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PAPER

Synthesis and characterization of biodegradable macroporous cryogels crosslinked by chitosan oligosaccharide-*graft*-acrylic acid†

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Biodegradable macroporous hydrogels were prepared using a biodegradable crosslinker based on chitosan derivatives. The chitosan crosslinker was synthesized through the amidating reaction of amino groups in the chitosan oligosaccharide (CSO) chains and carboxyl groups in acrylic acid (AA) under the catalysis of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimidehydro-chloride (EDC) and *N*-hydroxysuccinimide (NHS). The amidating reaction is able to provide polymerizable carbon–carbon double bonds for CSO. The chemical structure of the resulting compound chitosan oligosaccharide-*graft*-acrylic acid (CSO-*g*-AA) was characterized by Fourier transform infrared spectroscopy (FTIR) and ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy. The content of double bonds was measured by titration analysis. The measurement showed a grafting ratio of AA on CSO of about 3.5% (on average two acryoyl groups per CSO-*g*-AA chain) when the applied molar ratio of AA to CSO was 1.0. Polyacrylamide (PAM) cryogels were synthesized by the free radical polymerization of acrylamide using a modified freezing polymerization method crosslinked by CSO-*g*-AA. The obtained cryogels were macroporous hydrogels which could be degraded into solutions of linear polymer chains when treated with appropriate enzymes. Then we used snailase as a model enzyme to study the biodegradation process. The degradation was monitored by morphological studies using a confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM), mechanical strength, and swelling ratio. The duration of the degradation process was adjustable from one month to two months when using different concentrations of snailase solutions.

Introduction

Hydrogels are three-dimensional crosslinked polymer networks, which are able to absorb large amounts of water while maintaining their insoluble property. Due to their good biocompatibility, high swelling ratio and special structural properties, hydrogels are increasingly used in various biological applications including drug delivery and tissue engineering.[1,2] Macroporous hydrogels are gel matrices containing macropores. The unique structure makes them quite attractive in biotechnical applications such as cell scaffolds,[3] drug delivery,[4,5] enzyme immobilization,[6] cell separation,[7] and so on. Generally, macroporous hydrogels can be obtained utilizing the methods of microphase separation,[8] porogen leaching,[9] gas bubbling,[10] comb-type graft copolymerization,[11] lyophilization–hydration,[12] and also cryo-polymerization.[13–15] Gels prepared at temperatures below the melting temperature of the solvent are called cryogels. The dissolved reactants are concentrated in small nonfrozen regions (so-called 'liquid microphase') whereas most of the solvent is frozen when reaction temperatures are below zero. Gel formation occurs in this liquid microphase and the crystals of frozen solvents act as pore-forming agents. After melting the crystals of the solvents, pores are formed in the resulting gels. Cryo-polymerization is considered as the most efficient and easiest method to achieve porous hydrogels with high mechanical stability.

A modified cryo-polymerization method for preparing macroporous hydrogels was developed in our laboratory a few years ago.[16–18] The speciality of our method comparing with conventional cryo-polymerization methods is that a cation surfactant dodecyldimethylbenzylammonium bromide (DDBAB) is used during the cryo-polymerization process. The function of DDBAB is that it will react with the water soluble initiator ammonium persulfate (APS) to form a hydrophobic initiator dodecyldimethyl-benzylammonium persulfate (DDBAPS) and the cryo-polymerization reaction is initiated by DDBAPS aggregates with sizes of hundreds of nanometers. This modified method is able to enhance the microphase separation between the pores and the pore walls. Therefore, the resulting cryogels possess macropores with much thinner pore walls than conventional cryogels.

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On the other hand, biodegradable hydrogels have also attracted great attention because of their advantages in some biotechnical applications. An effective approach to the formation of degradable cryogels is the utilization of degradable crosslinkers. During the past decades, many researchers have fabricated biodegradable crosslinkers. Biodegradable crosslinkers such as phosphazene derivatives, polysaccharide derivatives, aliphatic polyesters or poly(amino acid)s have been employed to obtain biodegradable hydrogels. In this way, hydrogels are able to be degraded into solutions of linear polymers under moderate conditions. Grosse-Sommer *et al.*[19] synthesized a trimeric phosphazene crosslinker to make degradable gels, and the crosslinking points of the gels would be hydrolyzed in aqueous condition. Paris and Garrido[20] introduced modified polycaprolactone chains as a crosslinking agent to synthesize biodegradable hydrogels. Andac *et al.*[21] synthesized *N*,*N*′-bis(methacryloyl)-L-cystine containing a disulfide crosslinkage (–S–S–) which can be cleaved by reductants such as dithiothreitol. Cha *et al.*[22] modified alginate to achieve oxidized methacrylic alginate which was used to crosslink poly(ethylene glycol) methacrylate and poly(*N*-hydroxymethyl acrylamide) to form hydrogels. Among the biodegradable crosslinkers, natural polymers such as alginate show advantages for their nontoxic and biocompatible properties. Therefore, we also want to choose modified natural polymers as crosslinkers in order to synthesize biodegradable cryogels using our modified cryo-polymerization method. However, alginate is an anionic polymer which will react with the cation surfactant DDBAB in the method. This matter will lead to the failure of synthesis of the cryogels. Therefore, natural polymers without anion charges are preferred for the creation of cryogels, which inspires us to synthesize chitosan-based crosslinkers.

