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A novel microsphere with a three-layer structure for duodenum-specific drug delivery

Xi Zhu, Dan Zhou, Yun Jin, Yu-pin Song, Zhi-rong Zhang, Yuan Huang*

Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education, West China School of Pharmacy, Sichuan University, No. 17, Block 3, Southern Renmin Road, Chengdu 610041, Sichuan, PR China

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ABSTRACT

Owing to the quick elimination of drug from duodenum and the depth of *Helicobacter pylori* (*H. pylori*) colonized in mucus, antibiotic therapy often fails in the eradication of *H. pylori* infection for duodenal ulcer. A novel duodenum-specific microsphere (DSM) consisting of three-layer structure was developed to enhance the drug concentration and retention time in duodenal mucus layer. Firstly a core–shell mucoadhesive microsphere was prepared with a novel emulsification/coagulation coating method by introducing drug loaded Eudragit cores into a thiolated chitosan mucoadhesive layer. Then the obtained core–shell mucoadhesive microspheres were further coated with hydroxypropyl methylcellulose acetate maleate as the pH-sensitive layer for the trigger of mucoadhesion and drug release in duodenum. From the fluorescence microscopic and scanning electron microscopic images, the three-layer structure was successfully established. The microspheres exhibited a duodenum-specific trigger performance, good mucoadhesive property and pH-dependent drug release. *In vivo* study performed in rats demonstrated that DSM exhibited about 3-fold augmentation of AUC and about 5-fold augmentation of C_{max} for duodenal mucus drug concentration compared with free drug suspension. These results suggest that the three-layer structure microspheres may provide a promising approach for duodenum-targeting drug delivery system.

1. Introduction

Peptic ulcer is erosion in the lining of the stomach or the duodenum. More than 95% of patients with duodenal ulcer (DU) and more than 80% of patients with gastric ulcer are infected with *Helicobacter pylori* (*H. pylori*) (Walsh and Peterson, 1995). *H. pylori* infections are more prevalent in developing countries due to poor sanitation, overcrowded living conditions and a lack of clean water supplies (Mahady et al., 2008). Since *H. pylori* colonizes deeply within the gastric and duodenal mucus layer, antibiotic therapy often fails for its eradication due to the lack of sufficient drug concentration and contact time in mucus (Amiji, 2007). Although numerous gastro-retentive dosage forms were designed to prolong the local application of drugs over the last two decades (Shu and Zhu, 2000; Wang et al., 2001; Amiji, 2007), very few studies have been reported on duodenum-retentive systems. Duodenum, as a jointed tube connecting stomach to jejunum, is only 25 cm in length. Drugs taken by oral administration always go quickly through the duodenum and hardly to accumulate in this segment. Besides, a lot of drugs are sensitive to the highly acidic environment in the stomach. To develop a drug delivery system, which can protect the drugs from the strong acidity in stomach and specifically release the drug in duodenum to maintain an effective concentration is very important for the local treatment of duodenal *H. pylori* infection.

Due to the excessive gastric acid secretion of patients with DU, an extremely prolonged phase of migrating motor complex was observed (Kusano et al., 1993). This led to two major features that characterized the pH status of pathological duodenum: the duodenal lumen was nearly constantly acidified while duodenal pH was in a constant and rapid fluctuation (on time scales of several seconds) between very low to near neutral with the average pH of 4.2 at medium bulb segment (Rhodes and Prestwich, 1966; Wormsley, 1974; McCloy et al., 1984; Kusano et al., 1993). Thus a good mucoadhesiveness in a wide range of acidic environment as well as a specific trigger for both mucoadhesion and drug release in duodenum was requisite for a duodenum-specific drug delivery system.

Particulate systems were generally considered to be advantageous for mucoadhesive formulations (Chowdary and Rao, 2004).

Abbreviations: DU, duodenal ulcer; *H. pylori*, *Helicobacter pylori*; HPMCAM, hydroxypropyl methylcellulose acetate maleate; HPMC, hydroxypropyl methylcellulose; TCM, thiolated chitosan coated Eudragit microspheres; CCM, chitosan coated Eudragit microspheres; DSM, duodenum-specific microspheres; SGF, simulated gastric fluid; SPDF, simulated pathological duodenal fluid.

* Corresponding author. Tel.: +86 28 855016171; fax: +86 28 85501617.
E-mail address: huangyuan0@yahoo.com.cn (Y. Huang).

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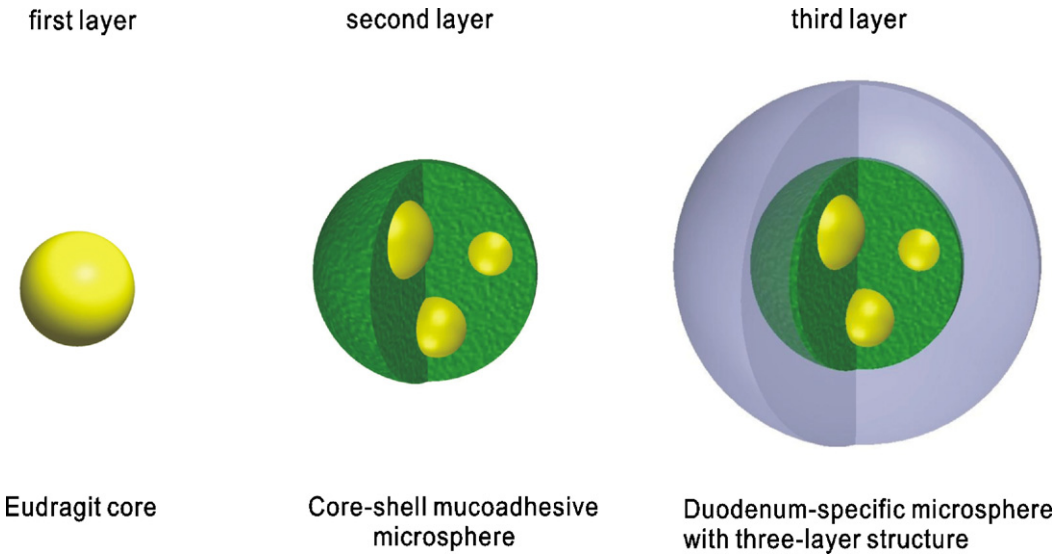

Fig. 1. Scheme of the Eudragit core, the core–shell mucoadhesive microsphere and the duodenum-specific microsphere with three-layer structure.

