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RESEARCH ARTICLE

pH-Sensitive and mucoadhesive microspheres for duodenum-specific drug delivery system

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Abstract

Particulate systems that could deliver drug specifically to duodenum have not yet been reported. The aim of this study was to develop a novel duodenum-specific drug delivery system based on thiolated chitosan and hydroxypropyl methylcellulose acetate maleate (HPMCAM) for the duodenal ulcer application. Berberine hydrochloride was used as model drug. Thiolated chitosan was synthesized and further used for the preparation of mucoadhesive microspheres. HPMCAM, which is insoluble below pH 3.0 was synthesized and used for the coating of thiolated chitosan microspheres (TCM). The resulting thiolated chitosan immobilized on chitosan was 268.21 ± 18 μmol/g. *In vitro* mucoadhesion study showed that the mucoadhesion property of TCM was better than that of chitosan microspheres. Morphological observation showed that the HPMCAM coating would maintain its integrity in simulated gastric fluid (SGF) for 2 h and dissolved quickly in simulated pathological duodenal fluid (SPDF; pH 3.3). *In vitro* drug release studies showed that only 4.75% of the drug was released in SGF for 2 h, while nearly 90% of the drug was released within 6 h after transferring into SPDF.

Keywords: Duodenum-specific; thiolated chitosan; mucoadhesion; pH-sensitive; enteric coating

Introduction

Duodenal ulcer is a multifactorial disease, with a lifetime prevalence of approximately 10%[1]. Numerous studies have demonstrated that *Helicobacter pyloyi* infection is strongly associated with duodenal ulcer[2]. Although *H. pylori* is very sensitive to many antibiotics *in vitro*, single antibiotic therapy fails to eradicate *H. pylori* infection and only approximately 75% eradication efficiency on average has been observed with triple therapy protocols[3]. The main reason is that sufficient antibiotic concentration and enough contact time in mucus are hard to achieve[3]. Besides, traditional high-dose oral administration of antibiotics also presents high risk for the development of microbial resistance and has profound influence on the entire microbial flora of the gastrointestinal (GI) tract[4,5]. Delivery of antibiotics locally in the duodenum and prolonging the residence time can be a good strategy to improve the efficacy, which can allow more of the antibiotics to diffuse through the duodenal mucus layer. However, to our knowledge, there were few reports about duodenum-specific drug delivery system. Furthermore, with only 25 cm in length, drugs taken by oral administration always go quickly through the duodenum. Thus, a drug delivery system which has duodenum-specific mucoadhesive properties is needed in the treatment of DU.

Enteric coating is an effective way to protect the drug against gastric acid and prevent the release of the encapsulated particles or the drug before reaching the site of action. Because of the abnormally prolonged period of secretion, the more rapid delivery of acid to the duodenum and the decrease in buffering capacity, lower mean pH levels and extremely increased percentage of time of acidity to pH < 4.0 were observed in patients with duodenal ulcer[6]. The average pH at the base of duodenal bulb was 3.3[7]. However, commercially available enteric coating agents are commonly soluble above the pH ranging from 5.0 to 7.0, such as cellulose acetate phthalate (CAP, pH 6.0 or higher), Eudragit L (pH 6.0 or higher) and

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Eudragit S (pH 7.0 or higher). Thus, the enteric pharmaceutical preparations based on these coating agents pass through at least the first portion of the duodenum prior to the complete release of the encapsulated particles. Our group had previously developed a novel duodenum-specific coating agent hydroxypropyl methylcellulose acetate maleate (HPMCAM) which can be dissolved at pH above 3.0–3.7, and successfully applied it to the "press-coated tablets"[8]. *In vitro* results demonstrated that the drug release of "press-coated tablets" was completely suppressed in simulated gastric fluid (SGF; pH 1.2) for 2 h while the tablets showed a rapid release profile in pH 3.4. However, due to the large volume and the relative small surface area in contact with mucosa, tablets had many drawbacks as GI site-specific preparations. Besides, the large mass of the dosage form as well as the vigorous movement of the GI tract will result in a large variation of GI distribution[9–11].

