Calcium-Carboxymethyl Chitosan Hydrogel Beads for Protein Drug Delivery System

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ABSTRACT: In this study, carboxymethyl chitosan (CMC) hydrogel beads were prepared by crosslinking with Ca^{2+}. The pH-sensitive characteristics of the beads were investigated by simulating gastrointestinal pH conditions. As a potential protein drug delivery system, the beads were loaded with a model protein (bovine serum albumin, BSA). To improve the entrapment efficiency of BSA, the beads were further coated with a chitosan/CMC polyelectrolyte complex (PEC) membrane by extruding a CMC/BSA solution into a $CaCl_2$/chitosan gelation medium. Finally, the release studies of BSA-loaded beads were conducted. We found that, the maximum swelling ratios of the beads at pH 7.4 (17–21) were much higher than those at pH 1.2 (2–2.5). Higher entrapment efficiency (73.2%) was achieved in the chitosan-coated calcium-CMC beads, compared with that (44.4%) in the bare calcium-CMC beads. The PEC membrane limited the BSA release, while the final disintegration of beads at pH 7.4 still leaded to a full BSA release. Therefore, the chitosan-coated calcium-CMC hydrogel beads with higher entrapment efficiency and proper protein release properties were a promising protein drug carrier for the site-specific release in the intestine. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 3164–3168, 2007

Key words: carboxymethyl chitosan; chitosan; hydrogels; pH sensitivity; drug delivery systems

INTRODUCTION

In the field of biomedical materials, hydrogels have been paid a great deal of attention especially as site-specific or controlled-release drug delivery systems. The key in the preparation of hydrogels is the formation of crosslinking structure, which maintains their stability in aqueous medium. Common crosslinking methods of hydrogels include chemical crosslinks and physical crosslinks. The main drawbacks of chemical crosslinks are the toxicity of residual covalent crosslinkers or unwanted side effects with drugs in chemical crosslinking reaction. To avoid these drawbacks, physical crosslinks are preferable for the synthesis of biocompatible hydrogels, because of the absence of covalent crosslinkers and chemical reactions.[1,2] Ionic crosslink, as an effective physical method, is usually carried out physically by dispersion of the polymer/drug solution into an aqueous gelation medium. In such a simple and mild process, the biological activity of drugs can be well retained.[3] In addition, because the reversible ionic bonds can be further modified by external conditions such as the pH and ions of the application medium, hydrogel networks with ionic crosslink generally exhibit pH-sensitive and ion-sensitive swelling and drug release properties. All these advantages endow ionically crosslinked hydrogels with huge potential for the application as site-specific or controlled-release drug delivery systems. To date, there are only a few kinds of suitable systems well developed, such as Ca^{2+}-crosslinked alginate[4–8] and tripolyphosphate-crosslinked chitosan.[9]

For the applications as carriers of macromolecules, the entrapment efficiency is a key parameter. To reduce macromolecules loss from gel beads, several approaches have been achieved, including forming a chitosan coating at the bead surface,[4,5] adjusting the pH value of polymer solution slightly below the isoelectric point of loaded enzyme,[6] using a higher degree of deacetylation and a higher molecular weight (MW) of polymer matrix,[9] and adding a certain amount of bentonite to hold back the water in the beads.[6]

Carboxymethyl chitosan (CMC), an important water-soluble derivative of chitosan, has many outstanding properties including nontoxicity, biodegradability, biocompatibility, antibacterial, and antifungal bioactivity. For these advantages, CMC has received considerable attention in biomedical applications.[10–14] For the delivery of protein drug, glutaraldehyde-crosslinked CMC hydrogels showed polyampholyte characteristics and pH-sensitivity.[15] Moreover, CMC has ever been used to blend with alginate to prepare Ca^{2+}-crosslinked hydrogel beads to improve the swelling ability of the beads at pH

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7.4.[8] Having carboxylate ions (—COO^-) on polymer chains, CMC should be a good candidate for Ca^{2+}-crosslinked hydrogel beads. However, it was reported that an instant precipitation with an irregular shape (rather than bead formation) was observed upon addition of pure aqueous CMC into the $CaCl_2$ solution.[8]

In this study, low MW CMC was crosslinked with Ca^{2+} to fabricate pH-sensitive hydrogel beads. The swelling, BSA loading, and release properties of the beads were investigated. Chitosan-coated calcium-CMC beads were used to improve the BSA entrapment efficiency.