Chitosan microsphere was one of the most widely studied mucoadhesive particulate systems. As cationic polymer, chitosan possesses extraordinary mucoadhesive potential in acidic environment due to the electrostatic interaction with mucus (Bhattarai et al., 2010). Thiolated chitosan, a chitosan derivative with thiol groups, was reported to lead a 5–100-fold augmentation in mucoadhesiveness compared with unmodified chitosan in perineutral environment (Bernkop-Schnurch, 2005; Sreenivas and Pai, 2008). Therefore, the thiolated chitosan seemed to be suitable mucoadhesive candidate. Nevertheless, in acidic environment, chitosan network was highly permeable to hydrophilic low molecular weight drugs due to its swelling property. A rapid drug release profile was commonly observed for some most commonly used antibiotics, such as tetracycline (Hejazi and Amiji, 2004) and amoxicillin (Giunchedi et al., 1998). Although the chemical cross-linking and reacetylation of chitosan were applied to modify the release performance of drugs, these methods were reported to be remarkably harmful for mucoadhesiveness due to the impairment of chain flexibility and reduction of free surface amino group (Huang et al., 2000; Mi et al., 2000; Wittaya-areekul et al., 2006). Therefore, the inconsistency between the control of drug release and the mucoadhesiveness of chitosan microspheres in acidic environment needed to be resolved.

Since the mean range of gastric pH in fasted state of DU patients were rarely over 2.3, polymer coating agent, with a pH-sensitive value around 2.3–4.2, may be used as a duodenum-specific trigger. However, nearly all of the commercially available enteric coating agents are soluble at pH above 5.0 (Huang et al., 2005). Our group had previously developed a series of hydroxypropyl methylcellulose acetate maleate (HPMCAM) with pH sensitive values (the pH below which the polymer is insoluble) ranging from 3.0 to 3.7. The material also showed good tablet press-coating properties (Huang et al., 2005). But the coating of particulate system was not reported.

Berberine hydrochloride, a hydrophilic low molecule weight drug, is an active principle extracted from the Traditional Chinese Medicine-Rhizoma coptidis (huang-lian). It was widely used in third world countries against gastrointestinal infection for its low price and reported to show good antibacterial activity against *H. pylori in vivo* with few side effects (Wang and Wang, 2003).

The aim of the present study was to establish a novel duodenum-specific microsphere (DSM) with a three-layer structure (Fig. 1). Berberine hydrochloride was encapsulated within a core of Eudragit S100 (inner layer) to control the drug release, then the cores were further surrounded by thiolated chitosan mucoadhesive layer (second layer) with a novel emulsification/coagulation coating method. The obtained core–shell mucoadhesive microspheres were further coated with HPMCAM (pH sensitive value of 3.0) as the pH-sensitive layer (third layer) for the trigger of both mucoadhesion and drug release in duodenum. The mucoadhesiveness as well as *in vitro* release of the microspheres were investigated and *in vivo* study was preformed in rats. To our best knowledge, this is the first report about a particulate duodenum-specific drug delivery system. The introducing of Eudragit S100 cores into chitosan microspheres for the modification of drug release performance is also reported for the first time.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Aokang Biotechnology Ltd. (China) and the deacetylation degree was 92.5% and MW was 600 kDa. Gelatin was obtained from Kermel Co. Ltd. (China). Eudragit S100 was obtained from Huzhou Zhanwang Pharmaceutical Co. Ltd. (China). Berberine hydrochloride was acquired from Xixiao Chinese Medicine Co. Ltd. (China). Mucin from porcine stomach (TYPE III) was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Sodium thioglycolate (TGA-Na), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCL) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). HPMC (E_6) was a gift from Colorcon Co. Ltd. (China). All other reagents were all commercially available and used as received.

2.2. Preparation and evaluation of core–shell mucoadhesive microspheres

2.2.1. Synthesis and characterization of thiolated chitosan

Thiolated chitosan (chitosan–thioglycolic acid conjugates) was synthesized by a method described previously (Hornof et al., 2003). Briefly, chitosan (500 mg) was hydrated in 4 ml of 1 M HCl and dissolved by the addition of demineralized water to obtain a 1% solution of chitosan hydrochloride. To this solution, 500 mg of sodium thioglycolate was added. Thereafter EDC·HCL dissolved in 1 ml demineralized water was added to a final concentration of 125 mM. The reaction mixtures were stirred for 5 h at room

temperature. In order to isolate the thiolated chitosan, the polymer solutions were dialyzed in tubings (molecular weight cut-off 8–14 kDa) for 3 days at 4 °C in the dark against 5 mM HCl, then twice against the same medium but containing 1% NaCl and twice against 1 mM HCl. Samples and controls (prepared in the same way but without EDC·HCL) were lyophilized and stored at 4 °C until further use.

The amount of thiol groups in thiolated chitosan was determined spectrophotometrically with Ellman's reagent as described before (Hornof et al., 2003). Firstly, 0.50 mg of thiolated chitosan was hydrated in 250 ml demineralized water. Then 250 ml phosphate buffer (0.5 M, pH 8.0) and 500 ml Ellman's reagent (3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) in 10 ml of 0.5 M phosphate buffer, pH 8.0) were added. The samples were incubated for 3 h at room temperature and then centrifuged (14,000 rpm, 15 min). The supernatant was measured at a wavelength of 450 nm. Thioglycolic acid standards were used to calculate the amount of thiol groups immobilized on the polymer.

2.2.2. Preparation of Eudragit cores

Eudragit cores were prepared by solvent evaporation technique (Haznedar and Dortunç, 2004). Berberine hydrochloride (300 mg) and aluminum stearate (400 mg) were dispersed into 20 ml of Eudragit S100 acetone solution (2%, w/v, containing 5% water). The resulting dispersion was added to liquid paraffin (saturated with acetone) and stirred at 600 rpm for 5 h at room temperature. After evaporation of acetone, the formed microspheres were collected by vacuum filtration, washed with n-hexane and air dried. Blank Eudragit microspheres were prepared without adding berberine hydrochloride.

2.2.3. Mucoadhesive coating of Eudragit cores

The thiolated chitosan coated Eudragit microspheres (TCM) were prepared by a novel emulsification/coagulation coating method, which was derided from the preparation of chitosan microspheres reported by Shu and Zhu (2000). Briefly, 200 mg of above prepared Eudragit microspheres were dispersed into 10 ml of acetic acid (3%, w/v) solution which containing thiolated chitosan (3%, w/v) and gelatin (1%, w/v). The mixture was emulsified into 80 ml liquid paraffin (containing 0.2%, w/v, Span 80) under mechanical stirring (600 rpm, 30 °C) for 20 min. Then the resultant mixture was cooled with ice-salt bath for coagulation of gelatin. The oil was discarded and the microspheres were dropped into cold sodium tripolyphosphate solution (1%, w/v) under agitation. After ionic cross-linking, the microspheres were separated by filtration, rinsed with distilled water and then n-hexane, subsequently dehydrated with acetone and finally air dried.

Chitosan coated Eudragit microspheres (CCM) were prepared similarly using unmodified chitosan. Blank TCM and CCM were also prepared with blank Eudragit microspheres.

