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pH-sensitive and mucoadhesive thiolated Eudragit-coated chitosan microspheres

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ABSTRACT

The aim of this study was using Eudragit–cysteine conjugate to coat on chitosan microspheres (CMs) for developing an oral protein drug delivery system, having mucoadhesive and pH-sensitive property. Bovine serum albumin (BSA) as a protein model drug was loaded in thiolated Eudragit-coated CMs (TECMs) to study the release character of the delivery system. After thiolated Eudragit coating, it was found that the release rate of BSA from BSA-loaded TECMs was observably suppressed at pH 2.0 PBS solution, while at pH 7.4 PBS solution the BSA can be sustainingly released for several hours. The structural integrity of BSA released from BSA-loaded TECMs was guaranteed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and circular dichroism (CD) spectroscopy. The mucoadhesive property of TECMs was evaluated and compared with CMs and Eudragit-coated chitosan microspheres (ECMs). It was confirmed that after coating thiolated Eudragit, the percentage of TECMs remained on the isolated porcine intestinal mucosa surface was significantly higher than those of CMs and ECMs. Likewise, gamma camera imaging of Tc-99 m labeled microsphere distribution in rats after oral administration also suggested that TECMs had comparatively stronger mucoadhesive characters. Therefore, our results indicated that TECMs have potentials to be an oral protein drug carrier.

1. Introduction

Recent advances in biotechnology and engineering made it possible to use peptides and proteins as therapeutic agents for clinical applications (Peppas and Kim, 2006). The successful delivery of these protein drugs has been an ongoing topic in the pharmaceutical research for many years. Among various delivery methods, the oral delivery is the most convenient and desired way of drug delivery, especially when repeated or routine administration is concerned (B.Y. Kim et al., 2005). It presents advantages of avoiding pain and discomfort associated with injections as well as eliminating contaminations (des Rieux et al., 2006), compared to parentally delivery method commonly used. To the peroral route, however, the peptides and proteins must resist the harsh environment of gastrointestinal tract. To improve the peroral absorption efficiencies, multifunctional polymers exhibiting pH dependent, permeation enhancing, enzyme inhibitory and mucoadhesive properties have been used. Among these features, the pH-sensitive and mucoadhesion seems more important. It has been reported that protein drugs were successfully delivered through oral administration using mucoadhesive or pH-sensitive microspheres (Sajeesh and Sharma, 2006; Thongborisute et al., 2006; Takeuchi et al., 2003; Meissner et al., 2007; Sarmento et al., 2006).

As mucoadhesive polymers, thiolated polymers or so-called thiomers have been investigated intensively in recent years. It has been thought that the thiomers are capable of forming covalent bonds with cysteine-rich subdomains of mucus glycoproteins (Leitner et al., 2003). The underlying mechanism is based on thiol/disulfide exchange reactions and an oxidation process between the reactive thiol groups of the mucoadhesive polymer and cysteine-rich subdomains of the mucin glycoproteins (Bernkop-Schnürch et al., 2001). This covalent bond extensively improved the mucoadhesive property. It has been reported that polycarbophil (PCP)–cysteine conjugate and chitosan–thioglycolic acid conjugate displayed more than two-fold and ten-fold higher adhesive properties on freshly excised intestinal mucosa than the corresponding unmodified polymer, respectively (Bernkop-Schnürch et al., 1999; Kast and Bernkop-Schnürch, 2001). In addition, thiolated polymers also exhibited strong permeation enhancing (Clausen et al., 2002) and enzyme inhibitory properties (Bernkop-schnürch and Thaler, 2000). This work employed Eudragit L-100–cysteine conjugate as thiolated polymer, which can be synthesized by the formation of covalent bonds between

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the amino groups of the cysteine and the carboxylic ones of the Eudragit® L-100. Eudragit® L-100 as an enteric coating material is soluble above pH 6.0 medium, which can protect the protein drugs from the acidic environment in stomach. Thiol groups of cysteine were introduced in the Eudragit to provide disulfide bond formation ability to this polymer. Therefore, the thiolated Eudragit can be expected to exhibit both pH-sensitive and mucoadhesive features.

