. 7. Blood loss weight from a bleeding rat liver treated with CSS/EPLM-6 hydrogel, and pure CSS solution (4 wt%), respectively ($^*p < 0.01$) (A); illustration of the bleeding site which is treated by CSS/EPLM-6 hydrogel, the circle illustrated where the hydrogel is (B).

This was attributed to the multi-interaction between CSS/EPLM designed by us and gelatin which was used as a soft tissue analog here. Namely, covalent bonding, ionic attraction, as well as hydrogen bonding which appeared among hydroxyl, thiol, amine, and maleimide groups on the above macromolecules provided a much stronger bonding force. From Fig. 5, the adhesive strength was also found to be improved with the increase of CSS concentration. This is because higher CSS concentration may result in the more introductions of thiol groups and maleimide groups to the hydrogel according to our recipe. Hence, more crosslinkage formed and the cohesive force of the hydrogel network was improved. On the other hand, the higher the concentration is, the more interactions between gelatin matrix and hydrogel components are, which could also do favor to enhance the adhesive force. Moreover, it was noteworthy that CSS solution alone was said to have good adhesive properties (Wang et al., 2009). Therefore, pure CSS solution (4 wt%) was also tested and compared with CSS/EPLM-6 hydrogel, the CSS solution concentration of which was also 4 wt%. Significant improvement of adhesive strength was observed in the case of hydrogel. One reason is that the thiol-maleimide linkages and the formation of hydrogel network greatly improve the cohesive force. The other reason lies in the enhancement of the interaction between gelatin and the adhesive materials due to the more cationic groups provided by EPLM. It can be found that CSS/EPLM-6 hydrogel exhibited the highest adhesion strength, approximately 87.5 kPa, which were *ca.* 4 times higher than that of the fibrin glue.

3.5. Cytotoxicity assay

An ideal bioadhesive should not leak toxic products or molecules, which could be evaluated through *in vitro* cytotoxic tests. Therefore, it is important in the present project to verify the innocuous nature of the hydrogel. Cytotoxicity of CSS/EPLM hydrogel was investigated using L929 cell line and the results are shown in Fig. 6. No statistically significant differences ($p < 0.05$) were observed in the cell viability within 48 h and 72 h in the presence of hydrogel extracts in comparison with the negative control. Cell viability of CSS/EPLM-4 hydrogel extracts even surpassed the negative control, suggesting no toxicity to L929. Although a decrease of cell viability was observed in CSS/EPLM-6 hydrogel extracts, it still reached nearly 100% of that of the negative control at 72 h, indicating CSS/EPLM hydrogels studied in this assay were almost non-toxic to L929 cells. The excellence cytocompatibility of the CSS/EPLM hydrogels was ascribed to their structural and compositional similarity to natural extracellular matrices, as well as the green fabrication approach of the hydrogels, proving that the Michael addition reaction is a promising method and that CSS/EPLM hydrogel is a good candidate for internal clinical use.

3.6. Hemostatic property of CSS/EPLM hydrogel

To effectively arrest bleeding, hemostatic materials are required to possess quick curing time, strong bonding force to the surrounding tissues, as well as good biocompatibility. CSS/EPLM hydrogels were further explored as hemostatic tissue adhesives based on their excellent properties that meet the need of hemostatic agent. Fig. 7B shows photographs of bleeding liver treated with CSS/EPLM-6 hydrogel. It can be seen that the hydrogel was adhered onto the liver and the bleeding was effectively arrested. The amount of blood loss with and without treatment of hydrogels on the bleeding site was measured. From Fig. 7A, the total blood loss from the untreated liver was about 106.7 mg for 3 min after the liver was pricked. In contrast, the bleeding was found to be significantly arrested by the applying of the hydrogel. The loss of blood was reduced to 28.3 mg due to the synergistic effect of the good adhesiveness of the hydrogels and the hemostatic property of chitosan. Besides, as chitosan itself is known to have good hemostatic ability, the hemostatic property of CSS solution (4 wt%) alone was also tested. It should be noted that CSS solution alone also showed similar hemostatic ability to that of CSS/EPLM hydrogel. This phenomenon was similar to the results reported by Ryu et al. (2011) when they investigated the hemostatic property of their novel catechol-functionalized chitosan/pluronic adhesive hydrogels. However, in practical application, because of the slower gelation kinetic rate, CSS solution dressing on the bleeding site was more easily detached and washed out by buffer or body fluids compared with CSS/EPLM hydrogel (Ryu et al., 2011). Hence, the result demonstrates that the CSS/EPLM hydrogels exhibit both elastic and adhesive properties when crosslinked *in situ*, thus serving as an effective anti-hemorrhaging agent.

4. Conclusions

An attractive new hydrogel adhesive sealant consisting of thiolated chitosan and maleimide modified ε-polylysine was

developed, taking the privilege of the mild condition offered by Michael addition. The hydrogel can formed rapidly *in situ* within several seconds due to higher crosslinking efficiency of maleimide groups and the reaction time scales are appropriate for bioadhesive sealant applications. Furthermore, the hydrogel can be seen as a synthetic analog of natural extracellular matrix and showed almost no cytotoxicity. Besides, the hydrogel demonstrated adhesion strength that is significantly superior to the commercially available fibrin based adhesives. When applied to a rat liver defect, the hydrogel showed excellent hemostatic property. These kinds of *in situ* forming hydrogels are expected to be applied for novel tissue sealants and hemostatic materials and also hold high potential for drug delivery and tissue regeneration.

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Appendix A. Supplementary data

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