Particulate systems were generally considered advantageous for mucoadhesive formulations as the small size enabled them to make intimate contact with a larger mucosal surface area[12]. Chitosan microspheres are one of the mostly studied mucoadhesive particulate systems[13–15]. The biopolymer chitosan is obtained by alkaline deacetylation of chitin, which is one of the most abundant polysaccharides in nature[16]. With good mucoadhesive properties, chitosan has been utilized widely as mucoadhesive material for oral, nasal, ocular, and buccal drug delivery[17–19]. Thiolated chitosan is a derivative of chitosan with thiol groups which is usually attached to the primary amino groups of chitosan. Due to the formation of disulfide bonds with mucus glycoproteins, the mucoadhesiveness is 6–100-fold augmented in thiolated chitosan compared with unmodified chitosan[16].

Berberine chloride (BER.HCL) is an active principle extracted from the Traditional Chinese Medicine-*Rhizoma coptidis* (huang-lian). Clinical experiments have shown that it has good antibacterial activity against *H. pylori in viv*o with fewer side effects than some commonly used antibiotics[20].

The aim of our study is to establish a novel duodenum-specific drug release system. Thiolated chitosan was synthesized by covalently attaching thioglycolic acid to the primary amino groups of chitosan *via* an amide bond. Then mucoadhesive microspheres were prepared with berberine chloride as the model drug and then coated with HPMCAM, which can be dissolved at pH above 3.0. The mucoadhesive properties of thiolated chitosan microspheres (TCM), morphology of HPMCAM-coated TCM in different pH medium and *in vitro* release were investigated.

Materials and methods

Materials

Chitosan was obtained from Aokang Biotechnology Ltd. (Fujian, China), and the deacetylation degree was 93.1% and MW was 400 KD. Berberine chloride was obtained from Xixiao Chinese Medicine Co. Ltd. (Sichuan, China). Sodium thioglycolate (TGA-Na), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich Co. (St. Louis, MO). HPMC (E6) was a gift from Colorcon Co. Ltd. (Shanghai, China). All other reagents were all commercially available and used as received.

Synthesis of thiolated chiotosan

Thiolated chitosan (chitosan-thioglycolic acid conjugates) was synthesized by a method described previously[21]. Briefly, 500 mg of chitosan 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. 500 mg Of sodium thioglycolate was added to this solution. Thereafter EDAC dissolved in 1 mL demineralized water was added to a final concentration of 125 mM. The reaction mixtures were incubated for 5 h at room temperature under constant stirring. In order to isolate the thiolated chitosan, the polymer solutions were dialyzed in tubing (molecular weight cut-off 8–15 kDa) for 3 days at 4°C in the dark in 5 mM HCl, then twice against the same medium but containing 1% NaCl. Then the samples were dialyzed twice against 1 mM HCl to adjust to pH 4. Thereafter samples and controls (prepared in the same way but omitting EDAC) were lyophilized and stored at 4°C until further use.

Characterization of thiolated chitosan

Fourier transform infrared (FT-IR) spectra of chitosan and thiolated chitosan were recorded on Bruker Vector 22 FT-IR Spectrometer using KBr method (Billerica, MA). The new amide bond formation and thiol group substitution in thiolated chitosan can be confirmed by FT-IR, based on the presence of the characteristic peaks of newly formed amide bond and thiol groups.

The amount of thiol groups in thiolated chitosan was determined spectrophotometrically with Ellman's reagent as previously described[21]. First, 0.50 mg of thiolated chitosan was hydrated in 250 mL demineralized water. Then 250 mL phosphate buffer saline (PBS; 0.5 M, pH 8.0) and 500 mL Ellman's reagent (3 mg of DTNB) in 10 mL of 0.5 M PBS, pH 8.0) were added. The samples were incubated for 3 h at room temperature. The supernatant was separated from the precipitated polymer by centrifugation (14,000 rpm, 15 min). Thereafter 300 μL of the supernatant were transferred to a microtitration plate and the absorbance was measured at a wavelength of 450 nm with a microtitration plate reader. Thioglycolic acid standards were used to calculate the amount of thiol groups in thiolated chitosan.

