Preparation and Release Behavior of Carboxymethylated Chitosan/Alginate Microspheres Encapsulating Bovine Serum Albumin

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ABSTRACT: Microspheres were prepared from carboxymethylated chitosan (CM-chitosan) and alginate by emulsion phase separation. Their structure and morphology were characterized with IR spectroscopy and scanning electron microscopy. Bovine serum albumin (BSA) was encapsulated in the microspheres to test the release behavior. The swelling behavior, encapsulation efficiency, and release behavior of BSA from the microspheres at different pHs and with a pH-gradient condition were investigated. The BSA encapsulation efficiency was calculated to be 80%. The degree of swelling of the microspheres without BSA loaded at pH 7.2 was much higher than that at pH 1.0. The encapsulated BSA was quickly released in a Tris–HCl buffer (pH 7.2), whereas a small amount of BSA was released under acid conditions (pH 1.0) because of the strong electrostatic interaction between —NH_2 groups of CM-chitosan and —COOH groups of alginic acid and a dense structure caused by a Ca^{2+} crosslinked bridge. For the simulation of the processing of the drug under the conditions of the intestine, the microspheres were tested in a pH-gradient medium, in which an acceleration of the release occurred at pH 7.4 after a lag time at a low pH (5.8–6.8). At pH 7.4, a large amount of BSA was released from the microspheres in a short time because of the rapid swelling of the microspheres. However, the release only depended on the diffusion of BSA at relatively low pHs, this resulted in a relatively low release. J Appl Polym Sci 92: 878–882, 2004

Key words: chitosan; alginate; microspheres; release; bovine serum albumin

INTRODUCTION

Recently, microcapsules and microspheres have attracted more attention in developing controlled and sustained drug-delivering systems with biopolymers because of their nontoxicity, biodegradability, and biocompatibility. Drugs are encapsulated to mask taste and odor, to stabilize the drug, to improve gastrointestinal tolerance, and to provide sustained release after oral administration.[1] Prolonging the residence time of drug carriers at the absorption site makes controlled-release drugs available.[2] Chitosan is a biopolymer derived from chitin. Chitosan has been used in agriculture, industry, and medicine[3] and extensively as a biomaterial[4] because of its immunostimulatory activities,[5] anticoagulant properties, antibacterial and antifungal action,[6] and its performance as a wound-healing promoter in the field of surgery.[7] As a permeation enhancer for drug delivery *in vitro*,[8] chitosan is able to open epithelial tight junctions to allow for an increase in paracellular-transport macromolecular drugs.[9] However, chitosan suffers from low solubility at a physiological pH of 7.4, which limits its use as an absorption enhancer in, for example, nasal peroral delivery systems. Therefore, cationic and anionic chitosan derivatives have been studied.[10] Carboxymethylated chitosan (CM-chitosan) has been paid more and more attention because of its good solubility in water, and it is more convenient to apply in medicine because it fits the neutral environment of the human body, thereby improving the permeability of drugs.[11] Moreover, the derivatives of chitosan have many of the same properties as chitosan, such as biodegradability, biocompatibility, and antibacterial and antifungal bioactivity.

Alginate is a natural biopolymer from marine materials, and it has been used successfully in the food and beverage industry as a thickening agent, a gelling agent, and a colloidal stabilizer for many years.[12] It presents the most outstanding separation performance through pervaporation for the dehydration of alcohol/water.[13,14] Microcapsules of alginate coated with a polycation have been widely investigated for applications in, for example, immmunoprotective containers for cell transplantation,[15–17] enzyme immobilization,[18–20] and drug-release systems.[21] Encapsulation membrane may be formed through the reaction of

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chitosan with alginate and the release of proteins from alginate matrices, such as albumin, bovine serum albumin (BSA), and CD40 ligands.[12] In our laboratory, we have prepared microcapsules from chitosan, and carboxymethylcellulose has been prepared.[22] Encapsulation can enhance the protein stability and retain the biological activity of the encapsulated materials. In addition, the microcapsules can protect the protein drug as it passes through the acidic and enzyme environment of the stomach and can release the protein via diffusion and capsule degradation once they reach the intestinal region, at which the drug is effectively absorbed into the blood stream.[23]

In previous works, we explored blend membranes from CM-chitosan and alginate,[24,25] which have shown good mechanical properties because of the strong electrostatic interaction caused by NH_2 groups of CM-chitosan with —COOH groups of alginic acid.[25] With two water-soluble materials, it is more convenient to prepare microspheres with the membrane permeability of the drugs and easier to purify the microspheres. On the basis of water-insoluble blend membranes prepared from water-soluble CM-chitosan and alginate, we attempted to prepare CM-chitosan/alginate microspheres, and we chose BSA as the model drug to test the release behavior of the microspheres. The microsphere properties and their release behavior were investigated to determine the potential applications of these natural materials.