Chitosan, a linear cationic natural polyaminosaccharide, obtained by N-deacetylation of chitin (the second most abundant natural macromolecule except cellulose), is of great importance in biological systems due to its biodegradability, biocompatibility, and antimicrobial abilities.[23] Providing functional groups as primary amines as well as hydroxyl in the repeating units, chitosan can be chemically modified without reducing the degree of polymerization.[24] However, chitosan cannot commonly be dissolved in pure water, so that chitosan oligosaccharide (CSO) is used in this work for the synthesis of a novel biodegradable crosslinker chitosan oligosaccharide-*graft*-acrylic acid (CSO-*g*-AA) due to its excellent solubility in water. Water solubility is an important property which allows polymerization of CSO-*g*-AA crosslinked cryogels in the 'liquid microphase' of a frozen state. Once there are more than two acryloyl groups (–CO–CH=CH_2) per CSO polymer chain, CSO-*g*-AA is able to act as a biodegradable crosslinker to synthesize most of the acrylic acid or acrylamide cryogels or their derivative cryogels. The obtained cryogels are able to be degraded into linear chains due to the degradation of the crosslinking point in appropriate enzyme solutions.

In this paper we aim to prepare a novel kind of biodegradable macroporous cryogel which might potentially be applied in structural scaffolds for the culture of living cells in the macropores and modified chitosan oligosaccharide was used as the crosslinker. We report the synthesis and characterization of CSO-*g*-AA and the CSO-*g*-AA crosslinked cryogels. The chemical characteristics of the CSO-*g*-AA crosslinker were analyzed by Fourier transformed infrared spectrometer (FTIR), nuclear magnetic resonance spectrometer (NMR) and KBrO/KBr titration method. The morphology of the cryogels during the degradation process was observed by confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM). Furthermore, the kinetics of the degradation of the CSO-*g*-AA crosslinked cryogels was followed by rheological measurements.

Experimental

Materials

Chitosan oligosaccharide (Mw < 10 000, degree of deacetylation 85%, Jinan Haidebei Marline Bioengineering, China), acrylic acid (AA, 98%, Shanghai chemical reagent purchase and supply Wulian chemical factory), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimidehydro-chloride (EDC, 98.5%, Aladdin-reagent, Shanghai, China), N-hydroxysuccinimide (NHS, 98%, J&K Chemical Ltd.) and fluorescein isothiocyanate, isomer I (FITC, 95%, Alfa Aesar), were employed as received. For the synthesis of the hydrogels, acrylamide (AAm, 99%, Shanghai Third Chemical Reagent Factory, China), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, 99%, Acros Organics, New Jersey), ammonium persulfate (APS, ≥98%, Sinopharm Chemical Reagent Co. Ltd., China) and dodecyl dimethyl benzyl ammonium bromide (DDBAB, 95%, Shanghai Jingwei Chemical, China) were used as received. Potassium bromate (KBrO), potassium bromide (KBr), sodium hyposulfite ($Na_2S_2O_3$), potassium iodide (KI) and soluble starch (Aladdin-reagent, Shanghai, China) were also used as received. Potassium dichromate ($K_2Cr_2O_7$, Aladdin-reagent, Shanghai, China) was dried before using. Snailase (Fujian Zhangzhou Jintian Bio-tech Co. Ltd., China) was used for the degradation of the hydrogels. Buffer solutions with pH = 4 were prepared by a combination of HAc and NaAc solutions. The ionic strength of all the buffers was 0.02 mol L^{-1}. The pH values were determined with a pH meter (pHS-3C, Hangzhou Dongxing, China).

Synthesis of CSO-*g*-AA

Chitosan oligosaccharide (1.1 g), EDC (0.7 g), and NHS (0.3 g) were dissolved in 25 mL dilute hydrochloric acid (HCl) solution with pH of 4.7. Then, 0.25, 0.39, 0.49, 0.59, 0.74, or 0.98 g acrylic acid, namely molar ratios of AA to the repeating unit of CSO (RUCSO) of 0.5, 0.8, 1.0, 1.2, 1.5, or 2.0 respectively, was added into the reaction medium respectively. The reaction was carried out at room temperature for 72 h under vigorous stirring. Successively, the solution was poured into ethanol to precipitate out the product CSO-*g*-AA. The product was filtered off and washed with ethanol several times to wash out unreacted compounds. Afterwards, the product was dried at room temperature until a constant weight was achieved.

Characterization of CSO-*g*-AA monomer

The FT-IR spectra were recorded in potassium bromide pellets using a FTIR-5700 spectrophotometer (Nicolet). The dried CSO, CSO-*g*-AA, and CSO-*g*-AA crosslinked cryogel were powdered, ground with potassium bromide powder and pressed into pellets

under reduced air pressure. The IR scans were recorded between 4000 and 500 cm^{-1}. CSO-*g*-AA was also characterized using ^1H -NMR (Bruker Avance 400) spectroscopy.

Determination of the amidation degree of CSO

For each experimental datum in the following experiments, in addition to those mentioned in particular, at least three values were averaged to obtain reliable data. The bromine titration in the form of the familiar potassium bromide–potassium bromate mixture has been applied to quantitate unsaturated carbon–carbon double bonds for other compounds[25,26] and was applied to measure that in CSO-*g*-AA after some modifications. Briefly, a standard solution of potassium bromide and potassium bromate was employed in acid solution to generate bromine, which reacted with the unsaturated double bonds. Then, the unsaturated double bonds were reacted with bromine for 120 min. After that, potassium iodide solution was added. The liberated iodine was titrated against thiosulphate solution using starch as the indicator towards the end point.

