Study of a novel injectable hydrogel of human-like collagen and carboxymethylcellulose for soft tissue augmentation

Bowen Liu,¹ Xiaoxuan Ma,¹ Chenhui Zhu,¹ Yu Mi,¹ Daidi Fan,¹* Xian Li,¹ Lan Chen²

¹Shaanxi R&D Center of Biomaterials and Fermentation Engineering, School of Chemical Engineering, Northwest University, Xi’an 710069, Shaanxi, China.
²Shaanxi Key Laboratory of Degradable Biomedical Materials, School of Chemical Engineering, Northwest University, Taibai North Road 229, Xi’an 710069, Shaanxi, China.

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Abstract: A novel injectable hydrogel was fabricated by human-like collagen (HLC) and carboxymethylcellulose (CMC) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and adipic acid dihydrazide (ADH) as cross-linkers. The morphology and structure of the hydrogels were characterized by scanning electron microscope and Fourier transform infrared spectroscopy. The results showed that the HLC and CMC were successfully cross-linked through amide bonds and HLC could enhance the pore size of the composite, whereas CMC could be a strong backbone in the hydrogel to keep its appearance. The results of thermogravimetric analysis showed that the thermostability of HLC/CMC was strengthened significantly as compared with that of CMC. The tests of the equilibrium swelling ratio and in vitro degradability indicated that the HLC/CMC hydrogel possesses good water absorbing ability and slow degradability in vitro. Finally, biocompatibility test provided the possibility that HLC/CMC hydrogels are suitable for biomedical applications such as soft tissue augmentation for their good biocompatibility.

Keywords: human-like collagen, carboxymethylcellulose, hydrogel, cross-linking

Introduction

As the age of people grow old, the soft tissue of face gradually lost fat and collagen, usually results in a decrease of elastic and volume of skin tissue. Therefore, more and more attentions are paid on the facial reconstitute or reshape and many studies are focusing on how to make perfect soft tissue filler for augmentation of skin or facial rejuvenation. The materials for preparing soft tissue filler are generally conclude fat, collagen [1, 2], synthesis or natural chemical polymer and so on [3, 4]. These different materials possess different chemical structures and physical properties, results in different biocompatibility and biodegradation speed [4–6]. However, no perfect soft tissue filler is currently available, although many options exist that are adequate for a given task, satisfy patients, and offer excellent safety profiles, the complication after injection of these fillers could not be totally avoided [2, 3, 7].

Cellulose is the most abundant natural polymer on Earth. Due to their low cost, biodegradability and biocompatibility, cellulose and its derivatives are highly attractive as suitable biopolymers for biomedical applications. Carboxymethylcellulose (CMC), one of the major cellulose derivatives, is a water-soluble polysaccharide and has
been widely applied in cosmetics due to its high water retaining ability, good biodegradability and lack of toxicity [8-10]. In addition, the CMC is easily available with high purity in low cost, results in broad applications in foods as dispersion stabilizer. The physical and chemical properties of CMC solutions dependent on pH, ionic strength and temperature have been also reported broadly to date [11].

Collagen, a major structural protein of the extracellular matrix that provides good mechanical strength to various tissues, is extremely important for tissues growth and maintaining configuration. However, owing to its infection risks of pathogens and some complications such as hypersensitivity and allergic reactions, the application of animal collagen was restricted [1]. Human-like collagen (HLC), produced by high cell density cultivation of recombinant *Escherichia coli* [12, 13], is a novel water soluble protein with lower immunogenicity and is free of pathogens; this could provide reliable compositions with predictable structure, and it is a novel biomaterial that has successfully been used for the construction of artificial bones [14–16] and artificial vascular scaffolds [17, 18].

