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Research paper

# Controlled release of a model protein drug ovalbumin from thiolated hyaluronic acid matrix

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ABSTRACT

The objectives of this work were to investigate the properties of the thiolated hyaluronic acid (HA) with different molecular weights (MWs) and to assess the effects of MW and dissolution medium on the release of ovalbumin from thiolated HA matrix tablets. Four HA-cysteine (HA-Cys) conjugates with increasing MWs (47, 101, 260 and 550 kDa) were synthesized. The resulting HA-Cys conjugates exhibited increased stability against hyaluronidase degradation, and such an effect became stronger with the increase of polymer MW. Swelling and erosion studies of HAs or HA-Cys conjugates based matrix tablets were performed in various media. We found that the water uptake capacity and erosion rate were strongly dependent on MW of HA, ionic strength and pH of the medium. Release studies showed that all investigated factors exerted effects on the drug release. Most of the release data based on HA matrix tablets followed super Case II transport but conformed to non-Fickian diffusion with HA-Cys conjugates. Taken together, this work managed to develop HA-Cys conjugates-based matrix tablets as controlled release systems for protein with tunable release rate through varying MWs of polymer.



## 1. Introduction

Oral delivery of peptide and protein drugs is of great convenience and patient compliance. However, development of oral controlled delivery for peptide and protein drugs faces tremendous challenges [1], including low stability, low permeability of large molecules, and short half-time. Moreover, these drugs can be easily destroyed by enzymes in the gastrointestinal tract, leading to extremely low oral bioavailability and therapeutic efficacy of most peptides and proteins. Fortunately, various approaches have been explored to enhance the oral bioavailability of therapeutic proteins, such as the use of absorption enhancers [2], enzyme inhibitors [3], mucoadhesive systems [4], and some special drug delivery system [5] et al.

During the last decade, thiolated polymers, the introduction of thiol functional groups to polymers, have displayed excellent mucoadhesive properties [6], permeation-enhancing effect and efflux pump inhibitory properties [7]. Additionally, thiomers showed effective resistance towards the activity of carboxypeptidase A or B, chymotrypsin and aminopeptidase, and therefore they can protect the incorporated drugs, especially peptides and proteins, from the enzymatic degradation in the intestine [8–10]. It has been demonstrated that polycarbophil–cysteine as carrier matrix of insulin can guarantee a controlled drug release over 10 h among thiolated polymers [11]. Release studies of insulin showed an almost zero-order release kinetic due to its in situ gelling properties [12]. Therefore, all above-mentioned advantages favor thiomers as a highly suitable hydrophilic matrix for the oral controlled delivery of proteins. However, it is known that drug release from hydrophilic matrix tablets is a complex process, including dissolution, diffusion and erosion mechanisms [13], and such a process depends on many factors (drug, polymer and dissolution medium) that affect the water diffuse through the matrix and erosion [14,15].

Hyaluronic acid (HA), a natural liner polysaccharide [16], is endowed with properties of strong hydration, high biocompatibility, viscoelasticity and low immunogenicity [17]. However, natural HA is sensitive to strong acid, alkali, heat, free radicals and hyaluronidase, and it is easy to be degraded, which limits its application in prolonged release formulations. Chemically modified HA [18] has been widely accepted as an alternative to efficiently solve this defect. For this purpose, thiol-modified HA has been synthesized and applied in wound healing [19], tissue engineering [20] and drug delivery [21]. This new system exhibited significant improvement in mucoadhesion, the inhibition effect of the

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conjugate towards peptidase and sustained release property [22]. Moreover, HA has a wide molecular weight (MW) range of $10^3$–$10^7$ Da [23], and it has been known to play different roles in the body depending on its MW [24]. However, matrix tablets of thiolated HA with different MWs have not been elucidated yet, and the drug release mechanisms remain unclear. Swelling and erosion behavior information of polymer matrix may be useful to understand drug release kinetics.

In the present study, we aimed to synthesize HA-cysteine (HA-Cys) conjugates with increasing MWs ($HA_{47k}$-Cys, $HA_{101k}$-Cys, $HA_{260k}$-Cys and $HA_{550k}$-Cys), to investigate the stability of thiol groups, enzymatic degradation and the effect of the MW and dissolution medium on the swelling and erosion, to determine the predominant release mechanisms via drug release in vitro, and to discuss their applicability as controlled release systems for protein.

## 2. Materials and methods

### 2.1. Materials

HA with MWs of 47, 101, 260 and 550 kDa were purchased was from Freda (Shandong Freda Co. Ltd., China). L-cysteine, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) and *N*-hydroxy-succinamide (NHS) were obtained from Aladdin (Shanghai, China). 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) was supplied from Sigma (St. Louis, MO, USA). Ovalbumin (OVA) was provided by Sinparm Chemical Reagent Co., Ltd. Hyaluronidase (type I, 300–500 U/mg) was purchased from bovine testes. All other reagents were of analytical grade unless otherwise stated.