The grafting ratio (GR) of unsaturated double bonds in CSO-*g*-AA can be estimated by titration analysis, and calculated from the following equation:

$$\mathrm{GR} = \frac{c(V_0 - V_1) \times M}{2m \times 1000} \times 100\%$$

where c stands for the molar concentration of $Na_2S_2O_3$ solution (mol L^{-1}), V_1 and V_0 respectively stand for the volume of $Na_2S_2O_3$ solution used in the sample and blank test (mL), M stands for molar mass of each CSO repeating unit (161 mol g^{-1}), and m stands for the mass of the CSO-*g*-AA sample (g). GR shows the molar ratio of grafted acryloyl groups to the repeating units.

Preparation of CSO-*g*-AA porous hydrogels

The CSO-*g*-AA product with a molar ratio of AA to CSO of 1.0 was used to prepare hydrogels. To form macroporous cryogels, 150 mg acrylamide, 50 mg CSO-*g*-AA and 25 mg DDBAB were dissolved in 1.5 mL deionized water in glass columns (i.d. 20 mm). The solution was precooled in a 4 °C refrigerator for 10 min followed by adding TEMED (10 μL) and APS (4 wt%, 100 μL). Polymerization was carried out at −12 °C for 12 h. Then, the defrosted cryogels were immersed in excess deionized water at ambient temperature for 48 h, and the water was refreshed every few hours to remove the unreacted materials. For the preparation of nonporous hydrogels (common hydrogels), 150 mg acrylamide and 50 mg CSO-*g*-AA were dissolved in 1.5 mL deionized water in glass columns (i.d. 20 mm). Then TEMED (10 μL) and APS (4 wt%, 100 μL) were added to initiate the polymerization. Polymerization was carried out at room temperature for 12 h. The common hydrogels were washed in the same way after the reaction. Special attention should be paid to the fact that the common hydrogels mentioned in this research work are nonporous degradable hydrogels.

The cryogels crosslinked by CSO-*g*-AA which were synthesized from the molar ratio of AA : RUCSO = 1 : 1 were applied in the following experiments such as mechanical strength measurements, swelling ratio measurements, and morphology observations, because these cryogels were more mechanically stable than the other cryogels crosslinked by CSO-*g*-AA which were synthesized from the other molar ratios of AA to RUCSO.

Mechanical strength of the cryogels with various amounts of crosslinker

The effect of composition on mechanical strength of the cryogels was investigated using dynamic viscoelastic measurements. When preparing cryogels with various amounts of CSO-*g*-AA, the procedures for the preparation were applied as described above. Here, other amounts of CSO-*g*-AA (30 mg, 40 mg, and 60 mg) were applied. The applied amounts of acrylamide were 170 mg, 160 mg, and 140 mg respectively, in order to maintain the total amount of the monomers at 200 mg.

The dynamic viscoelastic properties of the cryogels were measured with a rheometer (RS6000, HAAKE, Deutschland) using parallel plates of 20 mm diameter during the degradation experiments. The surface of the plates was coated with a film of vacuum silicon grease to avoid sample slip as well as evaporation of water from the samples. Small amplitude shear experiments were performed over a frequency (f) range of 0.1 to 10 Hz at a constant shear strain (γ) of 0.5%. All the rheology measurements were carried out at 25 ± 0.1 °C controlled by a Peltier plate.

Mechanical strength of the cryogels through the degradation process

Snailase was dissolved in HAc–NaAc buffer solutions at pH = 4.0. The CSO-*g*-AA cryogel with a diameter of 20 mm and height of 5 mm was placed in a 25 mL beaker and treated with different concentrations of snailase (in the range 0.2–0.8 mg mL^{-1}) at 37 °C without stirring. The snailase solutions were refreshed every 48 h. Dynamic viscoelastic measurements were performed in the same way as the above mentioned method.

The cryogels applied here were prepared by polymerization of 50 mg CSO-*g*-AA and 150 mg acrylamide. Hydrogels with the same composition were also used for the following characterization.

Swelling ratio of the cryogels

The macroporous cryogel samples which were immersed in 0.2 mg mL^{-1} snailase solutions during the degradation process were measured gravimetrically after wiping off the excess water with wet filter paper. The swelling ratio (SR) is calculated as W_s/W_d, where W_s is the weight of the water in the swollen sample at pH = 4 and W_d is the weight of the dry sample.

Morphology observations of the cryogels through the degradation process

The morphology changes of the cryogels in the fully swollen state during the degradation process were visualized by confocal laser scanning microscope (CLSM, A1R/A1, Nikon, Japan). Pieces of cryogel with a thickness of approximately 0.5 mm were immersed in FITC solution with a concentration of 5 mg L^{-1} for 72 h and then refreshed with deionized water every several hours until no fluorescent dye could be detected in the washings. The fully

stained samples were then cut into slices with a thickness of approximately 0.5 mm to perform further observations. Fluorescent images were obtained using a CLSM. The light sources were HeNe lasers with excitation wavelength 488 nm for FITC. All images were taken at 512 × 512 pixels.

The morphology of freeze dried samples during degradation was studied using scanning electron microscope (SEM). Before the observations, the dried samples were coated with platinum by coating equipment (IB-5 ION coater, EIKO, Japan) 5 min in advance.