Fluorescent labeled core–shell mucoadhesive microspheres were prepared according to above-mentioned method by incorporation of Nile Red into the Eudragit cores and using FITC-labeled chitosan to replace chitosan.

2.2.4. Surface morphology, fluorescence microscopy and particle size determination

The surface morphology of Eudragit cores and TCM were examined using scanning electron microscopy (SEM, Jeol LTD, Tokyo, Japan) at an accelerating voltage of 20 kV. Fluorescent labeled microspheres were incubated in phosphate buffer solution (pH = 6.8) for 30 min, then viewed under inverted microscope (Axiovert 40 CFL, Carl Zeiss, German). The mean particle size and size range of TCM were measured with a laser diffraction particle size analyzer (MALVEN, MASTERSIZER 2000, British). Anhydrate ethanol was used as dispersion medium.

2.2.5. Mucin absorption study

Surface mucin absorbing abilities of TCM, CCM and Eudragit cores were measured by an absorption experiment (Wang et al., 2001). 5 mg microspheres were suspended in 5 ml of mucin solution (0.5 mg/ml, type III, Sigma Co.) and incubated at 37 °C under shaking (100 rpm) for 2 h. After centrifugation (3600 rpm), the concentration of mucin in the supernatant was determined using Periodic acid/Schiff colorimetry method (Mantle and Allen, 1978). The mucin absorption experiments were performed with blank CCM and TCM as the berberine hydrochloride might interfere with the spectrophotometric determination. All experiments were done in triplicate.

To investigate the absorption ability of TCM and CCM in different acidic pH environment, the medium pH was adjusted to 1.5, 3.0, 4.2 and 5.0. The absorption in three different levels of ionic strength ($I \approx 0.005$, 0.05 and 0.25, adjusted with chloride sodium) and in the presence of 10% (v/v) ethanol in the medium ($I = 0.05$) was tested at each pH level.

2.2.6. In vitro mucoadhesive study on porcine duodenal mucosa

Mucoadhesive properties of TCM and CCM on isolated duodenal mucosa in different acidic pH environment were tested using a modified version of the rotating cylinder method (Grabovac et al., 2005). Freshly slaughtered porcine duodenum was washed with physiological saline. Then the duodenal mucosa was cut into pieces of 4 cm, threaded on a cylinder (diameter: 2 cm) and completely wetted by immersing the mucosa in the testing medium for 1 min. 50 microspheres of the same formulation were counted and scattered uniformly onto the mucosa without application of any force and then the whole mucosa was promptly placed in a chamber containing the testing medium (37 °C). After 20 min of incubation, the cylinder was rotated with 200 rpm for 30 min. The number of microspheres remaining attached to the mucosa was counted and the percentage of retention was calculated. The pH of testing medium was adjusted to 1.5, 3.0, 4.2 or 5.0. Another group of pH 5.0 was performed with 0.2% cysteine (w/v) as the disulphide bond breaking agent. Six parallels within each group were performed.

2.3. Preparation and evaluation of duodenum-specific microspheres

2.3.1. Synthesis of HPMCAM

Hydroxypropyl methylcellulose acetate maleate (HPMCAM) that used as the duodenum-specific pH-sensitive coating agent was synthesized according to our previous study and the pH-sensitive value (the pH below which the polymer is insoluble) was set at 3.0 (weight ratio of HPMC:maleic anhydrides:acetic anhydrides was 1:0.6:0.4) (Huang et al., 2005). Briefly, 5 g of HPMC (E_6) was dissolved in 30 ml of acetic acid at 85–90 °C, and then 2 g of maleic anhydrides, 3 g of acetic anhydrides and 2 g of sodium acetate were added. The reaction was allowed to proceed at 85–90 °C for 5 h, and then 10 ml of purified water was poured into the mixture to stop the reaction. After cooling to room temperature, 3 ml of concentrated hydrochloric acid was added to the mixture, which was then poured into an excess amount of purified water to separate the polymer. The crude polymer was washed with purified water, and then dried in vacuum.

2.3.2. Preparation of duodenum-specific microspheres

200 mg of HPMCAM was dissolved in 5 ml of acetone. After added with 40 mg of TCM and 20 mg of aluminum stearate, the mixture was dropped into 75 ml of liquid paraffin (saturated with

acetone) and stirred (600 rpm) at room temperature. After complete evaporation of acetone, the duodenum-specific microspheres (DSM) were formed. The microspheres were collected, washed with n-hexane and then dried in vacuum.

2.3.3. Surface and cross-section morphology, entrapment efficiency determination and particle size determination

The surface and cross-section morphology of DSM were examined using scanning electron microscopy (as above-mentioned). The drug contents were determined spectrophotometrically. Briefly, 10 mg of microspheres were extracted with 50% acetonitrile. After filtration through a 0.45 μm membrane filter (Xinya, Shanghai), the filtrate was measured at 345 nm using a UV–vis spectrophotometer (Varian Cary 100, California, USA) to determine the amount of drug. Experiments were performed in triplicate and loading efficiencies were calculated using Eq. entrapment efficiency (%) = (mass of drug present in microspheres/theoretical mass of drug) × 100. The particle size and size distribution of DSM were examined by measuring the Green diameters of 200 particles chosen at random using an inverted microscope (as above-mentioned).

2.3.4. Morphological analysis of DSM at different pH

To investigate the behavior of the duodenal-specific coating agent and the morphology of DSM in upper gastrointestinal, a morphological analysis was performed in simulated gastric fluid (SGF, pH 1.2) and simulated pathological duodenal fluid (SPDF, pH 4.2). Phosphate buffer saline of pH 4.2 was used as SPDF as the average pH at medium segment of duodenal bulb was 4.2 for patients with DU (Rhodes and Prestwich, 1966). A certain amount of microspheres was incubated in SGF or SPDF at 37 ± 0.5 °C. At 5, 30, 60 and 120 min, aliquot samples were withdrawn and the microspheres were collected by vacuum filtration, dried in vacuum and observed using scanning electron microscopy (as above-mentioned).

2.3.5. In vitro drug release study of DSM

To investigate the release behavior of DSM, 10 mg of microspheres were incubated in 50 ml of release medium (pH 1.2, 3.0, 4.2 and 5.0) at 37 ± 0.5 °C in a horizontal shaker at 100 rpm. To best simulate the environment of upper gastro-intestinal tract, the release behavior of DSM was also investigated in variable pH conditions, in which the microspheres were first incubated in 50 ml of SGF (pH 1.2) for 2 h, and then collected and transferred into 50 ml of SPDF (pH 4.2). At specific time points, the aliquot sample (1 ml) was withdrawn and replaced with same amount of fresh dissolution medium. The sample was filtered and the concentration of berberine hydrochloride was measured using UV spectrometer at 345 nm.

