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Different Content of EDC Effect on Mechanical Strength and Crosslinking Densities of a Novel CS-HLC/β-GP-EDC Hydrogel

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A novel injectable hydrogel for biomedical applications was successfully fabricated by human-like collagen (HLC) and chitosan (CS) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as cross-linker. The compression strength and crosslinking densities of the hydrogels were characterized by Electronic Universal Testing Machine. The results showed that the HLC and CS were successfully cross-linked through amide bonds and EDC could be a strong backbone in the hydrogel to keep its appearance. The results of the equilibrium swelling ratio and compression module of these hydrogels indicated that the compression moduli of these gels were directly proportional to their crosslinking densities and sol-gel transition was become true at 37°C. Therefore, CS-HLC/β-GP-EDC hydrogels are suitable for biomedical applications such as soft tissue augmentation for their good crosslinking densities.

Key words: Hydrogel, Human like collagen, Mechanical strength, Crosslinking densities.

In recent years, many hydrogels that are sensitive to changes in pH have been produced from synthetic polymers or natural polymers crosslinked by chemical (covalent bonds) or physical (hydrogen bonds, hydrophobic-hydrophilic interactions and electrostatic interaction) methods. These are widely used as supporting materials for biomedicine, drug delivery and soft tissue engineering owing to their easy adaptation to the environment of the body. Natural polymers, such as gelatin (Li, *et al.,* 2006), collagen (Ignatius, A, 2005), and chitosan (Adekogbe, 2005; Huang, 2005; Seo, 2005), generally have better biocompatibility and fewer latent toxic effects than synthetic polymers. 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 (Livaoglu, 2009; Gatti, 1999), collagen (Boulle, 2009; Landau, 2009), hyaluronic acid (Piacquadio, 1997;), calcium hydroxylapatite (Tzikas, 2004; Marmur, 2009), Poly-L-lactic acid (Humble, 2004; Rossner, 2009) or Polymethylmethacrylate (Lemperle, 2003; Hilinski, 2009). Chitosan, an

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amino-polysaccharide obtained by the alkaline deacetylation of chitin, is a natural polymer. Because of its good biocompatibility, biodegradability, non-toxicity, and the ability to release its degradation products, chitosan has been widely used in tissue engineering and in plastic and reconstruction surgery involving cartilage, liver, skin and spinal cord (In-Yong, *et al.,* 2008). Human-like collagen (HLC) (Fan, 2002; Hua, 2006), a recombinant protein with human-derived mRNA information, is highly expressed by recombinant Escherichia coli BL21 (E. coli BL21). 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(Tomihata, 1997; Lu, 2007).

The aim of this study was to prepare a novel injectable hydrogel of HLC and CS cross-linked by EDC for biomedical applications, and simultaneously, to evaluate the novel properties of the hydrogel. To clarify the effects of cross-linking, swelling ratio and mechanical strength were studied.

MATERIALS AND METHODS

Materials

The required HLC was expressed by E. coli with a cloned partial cDNA reversed from the mRNA coding for human collagen (Chinese patent number: ZL01106757.8). The CS (molecular weight 550,000, deacetylation degree 90-92%) was supplied by Qingdao Ocean Ltd. Co. (China). The β-sodium glycerophosphate (β-GP), EDC and glacial acetic acid were also supplied by Sigma Co, and all solvents and reagents were analytical grade.

Preparation of the CS-HLC/β-GP-EDC hydrogel

The CS-HLC/β-GP-EDC hydrogel was prepared by mixing 5% (w/v) CS dissolved in 0.1 mol/L acetic acid solution with an isocratic volume of 3% HLC solution. The mixture was stirred for 20 min in an ice-water bath until complete dissolution was achieved. Then, 1mL 40% β-GP and 0.02g, 0.04g, 0.06g and 0.08g EDC was added drop wise until the pH of the solution reached 7.4, respectively (Table 1). The solutions (sol) were placed in a thermo cell maintained 8 min for gelling at 37.0 ± 0.5 °C.

Swelling Measurement

The classical gravimetric method was employed to measure the swelling ratio of the hydrogels. For temperature-dependent swelling studies, gels were placed in triplicate in ultra-pure water (ddH_2O) and PBS buffer solutions with pH 7.4 at various temperatures for at least 24 h to reach equilibrium, after which the gels were weighed. The equilibrium swelling ratio was given as $(W_S-W_d)/W_d$, where W_S and W_d represent the weight of swollen gel and the dried gel weight, respectively.

