Preparation and Properties of Hyaluronic Acid Hydrogel Modified by L-cysteine Hydrochloride

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Abstract. Hyaluronic acid (HA) was modified by L-cysteine hydrochloride using chemical cross-linking method with horseradish peroxidase as catalyst, and HA was cross-linked intermolecularly by hydrogen peroxide to obtain modified hyaluronic acid hydrogel. The properties of modified HA hydrogel, such as viscosity, whiteness, swelling degree, porosity, water retention, texture were studied. The results showed that the viscosity, water retention capacity and hardness of the modified hydrogel increased, the swelling degree and porosity were reduced, and the hydrogel structure was dense by cross-linkage treatment.

1. Introduction
Hyaluronic acid (HA) had the advantages of good biocompatibility, easy degradability, and moisture retention for natural macromolecules. HA was widely used in the fields of cosmetics, food and biomedical materials, while natural hyaluronic acid was sensitive to strong acid and alkali solution, heat, free radicals and hyaluronidase, and its mechanical properties and stability were poor to application limits [1, 2]. It is necessary to modify hyaluronic acid to develop hyaluronic acid derivatives with new biological activity and functionality. L-cysteine hydrochloride was used to modify hyaluronic acid to improve the mechanical properties of hyaluronic acid and expand its application fields.

2. Materials and methods
2.1. Materials and instruments
Hyaluronic acid (MW 100-130, 200-400, 800-1,000, 1,300-1,500, 1,800-2,200 kDa, Shandong Freda Biomedical Co., Ltd.), L-cysteine hydrochloride (Sinopharm Chemical Reagent Co., Ltd.), 1-ethyl-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS, Bioengineering Co., Ltd.), disulfide thrreitol (Shanghai Lanji Biological Co., Ltd.), Horseradish Peroxidase (HRP, Sigma-Aldrich Shanghai Trading Co., Ltd.), CR-400 colorimeter (Konica Minolta, Japan), TA-XT.puls (Stable Micro System, UK), TENSOR 27 Fourier Infrared Spectrometer (BRUKER Scientific Instruments, Germany), DV-C Digital Viscometer (Brookfield, USA).

2.2. Test method
2.2.1. Preparation of modified hyaluronic acid. 1.0 g HA and 200 mL deionized water were stirred to swell and completely dissolved by Chen Lei's method with a slight modification [3]. 1.92 g EDC and
1.15 g NHS were added to 20 mL deionized water, and the pH value of the mixed solution was adjusted to 5.4 by 1 mol/L HCl solution, then activated using a magnetic stirrer to 80 r/min at room temperature for 0.5 h. 1.68 g L-cysteine hydrochloride was added to the mixed solution, and reacted at room temperature for 24 h. 4.6 g dithiothreitol was reacted at room temperature for 24 h using a magnetic stirrer at a stirring speed of 80 r/min. The reaction solution was transferred into a dialysis bag with a molecular weight of 3500 Da and dialyzed with deionized water for 3 days, and the modified hyaluronic acid were freeze-dried under a vacuum (<1 Pa) at -80°C.

2.2.2. Preparation of modified hyaluronic acid hydrogel. 0.5 g modified HA was dissolved in 10 mL phosphate buffered saline (PBS, pH 7.4), and mixed 250 μL PBS solution (containing 0.02 mg/mL HRP) after fully swelling and dissolving. 250μL of 0.02% hydrogen peroxide was added and stirred at room temperature to form a modified hyaluronic acid hydrogel.

2.3. Determination method

2.3.1. Determination of viscosity. The viscosity of hyaluronic acid hydrogel was measured by S63 viscometer rotor with 1mm/s test speed on torque of 10%~90%.

2.3.2. Determination of whiteness. The colorimeter was calibrated by whiteboard to L*=52.94, a*=-0.50 and b*=-4.85, and placed vertically on the surface of hydrogel for detection.

2.3.3. Determination of swelling degree. 20 mg hyaluronic acid gel was soaked in 10 mL of PBS buffer solution (pH 7.4). After the gel completely immersed, the moisture on gel surface was absorbed with filter paper in every 5 minutes, and immediately weighed until the quality of gel no longer changed [3].

2.3.4. Determination of porosity. 20 mg hyaluronic acid gel was added into 10 mL PBS buffer solution, and the total volume of the solution was recorded. The remaining volume of PBS buffer solution was recorded after sample taken out [4].

2.3.5. Determination of water retention. 2 g gel sample was centrifuged with 8,000 r/min for 3 min at 4°C, and the weigh of gel and centrifuge tube were determined after centrifuged water pour out [5].