EXPERIMENTAL

Materials

Low MW carboxymethyl chitosan (CMC) [MW $\sim 6.1 \times 10^4$, the degree of substitution of carboxymethyl group (DS) $\sim$0.66] was purchased from Aoxing Biotechnology (Zhejiang Province, China). Medium MW CMC (MW $\sim 5.3 \times 10^5$, DS ~ 0.65) was synthesized in our lab according to previous method.[15] Chitosan (MW $\sim 7.2 \times 10^5$; the degree of deacetylation, 85.5%) was purchased from Jinan Haidebei Biotechnology (Shandong Province, China). Calcium chloride (AR) was obtained from Tianjin Chemical Reagent (Tianjin, China). BSA was purchased from Huamei Biotechnology (Huamei, China). BCA™ Protein Assay Kit was obtained from Pierce Company (Rockford, IL).

Preparation of calcium-CMC gel beads

The calcium-CMC beads were prepared by dropping aqueous CMC solution (5, 3, and 2%, by w/w, respectively) into a gently stirred $CaCl_2$ solution (1, 3, 5, and 8%, by w/w, respectively) through a syringe (10 mL) with a 16-gauge needle. Gel formation in the shape of beads was formed instantaneously. The beads were allowed to crosslink with Ca^{2+} in solution for 1 h. After that, the beads were rinsed with deionized water several times to remove unreacted $CaCl_2$ on surface and subsequently dried at room temperature.

The morphology of fresh beads was recorded using a digital camera (Kodak DX6490).

Swelling characteristics of calcium-CMC gel beads

The swelling characteristics of calcium-CMC beads were determined by immersing dried test samples to swell at 37°C in 0.1*M* HCl (pH 1.2) for 2 h and subsequently transferred into a 0.05*M* trisHCl buffer (pH 7.4), simulating gastrointestinal tract conditions. At specific time intervals, samples were removed from the swelling medium and were blotted with a piece of paper towel to absorb excess water on surface. The swelling ratio of test samples were calculated from the following expression[8,15]:

$$\text{Swelling ratio} = (W_s - W_d)/W_d$$

where W_s is the weight of the swollen test sample and W_d is the weight of the dried test sample.

BSA incorporation

To load BSA in calcium-CMC beads, 0.35 g of CMC was added into 5 mL of 0.5% (w/v) aqueous BSA solution. After dissolution of CMC with stirring, the mixture was dropped into a gently agitated 20 mL solution of 5% (w/w) $CaCl_2$. The prepared beads were cured for 1 h and then were washed four times using 20 mL of deionized water every time. The decanted solution and washings were collected for further study.

BSA loading in calcium-CMC-chitosan beads was performed in a similar way except the gelation medium. A certain amount of chitosan (0.1, 0.3, and 0.5%, by w/w, respectively) and $CaCl_2$ (5% w/w) were added to a 0.1*M* HCl solution. After dissolution of chitosan and $CaCl_2$ with stirring, the pH was adjusted to 6.0 using 5% (w/w) NaOH solution. After filtration, a clear solution was obtained and used as the gelation medium for the preparation of BSA-loaded calcium-CMC-chitosan beads.

The same procedures were used to prepare placebo beads, which have no BSA.