Preparation of TCM

The TCM were prepared using the emulsification/chemical cross-linking technique. 240 mg Of thiolated chitosan was dissolved in 12 mL of 1% acetic acid solution (w/v). 60 mg Of berberine hydrochloride was dispersed in acidic

polymer solution. Then the resulted mixture was added into 50 mL liquid paraffin containing 1% Span 80 (w/v) under agitation (500 rpm, 40°C). After 30 min of stirring, formaldehyde saturated toluene was added to the formed W/O emulsion and the mixture was stirred for another 4 h. Then glutaraldehyde saturated toluene was added to further solidify the microspheres for another 20 h. The resulting microspheres were collected, washed several times with *n*-hexane, then dehydrated with acetone, and finally dried under vacuum at room temperature for 24 h. Five different batches were prepared with different amounts of formaldehyde and glutaraldehyde (Table 1).

Unmodified chitosan microspheres (UCM) were also prepared in the same way using unmodified chitosan.

In vitro release of TCM

To investigate the drug release behavior of microspheres with different amounts of cross-linking agent in simulated pathological duodenum fluid (SPDF, pH 3.3), 5 mg of microspheres were kept in 50 mL PBS (pH 3.3) and shaken at 100 rpm at 37 ± 0.5°C. At 0.5, 1, 2, 4, 6, 8, 12 h, 0.5 mL of the release mediums were withdrawn and replaced with equal volume of fresh SPDF. The amount of drug was detected using a ultraviolet (UV) spectrometer at 345 nm. Each experiment was repeated three times and the mean value was reported.

In vitro evaluation of mucoadhesiveness of TCM and UCM

The mucoadhesive properties of microspheres were evaluated by the method designed by Ranga Rao and Buri[22] with modifications. The fresh porcine duodenal mucosa was obtained from a local slaughter house and was washed with physiological saline. 20 mg Of TCM (batch B) or UCM were scattered uniformly on the surface of the duodenal mucosa and wetted by spraying with PBS (pH 3.3). Then, the duodenal mucosa with microspheres was placed in a chamber maintained at 93% relative humidity at room temperature. After 30 min, the tissues were taken out and fixed on a plate at a 45 degree angle. The duodenal mucosa was rinsed with PBS (pH 3.3) at a rate of 9 mL/min. The microspheres eliminated in 5 min, 10 min, and 30 min were collected, dried and weighed. Percentage of retention at time *t* [PR(*t*)] was calculated from the Equation 1:

$$PR(t) = \frac{T - W(t)}{T} \quad (1)$$

Table 1. The amounts of formaldehyde and glutaraldehyde added in different batches.

Batch	Formaldehyde saturated toluene (mL)	Glutaraldehyde saturated toluene (mL)
A	4	0.5
B	4	1
C	4	2
D	2	1
E	6	1

where T is the total weight of microspheres scattered and W(t) is the accumulated weight of eliminated microspheres at time *t*. The statistical significance of the differences between two groups of was analyzed using the two-tailed *t*-test and a *P* value of less than 0.01 was termed significant.

Synthesis of HPMCAM used as pH-sensitive coating agent

HPMCAM was synthesized according to our previous study and the pH-sensitive value was set at 3.0 (insoluble below pH 3.0, weight ratio of HPMC:maleic anhydrides:acetic anhydrides was 1:0.6:0.4)[8]. 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 used as a catalyst were added. The reaction was allowed to proceed at 85–90°C for 5 h, and then 10 mL of purified water were 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, and then filtered. The crude polymer was washed with purified water, and then dried in vacuum.

Preparation of HPMCAM-coated TCM

200 mg Of HPMCAM was dissolved in 5 mL of ethanol and acetone (1:4). After addition of 40 mg of TCM and 20 mg of aluminium stearates, the mixture was dropped into 20 mL of liquid paraffin and stirred at 600 rpm at room temperature. After complete evaporation of acetone and ethanol, the microspheres formed were collected, washed with *n*-hexane and dried under vacuum at room temperature for 24 h.