In order to make use of this multifunctional polymer for orally administered protein drugs, it is the aim of this study to develop an appropriate delivery system. Bovine serum albumin (BSA) as a protein model drug was incubated in chitosan microspheres (CMs) made by ionic gelation method, and these CMs were coated with thiolated Eudragit L-100. This formulation avoids the directly contact of protein with the thiolated polymer, therefore, the thiol group of the protein will not interact with the one of the polymer conjugate and no organic solution was used through the whole formulation procedure. All of these would be beneficial for the activities of protein drugs. In this study, the development and evaluation of this delivery system is described.

2. Materials and methods

2.1. Materials

Chitosan [low molecular weight (Brookfield viscosity 20,000 cps); deacetylation degree: 82%], L-cysteine hydrochloride monohydrate, fluorescein diacetate (FDA), tripolyphosphate (TPP) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Eudragit® L-100 was obtained from Röhm Pharma (Weiterstadt, Germany).

2.2. Preparation of thiolated Eudragit

The thiolated Eudragit was synthesized according to method reported previously (Quan et al., 2007). Briefly, Eudragit® L-100 was dissolved in dimethyl sulfoxide (DMSO) and the carboxylic acid moieties of the polymer had been activated by *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxyl succinimide (NHS) before L-cysteine hydrochloride was added. The reaction mixture was incubated at room temperature under nitrogen for avoidance of the oxidation of sulphydryl groups by atmospheric oxygen. To remove the unbound L-cysteine hydrochloride, the polymer was initially dialyzed against DMSO, and then against distilled water. After dialysis, the polymer solution was freeze-dried and the conjugate was stored in air tight containers at 4 °C until further use.

The amount of cysteine in the conjugate calculated by ^{1}H nuclear magnetic resonance (^{1}H NMR) (Avance™ 600, Bruker, Germany) was about 9.9 mol-%.

2.3. Preparation of BSA-loaded TECMs

BSA-loaded CMs were prepared according to a method described previously (Jiang et al., 2004). In brief, 1 ml of 15 wt-% TPP was dropped into 25 ml of 0.25 wt-% chitosan solution (chitosan was dissolved in 2% (v/v) aqueous acetic acid) under magnetic stirring and sonication. The CMs were obtained by centrifugation for 10 min at 3000 rpm. The BSA dissolved previously in distilled water was absorbed to the CMs before CMs were not completely hardened. After incubation, the suspension was centrifuged at 3000 rpm for 10 min to remove unloaded BSA, and then the thiolated Eudragit solution was added into the microspheres for coating. The mixture was stirred at speed of 400 rpm by magnetic stirrer for 15 min. The BSA-loaded thiolated Eudragit-coated CMs (TECMs) were collected by centrifuge at 3000 rpm for 10 min and washed with distilled water and then stored at 4 °C. Three kinds of TECMs were prepared: TECMs (1) BSA-loaded CMs were coated with the equal weight of thiolated Eudragit. TECMs (2) BSA-loaded CMs were coated with 2 times of weight of thiolated Eudragit. TECMs (3) BSA-loaded CMs were coated with 4 times of weight of thiolated Eudragit.

2.4. Morphology and size of microspheres

The particle sizes were measured using DLS-7000 (Otsuka Electronics. Ltd., Tokyo, Japan) with argon laser beam at a wavelength of 488 nm and the temperature was 20 °C.

To observe the microsphere morphology, the particles were gold-coated using coating chamber (CT 1500HF, Oxford Instruments, Oxfordshire, UK). The coated samples were observed using JSM5410LV field emission SEM (Tokyo, Japan).

