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Hyaluronic acid L-cysteine conjugate exhibits controlled-release potential for mucoadhesive drug delivery

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Hyaluronic acid-L-cysteine conjugate, a novel thiolated polymer, was synthesized and characterized for mucoadhensive drug delivery. L-Cysteine was covalently attached to hyaluronic acid via the formation of an amide bond. Adhesion studies on the mucosa indicated a 4.82-fold increase in the adhesion force of the obtained conjugate (containing 210.58 μmol thiol groups per gram polymer) versus unmodified hyaluronic acid. The results of a peptidase inhibition study revealed that the inhibitory effect of hyaluronic acid toward trypsin and chymotrypsin was significantly improved compared to hyaluronic acid. Permeation studies utilizing a MDCK cell monolayer system demonstrated a sustained drug release. Based on these features the novel thiolated polymer might represent a promising multifunctional excipient for various drug delivery systems.

1. Introduction

Mucoadhesive polymers that can prolong the residence time of drugs on various mucosae have received considerable attention in last decade. However, mucoadhesive polymers usually bind to the mucosal surface via the formation of non-covalent bonds such as hydrogen bonds, van der-Waal's forces, and ionic interactions, which in many cases are insufficient to guarantee adhesion (Bernkop-Schnürch 2005). Recently, a presumptive new generation of mucoadhesive polymers-thiolated polymers or so-called thiomers has been introduced for controlled drug delivery. The mechanism of improved mucoadhesion of thiomers is attributed to the formation of disulfide bonds between thiol-bearing side chains of the polymer and cysteine-rich subdomains of mucus glycoproteins (Bernkop-Schnürch et al. 2000). Improved mucoadhesive properties of thiomers, such as chitosan–thioglycolicacid conjugate, chitosan–2-iminothiolane conjugate and PHEA–thioglycolicacid conjugate, have been reported. In addition, thiomers have exhibited increased inhibitory effect toward peptidase (Bernkop-Schnürch and Thaler 2000; Bernkop-Schnürch et al. 2004).

Hyaluronic acid (HA) is a macromolecular linear polysaccharide consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by β-1–3 and β-1–4 glycosides bonds. HA has been widely used in medical fields due to its good viscoelasticity, plasticity, penetrability, moisture capacity, biocompatibility and biodegradability (Luo and Prestwich 2001; Zhang et al. 2001).

In this study, we described the modification of HA by L-cysteine (L-Cys), resulting in improved mucoadhesive features due to the immobilization of thiol groups on the polymeric backbone. Furthermore, the inhibition effect of the conjugate towards peptidase and the polymer's potential as a matrix for sustained release drug delivery system was investigated.

2. Investigations, results and discussion

2.1. Synthesis of hyaluronic acid-L-cysteine conjugates (HA-L-Cys)

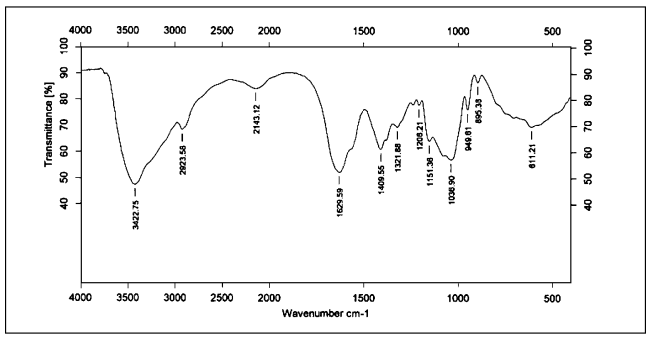
HA-L-Cys conjugates with high, medium and low content of thiol groups containing 210.58 μmol, 130.12 μmol and 73.21 μmol thiol groups/g polymer, respectively, were synthesized.