Characterization of microspheres

The surface morphology of TCM and HPMCAM-coated TCM were examined using scanning electron microscopy (SEM; Jeol Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV. The particle size of TCM and HPMCAM-coated TCM was measured using SEM photomicrographs ($n = 100$).

The drug content of HPMCAM-coated TCM was measured using high-performance liquid chromatography (HPLC). Briefly, 5 mg of microspheres were added to 2 mL of acetone to dissolve the coating and then the microspheres were washed three times with 5 mL of 1% nitric acid at 80°C. The supernatant was filtered through a 0.45 μm membrane and then measured by HPLC at 273 nm.

Morphological analysis of HPMCAM-coated TCM in different pH media

To investigate the morphological status of HPMCAM-coated TCM in the pathological gastroduodenal tract, 10 mg of microspheres were first placed in 5 mL of SGF (pH 1.2) and incubated by horizontal shaking at 100 rpm at 37 ± 0.5°C. After 2 h the microspheres were collected by filtration, then moved into 5 mL of simulated pathological

duodenal fluid (SPDF; pH 3.3) and continued shaking. Then at 5, 10, and 120 min, 1 mL of the medium were withdrawn and the microspheres were collected by filtration, dried in vacuum, and observed by SEM.

In vitro drug release study

5 mg Of HPMCAM-coated microspheres were incubated in 50 mL SGF (pH 1.2) at 37 ± 0.5°C in a horizontal shaker at 100 rpm during the first 2 h, and then the microspheres were collected by vacuum filtration and transferred into SPDF (pH 3.3). At 2.5, 3, 4, 6, 8, 10, 12 h, the 0.5 mL samples were withdrawn and replaced with same amounts of fresh dissolution medium. The samples were filtered and the concentration of berberine was measured using UV spectrometer at 345 nm.

Results and discussion

Synthesis and characterization of thiolated chitosan

The immobilization of thiol groups on the surface of the cationic polymer chitosan was achieved by covalent attachment of thioglycolic acid to the primary amino groups of chitosan *via* an amide bond. The coupling reaction was catalyzed by EDAC. The polymers obtained were white, odorless, and showed a fibrous structure.

Figure 1 represents the combined FT-IR spectra of thiolated chitosan and chitosan. According to spectrum of the thiolated chitosan (Figure 1A), -OH stretching peak at 3410 cm^{-1} indicated the presence of hydroxyl groups of chitosan; -SH stretching peak at 2400–2500 cm^{-1} was related to -SH groups of thioglycolic acid. The amide band peak at 1520 cm^{-1} confirmed the formation of amide bond in the chitosan — thioglycolic acid conjugates. The resulting amount of thiol groups immobilized was determined to be 268.21 ± 18 μmol/g chitosan using Ellman's protocol.

Preparation of TCM

Because of the good swelling properties of chitosan hydrogel, it is highly permeable to low molecular weight drugs in an acidic environment, which in turn leads to a rapid drug release profile[23]. Chemical cross-linking with formaldehyde or glutaraldehyde is the most widely used strategy to modify the release behavior of chitosan microspheres. Higher encapsulation efficiency and better spherical shape can be attained by using the toluene solution of the cross-linking agents compared with using the aqueous solution of the cross-linking agents[24]. In our preliminary study, the release behavior of microspheres cross-linked with formaldehyde was unsatisfactory because they showed severe burst release. However, microspheres cross-linked only with glutaraldehyde showed a shriveled shape which may be due to the great intensity of cross-linking. Thus, here, a combination of formaldehyde and glutaraldehyde was used for cross-linking. The microspheres were firstly cross-linked with formaldehyde to maintain their spherical shape and then further cross-linked with glutaraldehyde. The release profiles of the five batches of TCM in SPDF are presented in Figure 2.