EXPERIMENTAL

Materials

Sodium alginate was purchased from Shanghai Chemical Reagent Co. (Shanghai, China).Chitosan was supplied by Yuhuan Ocean Biochemistry Co., Ltd. (Zhejiang, China). The deacetylation degree of the chitosan was 92%. CM-chitosan was prepared according to the literature.[11] The chitosan, suspended in an aqueous 42 wt % NaOH solution, was reacted with monochloroacetic acid at 20°C for 6 h and then was neutralized with an aqueous HCl solution (pH 5). The product was washed with alcohol and then vacuum-dried to give CM-chitosan with a substitution degree of 1.0. The weight-average molecular weight of CM-chitosan in an aqueous 0.2 mol L^{-1} NaCl solution was determined to be 1.5×10^5 with a laser photometer (Dawn DSP, Wyatt Technology, Santa Barbara, CA) equipped with a Spectra P100 gel permeation chromatograph. Span-20 and sodium alginate were obtained from Shanghai Chemical Reagent Co. BSA was V-grade and was purchased from German Roche Co. (Germany). All the other materials were analytical-grade.

Preparation of the blend membranes

To clarify the properties of the microspheres, we prepared blend membranes from CM-chitosan and alginate according to our previous method.[25] A mixture of an aqueous 5 wt % CM-chitosan solution and an aqueous 8 wt % sodium alginate solution (1:1 w/w) was stirred for 15 min, filtered, and degassed. The blend solution was spread over a glass plate to a depth of 0.2 mm and was coagulated in a coagulation bath of an aqueous 5 wt % $CaCl_2$ solution for 15 min. After being washed with water, the membrane was regenerated in an aqueous 1 wt % HCl solution; a transparent blend membrane of CM-chitosan and alginic acid was obtained, and it was washed with running water. The blend membrane was dried on the glass plate in air.

Preparation of the microspheres

CM-chitosan (5 wt %) and 8 wt % sodium alginate in water were prepared and blended (1:1 w/w) to obtain a blend solution, and 0.5 g of BSA was dissolved in 40 mL of the blend solution. The microspheres were prepared by the emulsion phase separation at room temperature. The aqueous solution containing the blend solution and BSA was dispersed in liquid paraffin containing 1% span-20. With a mechanical stirrer at a speed of 900 rpm for 1.5 h, a paraffin system was formed into a water-in-oil emulsion. Then, an aqueous $CaCl_2$ solution (2 g of $CaCl_2$, 20 mL of water, 15 mL of ethanol, and 0.5 mL of acetic acid) was dropped into the system, and the dispersion was mixed for 2 h to form hard-shell microspheres. Isopropyl alcohol was then used to further harden the formed microspheres. The microspheres were collected by filtration, washed three times with 5% $CaCl_2$, deionized water, and ethanol, and vacuum-dried.

Characterization

IR spectra of the samples were recorded with a Fourier transform infrared spectrometer (170 SX, Nicolet, Minnesota). Scanning electron microscopy (SEM) was performed with a Hitachi (Japan) S-570 SEM instrument. The microspheres were coated with gold, and subsequently their surfaces were observed and photographed.

Swelling test

The dry microspheres (150 mg) containing no BSA were immersed in both 0.1*N* HCl (pH 1.0) and a Tris–HCl buffer (pH 7.2) for 24 h at room temperature until a swollen equilibrium was reached. The swollen samples were collected with a centrifuge, blotted with filter paper for the removal of the absorbed water on the surface, and then weighed immediately. The degree of swelling (SW) was calculated as follows:

$$\text{SW (wt \%)} = [(w - w_0)/w_0] \times 100\% \qquad (1)$$

where w and w_0 are the weights of the microspheres in the equilibrium swelling state and in the dry state, respectively.