2.2. Characterization of HA-L-Cys conjugates

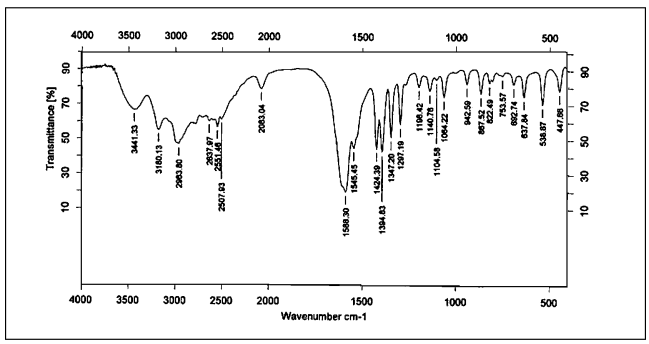
IR spectra of HA, L-Cys and HA-L-Cys conjugate are illustrated in Fig. 1. L-Cys exhibited an intense absorption at 2500 cm^{-1}. Thus, the formation of HA-L-Cys conjugate was confirmed by the appearance of the new band at 2500 cm^{-1} compared with unmodified HA, which was attributed to the absorption of the thiol group. Compared with the ^1H NMR spectrum of HA (Fig. 2), the ^1H NMR spectrum of HA-L-Cys (Fig. 3) showed a characteristic peak of methylene at δ2.95. Since the molecule of HA does not include methylene, this characteristic peak at δ2.95 indicated the linkage of the methylene to the thiol group. Consequently, the ^1H NMR spectra demonstrated the formation of an amide linkage between L-Cys and HA.

2.3. Mucoadhesive properties

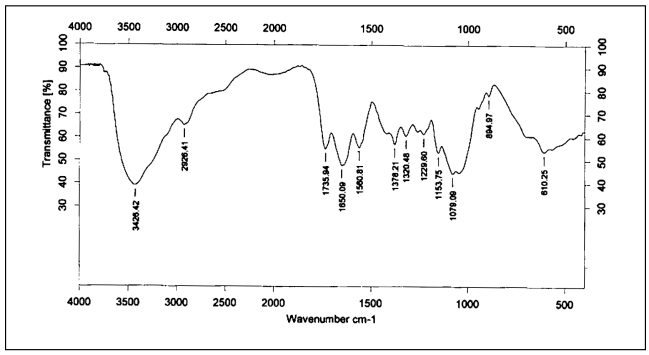
Mucoadhesive studies with self-made equipment showed that the adhesion of HA and HA-L-Cys conjugate with low, medium and high contents of thiol groups were 0.2193 N, 0.4635 N, 0.8087 N, and 1.2764 N, respectively (Fig. 4). Mucoadhesive studies with tablets containing HA-L-Cys conjugates demonstrated a clear positive correlation between the amount of immobilized thiol groups and the adhesion properties of the



(a)



(b)



(c)

Fig. 1: From (a) to (c): IR spectrum of HA, L-Cys and HA-L-Cys

polymer. HA-L-Cys conjugates with high content of thiol groups led to a 4.82-fold improvement in adhesion in comparison with the unmodified HA.

2.4. Enzyme inhibition studies

To avoid a presystemic metabolism caused by intestinal proteases, therapeutic polypeptides can be protected by incorporation in mucoadhesive polymers. Recent studies demonstrated that this protective effect can further be tremendously improved if the mucoadhesive polymer also displays enzyme inhibitory capabilities. The inhibitory effects of HA-L-Cys conjugate toward trypsin and chymotrypsin were evaluated. The results indicated that the covalent attachment of cysteine on the polymer led to a significant inhibitory effect toward trypsin (Table 1) and chymotrypsin (Table 2). The Ka of conjugates groups were significantly decreased comparing to unmodified HA groups in both enzyme-catalyzed reactions ($p < 0.05$). Also the enzyme inhibition study demonstrated that the increased amount of thiol groups in the conjugate resulted in decreased Ka, which can be explained by the immobilization of the enzyme on the HA-L-Cys conjugate.