The samples of common hydrogels were prepared and observed in the same way as the cryogels.

Results and discussion

Characterization of CSO-*g*-AA monomer

CSO-*g*-AA was synthesized as a result of the amidating reaction of amino groups on CSO chains with the carboxyl group on AA activated by EDC and NHS (Fig. 1). Using EDC, the carboxylic acid groups on AA were activated and o-acylisourea groups were given.[27] In the presence of NHS, the o-acylisourea group was converted to the NHS-activated carboxylic acid group, which was the reactive species towards the nucleophilic attack of the free amino group.[28–30] CSO-*g*-AA could be a usable crosslinker for the preparation of hydrogels which are synthesized from the polymerization of vinyl monomers, when CSO-*g*-AA contains two or more acryloyl groups. On the other hand, CSO-*g*-AA would lose its crosslinking capability when degradations take place between every two polysaccharide repeating units which are attached by acryloyl groups. Therefore, the CSO-*g*-AA crosslinked hydrogels could be biodegraded at the crosslinking point. The newly synthesized hydrophilic crosslinker CSO-*g*-AA has a good solubility in water, which allows the polymerization of acrylamide cryogels in the frozen state.

The degree of amidation of the CSO chains was controlled by the molar ratio of the carboxyl groups of AA to the amino groups on the CSO chains. The effect of the molar ratio on the amidation degree is shown in Fig. 2. It can be seen from Fig. 2 that the grafting ratio of CSO with AA shows a maximum of approximately 3.5% when the molar ratio of NH_2 (CSO) to COOH (AA) is 1 : 1. Therefore the molar ratio of AA to CSO of 1 : 1 was used in the following experiments. CSO-*g*-AA crosslinkers with different amidation degrees were used to prepare hydrogels, but only the products with a molar ratio of NH_2 (CSO) to COOH (AA) of 1.0 and 1.2 can form integrated hydrogels while the other four can only make the solution more viscous. Moreover, the hydrogel with the molar ratio of 1.0 showed much higher mechanical strength than the hydrogel with the ratio of 1.2. This indicates that CSO-*g*-AA can be used as an

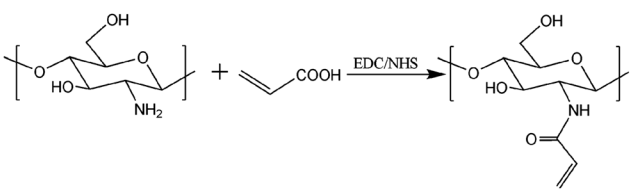

Fig. 1 Reaction diagram of the synthesis of the crosslinker CSO-*g*-AA.

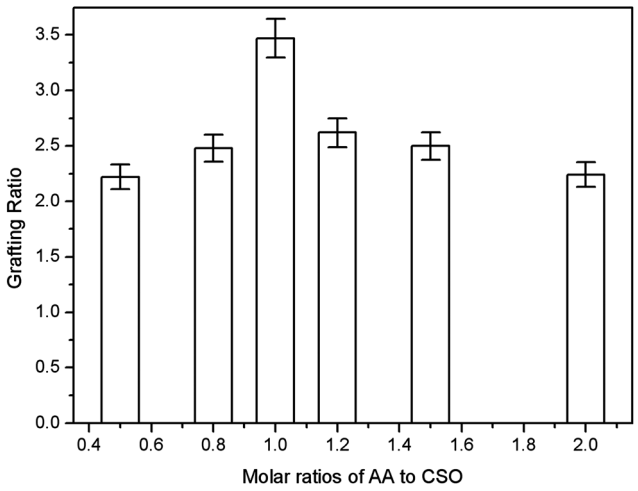

Fig. 2 The effect of molar ratio of AA to CSO on the grafting ratio.

efficient crosslinker only when there are enough acryloyl groups per CSO-*g*-AA chains. For the molar ratios of NH_2 to COOH of 0.5, 0.8, 1.5, and 2.0, the grafting ratios are all below 2.5%. Although the polymerization of CSO-*g*-AA without enough unsaturated bonds and acrylamide monomers also occurred, an integrated three-dimensional network can not form. On the other hand, we can calculate the approximate numbers of repeating units per CSO chain. The molecular weight of the applied CSO is approximately 10 000 and the molecular weight of the repeating unit of the polysaccharide CSO is 161. Therefore, there are approximately 62 repeating units for each CSO chain. The grafting ratio of the acryloyl group in CSO-*g*-AA is about 3.5%, which means *ca.* two (calculated as 62 × 3.5%) AA molecules were attached to one CSO chain. This can act as crosslinking to form three-dimensional networks. For the molar ratios of NH_2 to COOH of 0.5 and 0.8, the grafting ratios of CSO with AA were low. The possible reason is that less AA reacted with CSO when less AA was added. However for the molar ratios of NH_2 to COOH of 1.5 and 2.0, maybe the reason is different. This

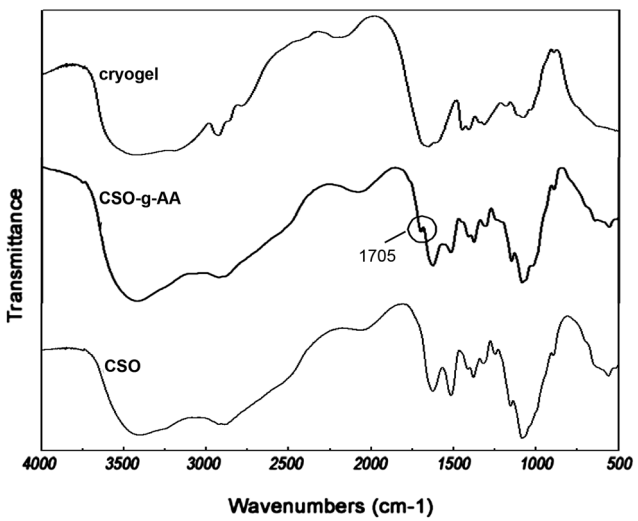

Fig. 3 FTIR spectra of CSO, CSO-*g*-AA and the CSO-*g*-AA crosslinked cryogel.