BSA encapsulation efficiency (AE) of the microspheres

For the calculation of the BSA AE, the amount of BSA released into the solution after 48 h was presumed to be the loading amount of BSA, which was determined by UV spectroscopy (160A, Shimadzu, Kyoto, Japan) at $\lambda = 279$ nm until the value stopped changing. The BSA AE of the microspheres was calculated as follows:

$$\text{AE} = (\text{Loading amount of BSA}/\text{Total amount of BSA}) \times 100\% \quad (2)$$

where the total amount of BSA was added to the original amount of BSA for the microspheres.

BSA release from the microspheres

The release *in vitro* of BSA from the CM-chitosan/alginate microspheres was carried out in a Tris–HCl buffer (pH 7.2) and 0.1*M* HCl at 37°C. Accurately weighed amounts (4.2 mg) of the microspheres were placed in a tube containing 5 mL of the buffer and were incubated at 37°C. At selected time intervals, the tubes were collected and centrifuged at 15,000 rpm for 5 min. The BSA concentrations in the supernatant were assayed with Lowry's method[26] for protein estimation. Each *in vitro* release study was performed twice to obtain the average value.

In another assay, the process was the same, but the pH of the release medium was gradually increased: pH 5.8 during the first 2 h, pH 6.8 during the following 3 h, and pH 7.4 until the end of the experiment. At specific time intervals (1, 2, 3, 4, 5, 6, 8, 10, 12, and 24 h), the microsphere dispersions were centrifuged at 15,000 rpm for 5 min, and the supernatant was assayed for drug release by UV spectroscopy to measure its absorbance at 279 nm.

RESULTS AND DISCUSSION

Structure and morphology of the microspheres

The IR spectra for the CM-chitosan, alginate, and blend membrane are shown in Figure 1. In comparison with chitosan (at 3442 cm^{-1}) and alginate (at 3421 cm^{-1}), the —OH stretching vibration bands of the microspheres and blend membrane were broader and shifted to a lower wave number (at 3400 cm^{-1}), suggesting that intermolecular hydrogen bonds existed in the microspheres and the blend membranes. The absorption bands of the microspheres were much stronger than those of chitosan and alginate.

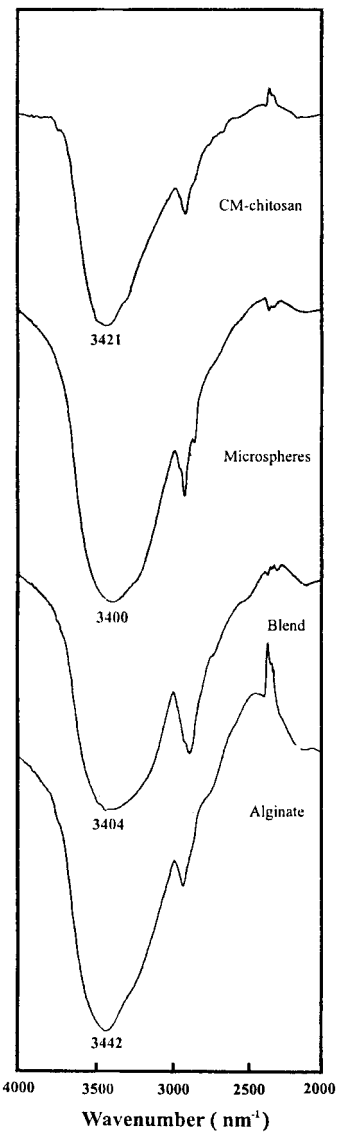

Figure 1 IR spectra of the alginate, blend membrane, microspheres, and CM-chitosan.

SEM images of the microspheres are shown in Figure 2; their surfaces and internal morphology are depicted. The SEM images of the microspheres displayed a smooth surface, which agreed with the blend membranes.[25] The microspheres basically exhibited sphere-like figures, whereas the shapes of the microspheres containing BSA were more irregular. Moreover, the mean size of the microspheres containing no BSA (4 μm) was smaller than that of microspheres containing BSA (7.6 μm). However, the microspheres were nonporous structure; this was the same as the surface of the blend membrane (i.e., a dense structure).