Compression Experiment

The hydrogels were tested by a gel strength instrument (Electronic Universal Testing Machine) as follows: firstly, the height of the compression plate was adjusted to 10 mm and its final height was adjusted to 4 mm. The disc of instrument was circular with a diameter of 10 mm and the compression speed was adjusted to 2 mm/min. The gel was cut to a length of 10 mm and placed on the instrument disc. When the compression displacement reached 6 mm, the test was stopped. The compression modulus and the relationship between the compression load and compression displacement were calculated. The effective crosslinking density (P_X) was determined by the compression modulus and polymer swelling ratio (Q), shown as follows (Lee,2001):

$$P_X=GQ^{1/3}/RT \quad \ldots (1)$$

G was the compression modulus, R was the gas constant (8.314×10^3 KPa·cm^3·mol^{-1}·K^{-1}) and T was 310 K.

RESULTS AND DISCUSSION

The hydrogel formation mechanism

EDC reaction with proteins have been reported by Tomihata K.[19] and Lu, G.Y.[20], EDC containing N=C=N functional group, 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

Table 1. The compositions of the CS-HLC/β-GP-EDC hydrogels

Sample	CS (mL)a	HLC (%)	β-GP (mL)b	EDC (g)
Gel1	2	3	1	0.02
Gel2	2	3	1	0.04
Gel3	2	3	1	0.06
Gel4	2	3	1	0.08

a CS is 5 wt.% aqueous solution.
b β-GP is 40 wt.% aqueous solution.

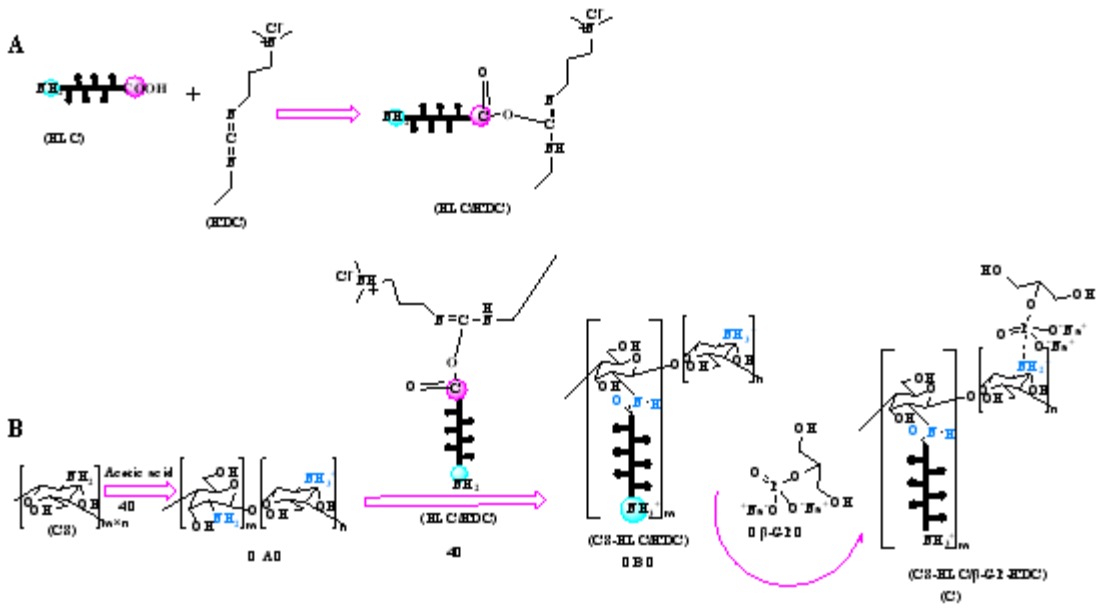
Table 2. The compression module and crosslinking density of gels

Gel	G (KPa)	Q	Q1/3	ρ×10-6mol/cm^3
Gel1	25.66	5.3	1.39	17.61
Gel2	28.00	7.43	1.64	1812.23
Gel3	30.00	8.4	1.64	1812.23
Gel4	35.14	9.5	1.71	16.97

and the acid (scheme. 1A). $–NH_2$ of a part of CS chain was protonated to $–NH_3^+$ under acidic conditions, and also $–NH_2$ of the end HLC chain was protonated to $–NH_3^+$. While part of $–NH_2$ of not protonated CS reacted with HLC/EDC to glycoprotein chain was connected by a new amide bond (scheme.1B). A new bound of $-NRH_2^+$ were appeared mainly due to conjugation of P atoms on $-OPO_3^{2-}$ with N atoms from $–NH_3^+$ of a part of CS chain (scheme. 1B).