Recently, many studies have found that the combination of CMC and other natural materials have many advantages such as enhancement of biocompatibility, greater resistance to enzymatic digestion and good mechanical properties when compared to CMC alone [19–23]. In this study, we assumed that the HLC could crosslink with CMC through amide linkages by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and adipic acid dihydrazide (ADH) as shown in the reaction scheme reported for CMC [24], where EDC is the carboxyl-activating agent and ADH is the crosslinker. The aim of this study was to prepare a novel injectable hydrogel of HLC and CMC cross-linked by ADH and EDC for biomedical applications, and simultaneously, to evaluate the novel structures and properties of the hydrogel. To clarify the effects of cross-linking, the infrared spectra, swelling ratio, thermogravimetric analysis and *in vitro* enzymatic degradation of the cross-linked matrix were studied, finally the biocompatibility of the hydrogel was also evaluated.

**Results and discussion**

**Preparation of the hydrogel**

EDC, a water-soluble carbodiimide with good biocompatibility and easy removability from reaction systems, was used to react with the carboxyl groups on proteins or polysaccharides to form the unstable intermediate *O*-acylisourea. This intermediate makes proteins and polysaccharides reactive with other side groups to form an amide linkage between the amine and the acid at pH 4.0–5.0 [26, 27].

Adipic acid dihydrazide (ADH), one of the widely used homobifunctional linkers in bioconjugate chemistry, could be used to react with the carboxylic acid groups of polysaccharides under mild conditions of pH 4.0–4.8 in the presence of EDC to form a hydrazide amino intermediate of ADH and polysaccharides. The other hydrazide group of ADH could be further modified with the carboxylic acid groups of polysaccharides or other chemical compounds to form an intramolecular or intermolecular cross-linked composite [24, 28, 29].

In our study, the effects of cross-linking with ADH and EDC on the HLC/CMC hydrogel were evaluated. The supposed cross-linking reaction scheme of HLC and CMC by EDC and ADH is shown in Figure 1. The carboxylic groups of CMC or HLC were activated by EDC to form the unstable intermediate *O*-acylisourea, which
subsequently reacted with ADH to form a hydrazide amino intermediate. Furthermore, the remaining carboxylic groups of CMC and HLC activated by EDC at the same time could react with another hydrazide group of the hydrazide amino intermediate or the amino groups of HLC, leading to a highly cross-linked hydrogel of reticular structure through amide linkage between CMC and HLC.

Fig. 1. Scheme of the cross-linking processes. The HLC, CMC or ADH in the figure are represented as complete molecules except for the reacted carboxylic group or amino group.

Fig. 2. Photographs of hydrogels: (a) original HLC/CMC hydrogels GEL20, GEL43, and GEL67 in inverted 10 mL centrifuge tubes, from right to left, (b) GEL50 after dialysis and washing by ultrapure water.

The photos of hydrogel are shown in Figure 2. The appearance of the original hydrogel was cylindrical owing to the mould of the tube, and it became relatively opaque when the concentration of HLC increased owing to the solution properties of HLC. However, when the concentration of HLC was higher than CMC, the HLC/CMC
hydrogel became less stiff, which could be attributed to the lesser carboxylic groups of HLC than CMC. Furthermore, the pure CMC hydrogel could be prepared by the same method but the pure HLC hydrogel could not, illustrating that the CMC molecule is much bigger and longer than the HLC molecule; thus, CMC was more inclined to act as a strong backbone in the hydrogel for keeping its appearance but HLC could not. Additionally, we found that the concentrations of HLC and CMC should reach a certain level to form a suitable hydrogel; when the HLC and CMC concentrations were too high, the obtained hydrogel was too stiff and fragile, and when the concentrations were not enough for gelation, no hydrogel could be fabricated.

**Characterization**

The HLC/CMC hydrogels after freeze-drying were observed by SEM. As seen in Figure 3, the surface of all samples is full of pores and the pore size ranges from several tens to several hundreds of micrometers, which could be attributed to the freeze-drying process and cross-linking degree of HLC and CMC; the higher the cross-linking degree of the composite, the smaller its pore size would be. Furthermore, the microstructures of the samples were also greatly affected by the composition of the hydrogel: as the HLC concentration increased, the size of the pores decreased, leading to a less open and looser structure and decreased water retention ability.