Determination of BSA entrapment efficiency

The decanted 20 mL of gelation medium and four 20 mL-washings during the course of BSA incorporation were analyzed using the bicinchoninic acid (BCA) protein assay, which is widely used to quantitatively determine the concentration of most proteins.[16] Briefly, 25 μL of each sample was drawn and mixed with 200 μL of working reagent. The mixture was incubated for 30 min at 37°C. The working reagent was a combination of reagent A (sodium carbonate, sodium bicarbonate, BCA, and sodium tartrate in 0.1*M* sodium hydroxide) and reagent B (4% cupric sulfate). The mixture of the decanted gelation medium and washings of the placebo beads was used as a blank. Nine different concentrations of 0–2000 μg/mL of BSA solution were used as standards. The concentration of BSA in each sample was quantified by measuring the absorbance at 570 nm using a microplate reader (Multiskan MK3, Labsystem).

The BSA entrapment efficiency in beads was calculated from the difference between the amount of BSA dissolved in aqueous CMC solution and that of BSA released in the gelation medium divided by the amount of BSA dissolved in aqueous CMC solution.

BSA release studies

To study the release profiles of test beads, dried test samples were immersed in 0.1*M* HCl (pH 1.2) for 2 h and subsequently in 0.05*M* trisHCl buffer (pH 7.4) at 37°C, simulating gastrointestinal tract conditions. At predetermined time points, 0.2 mL of sample was taken out and replaced each time by 0.2 mL of fresh solution. The cumulative concentration of released BSA was determined by using the BCA protein assay as described above.

All experiments were repeated in quadrates, and all data are presented as a mean value with its standard difference (mean ± SD) of four experiments.

RESULTS AND DISCUSSION

Preparation of calcium-CMC gel beads

To investigate the ability of CMC to form calcium-crosslinked gel beads, two different MWs of CMC with similar DS were used. When two kinds of CMC solutions were dropped into a $CaCl_2$ solution, different phenomena were observed as shown in Figures 1(a,b). Irregular shapes were formed when using 5% (w/w) medium MW CMC solution, as reported in the literature.[8] However, spherical gel beads could still be fabricated using lower concentrations of medium MW CMC solutions (3 and 2%, by w/w) [Fig. 1(a)]. The problem was that, due to their bad mechanical strength, the beads could not stand but extend once leaving the gelation medium. Moreover, these badly fragile beads tended to break when handled. The reason for this phenomenon may be due to the high viscosity of medium MW CMC solution, which limited the diffusion of calcium ion into CMC solution and subsequently leaded to an inadequate crosslinking density of gel beads and low mechanical strength to keep their original spherical shape.

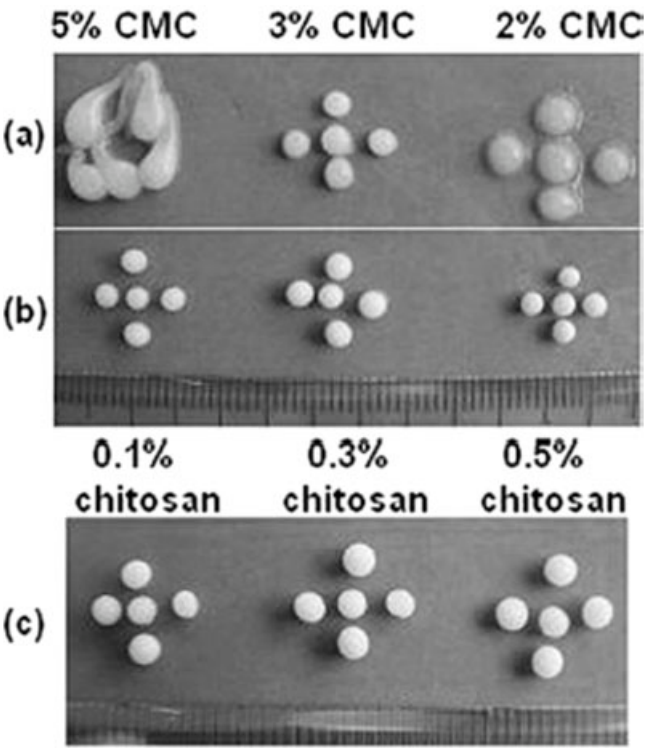

Figure 1 Photographs of fresh gel beads: (a) $CaCl_2$ (3%, w/w)-CMC (medium MW, by w/w) beads; (b) $CaCl_2$ (3%, w/w)-CMC (low MW, by w/w) beads; (c) $CaCl_2$ (5%, w/w)-CMC (low MW, 6.5%, w/w)-chitosan (by w/w) beads.