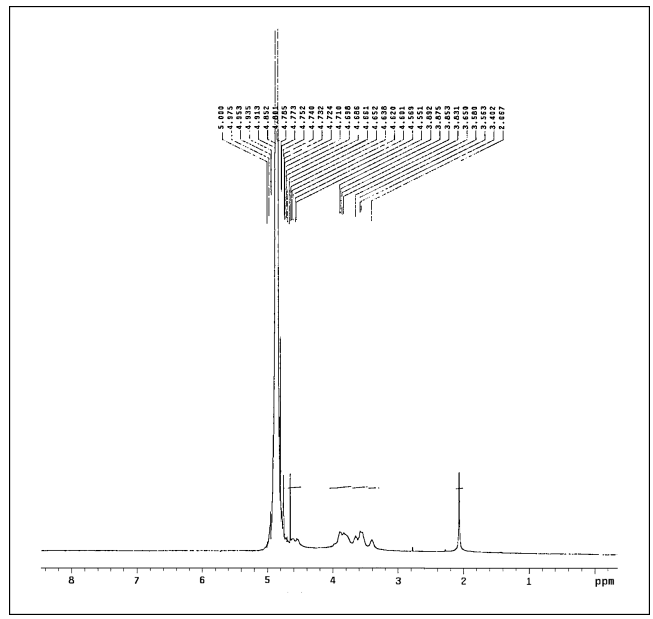


Fig. 2: 1H NMR spectrum of HA

2.5. Permeation studies utilizing a MDCK cell monolayer system

A comparison of the permeation rate demonstrated that unmodified HA groups lead to a much faster transport than the conjugates groups for both sodium fluorescein (Fig. 5) and insulin (Fig. 6). And the fact that increased amount of thiol groups in the conjugate resulted in decreased permeation rate can be found in the permeation studies of both model drugs. Such a difference in the release rate could be explained by the fact that a higher content of thiol groups could facilitate the formation of disulfide bonds within the thiolated polymers, providing an improved cohesiveness of the matrix-system, which becomes a barrier for drug release.

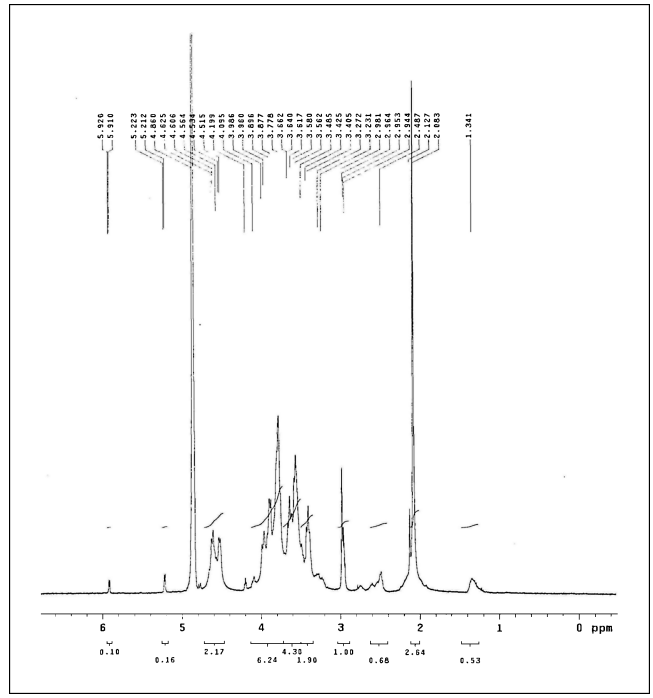


Fig. 3: 1H NMR spectrum of HA-L-Cys

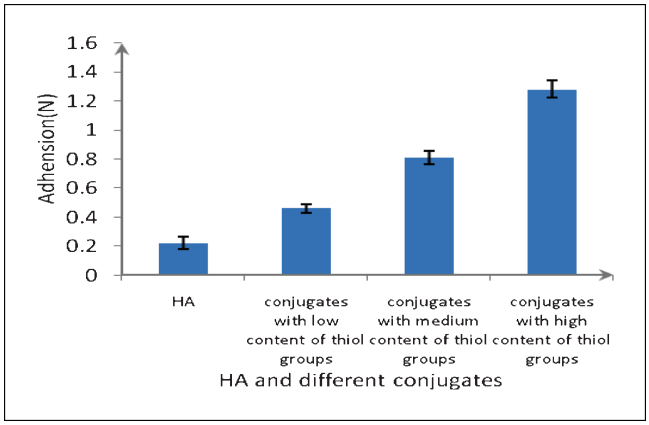


Fig. 4: Mucoadhesive properties of HA and HA-L-Cys conjugate

2.6. *Safety evaluation of HA-L-Cys conjugates by MTT method*

Cell viability in the presence of HA-L-Cys conjugate and HA was tested (Table 3). The results showed that 0.05%, 0.1%, 0.2% HA-L-Cys conjugates and HA showed no significant inhibition on cell growth compared with control group ($p < 0.05$), which indicated that the toxicity of HA-L-Cys conjugate for MDCK cells was trivial.