![SEM images of (a) GEL20, (b) GEL43, (c) GEL67.](image)

**Fig. 3.** SEM images of (a) GEL20, (b) GEL43, (c) GEL67.

The FT-IR spectra of hydrogel samples are shown in Figure 4. Pure CMC and pure HLC were set as controls. The significant absorption bands of the hydrogels at 3400–3500 cm\(^{-1}\) were caused by the stretching of hydroxyl groups. The bands attributed to COO\(^{-}\) at 1612 cm\(^{-1}\) and 1424 cm\(^{-1}\) in the CMC were decreased sharply when compared with the HLC/CMC hydrogels. Meanwhile, the amide bands (1656 cm\(^{-1}\) and 1542 cm\(^{-1}\)) were also observed in the HLC/CMC hydrogels, illustrating that CMC and HLC were successfully cross-linked with each other through amide bonds by ADH and EDC.

The thermogravimetric analysis curves of HLC, CMCNa and GEL50 are shown in Figure 5. The weight loss of HLC around 320 °C was the result of the decomposition of HLC, whereas the weight loss of CMCNa around 290 °C was attributed to the decomposition of CMCNa, illustrating that the thermostability of HLC is stronger than that of CMCNa.
**Fig. 4.** FT-IR spectra of GEL50, GEL CMC, pure CMCNa, and pure HLC. The dashed lines in the photograph indicated the same FT-IR wavenumbers of all samples while the “1612” and the dash indicated the specific wavenumbers.

**Fig. 5.** TGA analysis curves of pure HLC, pure CMCNa, and GEL50.
Furthermore, the major peak in the DTG curve of GEL50 shifted to 300 °C, showing a better thermostability when compared with the curve of CMCNa. This conforms that the HLC/CMC polymer was formed as a result of the cross-linking reaction, and also that the HLC/CMC polymer possessed enhanced stiffness than CMCNa due to the intermolecular cross-linking. The minor peak in the DTG curve of GEL50 around 200 °C could be probably attributed to the decomposition of the intermediate products in the cross-linking reaction.

**Swelling ratio determination**

High swelling ratio is one of the most important properties and requirements of hydrogels for biomedical applications such as soft tissue augmentation. The swelling ratios of different HLC/CMC hydrogels at 37 °C after soaked in ultrapure water and physiological saline solution for 24 h are shown in Figure 6. As we supposed, the swelling ratio of HLC/CMC hydrogels decreased a little with an increase in HLC content and these hydrogels with the highest swelling ratio were GEL20 and GEL CMC, which could be attributed to the highly hydrophilic carboxyl groups of CMC absorbing a lot of water to increase the volume of the hydrogel and the lesser carboxylic groups of HLC than CMC. However, the HLC also possessed comparatively good water retention ability for its hydrophilic regions, resulting in the decrease of swelling ratio of the HLC/CMC hydrogel was not linearly correlated with the decrease of CMC content in hydrogel.

![Graph showing swelling ratios](image)

**Fig. 6.** Equilibrium swelling ratios of different HLC/CMC hydrogels after immersing in ultrapure water and in physiological saline solution for 24 h at 37 °C (n = 5).

A significant difference was observed in the swelling ratio of HLC/CMC hydrogels and CMC hydrogels (p < 0.05), indicating that the different composition ratios of HLC/CMC composition significantly affected the structures and properties of
hydrogel. Further, a significant difference was also observed in the equilibrium swelling ratio in ultrapure water and in physiological saline solution ($p < 0.05$), indicating the equilibrium swelling ratio was also affected by the ionic strength as a result of the inhibition of the electrostatic effects caused by the charges of the carboxyl groups of CMC and HLC.

