Properties of Hyaluronic Acid Nanoparticles Modified by Histidine for Embedding Flavonoids

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Abstract. Hyaluronic acid (HA) was modified by histidine (His) to prepare His-HA conjugate, and flavonoid-containing mixed nanoparticles were embedded by formamide and dimethylformamide with ultrasonic treatment. The porosity, swelling degree, encapsulation efficiency, drug loading and in vitro release of nanoparticles were measured for embedding capacity. The results showed that the prepared His-HA conjugate with ratio of 9:1 had a high buffering capacity at endosome pH 6.0, and the rate of encapsulation and drug loading for embedding effect were 50%~80% and 8%~13% respectively.

Keywords: histidine; hyaluronic acid; rutin; nanoparticles.

1. Introduction

The functional role of natural foods and natural essential oils had attracted much attention with economic development and life improvement, and the plant extract industry was one of fast growing industries in the world. The waste water of plant extraction produced with physical, chemical or biological methods to extract certain substances of special effects from plants. The waste water of plant extraction was mostly discharged directly to more waste of resources and environmental pollution. The compositions of medicinal and edible plants had a long history of application in food, health care products, medicine, daily chemical production and other fields. The histidine modified HA was used to prepare conjugates of nanoparticles for embedding flavonoids in waste water. This study was to provide a reference for the comprehensive utilization of waste water extracted from medicinal and edible plants.

2. Materials and methods

2.1. Materials and instruments

HA was purchased from Shandong Freda Biomedical Co., Ltd., Rutin, apigenin and proanthocyanidin were from Aladdin Biochemical Technology Co., Ltd., Quercetin Hydrate, Carbodiimide (EDC), N-Hydroxysuccinimide (NHS) and Histidine were purchased from Shanghai Bioengineering Co., Ltd., Formamide, Dimethylformamide and Triethylamine were from Tianjin Guangfu Fine Chemical Research Institute. FD-27 vacuum freeze dryer (Beijing Detianyou Technology Development Co., Ltd.), TU-1901 dual-beam UV-Vis spectrophotometer (Beijing Puxi General Instrument Co., Ltd.), BS203i...
2.2. Test method

2.2.1. Preparation of His-HA conjugate. 1 g HA dissolved in distilled water and added cross-linking agents of EDC and NHS with a molar ratio of 1:1.2:1.2 by magnetic stirring. The histidine (His:HA =9:1) was added after 30 min and magnetically stirred for 24 h at room temperature and airtight conditions. The reacted material was transferred into a dialysis bag (Mr=8000~12000 D) to dialyze with distilled water for 4 d to remove histidine and cross-linking agent, and His-HA conjugate was freeze-dried after dialysis [1].

2.2.2. Preparation of His-HA Nanoparticle Solution. The self-aggregating His-HA nanoparticles were prepared by ultrasonic dispersion method. 5 mg His-HA conjugate was added to 5 ml phosphate buffer solution (pH =7.4) in 37°C water bath for 24 hours to achieve sufficient swelling. The conjugate was pulverized with probe-type ultrasonic waves in ice bath at 90 W for 1 min, and the working pulse was on for 2 s and off for 3 s, and repeated three times to obtain clear and transparent nano solution [2].

2.2.3. Preparation of His-HA nanoparticles containing flavonoids. 50 mg His-HA conjugate was dissolved in 2.5 ml formamide, and 5 mg mixture embedded in 1 ml dimethylformamide containing triethylamine added at molar ratio of 1.3. Both solution were mixed to 10% flavonoids to His-HA conjugates in the dark for 24 h, and dialyzed by dialysis bag with large amount of distilled water for 2 d. The organic solvents, triethylamine and embed flavonoids were removed and transferred the liquid in the dialysis tape to a vial. The flavonoid-loaded nanoparticle solution were treated by ultrasonic wave, and freeze-dried in vacuum and stored for later use.

2.3. Measurement method

2.3.1. Porosity of His-HA conjugate and entrapment. 10 mg freeze-dried His-HA conjugate and embed freeze-dried substances were dissolved in 10.0 mL PBS buffer solution, and completely immersed in PBS buffer solution. The sample was taken out after 10 min, and the volume of the remaining PBS buffer solution was recorded.

2.3.2. Swelling degree of His-HA conjugate and entrapment. 10 mg freeze-dried His-HA conjugate and embed freeze-dried substances were dissolved in in 10 mL of PBS buffer solution. The dry substance was completely submerged, and the surface moisture was absorbed by filter paper in every 5 min, and immediately weighed to unchanged mass [3].

2.3.3. Determination of buffer capacity of His-HA conjugate. His-HA conjugate and sodium chloride (control sample) were dissolved in 35 ml distilled water [4], and adjusted the pH of the solution to 12 with 1 mol/L sodium hydroxide, then used 0.5 mol/L hydrochloric acid titration.

2.3.4. Encapsulation efficiency of flavonoids/His-HA nanoparticles. 5 mg flavonoids/His-HA nanoparticles were dissolved in 5 ml formamide, and recorded the absorbance of the solution at the corresponding maximum absorption wavelength with an ultraviolet/visible spectrophotometer. The flavonoids content in the solution was calculated by standard curve of the substance to obtain encapsulation efficiency of flavonoids/His-HA nanoparticles.

2.3.5. Drug Capacity of flavonoids/His-HA nanoparticles. 5 mg flavonoids/His-HA nanoparticles were dissolved in 5 ml formamide, and recorded the absorbance of the solution at the corresponding maximum absorption wavelength with an ultraviolet/visible spectrophotometer. The flavonoids content in the
solution was calculated by standard curve of the substance, and the drug capacity of flavonoids/His-HA nanoparticles were measured.