2.5. Entrapment efficiency

BSA entrapment efficiency in microspheres was measured as follows: 0.25 ml BSA-loaded CMs and BSA-loaded TECMs suspensions were taken out and centrifuged at 2500 rpm for 15 min. CMs and TECMs were dissolved in 1 ml of 2% aqueous acetic acid and 1 ml of 0.1 M NaOH solution, respectively, and incubated at 37 °C for 24 h. Then microspheres were further broken down by homogenizer (T 25 basic ULTRA-TURRAX IKA LABORTECHNIK) and centrifuged at 12,000 rpm for 10 min. The supernatant was collected, and the concentration of BSA in the supernatant was measured by the Micro BCA protein assay method.

2.6. Drug release studies in vitro

The *in vitro* release of BSA from BSA-loaded TECMs was determined as follows. The BSA-loaded microspheres separated from 1 ml suspension by centrifugation were placed into 1.5 ml Eppendorf tube with 1 ml of phosphate buffer solution (pH 7.4 or pH 2.0) for 8 h at 100 rpm/min and 37 °C using shaking water bath. One ml of aliquot was withdrawn and replaced with an equal volume of release medium at predetermined time and the amount of BSA released was measured by micro-BCA method.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The primary structural integrity of released BSA was detected by SDS-PAGE in comparison with native BSA and reference markers. In brief, BSA-loaded TECMs was incorporated in pH 7.4 phosphate buffer for 8 h at 37 °C and 100 rpm. The samples were centrifuged at 2500 rpm in order to separate the released protein from the particles and an aliquot was then mixed with the loading buffer and treated for 10 min at 100 °C. The SDS-PAGE was performed with 10% separating gel and run in Tris–glycine buffer at constant voltage mode (120 V). Finally, the gels were stained with 1% Coomassie blue solution and destained with an aqueous solution of 10% methanol and 10% acetic acid.

2.8. Circular dichroism spectroscopy

The changes in the secondary structure of the released BSA with respect to the native BSA were measured by CD spectroscopy (Jasco J-715, Japan) with a quartz cylindrical cell. Solutions of native BSA and released BSA were scanned over the wavelength range 195–260 nm. Using the Jasco software, the background solution (PBS 7.4) was subtracted from each protein spectrum.