**In vitro enzyme degradation**

The slow degradation behavior of hydrogels is another important property and requirement of hydrogels for soft tissue augmentation, thus the degradation rate of prepared HLC/CMC composites in collagenase solution was investigated and the results are shown in Figure 7. The degradation rate was found to be closely related to the composition of the hydrogels: when the ratio of HLC in the hydrogels increased, the degradation rate of the hydrogels correspondingly increased; this could be attributed to the increased enzymatic sites of the hydrogel with the content of HLC increased. Furthermore, the HLC/CMC composites were decomposed very quickly in collagenase solution owing to the enzymatic hydrolysis of HLC in the first two or three days but much slow in the last several days owing to the slow hydrolysis of CMC, while the control HLC/CMC composite was decomposed very slowly only by hydrolysis. This confirmed that the HLC was much inclined to be grafted onto the CMC molecule which was the backbone of the HLC/CMC hydrogels. Additionally, the degradation rate of HLC/CMC hydrogel was much slower than the rates in some previous reports of the collagen hydrogel cross-linked by EDC alone [25, 30]; this was possibly owing to the intramolecular or intermolecular cross-linking of CMC and HLC.

![Degradation rates of different HLC/CMC hydrogels. (n = 3).](image)

Although the degradation rate of hydrogels in the enzyme solution could not exactly represent the actual degradation behavior *in vivo*, the *in vitro* studies could still
provide us comparatively useful information and guide us in improving the properties of hydrogels through different methods under different conditions. The *in vitro* degradation study proved that the HLC/CMC hydrogel cross-linked by EDC and ADH is a promising biomedical material, which can be applied widely in tissue engineering after careful examination of biocompatibility.

**In vivo biocompatibility**

The HLC/CMC hydrogel was implanted into the dorsal subcutaneous region of mice to investigate the initial biocompatibility *in vivo*. The animal experiments confirmed that GEL50 has good biocompatibility and comparatively slow biodegradability: as seen in the histological sections of the implanted hydrogel and nearby tissue in Fig. 8, no infection could be observed in the skin and subcutaneous tissue, and after 28 days, a significant amount of hydrogel could still be observed. Although a longer study should be conducted to evaluate the long-term biocompatibility of the hydrogel, the *in vivo* biocompatibility test could still illustrate the potential application possibility of the HLC/CMC hydrogel in biomedical fields and tissue engineering for its good biological properties.

**Fig. 8.** Photographs of HLC/CMC hydrogel: (a) immediately after injection, (b) 3 days after injection; and H & E staining of HLC/CMC hydrogels (magnification is 40 ×): (c) 14 days after injection, (d) 28 days after injection. T: tissue; H: hydrogel.

**Conclusions**

In this study, hydrogels with different composition ratios of HLC and CMC were successfully fabricated by cross-linking with ADH and EDC. These novel hydrogels could be potentially applied to tissue engineering and biomedical fields for their good
biodegradability and structural properties. The results of FT-IR analysis proved that the amide linkages are formed between HLC and CMC. SEM photographs showed that the HLC/CMC hydrogel has regular porous networks of sponge-like structure. The swelling ratio and degradation rate of the hydrogels were closely correlated to the composition ratio of these hydrogels: an increase in the ratio of HLC could decrease the swelling ratio and degradation rate of hydrogels to some extent. Good thermal stability and slow denaturation behaviors of the HLC/CMC copolymer were also detected by TGA. The in vivo biocompatibility test of the HLC/CMC hydrogel showed that the HLC/CMC hydrogel has good biocompatibility and can be widely applied in biomedical areas as suitable soft tissue filler.

**Experimental part**

**Materials**

CMC sodium (CMCNa, high viscosity, 2096.0 mPa·s at pH 7.2) was obtained from Merck, Germany; HLC, type II, produced in our laboratory, molecular weight 9.0 × 10^4; ADH was obtained from Shanghai Eutec Chemical Co., Ltd., China; EDC was obtained from Thermo Scientific, USA; Collagenase, type II (EC 3.4.24.3, from Clostridium histolyticum, 315.0 U/mg) was obtained from Merck, Germany.