### 2.2. Synthesis of HA-Cys conjugates

HA-Cys conjugates (47, 101, 260 and 550 kDa) were synthesized by the covalent attachment of cysteine to HA according to a previously described method [22]. Briefly, 1 g of each HA (47, 101, 260 and 550 kDa) was separately hydrated in deionized water followed by the addition of EDAC/NHS at a final concentration of 125 mM and 100 mM, respectively. Subsequently, the pH was adjusted to 5.5 using 0.1 M HCl. After incubation at room temperature for 45 min under stirring, L-cysteine ($n_{L-Cys}$:$n_{-COOH}$ = 5:1) was added, and the pH was adjusted to 5.0. Reaction mixtures were incubated at room temperature for 4 h under stirring. The resulting conjugates were dialyzed in dialysis bags (MW cut off of 12 kDa). Controls were prepared and isolated as the polymer conjugates using the same way except that EDAC and NHS were omitted during coupling reaction.

### 2.3. Characterization

#### 2.3.1. FT-IR spectroscopy

Samples pellets prepared with HA or HA-Cys conjugates and KBr were subjected to FT-IR spectroscopy using a Fourier-transform infrared spectrophotometer (Nicolet FT-IR, 20SXB, USA) with a frequency range of 4000–500 $cm^{-1}$.

#### 2.3.2. Determination of the thiol group and disulfide contents

The number of thiol groups immobilized on the polymer conjugates was spectrophotometrically determined using Ellman's reagent as previously described [25]. The disulfide contents were determined using $NaBH_4$ for reduction [26] and Ellman's method.

#### 2.3.3. Oxidation of thiol groups

HA-Cys (47, 101, 260 and 550 kDa) conjugates were respectively dissolved in phosphate buffer (pH 5.0) and phosphate buffer (pH 7.4) containing 0.9% NaCl at a final concentration of 5 mg/ml. The solutions were incubated at 37 ± 0.5 °C under continuous shaking (100 rpm). At predetermined time points, aliquots were withdrawn, and the amount of remaining free thiol groups was determined using a spectrophotometer with Ellman's reagent as abovementioned.

#### 2.3.4. Degradation by hyaluronidase

The amount of hyaluronic acid was carried out by colorimetric reaction of HA with cetyltrimethyl ammonium bromide (CTAB) according to the method described by Ferrante [27].

In order to determine the enzymatic degradation, solutions of HAs or HA-Cys conjugates (0.5 mg/ml) were prepared with 0.2 M acetate buffer (pH 6.0) containing 0.15 M NaCl, followed by addition of 1 mg/ml hyaluronidase solution (1.0 mL), respectively. The mixtures were incubated at 37 ± 0.5 °C under continuous shaking (100 rpm), and 1 mL aliquots of the mixture were withdrawn at different times. Percentage of enzymatic degradation was calculated using equation as follows Eq. (1):

$$P(\%) = 100*(M_0 - M_t)/M_0 \quad (1)$$

where P is the percentage of enzymatic degradation, $M_0$ is the original amount of HA, and $M_t$ is the amount of remaining HA after the degradation.

### 2.4. Preparation of matrix tablets and drug-loaded matrix tablets

Briefly, 30 mg freeze-drying HA-Cys (47, 101, 260 and 550 kDa) conjugates and the corresponding unmodified HAs were compressed into flat-faced tablets (5.0 mm diameter) by single-punch tablet press (Type TDP, Shang Hai).

In order to prepare OVA-loaded HA-Cys or HA matrix tablets, OVA was previously added to a polymeric solution of HA-Cys or HA in water, in a 1:5weight ratio (OVA: polymer). The solution was stirred for 10 min and freeze-dried. After that, the freeze-drying mixture were compressed into flat-faced tablets under similar conditions. The compaction pressure was maintained at 8–9 kN during the preparation of all tablets.

### 2.5. Swelling and erosion studies

Swelling studies were performed using a dissolution apparatus (ZRS-8G, Tianjin, China) with the basket method (2010 Chinese Pharmacopoeia) in the dissolution medium of distilled water, 0.1 M saline solution, 0.2 M saline solution, HCl (pH1.2), or phosphate buffer (pH6.8). First, the dry matrix tablets without drug were weighed, and then the matrix tablets were immersed in test medium at 37 ± 0.5 °C and stirred at 50 rpm. After 5, 10, 20, 40, 60, 90 and 120 min, the hydrated matrix was weighted after the surface solution was blotted with a tissue. The swelling ratio could be calculated at each time point via Eq. (2) as follows, where $W_0$ is the initial weight, and Wt is weight of the test tablets at the time point t [28].

$$\text{Swelling} = [(Wt - W_0)/W_0] \quad (2)$$

During the swelling studies, the amount of matrix erosion was analyzed at predetermined time periods via spectrophotometrically determining (UV1000, Techcomp Ltd., China) the cumulative contents of HA dissolved in test media as described above.

### 2.6. In-vitro drug release studies

OVA release experiments were conducted under “sink” conditions by immersing the above-mentioned drug-loaded matrix