2.3.6. Texture analysis of gel. The texture characteristics of the prepared gel were analyzed by a texture analyzer with the puncture distance to 10 mm, using cylindrical probe (P/0.5S, 10 mm) at 1 mm/s pre-test speed, 0.5 mm/s test speed, and 10 mm/s post-test speed, respectively. The trigger force was 10 g, and the data speed was 100 points/s [6].

3. Results and analysis

3.1. Viscosity change of modified hyaluronic acid hydrogel

![Figure 1. The viscosity changes of different molecular weight hyaluronic acid hydrogels](image-url)
The hydrogel with a molecular weight of 1.8-2.2 million Da had a high viscosity, and the modified viscosity reached a maximum of 3530 cp, while the hydrogel with a molecular weight of 100,000-130,000 Da had a low viscosity, as shown in Figure 1. The viscosity of hyaluronic acid hydrogel increased with molecular weight. The viscosity of the hydrogel prepared by Wang Yafei [4] increased with hyaluronic acid content, which was basically consistent with the results of this test.

The viscosity of modified hyaluronic acid hydrogel was higher than that of the hydrogel before modification. The viscosity of the modified hyaluronic acid hydrogel increased with molecular weight sequentially. The rigidity of the double helix network structure of hyaluronic acid molecules continued to increase with modified gel process of hyaluronic acid. The carboxyl group (-COOH) of HA and the amino group (-NH₂) of L-cysteine hydrochloride removed water molecules under the activation of EDC/NHS to generate amide bonds (-CONH-), and the carboxyl group protonated and the degree gradually strengthened. The double helix structure formed by the ionized hyaluronic acid chain was gradually replaced by the double helix structure formed by the protonated hyaluronic acid. The rigidity of the local network continued to increase, the meshes continued to decrease, and the rigid network hindered the system. The free movement of water molecules made the entire system viscous. The modified hyaluronic acid was cross-linked intermolecularly by HRP catalyst under the action of H₂O₂, and the viscosity of the hydrogel was increased. The viscosity of the GMAHA gel prepared by Liu He [7] was significantly higher than that before crosslinking, which was consistent with the results of this test.

3.2. Whiteness change of modified hyaluronic acid hydrogel

![Fig 2. The whiteness change of different molecular weight hyaluronic acid hydrogels.](image)

Whiteness had an important influence on the application of hydrogels, and higher whiteness expanded its applications in more fields. The difference in whiteness of hyaluronic acid hydrogels with different molecular weights was not significant, as shown in Figure 2. HA hydrogels with a molecular weight of 100-130 kDa had a high whiteness, while 1,300-1,500 kDa hydrogels had a little whiteness.

The difference in whiteness of different molecular weight gels before and after modification was not significant, and the difference in whiteness of gels with the same molecular weight before and after modification is not significant. However, the whiteness of the hydrogel after modification decreased compared with that before modification. The whiteness of the hydrogels with molecular weights of 100-130 kDa and 1,800-2,200 kDa changed the most, which was related to the measurement time on hydrogel formation. The hyaluronic acid solution was colorless and transparent, and had little effect on the whiteness of the hydrogel. HRP was used as a catalyst in the gel process to structure changed by L-cysteine hydrochloride modification, modified hyaluronic acid was cross-linked intermolecularly under the action of H₂O₂, and HRP produced a red substance to decrease whiteness of modified gel by H₂O₂. The red substance produced was not obvious with little amount of enzyme, and the modification had little effect on the whiteness of the gel.
3.3. Water holding capacity changes of modified hyaluronic acid hydrogel

Polysaccharide colloids were prone to dehydration, which reduced the quality of gels. The macromolecular chain of hyaluronic acid contained hydrophilic groups of hydroxyl and carboxyl. It was a linear macromolecular polysaccharide with high hydrophilicity, and hyaluronic acid contained a lot of negative charges to interact. The combination of salt ions increased the osmotic pressure around the molecule, which made hyaluronic acid to high water absorption and retention properties. The difference in water holding capacity of hyaluronic acid hydrogels was not significant between 100 to 1,500 kDa molecular weight. The hydrogel with a molecular weight of 1,800-2,200 kDa had a high water holding capacity, which was even stronger after hydrogel modification to reach 94.42%, as shown in Figure 3. A single gel system formed a uniform network structure and contained a large number of pores, and the gel network structure was sponge-like. The size and number of gel pores determined the water holding capacity of the gel, and a high-concentration gel system formed a dense network structure to high water holding capacity [4].