2.3.6. In vivo study of duodenal mucus drug concentration

Fifty Wistar rats weighing 200–250 g were randomly divided into control and test groups. Animals were fasted for 24 h with free access to water. All experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University and were performed to minimize animal suffering. A berberine hydrochloride dose of 1 mg/kg was intragastically administered in the form of DSM (test) or drug suspension (control). DSM was administered with a self-made intragastric administration device composed of a polyethylene tube and a metal push rod. At 0.5, 1, 2, 4 and 6 h post-administration, the rats were sacrificed by cervical dislocation ($n = 5$). Then, the duodenums were surgically excised. The duodenal mucus were removed carefully with tweezers and weighted, smashed, and then diluted to 1 ml. The mixture was added with 200 μl of internal standard solution (28.8 μg/ml palmatine chloride) and 200 μl of 10 M NaOH. Then the mixture was extracted with diethyl ether. The extraction solvent was evaporated to dryness and then re-dissolved with 200 μl of mobile phase. Drug concentration was determined by an HPLC method with ultraviolet detection at 345 nm. Separation was achieved on a Diamosil C18 column (150 mm × 4.6 mm, 5 μm) with mobile phase of acetonitrile–water (48:52, containing 0.025 mol/l KH_2PO_4, 5 mmol/l sodium lauryl sulfate and the pH was adjusted to 3.0). The flow rate was 1.0 ml/min.

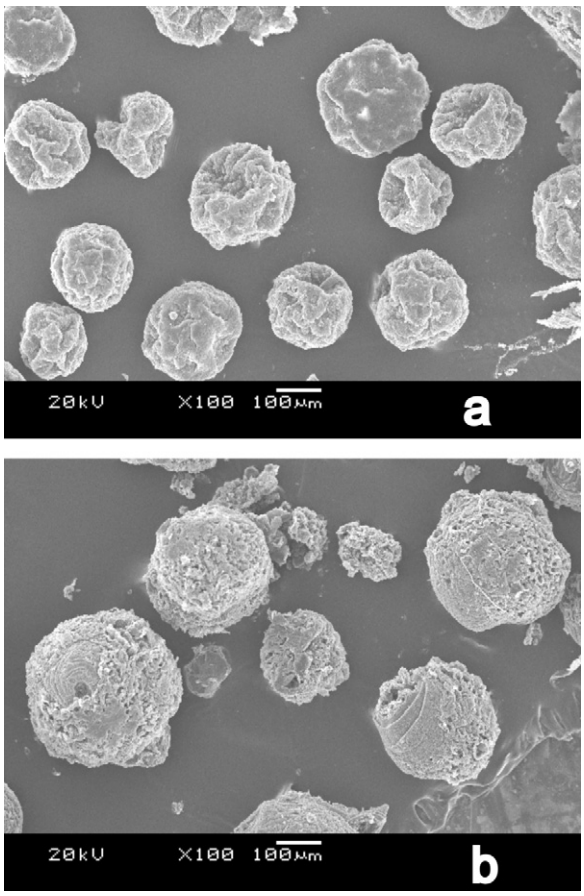

Fig. 2. SEM images of Eudragit cores (a) and TCM (b). The Eudragit cores had an extremely wrinkled surface while exhibited a smoother surface after coated with thiolated chitosan.

3. Results and discussion

3.1. Preparation and evaluation of core–shell mucoadhesive microspheres

3.1.1. Synthesis of the thiolated chitosan

The immobilization of thiol groups on the surface of the cationic polymer chitosan was achieved by the covalent attachment of thioglycolic acid to the primary amino groups of chitosan via an amide bond. The polymers obtained were white, odorless and showed a fibrous structure. The resulting amount of thiol groups immobilized on chitosan was determined to be 52.9 ± 0.3 μmol/g.

3.1.2. Preparation and morphology of core–shell mucoadhesive microspheres

Fig. 2 shows the appearances of Eudragit cores (a) and TCM (b). The former had an extremely wrinkled surface while exhibited a smoother surface with a mean diameter of 313 μm (89.2–554.2 μm) after coated with thiolated chitosan. Fig. 3 presented the fluorescence microscopy images of fluorescently labeled TCM. Nile Red was incorporated in the Eudragit cores (red light) and the FITC labeled chitosan was used as the mucoadhesive layer

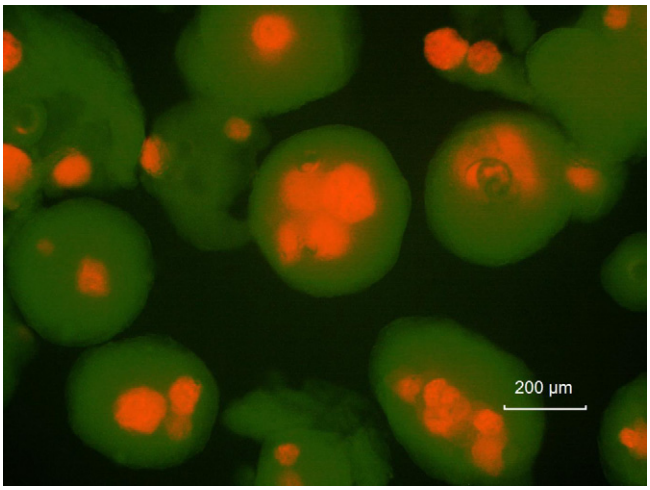

Fig. 3. Fluorescence microscopic images of fluorescently labeled mucoadhesive microspheres. One or several Eudragit cores (yellow-green light) were eccentrically encapsulated within a thick mucoadhesive chitosan layer (green light). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(green light). It can be observed that one or several Eudragit cores were eccentrically encapsulated within a thick mucoadhesive chitosan layer.

The high permeability of non-chemical crosslinked chitosan matrix in acidic environment restricted its utility as a carrier in upper gastrointestinal tract (Bhattarai et al., 2010). In our study, a core–shell structure was introduced to endow the non-chemical crosslinked chitosan microspheres with controlled release property. Eudragit S100, a polymer which is insoluble at pH below 7, was usually used as enteric coating agents. It can also be used as a sustained release material in acidic environment. Our preliminary experiments showed that Eudragit S100 microspheres exhibited satisfactory release profile for hydrophilic small molecular weight drug in acidic environment. The core–shell mucoadhesive microsphere with one or several drug-loading Eudragit cores (control the drug release) and an ionic-crosslinked chitosan/thiolated chitosan shell (maintain mucoadhesive property of microspheres), was reported for the first time.