Based on these features the HA-L-Cys conjugate represents a promising polymeric carrier matrix for delivery systems, which might provide a prolonged residence time and sustained release of the drug on the mucosa.

3. Experimental

3.1. *Materials*

Hyaluronic acid (medium molecular mass: 140 M) was purchased from Shandong Freda Co. Ltd. L-Cysteine (L-Cys) was purchased from Shanghai Huishi biochemical reagent Co. Ltd. Hyaluronidase (Type I, 400–1000 units/mg) was purchased from bovine testes. Chymotrypsin (≥ 800 U/mg from bovine pancreas) was obtained from China National Medicine Co. Ltd. Trypsin (≥ 250 U/mg from bovine pancreas) was obtained from Bio Basic Unit Co. Ltd. All chemicals were of analytical grade. Wistar rats were provided by the West China Laboratory Animal Center of Sichuan University.

3.2. *Synthesis of hyaluronic acid-L-cysteine conjugates (HA-L-Cys)*

400 mg HA was dissolved in 100 ml of demineralized water, followed by the addition of different amounts of ethyl-dimethyl-amino-propylcarbodiimide (EDCI) and *N*-hydroxy-succinamide (NHS). The pH was adjusted to 5.0 by the addition of 0.1 M HCl. The reaction mixture was stirred at room temperature for 30 min and then L-Cys was added. The reaction mixture was incubated at room temperature for 4 h under stirring. The pH value was adjusted and maintained at 5.0 with 1 M NaOH or 1 M HCl during the whole experiment. The resulting polymer conjugate was dialyzed twice against 2 mM HCl containing 0.9% NaCl for 6 h each time. Finally, the aqueous polymer solutions were frozen and lyophilized at −45 °C and 309 mbar for 24 h and stored at 4 °C until future use. HA-L-Cys conjugates with high,medium and low content of thiol groups were obtained when the ratio of n_{EDCI}:$n_{HA\text{-}COOH}$ was 5:1, 3:1 and 1:1, the ratio of n_{NHS}: $n_{HA\text{-}COOH}$ was 4:1, 1:1 and 0.5:1, and the ratio of $n_{L\text{-}Cys}$:$n_{HA\text{-}COOH}$ was 5:1, 1.5:1 and 1:1, respectively.

3.3. *Determination of thiol group content*

The degree of modification, i.e. the amount of immobilized thiol groups on HA-L-Cys conjugate, was determined spectrophotometrically using Ellman's reagent quantifying free thiol groups. Briefly, 20 mg conjugate was hydrated in 10 mL of 2 mM HCl. Following that, 500 µl of Ellman's reagent prepared by dissolvation of 3 mg 5,5'-dithiobis (2-nitrobenzoic acid) in 10 ml of 0.05 M Tris-HCl buffer pH 8.5 was added to a 500 µl aliquot of the resulting solution. Then the samples were incubated for 3 h at room temperature. After centrifugation at 12000 rpm for 15 min, the precipitated polymer was removed, and the supernatant was transferred to a microtitration plate and the absorbance was measured at a wavelength of 415 nm using a microtitration-plate reader. The amount of free thiol groups was calculated from a standard curve obtained by solutions with increasing concentrations of L-Cys.