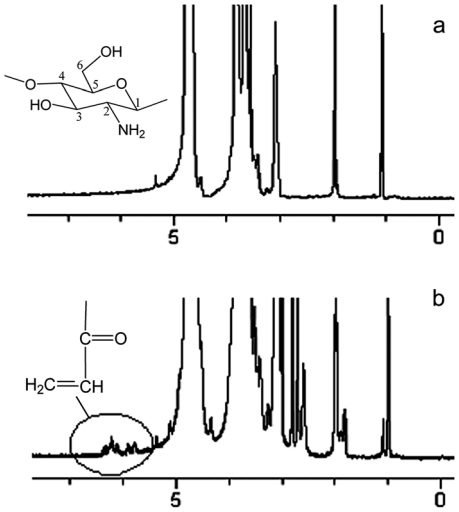

Fig. 4 ^{1}H NMR spectra of CSO (a) and CSO-*g*-AA (b).

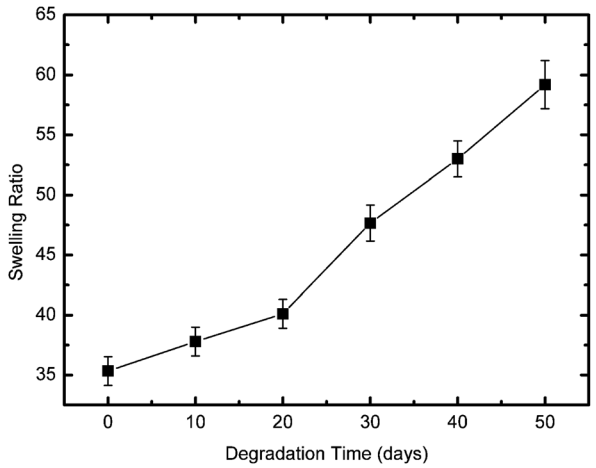

Fig. 5 Swelling ratio of the CSO-*g*-AA crosslinked cryogels during degradation.

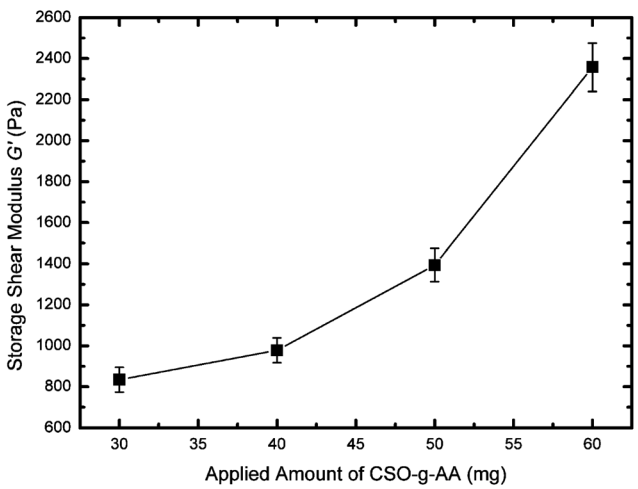

Fig. 6 Storage shear modulus G' of the cryogels with various amounts of CSO-*g*-AA crosslinker.

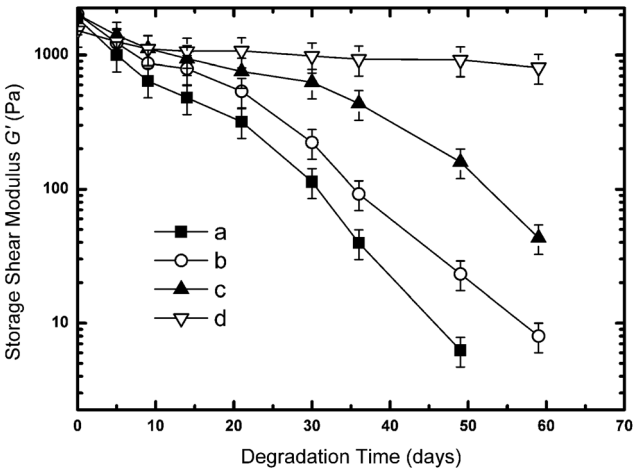

Fig. 7 Storage shear modulus G' of the CSO-*g*-AA crosslinked cryogels at various degradation times in different concentrations of snailase: 0.8 mg mL^{-1} (a), 0.4 mg mL^{-1} (b), 0.2 mg mL^{-1} (c), and 0 mg mL^{-1} (d).