Usually the chitosan layer in a core–shell structure was coated by a layer-by-layer method, which takes the advantage of electrostatic attraction between chitosan and core material (Gaserod et al., 1998). Although this method was reported to be simple and mild, it was only limited to the coating of negatively charged polymer. Other drawbacks, such as the leakage of incorporated drug in the coating process and thinness of chitosan layer were also unavoidable. In our study, a novel emulsification/coagulation coating method was applied to the chitosan coating of the Eudragit core. By the cooling coagulation of gelatin, emulsion droplets could be directly shaped into gelatinous microspheres. Meanwhile, Eudragit cores were immobilized and fixed into the chitosan and gelatin network. Since this method of core encapsulation does not involve electrostatic attraction between core material and chitosan, it probably can also be used in the chitosan coating of microspheres core composed of other polymer.

3.1.3. Mucoadhesion study

The core–shell mucoadhesive microsphere (TCM or CCM) was the existing form of the duodenum-specific microspheres (DSM) in duodenum after the dissolution of pH-sensitive outer layer. Thus the duodenal retention property of DSM was determined by the mucoadhesive properties of TCM and CCM. Duodenum, as a jointed tube connecting stomach to jejunum, is only 25 cm in length (Gray and Lewis, 1918). Drugs taken by oral administration always go quickly through the duodenum and are hardly to accumulate in this segment. Thus a particulate system targeting to the duodenum should possess strong mucoadhesive ability. Since the duodenal mucosa in patient with DU was nearly constantly acidified and the duodenal pH was in a constant and rapid fluctuation (on time scales of several seconds) between very low to near neutral (Wormsley, 1974; McCloy et al., 1984; Kusano et al., 1993), the particulates system should have good mucoadhesive property in a wide range of acidic environment. However, no thorough study about the mucoadhesiveness of thiolated chitosan in a series of acidic environment has been reported and the effect of different forces on the interaction between thiolated chitosan and mucin remained to be elucidated.

It is widely recognized that specific interactions between polymers and mucin play an important role on mucosal adhesion at the molecular level (Sogias et al., 2008). The mucin can be absorbed onto the surface of microspheres by various interactions while the amount of mucin absorbed can reflect the mucoadhesive potential. The absorption experiments were performed at three levels of ionic strength ($I \approx 0.005$, 0.05 and 0.25) adjusted by adding NaCl which was reported to disrupt interaction between oppositely charged polyelectrolytes at a high concentration (Khutoryanskiy et al., 2003). Lower molecular weight alcohols, such as ethanol, were known to disrupt hydrophobic effects and hydrogen bond through competition between biopolymer-biopolymer interactions (Nurkeeva and Mun, 2002). 10% (v/v) of ethanol was added in the medium ($I = 0.05$) to investigate the effects of hydrogen bond and hydrophobic effects on absorption.

The results were shown in Fig. 4. As compared to Eudragit cores, significantly larger amounts of mucin were adsorbed on both CCM and TCM at all tested pH ($p < 0.05$). With the increase of the ionic strength, the amounts of absorption were decreased for both CCM and TCM at pH 3.0, 4.2 and 5.0. However, the trend was opposite at pH 1.5 (testing medium with ionic strength of 0.005 could not be obtained). At pH 3.0 and 4.2, no significant difference was found between CCM and TCM at every ionic strength ($p > 0.05$). At pH 5.0, however, TCM exhibited better absorption abilities than CCM at ionic strength of 0.25 and the amount of mucin absorbed by TCM was 1.4-fold higher than that of CCM ($p \leq 0.05$). It also should be noted that TCM exhibited higher absorption abilities than CCM at each ionic strength at pH 1.5 ($p < 0.05$). When added with ethanol, the amounts of absorption of TCM and CCM were reduced at every tested pH compared with those without addition of ethanol at the same ionic strength ($I = 0.05$), especially at pH 1.5.

The results demonstrated both chitosan-coating and thiolated chitosan-coating can greatly enhance the mucoadhesiveness of Eudragit cores. The mucoadhesive interaction was reported to be a complex with contributions from electrostatic attraction, hydrogen bonding and hydrophobic effects, and the relative contributions of each interaction could be changed by environmental pH (Sogias et al., 2008). With the introduction of thiol groups, the disulfide bond that formed between the thiol groups of thiolated chitosan and the cysteine residuals of mucin may also make some contributions. With the increase of ionic strength, the electrostatic interaction could be partially "screen-off". The positive correlation between the ionic strength and the amount of mucin absorbed at pH 1.5 and the contrary tendency at pH 3.0–5.0 demonstrated that the electrostatic interaction might play a negative role on the absorption at pH 1.5 while showed positive effect among pH 3.0–5.0. The values of pK_a for sialic acid is 2.6 and pI for mucin is about 3.0–5.0 (Ping et al., 1998). Thus, mucin would be positively charged at pH 1.5, and therefore electrostatic repulsion may exist between mucin and chitosan/thiolated chitosan. However, the amount of mucin absorbed was not drastically reduced for both CCM and TCM at pH 1.5 with the reverse of the electrostatic interaction. This