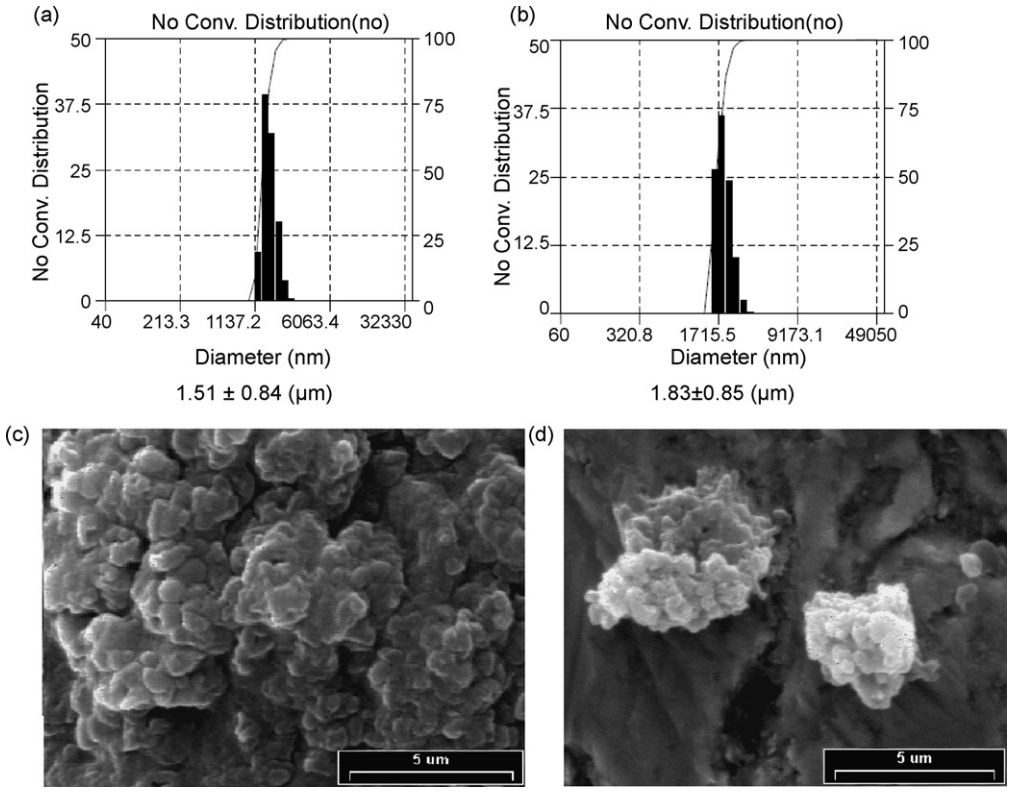

Fig. 1. Size distribution (a and b) and morphology (c and d) of BSA-loaded CMs and BSA-loaded TECMs: (a) BSA-loaded CMs, (b) BSA-loaded TECMs, (c) BSA-loaded CMs and (d) BSA-loaded TECMs.

2.9. Mucoadhesion studies in vitro

In order to evaluate the mucoadhesive property of TECMs, a method described in the literature (Maculotti et al., 2005) was used with a little modification.

Fluorescein diacetate (FDA) was incorporated in 6.0 mg CMs according to the method reported by Bernkop-Schnürch et al. (2006), then the microspheres containing FDA were coated with the equal weight of Eudragit or thiolated Eudragit.

Freshly excised porcine small intestine (5.5 cm × 2.5 cm) washed with 0.9% saline previously was attached on a microscopic slide. The FDA-loaded CMs, FDA-loaded Eudragit-coated chitosan microspheres (ECMs) and FDA-loaded TECMs were redispersed in 100 μl saline and transferred to the intestine. Then the microscopic slide was placed in a plastic cylinder vertically containing 40 ml of 0.9% saline. After 1 h of incubation under modest shaking condition at 37 °C, the microspheres remained on the mucosa were scrapped off and incubated in 5 M NaOH at 37 °C under shaking in order to quantitatively hydrolyze FDA to sodium fluorescein and the fluorescence was measured with a microplate reader at 490 nm.

2.10. Mucoadhesion studies in vivo

CMs were prepared as previously described method without BSA loading, and then labeled with Tc-99m as same method previously reported (E.M. Kim et al., 2005). The labeled CMs were coated with the equal amount of Eudragit or thiolated Eudragit.

Labeling efficiency of labeled microparticles was checked using ITLC-sg paper. Saline was used as mobile phase. The chromatographic strips were scanned on an automatic thin-layer chromatography (TLC) scanner (BIOSCAN, Washington, DC, USA).

Female SD rats weighing 200–250 g (6–7 weeks, Orient, Seoul, Korea) were kept in cages (3 mice per cage) and fed by standard laboratory chow and water. All animal experiments were approved by the Chonbuk National University School of Medicine Committee and were performed in accordance with their guidelines.

Prepared Tc-99m labeled microparticles were administrated to oral using oral zonde (200–300 μl). Then rats were anesthetized with isoflurane and positioned with prone. Images were acquired at 0.5, 1, 4, 6 h, respectively, using gamma camera (Camera name E.CAM, Simense, IL, USA). Static images were stored in a 256 × 256 matrix and acquisition time was 5 min.

2.11. Statistical data analysis

Statistical data analysis was performed using the Student's *t*-test with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Characterization of microspheres

Fig. 1(a and b) show the particle size distribution of BSA-loaded CMs and BSA-loaded TECMs measured by DLS, respectively. The results show that average particle sizes of BSA-loaded CMs and BSA-loaded TECMs are 1.51 ± 0.84 and 1.83 ± 0.85 μm, respectively.