Batch A that cross-linked with least amount of glutaraldehyde showed a severe burst release and more than 50% of the drug incorporated was released at first 0.5 h. From the release profiles of batch A, B, and C, it was obvious that with the increase of the glutaraldehyde, the drug release was greatly retarded. Batch B, E, and D were prepared with increasing amount of formaldehyde while the same amounts of glutaraldehyde were maintained. The drug release was only slightly decreased with the increase of formaldehyde. The difference between the two cross-linking agents on the release behavior could be the result of their structure. Glutaraldehyde is a dialdehyde with two aldehyde groups, so it can react with two amino groups of chitosan. Thus a network structure formed within the chitosan matrix and its swelling properties was reduced which in turn led to the decrease of permeability of chitosan matrix to drugs. Since the average turnover time of mucus is 6 h[25], we consider that it is helpful that most of the drug was released within 6 h for a mucoadhesive drug delivery system. Thus, batch B (Figure 2) possessed the most desirable release properties and approximately 78% of the incorporated drug was released within 6 h in a controlled manner. Therefore, batch B was further used in pH-sensitive encapsulation.

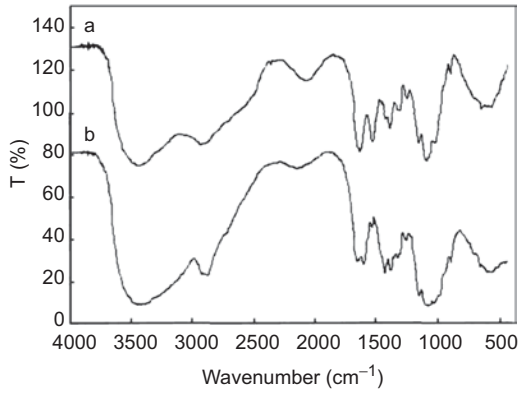

Figure 1. Fourier transform infrared spectra of thiolated chitosan (A) and chitosan (B).

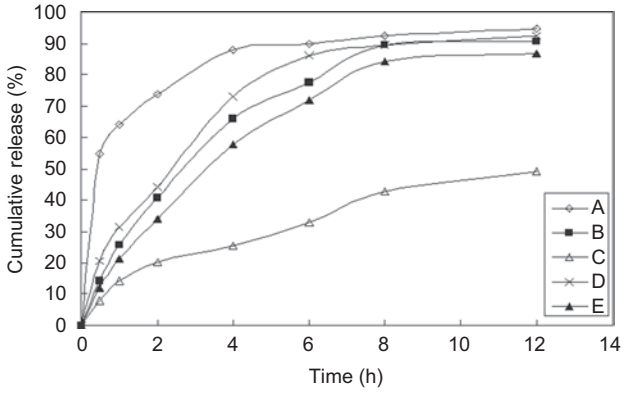

Figure 2. The release profiles of the five batches of TCM in simulated pathological duodenal fluid.

In vitro study of mucoadhesiveness

Figure 3 shows the results of the *in vitro* study of mucoadhesiveness. TCM showed better mucoadhesive properties than UCM in 10 min and 30 min ($P < 0.01$). No TCM was eliminated between 10 and 30 min most likely due to the strength of the disulfide bond.

Thiolated polymers which display thiol bearing side chains are a new generation of mucoadhesive polymers. Based on thiol/disulfide exchange reactions and/or a simple oxidation process, disulfide bonds are formed between such polymers and cysteine-rich subdomains of mucus glycoproteins building up the mucus gel layer. Thus their mucoadhesive properties are significantly improved[26]. As previously reported, disulfide bonds can only be formed above pH 5.0, in which the reactive form for oxidation of thiol groups, thiolate-anions, is represented[26]. In our study, pH 3.3 PBS was used to simulate the pathological condition of the duodenal bulb. The formation of disulfide bonds may be due to the fact the pH of mucus was little higher than that of the rinsing medium. Thiolated chitosan was also reported to display a high buffer capacity and can even act as a "microclimate"[26]. Thus the pH of the surrounding environment can be neutralized to some degree. The buffer capacity of thiolated chitosan can also be beneficial for the patient to relief some of the irritation caused by stomach acid.