2.3.6. In vitro release of flavonoids/His-HA nanoparticles. 3 mg flavonoids/His-HA nanoparticles were dissolved in 5 ml phosphate buffer (pH = 6.0 and 7.4), and the embedded nanoparticles solution was transferred to the dialysis bag with 50 ml phosphate buffer at corresponding pH value. This system was maintained in a constant temperature water bath at 37℃, and dialysis was performed at 100 r/min. Every 4 ml dialysate was taken out at 0.5, 1, 2, 3, 4, 6 and 9 h, and added the same amount of phosphate buffer at corresponding pH. The absorbance at the maximum absorption wavelength was measured with an ultraviolet spectrophotometer, the release amount was calculated according to the standard curve, and the cumulative release percentage was plotted against time to obtain the release curve of nanoparticles in vitro.

3. Results and analysis

3.1. Buffering capacity, porosity and swelling degree of His-HA conjugate

3.1.1. Buffer capacity of His-HA conjugate. The buffer capacity referred to the ability to alleviate the drastic changes of pH in liquid media (such as water, precipitation, etc.). Histidine had high buffering capacity under physiological pH conditions. The pKa value of histidine imidazole group was 6.0, and His-HA conjugate showed high buffering capacity within 5.0-6.5, as shown in Figure 1. The pH of the coupling changed gently with the addition of HCl, and Wang Xiaolei [5] obtained the same conclusions in the research of pH-responsive HA nanoparticles of anticancer drug carriers.

3.1.2. Porosity and swelling degree of His-HA conjugate. The porosity reflected the compactness of the material, and the conjugate and freeze-dried composite were analogous with porosity, as shown in Figure 2. The porosity of rutin and proanthocyanidins was similar with 75% to 71%. The porosity of quercetin and apigenin was close with 83.33% to 80%. The rutin and proanthocyanidins had similar molecular weights and main molecular structure of flavonols. Wang Yafei [6] used the same crosslinking agent of EDC and NHS to study on preparation and performance of silk fibroin/hyaluronic acid composite gel, and porosity of compound increased with HA addition. The spatial network framework of HA had high supporting effect to keep the stable shape of composite during the freeze-drying process.
The swelling degree of conjugate was obviously high to material after embedding, as shown in Figure 3. The amount of crosslinking agent increased the internal crosslinking of chitosan-HA composite gel, and the spatial network structure connected closely [7]. The resistance of molecules penetrating in the gel increased to external water, and the hydrophilic groups on the molecular chains of polysaccharide and HA were difficult to contact with water molecules. The extensibility of the gel polymer network structure was limited to decrease in the degree of swelling. The amount of cross-linking agent had molar ratio of HA to EDC and NHS cross-linking agent with 1:1.2:1.2, and the swelling degree was affected. Histidine connected to HA with increase of the amide bond in tight spatial structure, and the entry of external water molecule was hindered to reduce swelling degree.

Figure 2. Porosity changes of His-HA conjugate and composite

Figure 3. Changes in swelling degree of His-HA conjugate and composite

3.2. Encapsulation efficiency, flavonoid content and in vitro release of flavonoids/His-HA nanoparticles

3.2.1. Encapsulation efficiency of flavonoids embedded in His-HA nanoparticles. Encapsulation rate referred to the percentage of the encapsulated substance in the total amount of the drug in the liposome suspension. It was an important indicator for the quality control of liposomes and nanoparticles, and reflected the degree of drug encapsulation by the carrier. The encapsulation rate of rutin was
significantly lower than the other three groups of substances. The high encapsulation efficiency of proanthocyanidin was 86.76%, and the lowest apigenin was 57.85%, as shown in Figure 4.

![Figure 4. Changes in the encapsulation efficiency of flavonoids embedded in His-HA nanoparticles](image)

3.2.2. *Flavonoid content of flavonoids/His-HA nanoparticles.* Water-soluble polymers of amphiphilic molecules were hydrophobicized. The nanoscale self-organized body prepared by the hydrophobically modified water-soluble polymer was a core-shell structure. The core of the hydrophobic micro-structure domain existed in the nanoparticle, and the outer shell was composed of a hydrophilic skeleton of HA polysaccharide. It was characterized by a large load, and its distribution in organisms was mainly determined by the physical and chemical properties of the particles [5]. The drug loading rate of rutin was obviously low at 1.07%, as shown in Figure 5. The other three groups of data were not significantly different with 10.83%, 13.98% and 8.77% respectively.

![Figure 5. Changes in the amount of flavonoids in His-HA nanoparticles after embedding](image)

3.2.3. *In vitro release of flavonoids/His-HA nanoparticles.* The cumulative release of rutin was high of 30%, and the cumulative release of apigenin was 10%, as shown in Figure 6. The cumulative release percentage at physiological pH 7.4 was lower than acidic pH value of 6.0. Histidine had protonation with responsiveness of the nanoparticles to pH changes after embedding at pH 6.0, and the nanoparticles reduced the interaction balance of hydrophobic and hydrophilic forces. The originally stable core-shell structure was destroyed, and the release of materials inside the nanoparticles greatly accelerated.
Figure 6. The cumulative release of flavonoids at pH 6.0 and 7.4

4. Conclusion
The porosity and swelling degree of composite synthesized by histidine-modified HA had changed slightly, and exhibited high buffering capacity at physiological pH. The encapsulation efficiency of the nanoparticles with composited flavonoids was similar to the amount of flavonoids. The cumulative drug release percentage at the acidic pH of the endosomes of 6.0 was higher than that of the pH of 7.4, and the changes of responsive pH promoted release in vitro.

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