The morphologies of BSA-loaded CMs and BSA-loaded TECMs were observed as almost spherical shapes but a little aggregated, as shown in Fig. 1(c and d).

3.2. In vitro release of BSA from BSA-loaded TECMs

Fig. 2 shows the *in vitro* release profiles of BSA from BSA-loaded microspheres up to 8 h. The encapsulation efficiencies of CMs,

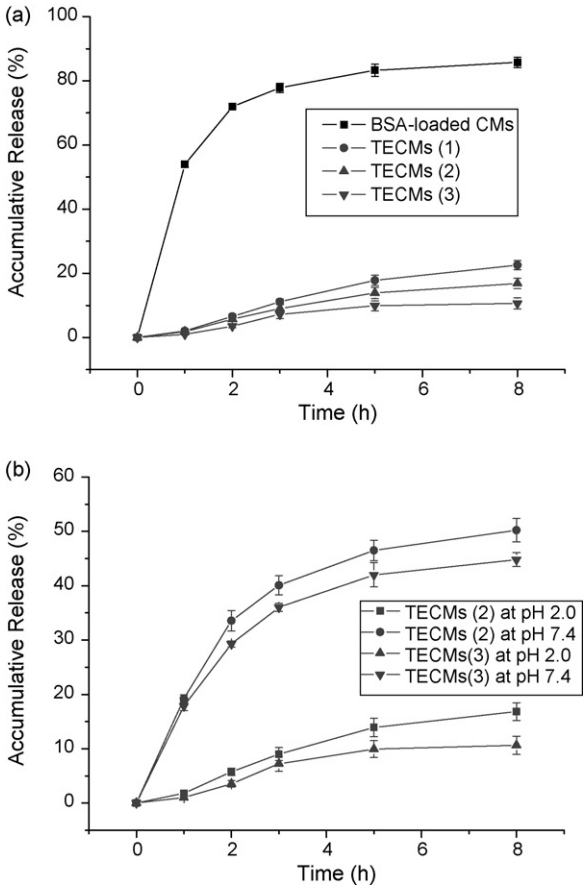

Fig. 2. *In vitro* release profiles of BSA from BSA-loaded TECMs (a) at pH 2.0 with different thiolated Eudragit coating amount and (b) at different pH. The results are shown as mean ± S.D. (n = 3 sample numbers).

TECMs (1), TECMs (2) and TECMs (3) were 45, 36, 30 and 29%, respectively. The encapsulation efficiencies are a little lower, which may be because of the better solubility of BSA in water. During the thiolated Eudragit-coating and washing process, some of BSA which had been encapsulated in the microspheres were re-dissolved in the coating and washing medium.

It was found that chitosan micropsheres were collapsed in the simulated gastric solution (pH 2.0). Around 70% BSA were released within 2 h and almost all of the formulated BSA were released during the period of 8 h (Fig. 2a), suggesting that CMs failed to protect the BSA in the simulated gastric solution, which would result in poorly delivered BSA to the intestine. On the contrary, the release of BSA from CMs was markedly suppressed after thiolated Eudragit coating. And compared to the TECMs (1), the TECMs (2) and TECMs (3) further retarded the BSA release amount as shown in Fig. 2(a), indicating that the high coating amount of thilated Eudragit shows high coating density of thiolated Eudragit on the CMs. However, a slight leakage of protein was obtained over 8 h even in the TECMs (3) formulation, suggesting that the coating might not be complete. Some protein may be located near or on the microspheres surface, which is dissolved with time and probably account for the protein released in the acidic pH.

At pH 7.4 less than 50% BSA was released from TECMs within 8 h owing to the coating of CMs with thiolated Eudragit although almost 65% of BSA was released from the BSA-loaded CMs within 8 h at pH 7.4 (data not shown). The amount of BSA released from TECMs was much higher than that of BSA released at pH 2.0 as shown in Fig. 2(b), which is in agreement with the report of Hori et al. (2005) that a small amount of ovalbumin (OVA) was released from Eudragit L100-coated chitosan-OVA microspheres on incubation in JP14 first fluid, when the incubation medium was changed to JP 14 s fluid, OVA was slightly released in JP 14 s fluid. It may be supported by the fact that the Eudragit L-100 is water-soluble with a pH-dependent property. Eudragit L-100 is an anionic polymer synthesized from methacrylic acid and methacrylic acid methyl ester. At high pH, the carboxylic groups become progressively ionized and hydrated which causes particles to swell and makes the drug release.