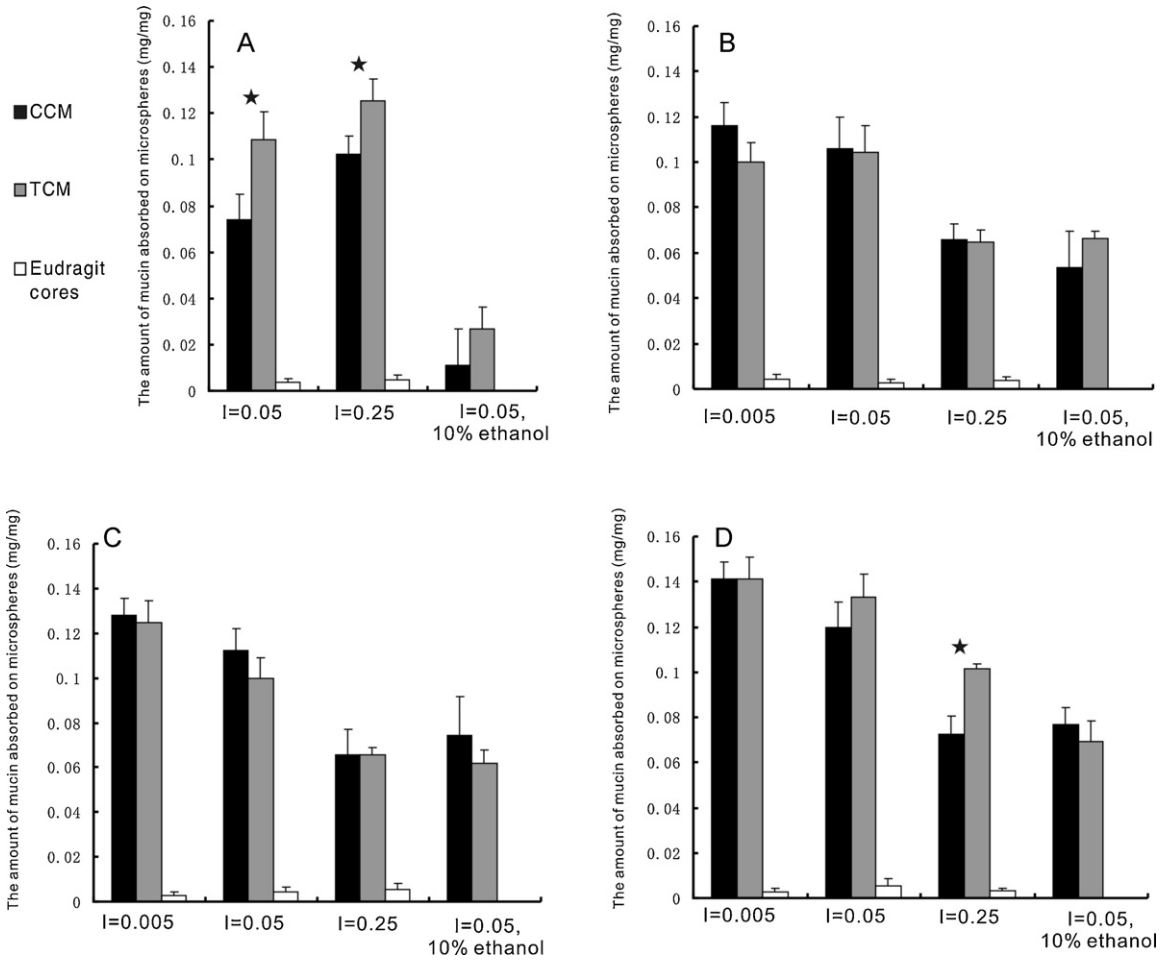

Fig. 4. The amount of mucin adsorbed to per weight of microspheres at pH 1.5 (A), 3.0 (B), 4.2 (C), 5.0 (D) with ionic strength of 0.005, 0.05 (with or without 10% ethanol) and 0.25 ($n = 3$). The group within which CCM and TCM showed significance difference is indicated by asterisks ($p < 0.05$).

may be because the loss of electrostatic attraction can be compensated by the increase of other forces. At pH 5.0, the amount of mucin absorbed by TCM was less affected with the increase of ionic strength compared to CCM and the amount of absorption was 1.4-fold than that of CCM at ionic strength of 0.25. This could be the evidence that the disulfide bonds formed and contributed to the absorption. Thiolated-anions were the reactive form for thiol groups, and its concentration was low in pH below 5 (Bernkop-Schnurch, 2005). Therefore, at pH 3.0 and 4.2, the formation of disulfide bonds would contribute little to the mucin absorption. Since the intensity of disulfide bond was much higher than that of non-covalent bond (Bernkop-Schnurch, 2005), its formation would be helpful for mucoadhesive behavior of TCM. The reason of the higher mucoadhesive potentials of TCM than CCM at pH 1.5 was not clear.

The great reduction of the amounts of mucin absorbed with the addition ethanol at pH 1.5 indicated that the hydrogen bonding and hydrophobic effects might be the main driving forces for the absorption at this pH. These two forces may also take some part in the absorption at other pH.

Mucoadhesive properties of TCM and CCM on isolated porcine duodenal mucosa were tested using a rotating cylinder method. The results were shown in Fig. 5. CCM and TCM bore a resemblance in their mucoadhesive properties at pH 1.5 and pH 3.0. At pH 4.2, TCM showed a better mucoadhesive property than CCM but the difference was not significant ($p > 0.05$). Significant difference was observed at pH 5.0 ($p < 0.05$) and the percentage of TCM remaining on mucosa was 1.6-fold higher than that of CCM. When the testing medium was added with 0.2% (w/v) of cysteine, the mucoadhesive properties of TCM was significantly reduced ($p < 0.05$) and no significant difference was observed between TCM and CCM ($p > 0.05$). As unbound cysteine acts as a disulphide bond breaking agent, it may cleave disulphide bonds formed between thiolated polymer and mucin (Leitner et al., 2003). Accordingly, the decrease in the mucoadhesive property of TCM further proved the formation of disulphide bonds between thiolated chitosan and the mucus layer at pH 5.0.

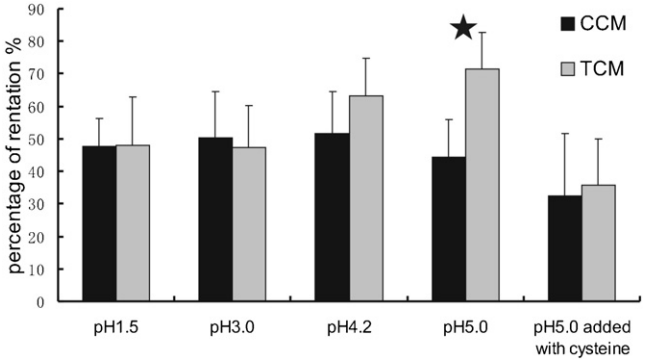

Fig. 5. *In vitro* mucoadhesion study of in different acidic pH environment ($n = 6$). Significance between CCM and TCM in the same group is indicated by asterisks ($p < 0.05$).

Thiolated chitosan was often reported to lead to 5–100-fold improvement for mucoadhesiveness compared to unmodified chitosan when evaluated in the forms of tablets in neutral environment (Sreenivas and Pai, 2008; Bernkop-Schnurch, 2005). In our study, however, the mucoadhesiveness of TCM was only 1.6-fold higher than that of CCM at pH 5.0. The relative low effectiveness of thiolation on the improvement of mucoadhesiveness may be the results of the variation of formulations rather than the difference of pH. Our results were similar with two other studies in which the mucoadhesiveness of particulate system based on thiolated polymers was 2-fold or 1.4-fold higher than that based on unmodified polymer (Bernkop-Schnurch et al., 2006; Quan et al., 2008). Particulate systems, such as microspheres were regarded to possess better mucoadhesive properties due to their small size which enabled them to make intimate contact with a larger mucosal surface area (Chowdary and Rao, 2004). Thus chitosan microsphere itself could provide good adhesion, and therefore the effect of thiolation was less significant and mucus exfoliation would probably contribute more to the removal of the microspheres.

The results of *in vitro* mucoadhesive study demonstrated that TCM exhibited similar mucoadhesive property with CCM at pH 1.5, 3.0 and 4.2, while exhibited better mucoadhesive property at pH 5.0 due to the formation of disulfide bond. This result was in accordance with the result observed in mucin absorption study. The mucoadhesive properties of TCM would be helpful for its retention in the pathological duodenum in which the pH was in a constant and rapid fluctuation between very low to near neutral. Thus the TCM was further used for the preparation of duodenum-specific microspheres.