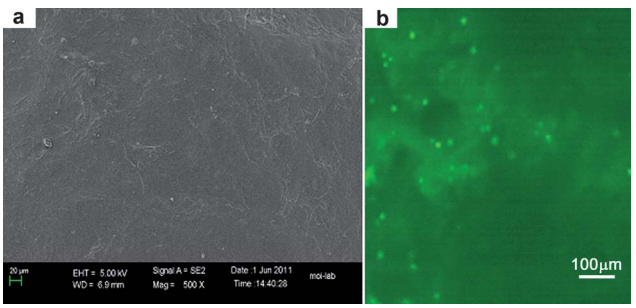

Fig. 8 SEM (a) and CLSM (b) micrographs of CSO-*g*-AA nonporous hydrogels.

reaction is related to the pH value of the reactant solution.[31] Adding more AA resulted in a decrease of the pH value of the reactant solution, which could probably show a negative effect for the reaction of CSO with AA. Although the grafting ratio is very low, it is sufficient for the formation of desirable cryogels since there are on average two carbon–carbon double bonds per CSO-*g*-AA chain. It is presumed that a higher grafting ratio of the acryloyl group on CSO-*g*-AA is not necessary or is even disadvantageous for obtaining appropriate cryogels as a higher grafting ratio would result in more crosslinking points in the obtained cryogels. Then, the pore walls in the cryogels would be more compact, and on the other hand, CSO needs to be cut into much shorter chains before the cryogels could be totally degraded into solution. These two matters would certainly make the cryogels much harder to degrade when the grafting ratio is higher.

FTIR analysis was used to confirm the chemical changes after the amidating reaction. It can be seen from Fig. 3 that the FTIR spectra of CSO-*g*-AA and CSO are almost the same. However, a new peak at 1705 cm^{-1} can be observed for CSO-*g*-AA revealing C=O of secondary acylamide which can not be observed in the CSO spectrum. This finding indicates that AA was attached to the chitosan chain. The peak at 1705 cm^{-1} disappeared in the spectrum of the CSO-*g*-AA crosslinked cryogel, demonstrating that most of the carbon–carbon double bonds were polymerized. ^{1}H NMR can provide more detailed

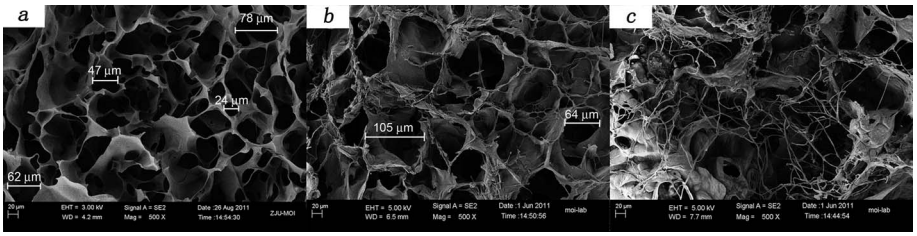

Fig. 9 SEM micrographs of CSO-*g*-AA cryogels before (a) and after treatment by 0.2 mg mL^{-1} snailase solution for 30 days (b) and 60 days (c).

information about the molecular structure than FTIR. The ^1H NMR (D_2O, ppm) spectrum of CSO-*g*-AA is shown in Fig. 4. The resonance signals at δ 5.75–6.38 ppm correspond to the protons of –CO–CH=CH_2. The signals observed between 4.4 and 5.0 correspond to the hydrogen bonded to the carbon atom C1. The signals between 3.30 and 4.00 ppm correspond to hydrogen atoms bonded to the carbon atoms C3, C4, C5 and C6 of the glucopyranose that are overlapped. The signal centered at 3.11 ppm corresponds to the hydrogen bonded to the carbon 2 of the glucopyranose ring, while the signal centered at 1.978 ppm corresponds to the hydrogen atoms of the methyl moieties belonging to the acetamido groups (–N–CO–CH_3).[32,33] The degree of deacetylation of applied CSO is 85% as mentioned in the experimental part, so, there are groups retained in CSO. Since the grafting ratio of the acryloyl group in CSO-*g*-AA is only about 3.5%, the changes in both the FTIR and NMR spectra after the amidating reaction are not very significant. However, the evident changes indicate CSO-*g*-AA is an efficient crosslinker.

Swelling ratio of the cryogels

The swelling ratios of the CSO-*g*-AA crosslinked cryogels during the degradation process are shown in Fig. 5. It can be seen that with an increasing degradation time period, the swelling ratio of the cryogels becomes higher and higher. The swelling ratios of undegraded samples are approximately 35 and that of the samples increases up to approximately 60 after being degraded for 50 days. A possible reason for the increase of the swelling ratio is that the partial degradation of the crosslinkers in the network result in a loosening of the compact pore walls accompanied by an extension of the crosslinked polymeric chains and a further swelling of the cryogels. As a result, the cryogels could retain more water. The swelling ratio was hard to measure accurately after 50 days because small gel fragments began to break off from the gel monolith and the gels were too weak to be taken out of the solution as an intact monolith. Therefore the data after 50 days are not presented.