Characterization of microspheres

Figure 4 shows the appearance of TCM (Figure 4A) and HPMCAM-coated TCM (Figure 4B). TCM had spherical shape with extremely rough surfaces, while the surface became smooth after coating with HPMCAM. As shown in Figure 4, the HPMCAM coating was integrative, continuous and free from large pores or cracks. TCM of batch B had a mean diameter of 275 μm. HPMCAM-coated TCM had a mean diameter of 746 μm and a drug content of 2.36 ± 0.46%.

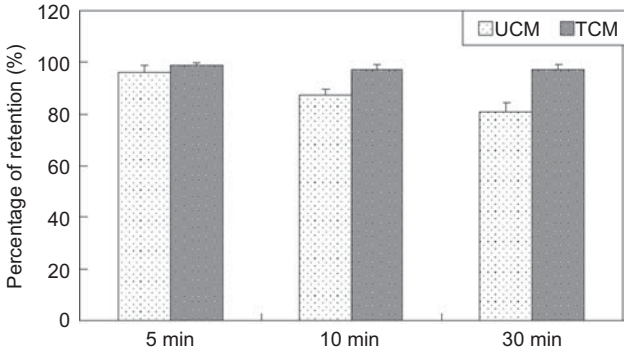

Figure 3. The results of *in vitro* mucoadhesive study.

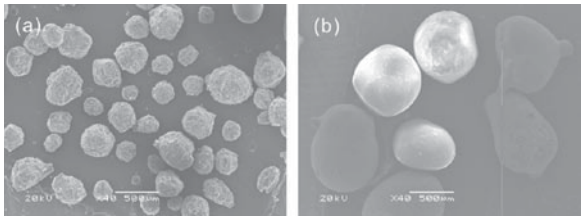

Figure 4. Scanning electron microscopy micrographs of the appearance of TCM (A) and hydroxypropyl methylcellulose acetate maleate-coated TCM (B).

Morphological analysis of HPMCAM-coated TCM in different pH media

To investigate the morphological status of HPMCAM-coated TCM in the pathological gastroduodenal tract, the microspheres were first incubated in SGF for 2 h and then incubated in SPDF (pH = 3.3) for 2 h. The morphologies of the microspheres before and after incubation are shown in Figure 5. The HPMCAM coating maintained its integrity after 2 h of incubation in SGF and no noticeable pores and cracks were found as compared with its initial state. After transferring into SPDF, most of the coating was corroded or began to peel within 5 min, but the exposed TCM essentially kept its original shape. The coating completely disappeared within 10 min of incubation in SPDF. The surface of TCM was smoother after 2 h of incubation in SPDF which may be the result of swelling.

A successful enteric coating agent must resist the low pH of the acidic medium in stomach and maintain its integrity. As the half period of stomach emptying was 64.2 min in patients with duodenal ulcer[27], an HPMCAM coating which could maintain the integrity for more than 2 h in SGF would effectively protect the encapsulated drug from the gastric acid before gastric emptying.

Lower mean pH value and increased time below pH 4.0 were observed in the duodenal lumen of patients with duodenal ulcer, owning to their abnormally prolonged period of secretion, more rapid delivery of acid of the duodenum and the decrease in buffering capacity of duodenum[6]. The average pH at the base of the duodenal

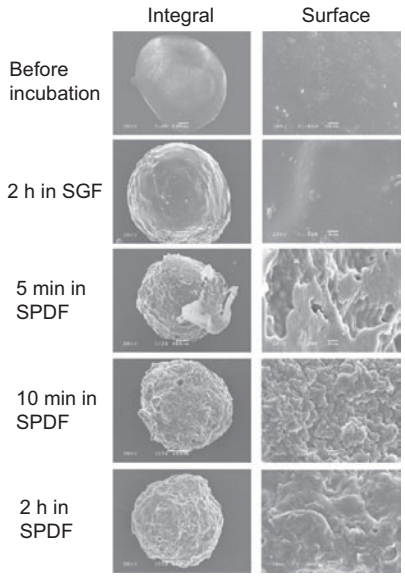

Figure 5. Scanning electron microscopy micrographs of the morphologies of hydroxypropyl methylcellulose acetate maleate-coated TCM before and after incubation. SGF, simulated gastric fluid; SPDF, simulated pathological duodenal fluid.