3.2. Evaluation of duodenum-specific microspheres

3.2.1. Characterization of DSM

Fig. 6 shows the appearance (a) and section morphology (b) of DSM (HPMCAM coated TCM). The microspheres had smooth surfaces and spherical shape with a mean diameter of 593.3 μm (213.4–925.6 μm). The HPMCAM coating was integrative, continuous and free from large pore or crack from appearance. A three-layer structure can be seen from the section morphology. The TCM was encased asymmetrically in a thick layer of HPMCAM. One Eudragit core that located in the TCM was observed. DSM had an encapsulation efficiency of 86.1 ± 3.6%.

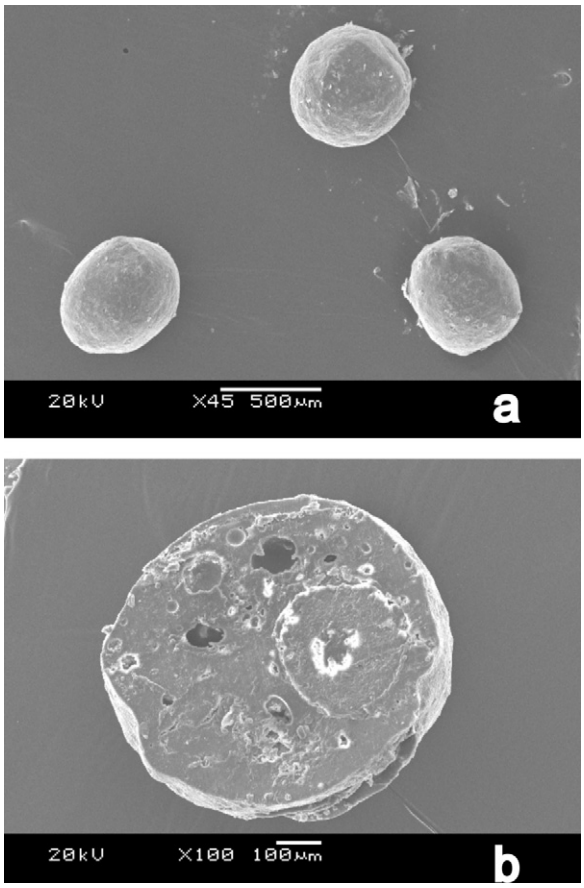

Fig. 6. SEM images of the appearance (a) and section morphology (b) of DSM.

3.2.2. Morphological analysis of DSM at different pH

Our group had previously reported the synthesis of a series of HPMCAM with pH sensitive values ranging from 3.0 to 3.7 by adjusting the ratios of HPMC/Maleic anhydrides/acetic anhydrides (Huang et al., 2005). In the present study, HPMCAM with pH-sensitive value of 3.0 was synthesized and further used as the duodenum-specific coating agent. To investigate the morphological status of the DSM at different pH, the microspheres were incubated in simulated gastric fluid (SGF, pH = 1.2) and simulated pathological duodenal fluid (SPDF, pH = 4.2) for 2 h. Since the duodenal mucosa was nearly constant acidified with the average pH of 4.2 at medium bulb segment for patients with DU (Rhodes and Prestwich, 1966), we chose 4.2 as the pH of SPDF. The morphologies of the microspheres after different times of incubation were shown in Fig. 7. The HPMCAM coating could maintain their integrity after 2 h of incubation in SGF. Although after 60 min of incubation some wrinkles were shown and the microspheres were no longer very spherical, no noticeable pores and cracks were found. After 5 min of incubation in SPDF, the bulk of HPMCAM coating was corroded and the TCM was revealed. The surface of TCM became smoother after 2 h of incubation in SPDF and that may be the result of swelling.

A successful enteric coating agent must maintain its integrity in the low pH of stomach, while dissolved quickly at the targeted site. As the time required to empty half of the stomach content was 64.2 min for patients with DU (Supe et al., 1986), the HPMCAM coating which could maintain its integrity for more than 2 h in SGF would successfully protect the encapsulated particle from the gastric acid before gastric emptying. The rapid dissolution of the pH-sensitive coating could enable a quick triggering of both mucoadhesion and drug release at the duodenum. It is highly possible that the HPMCAM coating will be corroded more quickly *in vivo*, owing to the mechanical vermiculation of duodenum.

3.2.3. In vitro release study

The release behaviors of DSM were investigated in different pH and variable pH condition. As shown in Fig. 8, DSM exhibited a pH-dependent drug release profile. The release rate was relatively low in SGF and approximately 10% of the drugs were released within 2 h, while approximately 50% and more than 80% of the drugs were released in 2 and 6 h in SPDF, respectively. In variable pH condition, the release was relatively suppressed during the first 2 h in SGF, while a higher release rate was observed when the microspheres were transferred into SPDF. Though the DSM exhibited a faster release rate as the pH changing from 3.0 to 5.0, most of the drug was released within 6 h in a controlled manner. Since the average turnover time of mucus was 6 h (Marriot and Hughes, 1990), we consider that it was helpful with most of the drug released within 6 h in gradually for a mucoadhesive drug delivery system. Thus the release rates of DSM in SPDF were satisfactory. The results demonstrated that the three-layer structure could effectively inhibit the drug release in gastric environment and reasonable controlled the drug release in pathological duodenal environment.