The water absorption capacity of the composite gel was related to the size of pores in gel, the connectivity between pores and porosity. The water absorption capacity of the modified hyaluronic acid hydrogel was higher than that pre-modification, as shown in Figure 3. HRP was used as a catalyst under the action of hydrogen peroxide, and modified hyaluronic acid was cross-linked intermolecularly to form a dense network structure to little pores and porosity. The dense network structure caused gel with high water holding capacity. The silk fibroin/hyaluronic acid hydrogel had good water absorption capacity, and its water absorption capacity gradually increased with hyaluronic acid content, which was consistent with the results of this test [4].

3.4. Texture analysis of modified hyaluronic acid hydrogel

Figure 3. Water retention changes of different molecular weight hyaluronic acid hydrogels

Figure 4. Texture changes of different molecular weight hyaluronic acid hydrogels
The texture characteristics of the gel formed by the different molecular weight HA were investigated by the texture analyzer, and the gel strength was expressed by the maximum induction force during the downward pressing of the texture analyzer probe, as shown in Figure 4.

The hardness of hydrogel first heightened and then decreased with higher molecular weight of hyaluronic acid. Hydrogels with a molecular weight of 1,300-1,500 kDa had a high hardness, while hydrogels with a molecular weight of 1,800-2,200 had a weak hardness. The HA solution of molecular weight had a higher viscosity, and had obvious steric hindrance during the reaction process, which was the crosslinking reaction to a certain extent and limited the increase in its hardness. Chen Yonghao [8] studied the texture of sodium alginate/hyaluronic acid hydrogel and found that high molecular weight reduced the hardness of the gel, which was consistent with the results of this test. The hardness of the modified hydrogel was obviously improved, which could further broaden the application field.

3.5. Changes in swelling degree of modified hyaluronic acid hydrogel

The gel was a porous macromolecular network structure, and pore diameters increased the specific surface area of the gel, which was beneficial for small molecules to pass gel. Water molecules existed in the form of free water and bound water in the hydrogel macromolecular network structure, which provided a structural basis for hydrogel to high water absorption, penetration and water holding capacity [4].

The swelling degree before and after modification showed a similar trend during the molecular weight of hyaluronic acid gradually increased. The swelling rates of hydrogel before and after modification were high to 8.18 and 10.54 for hyaluronic acid of 1,800-2,200 kDa molecular weight, as shown in Figure 5. It showed that the swelling rate of the hydrogel increased with molecular weight of hyaluronic acid. HA had strong water absorption properties during molecular weight of modified hyaluronic acid gradual increase, the water absorption and swelling properties of hydrogel enhanced. However, when molecular weight of hyaluronic acid in gel precursor increased to a certain extent, the swelling performance of gel reached the maximum.

The swelling degree of modified gel was significantly reduced in all of the molecular weight range before modification. The mass fraction of hyaluronic acid gradually increased during tyramine hydrochloride modified hyaluronic acid, and the increase of gel crosslinking density decreased in the water-absorbing swelling performance of the gel, which was basically the same change trend of swelling degree in this experiment [3]. EDC activated carboxyl groups on more polysaccharides during HA is
modification, and the cross-linking density of the gel increased and space inside for hold water decreased under the action of NHS.

The composite hydrogel formed more pores and smaller pore diameters, the raise in the degree of crosslinking leaded to a tighter hydrogel structure, which was not conducive to large water absorption. The high cross-linked density network structure and little pore size generated lower swelling capacity.

3.6. Porosity change of modified hyaluronic acid hydrogel

The porosity of hydrogel was high to 83% of hyaluronic acid with molecular weight 1,800-2,200 kDa before modification. The hyaluronic acid had low porosity to 16.7% with molecular weight of 100-130 kDa after modification, as shown in Figure 6.

The porosity of hydrogel showed an increasing trend with high molecular weight of hyaluronic acid. The volume of hydrogel tended to shrink during the freezing process. The spatial network framework of hyaluronic acid macromolecules had a high supporting effect and maintained original shape, and the pore size decreased after freeze-drying.

The porosity of hyaluronic acid with the same molecular weight after modification was lower than that before modification. The modified hyaluronic acid was cross-linked intermolecularly by L-cysteine hydrochloride, and hydrogel viscosity increased and hydrogel structure was denser using HRP catalyst under the action of H2O2. The small ice crystals were more difficult to move to generate large ice crystals, and the gap was smaller after modification. The volume of hydrogel tended to shrink, however the porosity decreased during the freezing process.

4. Conclusion
The viscosity of modified hydrogel and water holding capacity increased with HA molecular weight by the formation of new amide bonds to cross-linking. The hyaluronic acid hydrogel increased hardness and tighter structure after modification, and the pore size of hydrogel reduced after freeze-drying. The HA hydrogel increased viscosity and denser structure with smaller porosity by high cross-link density network structure and smaller pore size after L-cysteine Hydrochloride modification.

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