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Delivery of Positively Charged Proteins Using Hyaluronic Acid Microgels

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***Abstract*—**In this study, hyaluronic acid (HA) microgels were developed for the goal of protein delivery. First, a hyaluronic acid-tyramine conjugate (HA-TA) was synthesized with a degree of substitution of 13 TA moieties per 100 disaccharide units. Then, HA-TA microdroplets were produced using a water in oil emulsion method and crosslinked in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). Loading capacity and the release kinetics of lysozyme and BSA, as model proteins, were investigated. It was shown that lysozyme, a cationic protein, can be incorporated efficiently in the HA microgels, while the loading efficiency for BSA, as a negatively charged protein, is low. The release profile of lysozyme showed a sustained release over a period of one month. The results demonstrated that the HA-TA microgels are a good carrier for spatial delivery of cationic proteins for biomedical applications.

***Keywords*—**Microgel, inverse emulsion, protein delivery, hyaluronic acid, crosslinking.

I. Introduction

ONE issue in regenerative medicine and treatment of many diseases is making a proper vehicle for delivery of proteins and growth factors needed. A bolus injection of proteins would not provide the proper effect, as their half-lives in the body is short, and also the delivery of high doses of proteins may cause toxic effects. Therefore, to improve the efficacy, a controlled release system should be defined to prolong the presence of the proteins at the desired site [1].

In recent years, microgels have been introduced as suitable carriers for protein and drug delivery. Microgels are micrometer sized crosslinked polymer networks with a high water content that are suitable for delivery of hydrophilic molecules [2]. The high surface to volume ratio of the microgels leads to an efficient protein uptake and release [3].

Polysaccharides are good candidate for microgel fabrication as they are biocompatible, non-immunogenic, and biodegradable. Moreover, different functional groups present on their molecular chain makes the material suitable for various chemical modifications [4], [5]. HA, a natural glycosaminoglycan present in the body, is one of those polysaccharides that has attracted attention in the recent decade [6]-[8].

There are different methods to fabricate microgels. Water in oil gelation method involves the emulsification of an aqueous solution in an oil phase and subsequent crosslinking by the addition of a crosslinking reagent [9]. Recently, enzymatic crosslinking has been extensively explored in tissue engineering [10] and drug delivery [11] applications. Applying this methodology, the network is formed under mild conditions by addition of an enzyme, like peroxidases, and H_2O_2.

Herein, polysaccharide-based microgels were fabricated by enzymatic crosslinking of tyramine conjugated HA in an inverse microemulsion system. Two model proteins, lysozyme and BSA, were incorporated in the microgels by absorption. High loading capacity and sustained release of lysozyme, as a positively charged protein, provided HA microgels as a promising carrier for the delivery of cationic proteins.

II. Materials and Method

A. Materials

Sodium hyaluronate (MW=25 kDa) was purchased from Contipro Pharma, the Czech Republic. Tyramine hydrochloride (TA.HCl) (99%), anhydrous N,N-dimethylformamide (DMF) (99.8%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), H_2O_2, HRP, MES hemisodium salt dry powder, Span 80, bovine serum albumen (BSA), and lysozyme from chicken egg white were purchased from Sigma-Aldrich. All other solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and used as received. 2,2,4-Trimethylpentane (isooctane) was purchased from Honeywell.

B. HA-TA Synthesis

Sodium hyaluronate was dissolved in 30 mL of PBS. EDAC (1.0 g) and NHS (1.3 g) were added, and the solution was stirred at room temperature for 2 h. The product was precipitated in ethanol, filtered, and dried in the fume hood. 1 g of the synthesized polymer was dissolved in 30 ml of 0.1 M MES buffer (pH 6.1). Tyramine. HCl (100 mg) was added to the solution and the pH was maintained constant at 6.1. After overnight stirring, the resulting solution was precipitated in cold ethanol, filtered and washed with ethanol and finally diethyl ether. The final white powder was dissolved in MilliQ-

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water and dialyzed (MWCO 1000) against 100 mM sodium chloride solution for 2 days, a mixture of distilled water and ethanol (5:1) for 1 day and distilled water for 1 day. The HA-TA conjugate was obtained after freeze drying. The degree of substitution (DS) defined as the number of tyramine groups per 100 disaccharide units, was calculated from ^{1}H NMR (D_2O) spectral data by comparing the integral values of the aromatic protons of tyramine groups (6.8 and 7.2 ppm) and the methyl protons of HA (1.9 ppm).

C. Hydrogel Formation

The HA-TA was dissolved in PBS at a concentration of 5% w/v. To 1 ml of HA-TA solution, 20 µL of H_2O_2 (1.5 % v/v) and 10 µL HRP (2 mg/ml) in PBS were added and the mixture was gently mixed to form a gel. Gelation was determined with the vial tilting method. When the polymer solution does not flow upon inverting the vial, it was considered as a gel state.