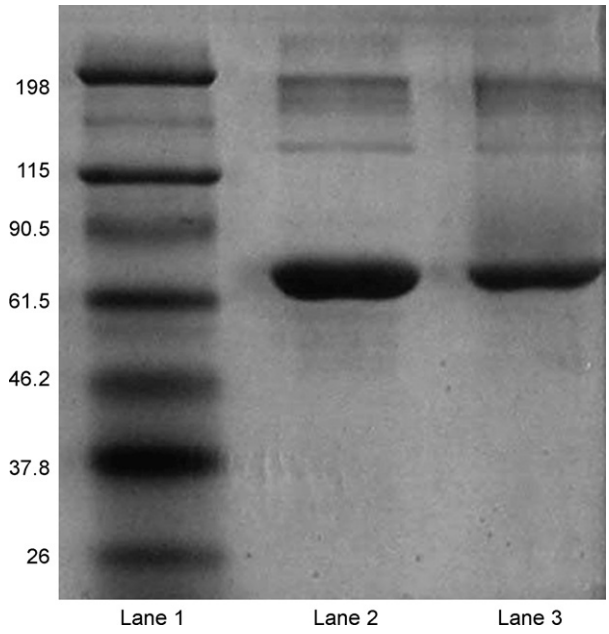

Fig. 3. SDS-PAGE. Lane 1: markers, lane 2: native BSA and lane 3: BSA released from BSA-loaded TECMs after incubation in PBS (pH 7.4) for 8 h.

Based on these *in vitro* results, a transport of the incorporated protein towards the intestine might be expected.

3.3. Conformational change of released BSA

The structural change of BSA determined by SDS-PAGE before and after microencapsulation is shown in Fig. 3. Lane 1 is a marker, Lane 2 is native BSA and Lane 3 is BSA released from the BSA-loaded TECMs after incubation in PBS for 8 h. The results indicated

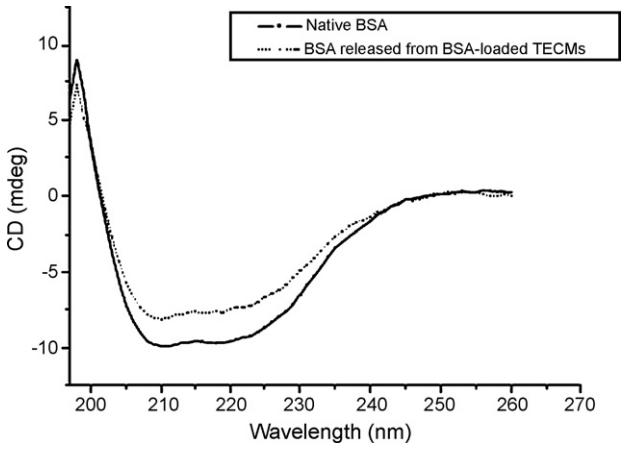

Fig. 4. Circular dichroism spectra of native BSA and released BSA from BSA-loaded TECMs.