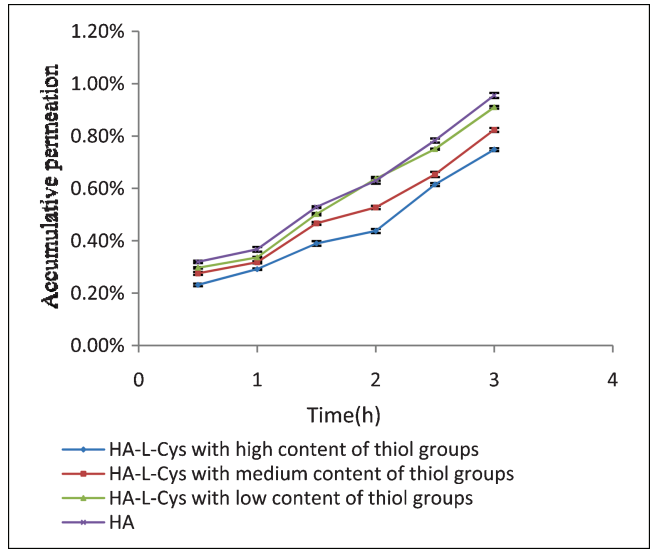


Fig. 5: Permeation studies of sodium fluorescein carried out with 0.5% (m/v) HA-L-Cys conjugates and 0.5% (m/v) HA

3.4. *Characterization of the HA-L-Cys conjugate*

IR spectral data were obtained using Nicolet 20SXB –IR and 1H NMR spectral data were collected using an UNITY INOVA NMR spectrometer at 400 MHz.

3.5. *In vitro evaluation of mucoadhesive properties*

In vitro mucoadhesive studies using self-made equipment was carried out as follows (Xiang et al. 2002; Zhou et al. 2001). Freshly excised small intestine segment from a Wistar rat was glued to a slide and a culture dish respectively. The slide was hanged at the right side of a scale and the culture dish was fixed below the slide. A beaker was put on the left scale pan to keep balance. Unmodified and thiolated HA tablets were incubated on the mucosa of the culture dish in 0.1 M PBS buffer at pH 6.8 for 15 min, followed by the addition of 50 g pressure on the slide for 15 min. Then water was added to the beaker at a speed of 3 drops per minute until the slide and the culture dish were separated. Mucoadhesion was calculated from the equation:

$$F = 9.8\,m$$

Where m is the weight of water in the beaker.

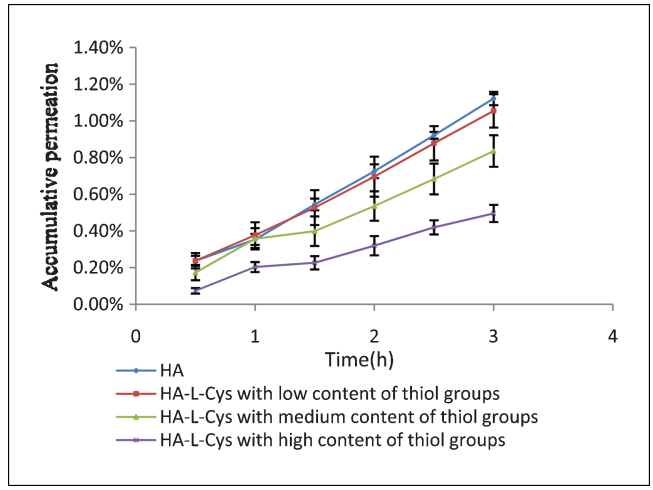


Fig. 6: Permeation studies of insulin carried out with 0.5% (m/v) HA-L-Cys conjugates and 0.5% (m/v) HA

Table 1: Inhibitory effect of HA-L-Cys conjugate toward trypsin

Groups	Ka × 10^{-4}	Inhibitory Rate(%)
Blank	7.26 ± 0.08	—
0.4% HA	7.04 ± 0.11	3.03% ± 1.52%
0.6% HA	6.99 ± 0.06	3.72% ± 0.83%
0.8% HA	7.03 ± 0.12	3.17% ± 1.65%
0.4% HA-L-Cys with low content of thiol groups	6.35 ± 0.07	12.53% ± 0.96%
0.6% HA-L-Cys with low content of thiol groups	4.49 ± 0.07	38.15% ± 0.96%
0.8% HA-L-Cys with low content of thiol groups	2.65 ± 0.10	63.50% ± 1.38%
0.4% HA-L-Cys with medium content of thiol groups	5.75 ± 0.11	20.80% ± 1.52%
0.6% HA-L-Cys with medium content of thiol groups	4.40 ± 0.08	39.39% ± 1.10%
0.8% HA-L-Cys with medium content of thiol groups	2.52 ± 0.06	65.29% ± 0.83%
0.4% HA-L-Cys with high content of thiol groups	4.43 ± 0.07	38.98% ± 0.96%
0.6% HA-L-Cys with high content of thiol groups	2.89 ± 0.03	60.19% ± 0.41%
0.8% HA-L-Cys with high content of thiol groups	1.51 ± 0.11	79.20% ± 1.52%