The appearance of a same piece of cryogel during the degradation process is presented in Supplementary Information (Fig S1†). The size of the sample increased with an increasing degradation time period. The diameter of the sample is around 2 cm before degradation, while the diameter is nearly 3 cm and 4 cm after being degraded for 20 days and 40 days respectively. After about 50 days, the cryogel could not expand as an intact monolith and began to break into small pieces. All these results are in accordance with those in the swelling ratio measurements. It can also be seen from Fig S1† that the colour of the cryogels

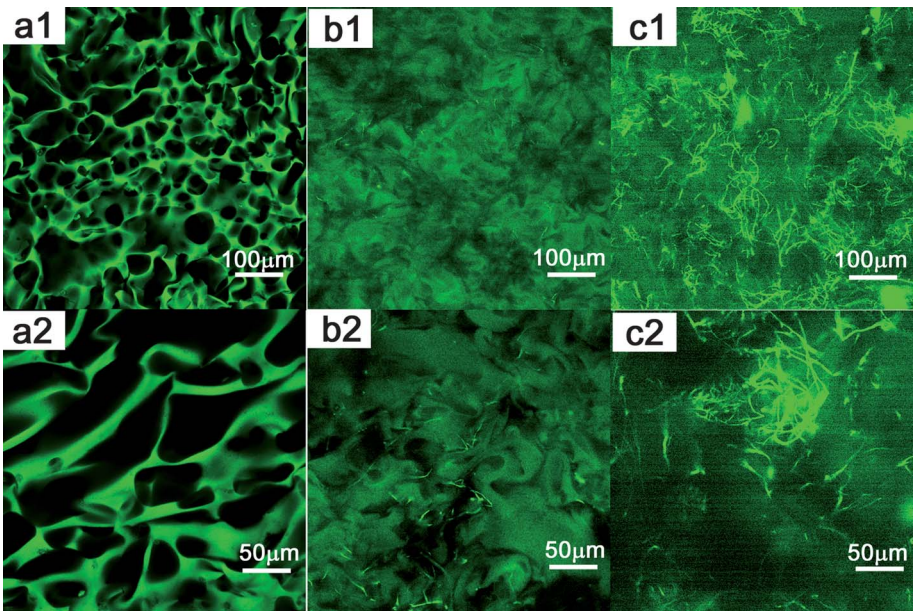

Fig. 10 CLSM micrographs of CSO-*g*-AA cryogels before (a1, a2) and after treatment by 0.2 mg mL^{-1} snailase solution for 30 days (b1, b2) and 60 days (c1, c2).

became duller and duller as the degradation went on. The probable reason is that the chitosan chains became shorter during the degradation process. Some researchers have suggested that the colour of chitosan with a lower molecular weight would be darker than that of the ones with higher molecular weight.[34] Therefore, the cryogel color became deeper.

Mechanical properties of the cryogels with various amounts of crosslinker

Fig. 6 shows the storage shear modulus G' of the cryogels with various amounts of CSO-*g*-AA crosslinker. It can be seen that G' of the cryogels increases evidently when the applied amount of CSO-*g*-AA increases. When the applied amount of CSO-*g*-AA is 30 mg, G' of the cryogel is approximately 800 Pa. When the applied amount of CSO-*g*-AA is 60 mg, G' increases up to approximately 2400 Pa. This indicates that the cryogel becomes more compact and tougher due to the increase of the amount of crosslinkages.

Mechanical properties of the cryogels during degradation

The cryogels with CSO-*g*-AA crosslinker degraded in snailase solutions. Chitosan is a linear cationic heteropolymer of randomly distributed GlcNAc and GlcN residues with β-1,4-glycosidic linkages. Snailase is a complex enzyme of cellulase, invertase polygalacturonase, amylase, hemicellulase, protease and pectinase. Most of them can hydrolyze β-1,4-glycosidic linkages, thereby degrading CSO.[35] Snailase was chosen as a model enzyme because it is efficient to degrade the cryogels at an economic price. When other single enzymes such as lysozyme and lipase were used for degradation of the cryogels, the rate of the degradation was much slower compared to snailase, which can be explained by synergistic effects of the complex enzyme system which may increase the efficiency of degradation of CSO. Moreover, complex enzymes would probably be much more similar to the enzyme systems in living bodies than single enzymes.

Fig. 7 shows the storage modulus G' of the cryogels *versus* the degradation time at a frequency (f) of 0.1 Hz, 25 °C and $\gamma = 0.005$. It can be seen that the rate of degradation is faster when the cryogels are treated with a high concentration of snailase solution. The storage modulus G' of the cryogels are as high as around 1800 Pa for all of the samples before degradation. It seems that the macroporous structure is tough enough to support the entire gel. As the cryogels degraded, the density of the elastically effective crosslinking points in the pore walls drops, and so does the storage modulus G'. After 30, 50, and 60 days' treatment with concentrations of snailase solution of 0.8 mg mL^{-1}, 0.4 mg mL^{-1}, and 0.2 mg mL^{-1}, respectively, the value of G' dropped to about 0.1% of the original value. Then, the cryogels disintegrated into small pieces and the mechanical properties could hardly be measured, and the cryogels dissolved in the snailase solution completely after about ten days. The cryogel samples were immersed in the same buffer solutions (HAc–NaAc, pH = 4.0 at 37 °C) without snailase as parallel experiments (Fig. 7(d)). It can be seen that the storage modulus G' dropped slightly over the 60 day period, but by much less than the substantial decreases in G' of the cryogels as shown in Fig. 7(a, b, and c). The storage modulus G' dropped slightly because chitosan, as a biodegradable nature polyaminosaccharide, can be degraded in natural conditions slightly. When treated with snailase, the rate of degradation of the gels becomes obviously faster.