bulb was 3.3[1], thus a PBS of pH 3.3 was used as SPDF. Since the gastric pH in fasting state of duodenal ulcer patients were rarely over 2.3[28], coating bases that can be dissolved at a pH ranging from 2.3 to 3.3 were suitable for a duodenum-specific drug delivery system. Our group had previously synthesized a series of HPMCAM with various levels of substitution by introducing acetyl group and maleyl group into hydroxypropyl methylcellulose (HPMC)[8]. By adequately altering the ratio of acetyl group and maleyl group per glucose ring of HPMC, HPMCAM with pH-sensitive values (the threshold value below which the polymer are insoluble) ranging from 3.0 to 3.7 could be obtained. In this study, HPMCAM with pH-sensitive value of 3.0 was synthesized (weight ratio of HPMC:maleic anhydrides:acetic anhydrides was 1:0.6:0.4) and further used as the duodenum-specific coating agent. Since the duodenum is only 25 cm in length[29], the duodenum-targeting coating would be effective only when it dissolved quickly. The *in vitro* study showed that the bulk of the HPMCAM coating was corroded within 5 min. It is highly possible that the HPMCAM coating will be corroded more quickly *in vivo*, owing to the mechanical vermiculation of duodenum.

In vitro release study

To better simulate the conditions of the pathological gastroduodenal tract, the *in vitro* release study was performed by firstly incubating the HPMCAM-coated TCM in SGF for 2 h and then transferring the microspheres into SPDF. The drug release profile of HPMCAM-coated TCM is shown in Figure 6. Berberine hydrochloride was released very slowly in SGF for the first 2 h and only 4.75% of the drug was released. When transferred into SPDF, the release was much faster and more than 90% of the drug was released in 6 h after transferring. The results demonstrated that the HPMCAM coating could effectively retard the release of the drug from the encapsulated particles and agreed satisfactorily with the phenomenon observed in the morphological study that the HPMCAM coating would be dissolved quickly in pH 3.3. Compared with the release profile of TCM (batch B) in SPDF, the release rate of the HPMCAM-coated TCM was a little higher when transferred into SPDF. More than 90% of the drug was released in 6 h after transferring into SPDF for HPMCAM-coated TCM while less than 80% of the drug was released in 6 h for TCM when directly dispersed into SPDF (Figure 2). This phenomenon was probably caused by swelling. Although morphology analysis showed that the HPMCAM coating could maintain its integrity for 2 h in SGF, some water could still penetrate through the coating and cause swelling of the TCM. Thus the permeability of the TCM after 2 h of incubation in SGF would be higher than the TCM that directly dispersed in SPDF.

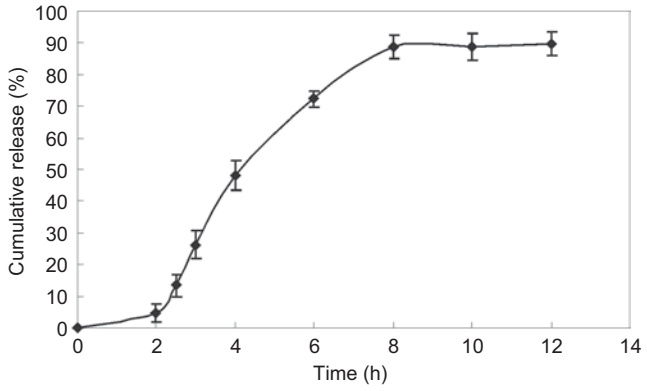

Figure 6. The *in vitro* drug release profile of hydroxypropyl methylcellulose acetate maleate-coated TCM.

Conclusion

A novel duodenum-specific drug delivery system was established using a novel pH-sensitive material HPMCAM and thiolated chitosan. Both *in vitro* morphological observation and *in vitro* release study of the HPMCAM-coated TCM showed favorable characteristics for the duodenum-specific delivery. In the future, this system should be subjected to *in vivo* study for further evaluation of its feasibility.

Declaration of interest

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