**Preparation of the hydrogel**

HLC and CMCNa were added into 10 mL centrifuge tubes containing 5 mL ultrapure water such that the quantity ratio of CMCNa and HLC was 4/1, 2/1, 4/3, 1/1, and 1/2 in tubes labelled GEL20, GEL33, GEL 43, GEL50 and GEL67, respectively. Then CMCNa and HLC were homogenized before 0.1 g ADH was added. After the pH of the solution was adjusted to 4.70 ± 0.05 by 0.1 mol/L HCl or NaOH, 0.04 g EDC was added and the pH of the solution was kept at 4.70 ± 0.05. The reaction was performed under room temperature for 12 h before the pH of the solution was adjusted to 7.0 to stop the reaction, and finally, the cross-linked HLC/CMC hydrogel was obtained. To eliminate the unreacted cross-linkers, the hydrogel was dialyzed against 0.1 mol/L NaCl solution and ultrapure water for 1 day each. Then, the hydrogel was soaked in ultrapure water for 1 day to eliminate the non-covalently linked materials, with the water refreshed every 2 h. Furthermore, in order to analysis the physical and chemical properties of these hydrogels, they were frozen at −80 °C for 3 h and lyophilized at −50 °C to form dry samples. The pure CMC hydrogel was fabricated according to the same protocol as the control and labelled as GEL CMC.

**Characterization**

The morphology of the dry hydrogel sample was studied by scanning electron microscopy (S-570, Hitachi, Japan). Small piece of different dry hydrogel samples were sputtered with an ultrathin layer of gold in a coating system, and analysed by SEM at an accelerated voltage of 15 kV. The FT-IR analysis were determined with a resolution of 2 cm⁻¹ and spectral range of 4000–400 cm⁻¹ (infrared spectrometer, QUIOX 55/HYPERON 2000, German) after these samples were ground into micropowders and prepared by pressing potassium bromide troche. Thermal stability of HLC/CMC hydrogel was assessed from 20 to 450 °C at a constant heating rate of 10 °C min⁻¹ in a nitrogen atmosphere (NETZSCH STA 449C). Weighed samples of
mass of 4–5 mg were introduced into aluminum pans; at the same time, an empty aluminum pan was set as the reference probe.

**Swelling ratio determination**

The equilibrium swelling ratio of the hydrogel with different ratios of HLC and CMC were determined as follows: 15 mg of each dry hydrogel sample was immersed in 10 mL ultrapure water and in 10 mL physiological saline, and after 24 h, the quantity of each sample was determined (electronic balance, Mettler AE50, Switzerland) after carefully absorbing the water on the surface of the hydrogel with filter papers. Additionally the swelling ratio of the cross-linked CMC hydrogel was determined using the same procedure as the control. Then the swelling ratio (SR) value was calculated as

\[ SR = \frac{W_s}{W_d} \]  (1)

where \( W_s \) was the weight of the wet hydrogel at swelling equilibrium at 37 °C, and \( W_d \) was the weight of the dry hydrogel.

**In vitro enzyme degradation**

The in vitro degradation test was performed according to a previous method with little modification [25]: different dry hydrogel samples were incubated at 37 °C in 20 mL collagenase solution (dissolved in pH 7.4 tris-buffer containing 10mm CaCl₂, 100 U/mL) and the samples were retrieved at fixed time intervals and dipped into 30 mL of distilled water three times and freeze-dried. The remaining weight was determined at each time point as \( W_t \) (t = 1, 2, 3... n). The degradation rate (\( DR_t \)) was calculated as

\[ DR_t = \frac{W_0 - W_t}{W_0} \]  (2)

where \( W_0 \) was the initial weight of dry hydrogel before collagenase treatment. Additional dry GEL50 hydrogels were set as controls and stored in the same conditions for all the duration of the experiment, without the addition of collagenase.

**In vivo biocompatibility**

To evaluate the initial in vivo biocompatibility of the HLC/CMC hydrogel, GEL50 samples (0.5 mL in volume) were implanted into the back of 6 mice, and 6 other untreated mice were set as controls. After 3, 14 and 28 days of implantation, 2 implanted mice and 2 control mice were sacrificed, the specimens were fixed in 10% phosphate-buffered formalin for 1 day, then dehydrated in a series of ascending concentrations of ethanol, and finally cleared in xylene before embedding in paraffin. Finally, the samples were sectioned (5 mm) and observed under a light microscope (Nikon eclipse 80i, Japan) after hematoxylin and eosin (H & E) staining.

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