D. Microgel Preparation and Characterization

HA microgels were prepared using a water in oil emulsion method. To prepare the microdroplets, first a HA-TA solution containing HRP (1.5 U/ml) was added dropwise to isooctane containing Span 80 as a surfactant. The water/oil ratio was 12. The mixture was homogenized using an Ultra-Turrax (T25, IKA Works Inc., USA) at a speed of 13500 rpm. Subsequently, 20 µL of a H_2O_2 solution was added to the microemulsion to crosslink the droplets. After 30 min homogenization, the particles were collected by centrifugation at 8000 rpm for 5 min, washed with acetone and ethanol, and dried under reduced pressure.

To visualize the swollen microgels, they were dispersed in water and imaged using phase contrast microscopy. Scanning electron microscopy was used to see the microgels morphology at dry state. Dry microgels were placed on a SEM mounting stud and imaged using a FEI Quanta 650 ESEM with an acceleration voltage of 10 kV.

E. Protein Loading

Two model proteins, lysozyme (14 kDa, pI=11.35 [12]), as a positively charged protein, and bovine serum albumin (BSA, 60 kDa, pI=4.7 [13]), as an anionic protein, were used to investigate the loading capacity of the microgels. Then, 1 mL of a protein solution at a concentration of 500 µg/mL was incubated with 2 mg of dry microgels, overnight on a shaker. The microgels were centrifuged (7000 rpm, 10 min) and the supernatant was removed and analyzed for its protein content using a Bradford Assay (Coomassie protein assay kit, Thermo scientific). Also, different amounts of dry microgels, from 1 to 5 mg, were incubated with a fixed amount of protein solution and the loading efficiency was calculated by subtracting the protein concentration in the supernatant from the initial amount of protein.

F. Protein Release

To investigate the release profile, loaded microgels were incubated with 1 ml of PBS (pH=7.4) containing 0.05% w/v sodium azide. At predetermined time points, the release medium was collected after centrifugation (7000 rpm, 10 min) and replaced with fresh medium. The amount of the protein was measured using a Bradford Assay.

III. Results and Discussion

A. HA-TA Synthesis and Hydrogel Formation

The HA-TA conjugate was synthesized through EDC/NHS chemistry. First, part of the carboxylic acid groups on the HA backbone was converted to NHS esters. In the second step, the NHS esters were reacted with tyramine to provide the HA-TA (Fig. 1).

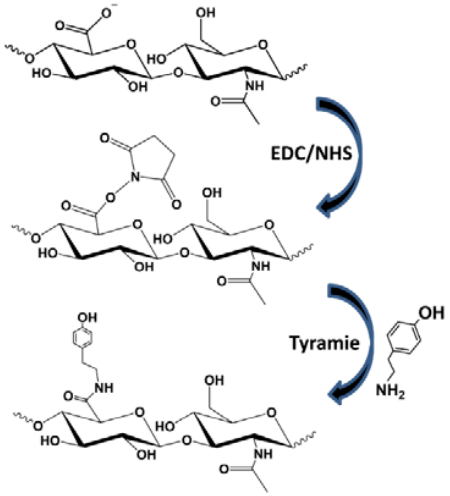

Fig. 1 Schematic representation of HA-TA synthesis

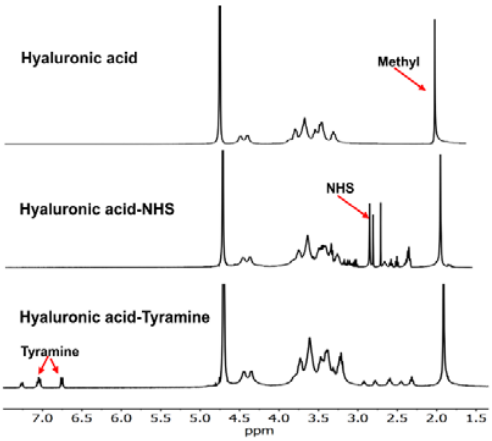

Fig. 2 ^{1}H NMR spectral data of HA, HA-NHS, and HA-TA

Fig. 2 shows the ^{1}H NMR spectral data of HA, HA-NHS, and HA-TA. The degree of substitution for conjugated NHS ester was 24% and for tyramine, calculated from the integral ratio of the tyramine phenolic protons and the HA methyl protons, was 13% (Fig. 2).

Gelation occurs by addition of HRP enzyme and H_2O_2. The gelation time can be tuned by adjusting the amount of HRP and faster gelation will be achieved by increasing the HRP concentration.