3.6. Enzyme inhibition studies

To evaluate the inhibition effect of HA-L-Cys conjugate towards chymotrypsin, chymotrypsin was dissolved in 0.2%, 0.4%, 0.6% HA-L-Cys conjugate solution (50 mM Tris-HCl pH 6.8) or HA solution (50 mM Tris-HCl pH 6.8) to get a final concentration of 0.06 mg/ml. Chymotrypsin dissolved in 50 mM Tris-HCl pH6.8 was used as control. After an incubation period of 60 min at 37 °C, aliquots of 0.24 mL were transferred to 3 mL of the substrate solution (10 mg N-benzoyl-L-tyrosine ethylester dissolved in 25 mL of methanol and 25 mL of demineralized water) and the increase in absorbance (ΔA 256 nm) was immediately recorded in 30 s intervals for 210 s at 20 °C. The Ka of enzyme-catalyzed reactions were calculated by plotting ΔA 256 nm versus time and determining the slope of these plots.
To evaluate the inhibition effect of HA-L-Cys conjugates towards trypsin, trypsin was respectively dissolved in 0.2%,0.4% or 0.6% HA-L-Cys conjugate solution (50 mM Tris-HCl pH 6.8) or HA (50 mM Tris-HCl pH 6.8) to get a final concentration of 0.2 mg/ml. Trypsin dissolved in 50 mM Tris-HCl pH 6.8 was used as control. After an incubation period of 60 min at 37 °C, aliquots of 0.4 mL were transferred to 3 mL of the substrate solution (5 mg N-benzoyl-L- arginine ethylester dissolved in 50 mL of 50 mM Tris-HCl pH 6.8) and the increase in absorbance (ΔA 253 nm) was immediately recorded in 30 s intervals for 240 s at 20 °C. The Ka of enzyme-catalyzed reactions was calculated by plotting ΔA 256 nm versus time and determining the slope of these plots.
Inhibitory rate was calculated from the equation:
Inhibitory Rate = $(Ka_{groups} - Ka_{control})/ Ka_{control}$

3.7. In vitro release studies

Cell culture medium was prepared by using DMEM powder 13.4 g/l, 3.7 g/l sodium bicarbonate, penicillin/streptomycin solution (100 U penicillin and 0.1 mg of streptomycin per liter medium) and 10% fetal calf serum.
MDCK cells were maintained in the media described above at 95% humidity and 37 °C in an atmosphere of 5% CO_2. The media were changed daily and cells were split twice a week. Cells were plated directly after splitting in a density of 1×10^5cells onto the membrane inserts of 12-well plates. The cells were allowed to grow and differentiate for 7 days, during this time the media mentioned above were changed every 48 h. Transepithelial electrical resistance (TEER) of the monolayers was measured with the Millicell-ERS. Release studies were carried out in the transwell monolayer system, displaying a volume of 0.75 ml of donor chamber, 1 ml of acceptor chamber and a permeation area of 1.1 cm^2. The pH of the prepared incubation medium Phosphate Buffered Saline (PBS) was 7.2. All experiments were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. After 1 h of preincubation with PBS described above, the media of the donor compartment were substituted by the HA-L-Cys conjugate with high, medium or low content of thiol groups solutions (0.5%, w/v) and control containing 0.5% (w/v) of unmodified HA. Sodium fluorescein and insulin were used as hydrophilic micromolecule and polypeptide model compound in a final concentration of 0.002% (w/v) and 700 μg/ml, respectively (Grabovac et al. 2008). Over 2 h incubation time, aliquots of 200 μl were taken from the acceptor compartment every 20 min, and the volume was substituted by 200 μl PBS at 37 °C.
Sodium fluorescein was determined fluorimetrically at an emission wavelength of 514 nm and excitation wavelength of 490 nm. Insulin was determined spectrophotometrically using Coomassie Brilliant Blue G250. The amount of permeated sodium fluorescein and insulin was calculated from a standard curve obtained before. The cumulative percentage of the model drugs released was calculated.