Morphology of the hydrogels

Fig. 8 shows the surface morphology of the common hydrogels before degradation. It confirms that the common hydrogels are nonporous in both the dried and swollen state. In the additional experiments, no obvious changes of the surface morphology could be found after degradation. It is speculated that the degradation reactions took place on the surface of the hydrogels. The former surface is peeled off continuously during degradation and the newly exposed surface is still nonporous.

The morphological changes in the degradation process were observed by SEM and CLSM. Fig. 9 shows the morphology of the freeze dried cryogels during the degradation experiments. It can be seen from Fig. 9(a) that the pore walls are smooth and the sizes of most pores are around 50 μm. Fig. 9(b) shows the morphology of the cryogels after 30 days' degradation. The pore walls become uneven and some fibriform objects begin to appear. At the same time, the pore size becomes larger as a result of further swelling of the cryogels during the degradation. The pore walls degraded partly due to degradation of the crosslinkers, so that the pore walls would probably be broken which resulted in an uneven surface. Meanwhile, the macroscopic volume of the cryogels became larger as shown in Fig. S1.† Fig. 9(c) shows the morphology of the cryogels after degradation for 60 days. There are more fibers and the pores become even larger. It is supposed that the structure of the cryogels is supported by the fibers rather than the pore walls close to the end of the degradation.

Fig. 10 shows the CLSM observations of the cryogels in the fully swollen state. Fig. 10(a) shows that the pores are smooth and uniform before degradation, which is similar to Fig. 9(a). It can be seen from Fig. 10(b) that the boundary between the pore walls and the pores is not so obvious and a few fibers appear. The size of the pores becomes smaller. Although the phenomenon is distinct from that in Fig. 9(b), the substances are not in conflict. As a result of the degradation of the crosslinkers in the pore walls, the crosslinking density decreased. Therefore, the pore walls can swell better in the solutions. So the pore walls expanded and occupied a partial area of the pores in the fully swollen state, while the pore size became smaller. On the other hand, the pore walls huddled together again after been freeze dried, so the pore size seemed larger in Fig. 9(b). Fig. 10(c) shows the CLSM observations of the cryogels after 60 days degradation. The macroporous structure disappeared leaving only many fibers. The remaining structure is supported by the fibers which can not keep the pores in the swelling state, and the entire cryogel cannot be supported any more.

Proposed degradation mechanism of the cryogels

Immersed in snailase solutions, CSO-*g*-AA cryogels disintegrate into small pieces and finally dissolve in the solution after certain periods. Chitosan is a linear cationic heteropolymer of randomly distributed GlcNAc and GlcN residues with β-1,4-glycosidic linkages, which can be hydrolyzed by snailase (a complex of

cellulase, invertase polygalacturonase, amylase, hemicellulase, protease and pectinase).[35] The network structure of the cryogels is destroyed through the degradation of the chitosan crosslinker. According to the experimental results we infer that the degradation reactions took place on the surface of the gels. For the common hydrogels without pores, snailase is too large to diffuse into the network. Therefore, the degradation reactions happen on the surface of the hydrogels. In the additional experiments, we find that the rate of degradation of the nonporous hydrogels was remarkably slower than that of the porous cryogels in the same volume. Furthermore, a shorter degradation time period was needed for smaller pieces of nonporous hydrogels to be degraded and dissolved in the solution. It seems that the rate of degradation is proportional to the specific surface area of the gels which could lead to the assumption that the degradation reactions happen on the surface of the gels. For the cryogels, although snailase also cannot diffuse into the pore walls, it can diffuse into the pores. Then, the pore walls are degraded from their surface. The surface area of the cryogels is much larger than that of the nonporous hydrogels, so the degradation rate of the cryogels would be faster than that of the nonporous hydrogels. Although degradation occurs on the surface of the pore walls, the complete degradation occurs in both the surface and the interior of the cryogels in a macroscopic view due to the porous structure. The crosslinking degree reduced, while the pore walls became loose. Some of the polyacrylamide linear chains break off and dissolve in the solution, while some of the linear chains remain twisted together to form the fibriform structure. However, the exact reason for the formation of fibriform structure is still being investigated. Finally, all the crosslinking points are degraded and the cryogels are completely dissolved in solution as linear chains. If necessary, the PAM cryogels could be quickly degraded into PAM chains and dissolved in the snailase solution in 72 h (40 °C, pH = 4.0) with slow stirring, which may promote the diffusion of snailase.

Conclusion

A novel water soluble CSO-*g*-AA crosslinker for the preparation of hydrogels was synthesized. A small quantity of acryloyl groups was attached onto the CSO chains through the amidating reaction of CSO and AA under the activation of EDC and NHS. The chemical structure of CSO-*g*-AA was confirmed by FTIR and ^{1}H NMR. The result of titration analysis showed that the grafting ratio of acryloyl groups was approximately 3.5%, namely, about two acryloyl groups per CSO-*g*-AA chain on average. The CSO-*g*-AA was successfully used to prepare biodegradable PAM-based macroporous hydrogels using a modified cryo-polymerization method. When immersed in snailase solutions, the swelling ratio of the cryogels increased at an early degradation stage due to the reduction of crosslinking density, while it decreased at the terminal degradation stage since the resulting linear polymer chains broke off from the cryogels. Finally the cryogels could be degraded into linear PAM chains and dissolved in the solution. The degradation time could be controlled from one to two months according to the enzyme concentration. It is considered that the cryogels undergo an entire degradation as a monolith while degrading from the surface of the pore walls in the microstructure.

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