Effect of pH on the swelling ratio

The pH dependence of the degree of swelling for the microspheres is shown in Figure 3. The microspheres

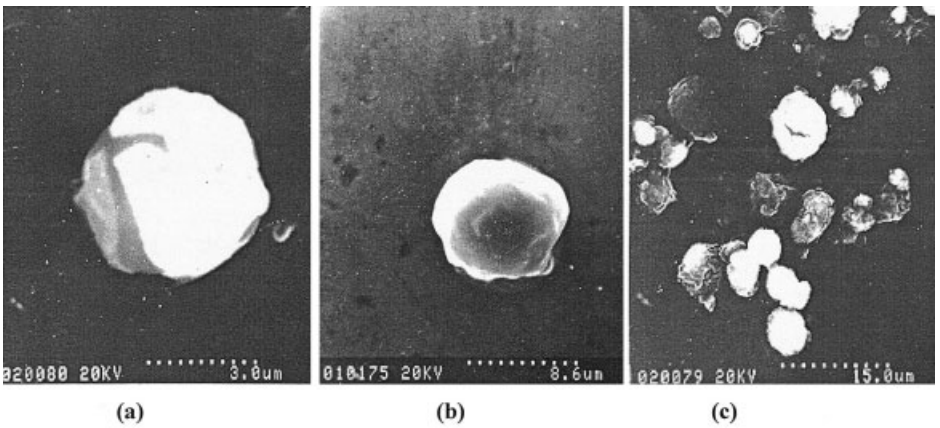

Figure 2 SEM images of (a) the microspheres and (b,c) the microspheres loaded with BSA.

in 0.1*M* HCl had a much lower percentage of swelling than those in a Tris–HCl buffer. Therefore, the swelling ability of the microspheres was weakened under an acid environment. The degree of dissociation of CM-chitosan was suppressed, and chitosan may have formed some kinds of loops. This loop formation resulted in less dense CM-chitosan/alginate membranes at pH 7.2. However, each polysaccharide could sustain the rigid main conformation at a low pH, and the protonated amino groups of CM-chitosan could enhance the electrostatic interaction between the —COOH groups of alginic acid and the —NH_2 groups of CM-chitosan, leading to a dense structure.

BSA release from microspheres in different pH environments

The BSA AE was calculated to be around 80%. Figure 4 shows the release *in vitro* of BSA from microspheres in different pH environments (pH 1.0 and pH 7.2). In comparison with the release curve at pH 7.2, a small amount of BSA was released at pH 1.0. The initial release within the first 5 h under acid conditions was about 17%, whereas more then 50% BSA was released from microspheres at pH 7.2. More than 95% of the BSA was released from microspheres at pH 7.2, whereas just 30% was released at pH 1.0, within 24 h. The results agreed with the order of the swelling degree because of the stronger interaction between CM-chitosan and alginate in the acid environment. The fast release of BSA at pH 7.2 was attributed to the higher permeability of the buffers in the microspheres, which resulted in the swelling of the polymers. The process of the model drug release from the microspheres was diffusion. At pH 1.0, the drug dissolved more slowly and diffused into the outer aqueous phase. Therefore, under acid conditions, the process of BSA release was mainly controlled by the diffusion process. At pH 7.2, the swelling value was much higher than that at pH1.0, and this resulted in a large amount of BSA released from the microspheres via diffusion.

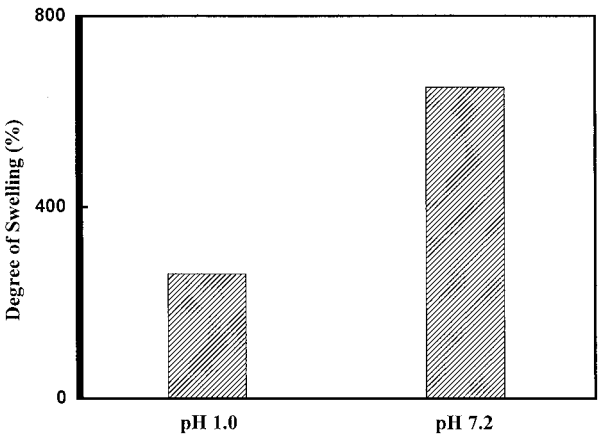

Figure 3 Degree of swelling of the microspheres at different pHs.