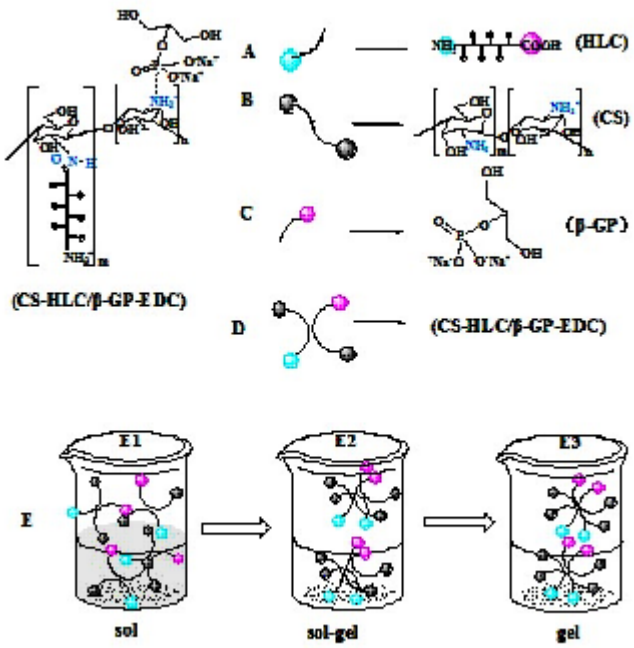
Swelling ratio of hydrogels

High swelling ratio is one of the most important properties and requirements of hydrogels for biomedical applications such as soft tissue



Scheme 1. Proposed interaction mechanisms for CS, HLC, EDC and β-GP: A, interaction of HLC and EDC; B, the CS-HLC/β-GP-EDC hydrogel formation process

augmentation. The swelling ratios of different CS-HLC/β-GP-EDC hydrogels at 37°C after soaked in physiological saline solution for 14 h are shown in Figure 1. As we supposed, the swelling ratio of CS-HLC/β-GP-EDC hydrogels decreased a little with an decrease in EDC content and these hydrogels with the highest swelling ratio were Gel4, which could be attributed to the highly hydrophilic carboxyl groups of EDC absorbed a lot of water to increase the volume of the hydrogel and the lesser



Scheme 2. Schematic diagram of the CS-HLC/β-GP-EDC hydrogel during gelatin process dependence on temperature. The solution of CS-HLC/β-GP-EDC in hydrogel system exhibits a sol-gel transition at 37°C

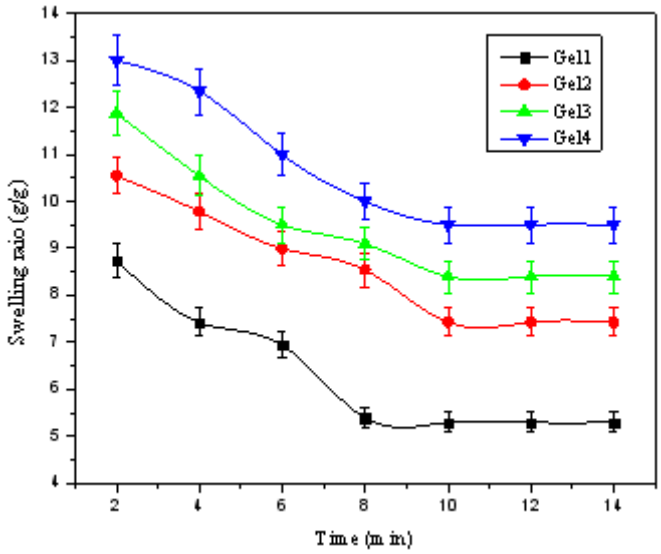


Fig. 1. The swelling ratio of CS-HLC/β-GP-EDC hydrogel with different content of EDC

carboxylic groups of HLC than EDC. However, the HLC also possessed comparatively good water retention ability for its hydrophilic regions, resulting in the decrease of swelling ratio of the CS-HLC/β-GP-EDC hydrogel was not linearly correlated with the decrease of EDC content in hydrogel.