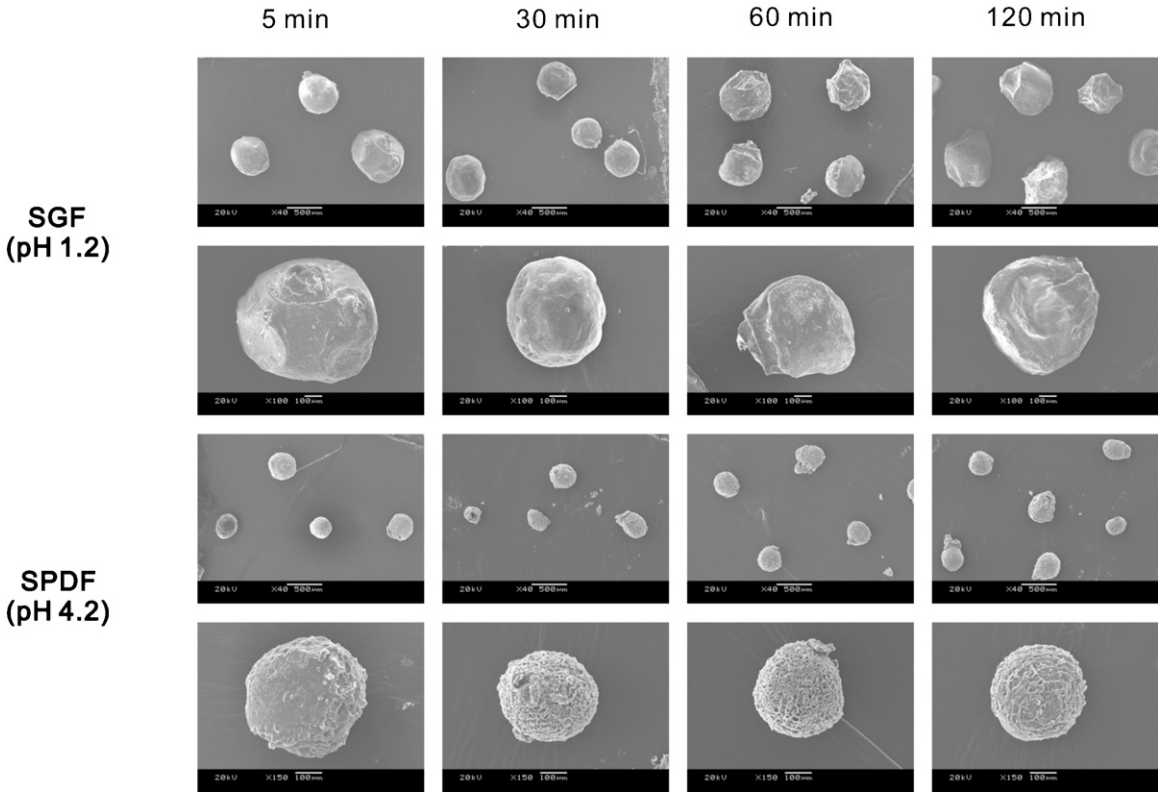

Fig. 7. The morphologies of DSM after various times of incubation in SGF or SPDF.

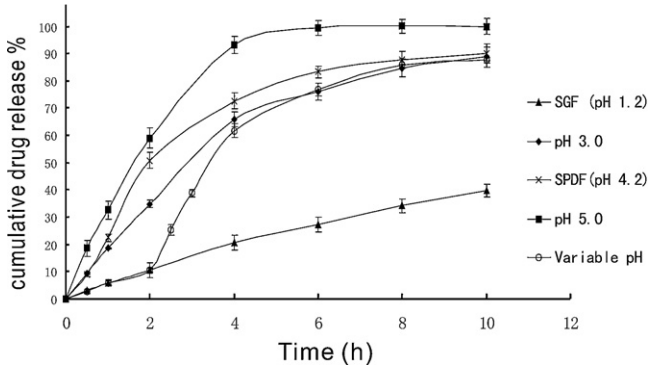

Fig. 8. *In vitro* release profiles of DSM in different pH conditions.

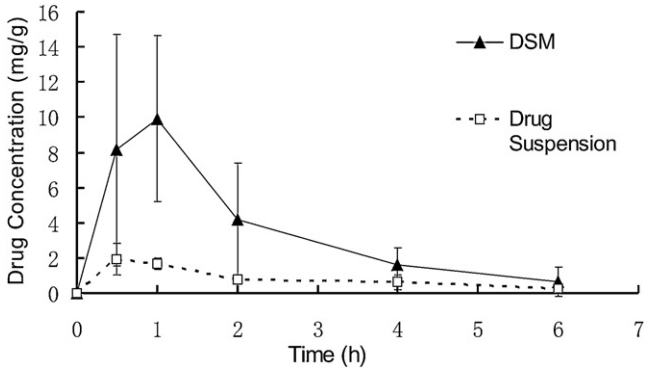

Fig. 9. Berberine hydrochloride concentrations in the rats duodenal mucus layer represented by mg of drug per gram of mucus after administration of DSM (▲) or drug suspension (□) ($n = 5$).

3.2.4. *In vivo study of duodenal mucus drug concentration*

To evaluate the effectiveness of the DSM for the augment of duodenal mucus drug concentration, an *in vivo* study was performed on rats. Concentration of berberine hydrochloride in duodenal mucus was measured after the administration of DSM or free drug suspension. Drug concentrations in the duodenal mucus, as mg per gram of mucus, were shown in Fig. 9. Table 1 shows the $AUC_{(0-\infty)}$, CL, C_{max} and T_{max} values for the drug suspension as well as the DSM.

As shown in Fig. 9, the drug concentration was greatly improved by the DSM in every tested time. After the administration of DSM, the drug concentration could be immediately boosted to a high level. The $AUC_{(0-\infty)}$ and C_{max} for DSM were 3.36 and 5.09 times higher than that for drug suspension ($p < 0.05$), respectively. This indicated the effectiveness of DSM for the enhancement of both drug concentration and the total amounts drug accumulated in duodenal mucus. Meanwhile, DSM has a decreased CL when compared to free drug suspension, indicating that DSM could enhance the retention of the drug in duodenal mucus.

The increase of the $AUC_{(0-\infty)}$ and C_{max} for DSM could be the combined result of the duodenum-specific coating of HPMCAM and mucoadhesive property of the incorporated TCM. Since the drugs

Table 1
AUC, CL, C_{max} and T_{max} values of drug in duodenal mucus after the administration of the drug suspension or DSM.

Formulation	AUC, mg/(g h) of mucus	CL, g/(h kg)	C_{max}, mg/g	T_{max}, h
Drug suspension	7.765 ± 3.162	2.926 ± 1.100	1.945 ± 0.892	0.700 ± 0.274
HPMCAM coated TEM	26.127 ± 14.374	0.906 ± 0.339	9.906 ± 4.724	0.800 ± 0.274

taken by oral administration always go quickly through the duodenum, a site-specific mucoadhesive particulate system could greatly enhance the drug concentration. The augments in both drug concentration and contact time would be helpful for the eradication of *H. pylori*, considering the topical action of drug on the mucus played an important role in its clearance. However, although a noticeable augmentation of drug concentration in duodenal mucosa was observed, there was a variance between the *in vivo* study and the *in vitro* release study. This variance indicated that a great proportion of the particles might be eliminated out of the duodenal section after 1 or 2 h. For this reason, there may be still a potential for a better duodenal concentration augmentation effect by upgrading the duodenal retention properties of the particles. Besides, the duodenal specific drug delivery system would be submitted to different pathological models in future.

4. Conclusion

A novel three-layer duodenum-specific microsphere was developed with one or several drug loaded Eudragit cores, a thiolated chitosan mucoadhesive layer and a pH-sensitive duodenum-specific trigger layer. The mucoadhesive layer showed good mucoadhesive potential from strong to weak acidic environment. The duodenum-specific coating can maintain its integrity in stomach and endowed the microspheres with a duodenum-specific drug delivery behavior. *In vivo* experiments demonstrated that the AUC and C_{max} of the drug in rat duodenal mucus were 3.36 and 5.09 times augmented for DSM compared with drug suspension, respectively. These results suggested that the three-layer structure microspheres might provide a promising approach for duodenum-targeting drug delivery and would be loaded with other antibiotics for further evaluation.

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