3.8. Evaluation of the safety of HA-L-Cys conjugates by MTT method

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was dissolved in phosphate buffered solution (PBS) pH7.4 at 5 mg/ml and filtered through a 0.22 μm filter. MDCK cells were maintained in the media described under section 3.7 at 95% humidity and 37 °C in an atmosphere of 5% CO_2. Cells were detached from culture flasks by trypsination and resuspended in culture medium to give a cell number of 3.5×10^4 cells/ml. 200 μl of the cell suspension were dispensed in a 96-well tissue culture

Table 2: Inhibitory effect of HA-L-Cys conjugate toward chymotrypsin

Groups	Ka × 10^{-4}	Inhibitory Rate(%)
Blank	3.47 ± 0.03	—
0.4% HA	3.43 ± 0.06	1.15% ± 1.73%
0.6% HA	3.47 ± 0.06	0.00% ± 1.73%
0.8% HA	2.96 ± 0.05	14.70% ± 1.44%
0.4% HA-L-Cys with low content of thiol groups	3.49 ± 0.04	-0.14% ± 1.15%
0.6% HA-L-Cys with low content of thiol groups	3.42 ± 0.01	1.44% ± 0.29%
0.8% HA-L-Cys with low content of thiol groups	2.81 ± 0.10	19.02% ± 2.88%
0.4% HA-L-Cys with medium content of thiol groups	2.88 ± 0.06	17.00% ± 1.73%
0.6% HA-L-Cys with medium content of thiol groups	2.81 ± 0.04	19.02% ± 1.15%
0.8% HA-L-Cys with medium content of thiol groups	2.35 ± 0.05	32.28% ± 1.44%
0.4% HA-L-Cys with high content of thiol groups	2.59 ± 0.05	14.99% ± 1.44%
0.6% HA-L-Cys with high content of thiol groups	2.16 ± 0.04	37.75% ± 1.15%
0.8% HA-L-Cys with high content of thiol groups	1.89 ± 0.03	45.53% ± 0.86%

Table 3: Effect of HA-L-Cys conjugate and HA on cell vialbility of MDCK cell

Groups	Viability rate(%)
0.05% HA	98.49 ± 6.27
0.1% HA	98.80 ± 8.11
0.2% HA	100.00 ± 4.69
0.05% HA-L-Cys with low content of thiol groups	94.66 ± 6.30
0.1% HA-L-Cys with low content of thiol groups	104.60 ± 5.15
0.2% HA-L-Cys with low content of thiol groups	96.25 ± 7.98
0.05% HA-L-Cys with medium content of thiol groups	106.14 ± 8.81
0.1% HA-L-Cys with medium content of thiol groups	94.90 ± 2.33
0.2% HA-L-Cys with medium content of thiol groups	107.41 ± 5.25
0.05% HA-L-Cys with high content of thiol groups	102.87 ± 8.80
0.1% HA-L-Cys with high content of thiol groups	100.72 ± 8.87
0.2% HA-L-Cys with high content of thiol groups	96.18 ± 7.63

tray. Incubation was carried out at 37 °C in an atmosphere of 5% CO_2 for 36 h. Five wells of the plate remained empty and served as blanks, receiving 200 µl of medium only. After incubation, decant the cell culture medium from the microtiter plates, and 200 µl of HA-L-Cys conjugates with high, medium, low content of thiol groups and unmodified HA were added to the wells, five wells for each group. And 200 µl of PBS was added to 5 wells as control group. All plates were incubated for 4 h. A volume of 20 µl of the MTT solution was then added to each well and plates were incubated for another 4 h. Supernatants were then removed and 150 µl DMSO was added to each well in order to dissolve the dark formazan crystals. The plates were thoroughly mixed for 5 min by using oscillator, and the optical density (OD) of each well was measured spectrophotometrically with a microplate reader at a wavelength of 490 nm. Cell viability was calculated according to the equation:

$$\text{Cellviability} = (OD_{\text{Polymers}} - OD_{\text{Blank}})/(OD_{\text{Control}} - OD_{\text{Blank}}) \times 100\%$$

3.9. Statistical analysis

Statistical data were analyzed using the One-factor Analysis of Variance (One-Way ANOVA). A p-value < 0.05 was considered statistically significant.

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