A significant difference was observed in the swelling ratio of CS-HLC/β-GP-EDC hydrogels, indicating that the different composition ratios of CS-HLC/β-GP-EDC composition significantly affected the structures and properties of hydrogel. Further, a significant difference was also observed in the equilibrium swelling ratio in ultrapure water, 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 EDC and HLC.

Hydrogel formation process

The system of CS-HLC/β-GP-EDC hydrogel shown in Scheme. 2. The hydrophilic group was represented by globe sign(⚬), the hydrophobic chains was represented by the shape of the arc of semi-circle(⌒). The HLC chain was represent by Scheme. 2A, the CS chain was represent by Scheme. 2B, the β-GP chain was represent by Scheme. 2C, and the CS-HLC/β-GP-EDC was represent by Scheme. 2D. The CS-HLC/β-GP-EDC was exhibit in sol show in Scheme. 2-E1, with temperature increasing, the hydrophilic groups of the hydrogel played an important role in the interaction, and hence the molecular chains in the gel were re-arranged to form an orderly and regular structure (Scheme. 2-E2). When the temperature approached to 37°C, the interaction of hydrophobic groups in the molecular chains gradually increased, becoming similar to that of the hydrophilic interactions and the molecular chains twisted with one other (Scheme. 2-E3), lead to sol-gel transition was come true.

Strength and Crosslinking Densities of the Hydrogels

The compression modulus of the hydrogels is listed in Table 2. As shown in Fig. 2, the compression modulus of Gel4 (35.14 KPa) was higher than that of Gel3 (30 KPa) and Gel2 (28 KPa) with the same compression displacement at 37°C. This can probably be ascribed to the influence of both structure and porosity on the flexibility and plasticity of the gel. The compression modulus was directly proportional to compression stress; the better the flexibility of gel, the higher the compression modulus value. Table 2 shows that the compression modulus of the hydrogels increased with their cross-linking densities at the same temperature. This could be explained in Eq. (1), where the compression modulus depended on the crosslinking density and the swelling ratio at the same temperature. As the values of $Q^{1/3}$ were nearly equal to 2 for Gel1, Gel2, Gel3and Gel4 (see Table 2), the compression moduli of these gels were directly proportional to their crosslinking densities. Therefore, crosslinking density played an

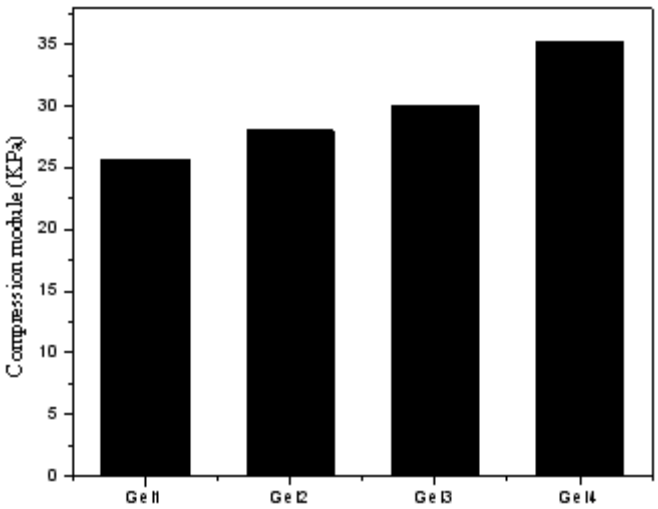


Fig. 2. The compression module of CS-HLC/β-GP-EDC hydrogel with different content of EDC

important role in the formation of the pH/temperature-sensitive hydrogels. The CS-HLC/β-GP-EDC hydrogels exhibited more adequate mechanical strength (35 KPa) and crosslinking densities (1.812×10^{-3} moL/cm^3) shown in Table 2, therefore CS-HLC/β-GP-EDC hydrogels are suitable for soft tissue defect filling.

CONCLUSION

In this study, hydrogels with different composition ratios of CS-HLC/β-GP-EDC were successfully fabricated by cross-linking with EDC. These novel hydrogels could be potentially applied to tissue engineering and biomedical fields for their good swelling ratio and crosslinking density. Furthermore, the swelling equilibrium of CS-HLC/β-GP-EDC hydrogels was reached at approximately 10h, which could easily be adapted to body fluid, and sol-gel transitions was formation. Additionally, the CS-HLC/β-GP-EDC hydrogels had a highly compression strength which would provide more space for environment pressure. In summary, the CS-HLC/β-GP-EDC hydrogel was proven to be highly suitable as an injectable material, as well as a tissue engineering scaffold.

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