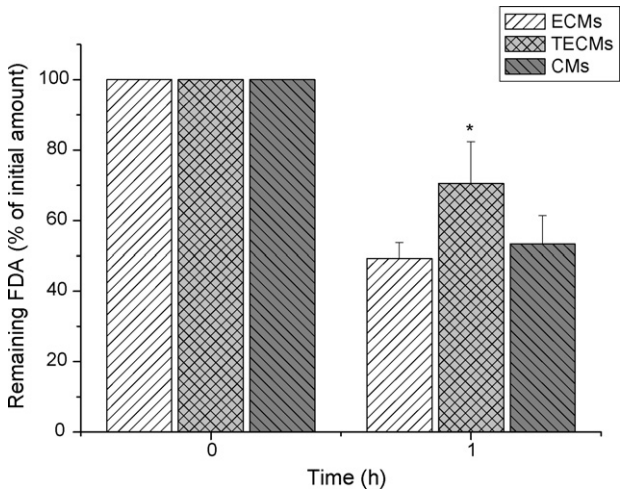

Fig. 5. Amount of FDA remained on excised porcine small intestinal mucosa. All results are shown as mean ± S.D. ($n = 3$ sample numbers, (*) differs from CMs and ECMs, $p < 0.05$).

that the encapsulation process used did not provoke any structural change in BSA. Fig. 4 shows the CD spectra of BSA before and after microencapsulation. As expected, the conformational change of BSA released from microspheres did not occur compared to that of native BSA.

3.4. Mucoadhesion property in vitro

To evaluate the mucoadhesive property, a marker compound FDA was incorporated into the microspheres. FDA is not soluble in water, once it is embedded within the particles, it would not be released from the particles in aqueous solution (Bernkop-Schnürch et al., 2006). Therefore, the remaining percentage of FDA on the mucosa surface represents the percentage of microspheres remained on the mucosa surface.

The embedded FDA in TECMs remained on the mucosal surface was significantly more than that in ECMs and CMs as shown in Fig. 5. The results revealed that the mucoadhesive property of Eudragit was improved due to the introduction of sulphydryl groups to Eudragit. The sulphydryl group can form a disulphide bonds with the mucus glycoprotein, which is supposed to be responsible for the enhanced mucoadhesive properties (Bernkop-Schnürch et al., 1999). The similar results were reported by the other groups. When using rotating cylinder method to evaluate the mucoadhesive property, adhesion time of thiolated 450 kDa poly(acrylic acid) and chitosan–TEA was significantly longer than unmodified ones (Bernkop-schnürch et al., 2003; Kafedjiiski et al., 2005).

