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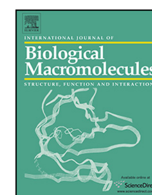

Preparation and characterization of a novel polysialic acid–hyaluronan graft copolymer potential as dermal filler

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ABSTRACT

Polysialic acid (PSA) and hyaluronan (HA) are non-immunogenic and biodegradable natural polysaccharides, but HA belongs to glycosaminoglycans and can be immediately degraded by human enzymes. In this study, we synthesized a novel PSA-HA draft copolymer to improve HA stability in vivo. This draft copolymer was characterized by SEM, element analysis and Zeta potential. Cytotoxicity assays, as well as pyrogen and hemolysis tests, were also conducted to test its biological functions further. Results showed that PSA-HA draft copolymer satisfies the medical requirement for biomaterials. In vivo degradation test proved that this copolymer can reduce the irritation rate and prolong the duration of cross-linked HA in skin. These results indicated that PSA-HA draft copolymer can be potentially used as an alternative for free HA in dermal filler (dual-phase cross-linked HA system).

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1. Introduction

Polysaccharides from microorganisms, such as chitosan, alginate, polysialic acid (PSA) and hyaluronan (HA), are an important class of biomaterials. Among them, HA is a natural polysaccharide composed of disaccharide units of d-glucuronic acid and *N*-acetyl-D-glucosamine with β-1,4 and β-1,3 glycosidic bonds ubiquitous in the human body [1]. HA is a highly hydrophilic polysaccharide with considerable potential as a biomaterial because of its physicochemical and biological properties, such as biocompatibility, biodegradability, and non-immunogenicity [2]. However, HA belongs to the class of glycosaminoglycans (GAGs) and is easily degraded in the human body [3]. Modifications by chemical or physical methods are applied to improve the physicochemical stabilities of HA; simultaneously, these methods also retain its natural biocompatibility, biodegradability, and non-immunogenicity [4–9].

In the molecular structure of HA, the groups available for chemical modification are the hydroxyl and carboxyl groups. The general methods to produce HA derivatives include esterification, cross-linking, grafting, and amidation. Grafting modification is attracting considerable attention because the composites of HA with other macromolecular materials possess the advantages of the two original molecules, including improved physicochemical properties and high biocompatibility. To date, the polymers used for HA grafting include chitosan [10], poly[lactic-*co*-(glycolic acid)] [11], polyleucine [12], high-density polyethylene [13], and chondroitin sulfate [14]. These graft HA composites can be used as wound dressing, scaffold material for tissue engineering, encapsulation material for drug delivery, and dermal filler material.

With regard to HA modification by cross-linking, the molecular weight, viscoelasticity, mechanical strength, and resistance to hyaluronidase of HA increase with the reduction in water solubility. Common chemical agents, such as 1,4-butanediol diglycidyl ether, divinyl sulfone, carbodiimide, and adipic dihydrazide, are used for HA cross-linking [15,16]. Some of these cross-linked HAs are widely used as dermal filler in the beauty industry and show the property of persistent filler. In typical products, such as Restylane, a dual-phase system is employed with exterior natural HA coating the cross-linked HA granules. Nevertheless, the exterior HA will be degraded quickly after injection, and the exposed residual unreacted group of chemicals in the cross-linked HA will cause unfavorable symptoms, such as rejection reaction and skin irritation [17].

PSA is a biodegradable polysaccharide with α-2,8 linkage of *N*-acetyl neuraminic acid (Neu5Ac) produced by *Escherichia coli* fermentation. Neu5Ac is widely distributed in the terminal of oligosaccharides in human and animal cells, and PSA can be found in neural cell adhesion molecules [18–20]. PSA is a non-GAG polysaccharide, unstable in acidic condition and can be hydrolyzed by

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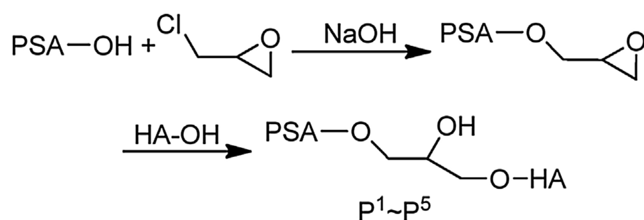

Scheme 1. Synthetic routes of polysialic acid (PSA)–hyaluronan (HA) graft copolymer.

sialidase. As a potential biomaterial, PSA exhibits no immunogenic response in the human body and has been used for protein/peptide modification known as polysialylation [21]. Furthermore, PSA based biomaterial was found to enhance neural cell proliferation and offer promising features as conduit additive in regard to peripheral nerve regeneration during neurogenesis and peripheral nerve repair in vivo [22].

The present study mainly aims to develop a biocompatible hydrogel system prepared by grafting HA with PSA, which can aid non-immunogenicity for the hydrogel. Also, PSA chains in the copolymer would repel each other due to strong negative charge and arrest hyaluronidase approachment to prolong half-life. The resultant PSA-HA draft copolymer is used to coat the cross-linked HA granule as a dual-phase dermal filler to lower the skin irritation rate and prolong the half-life of HA in the skin.