However, spherical and solid gel beads were successfully synthesized using low MW CMC [Fig. 1(b)]. Since the DS values of two CMC samples were almost the same, the different formation abilities of gel beads should be attributed to their difference in MW. It seems that low MW CMC is preferable to prepare practical gel beads crosslinked by calcium. For low MW CMC solution, 1% (w/w) was the least concentration for gel bead formation. When 0.5% (w/w) aqueous CMC was dropped into gently-stirred $CaCl_2$ solution, flocculent precipitates instead of spherical beads were formed and meanwhile the $CaCl_2$ solution became milky. In addition, high concentration of low MW CMC solution could be easily prepared and extruded from syringe, which provides a convenient way to make gel beads. In following experiments, 6.5% (w/w) low MW CMC solution was used to prepare various gel beads. These gel beads could quickly harden in about 30 min. After 60 min of curing time, an apparent shrinkage of gel beads was observed and these gel beads were solid enough to be handled for further studies.

Swelling characteristics of calcium-CMC gel beads

The swelling behaviors of calcium-CMC beads were determined by simulating gastrointestinal pH conditions. The swelling results were shown in Figure 2. The maximum swelling ratios of calcium-CMC beads at pH 7.4 (17–21) were much higher than those at pH 1.2 (2–2.5). This may be attributed to the presence of hydroxide ion (OH^-) in pH 7.4 medium. Free calcium ion and free hydroxide ion tend to form a precipitate when encountering each other in aqueous solution because of the extremely low solubility of $Ca(OH)_2$ in water. At pH 7.4, hydroxide ion could induce calcium ion to diffuse from calcium-CMC beads to external medium, which lowered the crosslinking density of the beads and subsequently caused the beads to swell. On the other hand, free carboxyl groups at pH 1.2 became dissociated at pH 7.4, inducing hydration around the carboxylate ions and increasing internal osmotic pressures induce hydrogel beads swelling.

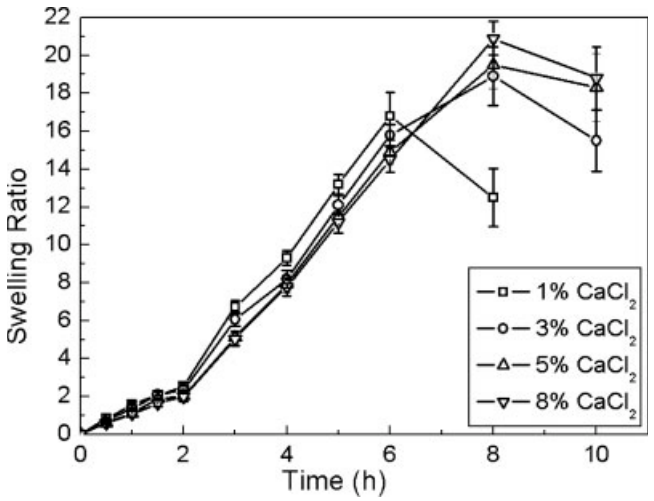

Figure 2 Swelling behaviors of calcium-CMC beads prepared in distinct concentrations of $CaCl_2$ solution, determined by immersing dried test samples to swell in 0.1*M* HCl (pH 1.2) for 2 h and subsequently in 0.05*M* trisHCl buffer (pH 7.4) at 37°C.