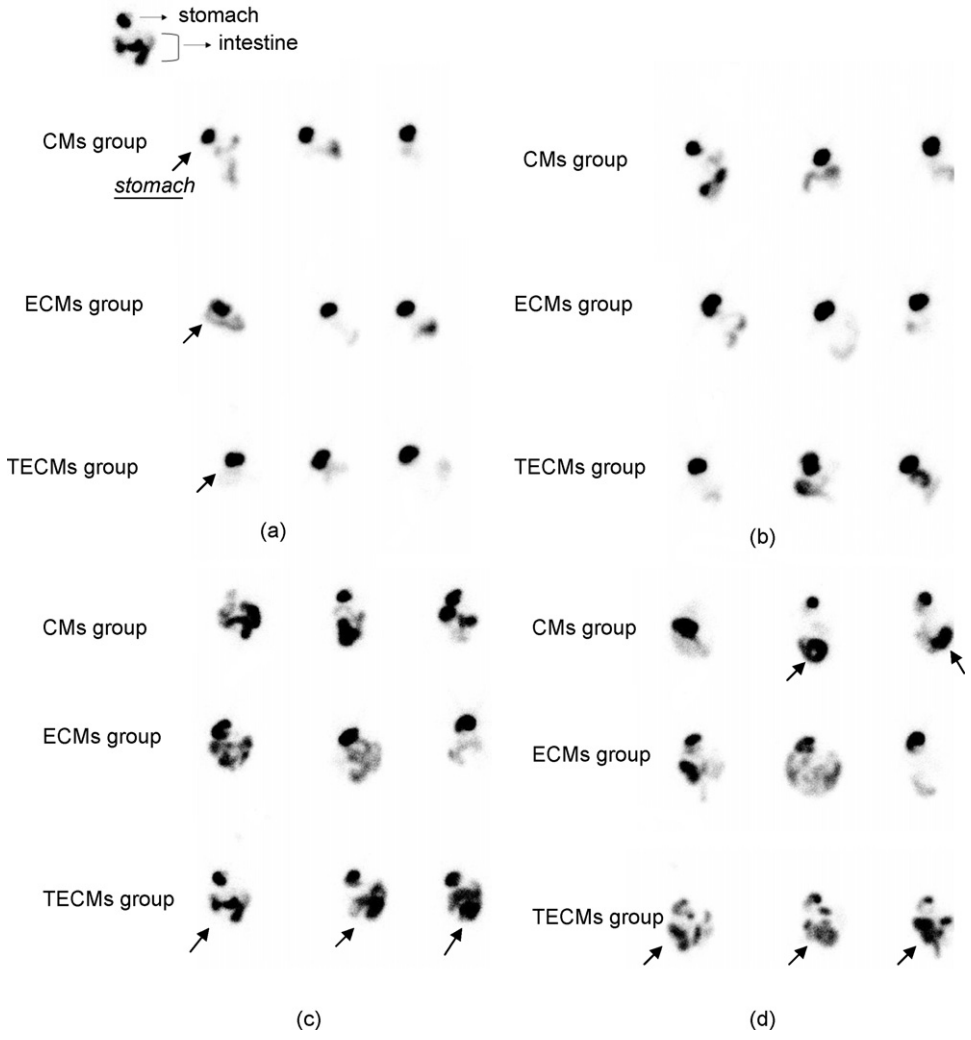

Fig. 6. Gamma camera imaging of Tc-99m labeled microsphere distribution in rats after oral administration (a) 0.5 h, (b) 1 h, (c) 4 h and (d) 6 h.

3.5. Evaluation of mucoadhesion in vivo

The Tc-99m labeling efficiency of microspheres was checked using ITLC-sg paper. Free Tc-99m shows a peak at 90 mm. After labeling, the peak shift to 30 mm and the peak at 90 mm cannot be detected (Figure not shown), suggesting that there was no free Tc-99m in the labeled chitosan microspheres and the labeling efficiency was high.

Fig. 6 shows gamma camera imaging of Tc-99m labeled microsphere distribution in rats at 0.5, 1, 4, and 6 h after oral administration.

From the distribution patterns of these radiotracers we found that a majority of microspheres of all the groups existed in stomach at 0.5 and 1 h after oral administration as shown in Fig. 6(a and b). After 4 h, the microspheres were transferred to the intestine. From the shade of color, it can be presumed that the more TECMs and CMs were adhered to the intestine than the ECMs (Fig. 6c). After 6 h, in all of the three groups, the amount of adhered microspheres was a little less than that at 4 h as observed in Fig. 6(d), whereas compared with the CMs and ECMs, the TECMs were still adhered to the whole intestine, suggesting that the mucoadhesive property of the TECMs is stronger than that of CMs and ECMs. Moreover, the results are in agreement with the *in vitro* mucoadhesion study.

The residence time of the drug carrier systems in the gastrointestinal tract is an important factor controlling the bioavailability of a drug (Takeuchi et al., 2003). The TECMs delivery system has strong mucoadhesive properties which can prolong the residence time for the protein drug at the site of absorption. Thus a strongly improved oral bioavailability will be expected although it has to be verified by *in vivo* studies in future.

4. Conclusion

BSA-loaded TECMs were prepared by coating negative charged thiolated Eudragit with BSA-loaded positive charged CMs. This system offers a mild formulation procedure which benefits for delivery and therapeutic activity of polypeptides. The release of BSA from BSA-loaded TECMs were decreased compared with that of CMs and the higher amount of thiolated Eudragit coating further retarded the BSA release from micropsheres at pH 2.0. The mucoadhesive property of TECMs was significant higher than that of ECMs and CMs both *in vitro* and *in vivo*. Therefore, the TECMs will be suitable for oral protein drug delivery.

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