2. Materials and methods

2.1. Materials

HA (pharmaceutical grade, Mw = 1.7×10^6 Da) was purchased from Bloomage Freda Pharm (Shandong, China), and PSA (Mw = 1.4×10^5 Da) was prepared and purified in our laboratory [23]. Other chemicals were analytical reagents from Sinopharm Chemical Regent (Shanghai, China). Human umbilical vein endothelial cell (HUVEC) was obtained from the China Academy of Science.

2.2. Synthesis of PSA-HA graft copolymer

Approximately 1.5 g of PSA was dissolved in 10 mL of 0.7% NaOH, and 0.4 mL of epichlorohydrin (ECH) was added to react at 35 °C for 6 h under mixing. At the end of the reaction, excess ECH was removed and concentrated by a vacuum. The activated PSA was purified by precipitation with 80% ethanol solution repeatedly.

Furthermore, the activated PSA was dissolved in PBS (pH 5.0, 20 mM) to yield a 1.5% (w/v) solution. Subsequently, a certain amount of HA was added, and the reaction was conducted for 56 h at 30 °C. The mixture was concentrated and dialyzed against deionized water for two days with 500 kDa membrane, until no PSA was detected by resorcinol method [24]. The final PSA-HA draft copolymer was obtained through lyophilization. The modification density of epoxy group was determined using sodium hyposulfite titration method [25] (Scheme 1).

2.3. Morphological analysis

The lyophilized PSA, HA and PSA-HA copolymer were surface-treated with spray-gold and observed by SEM (Quanta 200, FEI, Oregon, USA).

2.4. Zeta potential determination

The PSA-HA draft copolymer was dissolved in 50 mM Tri-HCl buffer with different pH (1.97, 3.25, 4.54, 6.06 and 7.20) and subject to Zeta potential analysis (ZetaPALS, BIC, USA). The untreated PSA and HA were used as control.

2.5. Element analysis

Dried sample of PSA, HA and PSA-HA copolymer were subject to elemental analysis using an elemental analyzer (Vario EL III, Elementar, Germany) and fractions of C, H and N element were determined. Content of PSA and molar ratio of PSA to HA in the PSA-HA copolymer were calculated according to the following equations.

$$\text{Content of PSA } X = 1 - [C_{\text{PSA-HA}} - C_{\text{PSA}}]/C_{\text{HA}}$$

$$\text{Molar ratio of PSA to HA } = X \times m \times M_{wHA}/\mathrm{M}_{\text{wPSA}}$$

C_{PSA}, C_{HA}, $C_{PSA\text{-}HA}$, are fraction of C element in PSA, HA and PSA-HA copolymer. M_{wHA}, M_{wPSA}, m, are molecular weight of HA, molecular weight of PSA, and mass ratio of PSA to HA.

2.6. Cytotoxicity assays

The PSA-HA copolymer was sterilized by γ-radiation for 20 s in a low-energy electron accelerator (AB5.0 Wuxi El Pont, China). Subsequently, 0.2 g copolymer was immersed in 1.0 mL DMEM medium with 10% fetal bovine serum (FBS) for 48 h to obtain an aqueous extract. DMEM medium with 10% FBS was used as a control. Exponential phase HUVECs were diluted to produce approximately 8.0×10^4 CFU cell suspension. The cell suspension was inoculated in 96-well plates with 100 μL of aliquots for each well. After cultivation for 24 h at 37 °C with 5% CO_2, the supernatant was discarded. Afterward, 100 μL of the control solution and the solutions with 100% 75%, 50%, and 25% aqueous extracts were added to the wells. Each group consisted of six wells, and the plates were cultivated for 48 h. Subsequently, 10 μL of MTT solution (5 mg/mL) was added to each well, and the cells were cultivated for 4 h. The supernatant was again discarded, and 100 μL of DMSO was added to the wells. After mixing for 10 min at room temperature, the absorbance at OD_{570} was recorded to calculate the relative growth rate (RGR).

2.7. Pyrogen test

The PSA-HA copolymer (0.2 g/mL) was leached with saline for 72 h, and the aqueous extract was used for pyrogen test, with saline as a control. Six healthy, mature New Zealand rabbits (clean grade, Animal Experimental Center of Jiangnan University, China) were used for pyrogen test. The rabbits were cared for in accordance with local animal welfare regulations in an AECJU-accredited facility, and the study was approved by the Institutional Animal Care and Use Committee and conducted at AECJU (Wuxi, China). Food and water were allowed ad libitum, room temperature was maintained at 17–21 °C, and relative humidity was between 40% and 70%.

Before experiments, food and water were stopped for 2 h. The leached solution of PSA-HA was injected into the auricular veins of rabbits, and saline injection was used as a control. The anal temperature of each rabbit was determined every 1 h after injection. The mean temperature of three measurements was used. Afterward, the

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