To investigate the effect of $CaCl_2$ concentration, the calcium-CMC beads were prepared in different concentrations of $CaCl_2$ gelation media (1, 3, 5, and 8%, by w/w, respectively). As shown in Figure 2, the maximum swelling ratios of all beads (2.0–2.5) were similar at pH 1.2 for 2 h. At pH 7.4, the maximum swelling ratios increased with $CaCl_2$ concentration, and disintegration took place earlier for those beads prepared in 1% $CaCl_2$ solution than in $CaCl_2$ solutions of other concentrations. In the preparation of calcium-CMC beads, calcium ion concentration may affect the crosslinking density of beads. In a certain range, a higher calcium ion concentration means the formation of a higher crosslinking density of gel beads, unless the crosslinking density reaches its highest value. Among all beads, the crosslinking density of the beads was the lowest when prepared in 1% $CaCl_2$ solution, leading to their earliest disintegration before further swelling. In the whole test time, the swelling ratios of the beads prepared in 5% $CaCl_2$ solution were very similar to those prepared in 8% $CaCl_2$ solution. This may be because the crosslinking density of beads had reached their highest value when prepared in 5% $CaCl_2$ solution. For this reason, 5% $CaCl_2$ solution was used to encapsulate BSA.

Entrapment efficiency of BSA in gel beads

Entrapment efficiency is a critical parameter to be considered in the application of hydrogels as drug delivery systems.[17] Since the candidates (including enzymes, peptides, and proteins) for such hydrogel systems can be very expensive, low entrapment efficiency would cause a waste and limit the use of such systems. The entrapment efficiencies of a number of enzymes in some hydrogel systems have been found to be 25–45%.[6,18,19]

In present research, by extruding CMC solution into a $CaCl_2$/chitosan gelation medium, the surface of calcium-CMC beads was further coated with a PEC membrane formed through interpolymeric ionic interactions between CMC and chitosan. The morphologies of calcium-CMC-chitosan beads were shown in Figure 1(c). The effect of chitosan concentrations on the entrapment efficiencies of BSA in calcium-CMC-chitosan beads was shown in Figure 3. The entrapment efficiency increased obviously with the chitosan concentration in gelation medium. Only 44.4% BSA was retained in the bare calcium-CMC beads, while 73.2% BSA was retained in the calcium-CMC-chitosan beads prepared in $CaCl_2$/chitosan (0.5%) gelation medium. The calcium-CMC-chitosan beads seem to be a better system for BSA encapsulation than calcium-CMC beads. The reduction of BSA leakage may be due to a higher hindrance for BSA diffusion. The higher hindrance mainly resulted from three factors, namely, the crosslinking of CMC with calcium ion, the ionic interaction between CMC and chitosan, and the higher viscosity of chitosan-containing gelation medium. Hari et al.[4] and Huguet et al.[20] also reported similar results with the use of chitosan coating.

Release behaviors of BSA from gel beads

The BSA release behaviors from various test beads, prepared in $CaCl_2$/chitosan gelation medium with different concentrations of chitosan, were shown in Figure 4. For all examined hydrogel beads except those modified with 0.5% chitosan, significant early release of BSA was observed. This could be because of the heterogeneous distribution of BSA in the gel beads.[21,22] Diffusion and migration of BSA may

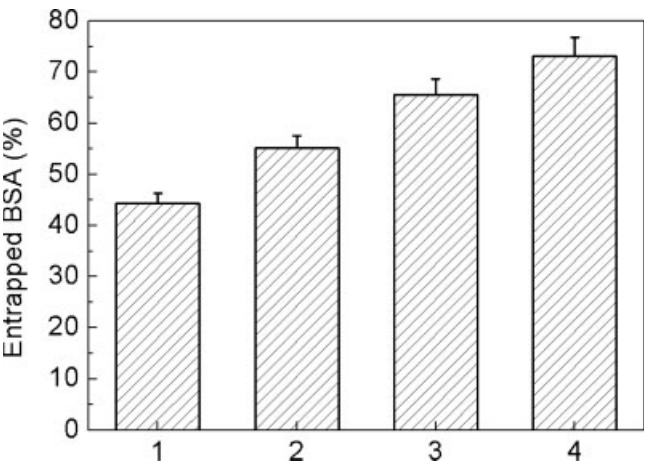

Figure 3 Percentage of BSA retained in various beads. (1) calcium-CMC beads; (2) calcium-CMC-chitosan (0.1%) beads; (3) calcium-CMC-chitosan (0.3%) beads; (4) calcium-CMC-chitosan (0.5%) beads.