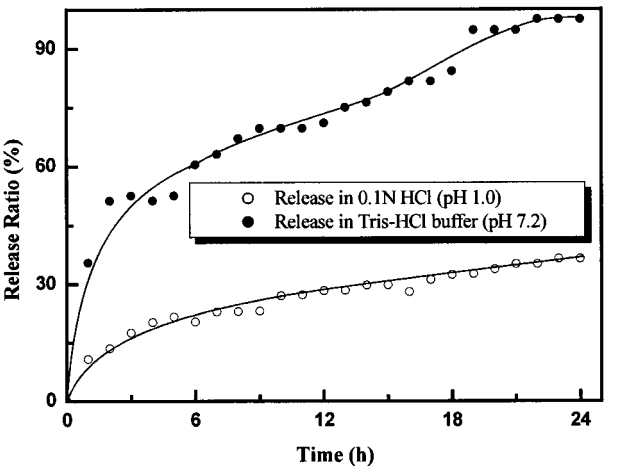

Figure 4 Dependence of the release ratio of BSA from microspheres in a Tris–HCl buffer (pH 7.2) and 0.1*M* HCl (pH 1.0) on the time.

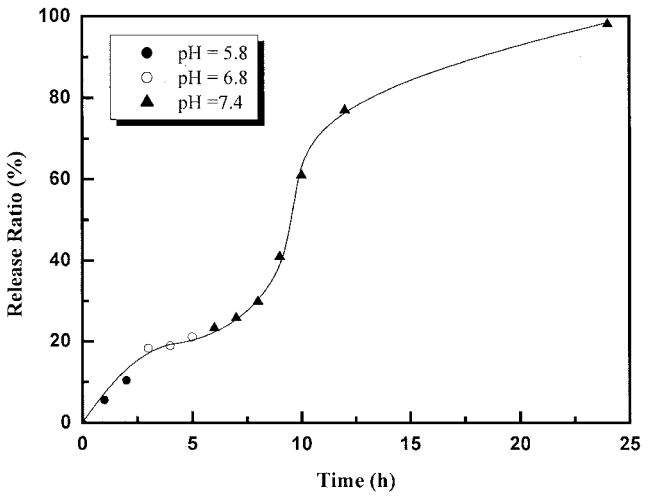

Figure 5 Dependence of the release ratio of BSA from microspheres at different pHs on the time.

BSA release in a pH-gradient environment

The *in vitro* release behavior of BSA microspheres is shown in Figure 5. Interestingly, the pH-gradient environment can be simulated as the environment from the small intestine to colon. The results of the BSA released in a pH-gradient (from 5.8 to 7.4) medium indicated that below the solubility pH of the matrix polymers (pH 7.4), the amount of BSA released was very low. After this lag time, the release occurred continuously, following almost zero-order kinetics. Once the release started, more than 90% of the entrapped BSA was released completely in 24 h. Although the BSA release in the CM-chitosan/alginate microspheres started at a low pH, an S curve indicated that there was an acceleration of release at pH 7.4. This was also confirmed with the results of the effect of pH on BSA release in Figure 5. In the intestine environment, the microspheres were swollen rapidly, and this resulted in a large amount of BSA released in a short time. Lorenzo-Lamosa et al.[28] reported the same result, showing a new system that combined the pH-gradient release of sodium diclofenac from chitosan/alginate microencapsulation for colonic drug delivery. On the basis of their results and all the results for CM-chitosan and alginate, the mechanism can be described as follows. When entering the intestine, the microspheres slowly swell at pH 5.8, accompanied by a very slow release, which depends on the diffusion mechanism of BSA from the microspheres. After 3–4 h, the microspheres reach the colonic region, and there the matrix materials swell rapidly via diffusion at pH 7.4. This pH dependence of the microsphere composition may beneficially trigger the release of entrapped BSA.

CONCLUSIONS

Microspheres encapsulating BSA were prepared from CM-chitosan and alginate with emulsion phase separation. The AE of BSA was about 80%. The microspheres were spherelike and had dense structures on a smooth surface. The degree of swelling of the microspheres without BSA loaded at pH 7.2 was much higher than that at pH 1.0. The BSA encapsulated in the microspheres was quickly released in the Tris–HCl buffer (pH 7.2), whereas there was just a small amount of BSA released under the acid conditions (pH 1.0) because of the strong electrostatic interaction between the —NH_2 groups of CM-chitosan and the —COOH groups of alginic acid and a dense structure caused by a Ca^{2+} crosslinked bridge. When the microspheres were immersed in a pH-gradient medium, after a lag time at a low pH, the release occurred continuously, following almost zero-order kinetics at pH 7.4. The release of BSA from the microspheres was slow at a relatively low pH and depended on the diffusion of BSA.

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