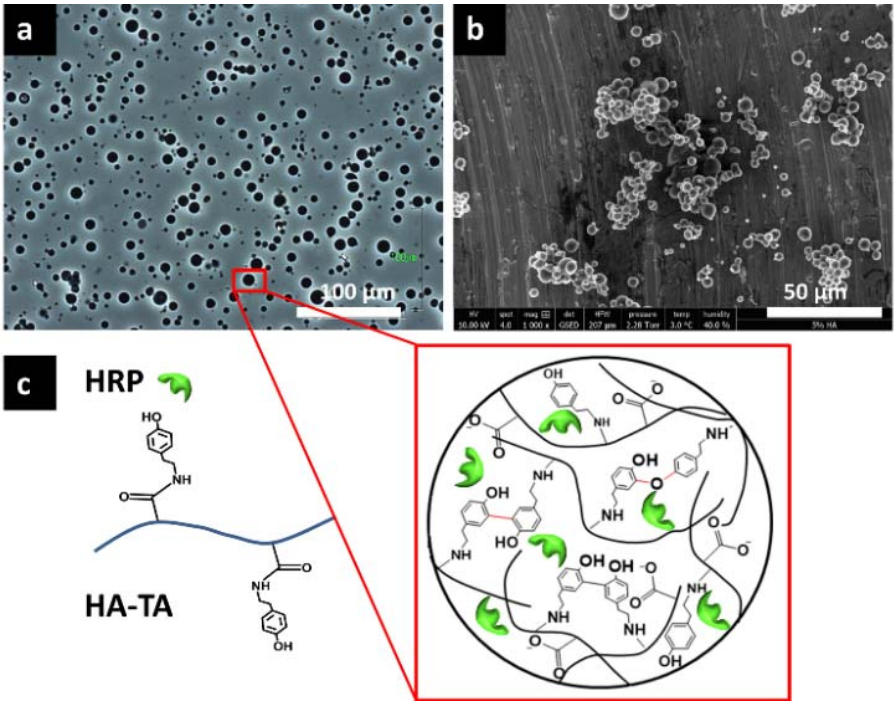

Fig. 3 (a) Microscopic image (b) SEM image of the HA microgels (c) A crosslinked droplet

B. Microgel Size and Morphology

Microgels were prepared through crosslinking of HA-TA microdroplets containing HRP by addition of H_2O_2. The oil phase was stabilized using Span 80 as a surfactant. The dry microgels were isolated as a fine powder that is easily redispersed in water. A microscopic image of the HA microgels is shown in Fig. 3 (a). Fig. 3 (b) shows a SEM image of the microgels. A spherical shape and a smooth surface of the microgels were clearly demonstrated. A schematic representation of the crosslinked droplets is depicted in Fig. 3 (c).

C. Protein Loading and Release

To show the capacity of HA microgels for protein uptake, lysozyme and BSA were used. The amount of proteins absorbed was calculated by measuring the protein content in the supernatant and subtracting it from the initial protein amount. The loading efficiency of lysozyme and BSA is shown in Fig. 4 (a). Lysozyme loading (~60%) was significantly higher than BSA (~7%) due to the electrostatic interaction between the negatively charged HA-TA [14] and the cationic protein. However, BSA is an anionic protein, and thus, the repulsive forces hinder the protein penetration into the microgel structure.

The release profiles of lysozyme from HA microgels at protein loadings of 90 and 150 µg/mg of dry microgels were shown in Fig. 5. Lysozyme was released from microgels in a sustained manner after an initial burst release. The sustained release of lysozyme was monitored for four weeks.

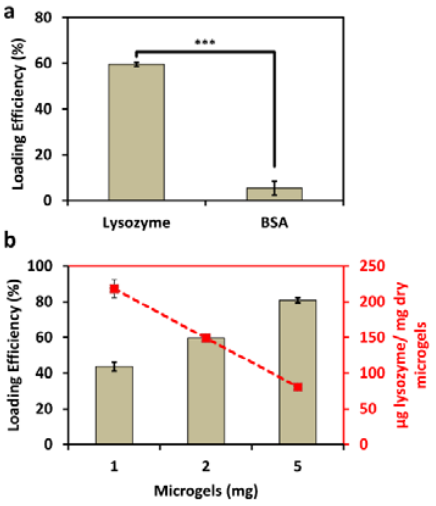

Fig. 4 (a) Loading capacity of HA microgels for lysozyme and BSA. (b) Loading efficiency and µg of lysozyme loaded per mg of dry microgels at different amount of microgels. *** p-value < 0.001

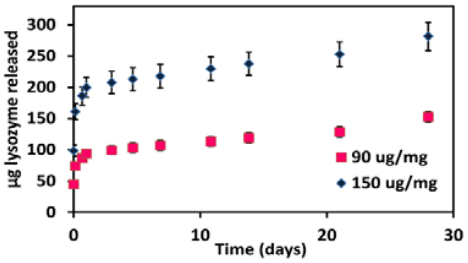

Fig. 5 Release profile of lysozyme at two different loadings

IV. Conclusion

In this study, HA microgels were prepared through enzymatic crosslinking of HA-TA microdroplets. Lysozyme was loaded with a high efficiency in the microgels by absorption. The electrostatic interaction between the cationic protein and the negatively charged HA-TA provides efficient protein loading and also a sustained release of the encapsulated proteins during a period of four weeks. The results demonstrated that the proposed HA-TA microgels are a good platform for the delivery of cationic proteins for biomedical applications.

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