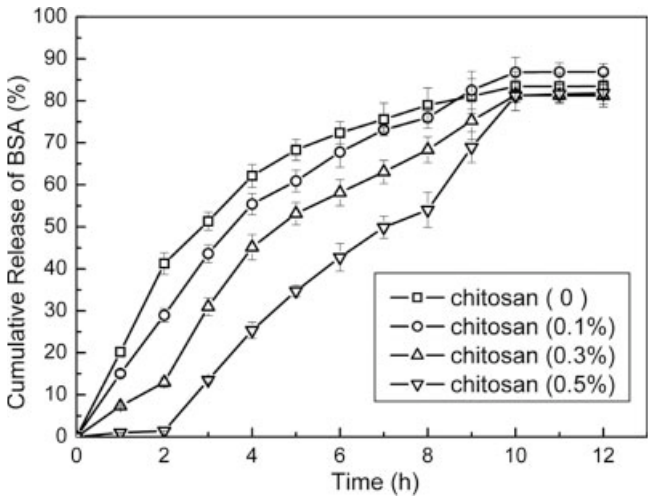

Figure 4 BSA release profiles from gel beads prepared in 5% (w/w) $CaCl_2$ solution with distinct concentrations of chitosan, determined by immersing dried test samples to swell in 0.1*M* HCl (pH 1.2) for 2 h and subsequently in 0.05*M* trisHCl buffer (pH 7.4) at 37°C.

occur during the drying process as water moved to the gel surfaces and evaporated. BSA may diffuse by convection with the water, leaving an uneven drug distribution across the gel, with higher concentrations at the surface, which leaded to early burst-like release as in Figure 4.

At pH 1.2, the cumulative release amount of BSA decreased apparently along with the increase of chitosan concentration in gelation medium. For example, 41% BSA was released in 2 h from the beads prepared in pure $CaCl_2$ solution, whereas only 1.4% BSA was released from the beads prepared in $CaCl_2$/chitosan (0.5%) gelation medium. Similarly, at pH 7.4, BSA release abided by the same trend mentioned above. The evident difference in the cumulative release amount of BSA from various beads may result from the influence of chitosan/CMC PEC membrane at the surface of calcium-CMC beads. That is to say, the PEC membrane limited the swelling of beads and reduced the release rate of BSA. Moreover, with the increase of chitosan concentration in gelation medium, the PEC membrane would become thicker, which restricted the BSA release to a greater extent.

A noticeable phenomenon is that a rapider BSA release happened between 8 and 10 h for any group of test beads. This is due to the fact of the disintegration of various beads from 8 to 10 h. Despite the protection of PEC membrane outside of beads, the disintegration still took place as observed in the swelling process of calcium-CMC beads (Fig. 2). Along with the disintegration of beads, BSA was released into the surrounding medium as much as possible. Subsequently, the cumulative release values of BSA became constant after 10 h for each group of beads.

CONCLUSIONS

In this study, spherical and solid calcium-CMC hydrogel beads were successfully prepared from low MW of CMC by ionically crosslinking with calcium ion. The beads exhibited pH-sensitivity. The entrapment efficiency of BSA in beads was greatly improved (from 44.4 to 73.2%) when the beads were coated with a chitosan/CMC PEC membrane. The PEC membrane limited the BSA release, yet BSA could still be fully released along with the disintegration of gel beads in pH 7.4 medium. Thus, the chitosan-coated calcium-CMC hydrogel beads with higher entrapment efficiency and proper protein release properties were a promising protein drug carrier for the site-specific release in the intestine.

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