Preparation of Porous Chitosan/Agarose Microsphere and Its R-Phycoerythrin Release Properties

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ABSTRACT: The chitosan microspheres (CS-CL) were prepared by suspension crosslinking method and used as carriers of R-phycoerythrin (R-PE). In this study, R-PE was loaded in the microspheres and released *in vitro*. The effects of pH value, temperature, ionic strength, and R-PE concentration on loading efficiency and release behavior were discussed. A novel microsphere that contained agarose (CS-AR MP) was prepared and the basic loading and releasing behavior for R-PE of this kind of new microspheres were also investigated. The results showed that all these chitosan microspheres have the ability to control-release R-PE. The addition of agarose may somewhat accelerate the release rate of R-PE from microspheres and reduce the capacity of adsorption for R-PE. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 2759–2766, 2007

Key words: chitosan microspheres; R-phycoerythrin; control-release; agarose

INTRODUCTION

Phycobiliproteins, including phycoerythin, phycocynin, allophycocyanin, and phycoerythrocyanin are the main light-harvesting chromoproteins existing in some algae such as Rhodophyceae, Cyanobacteria, Cryptophyceae, and some Pyrrophyceae.[1] They are water-soluble and are composed of α, β, and γ subunits (γ subunit appears only in phycoerythrin). Phycoerythin is the most stable of all phycobiliproteins, and its apoproteins carry two different, covalently bound tetrapyrrole prosthetic groups: phycoerythrobilin and phycourobilin; so, it normally has three absorption peaks or shoulders at 498, 535 (545), and 565 nm, and a fluorescence emission maximum at 580 nm.[2,3] Phycobiliproteins have been widely used in various fields such as natural food colorants, fluorescence probes called phycofluors in fluorescence immunoassays, fluorescence-activated cell sorting, and tumor photodynamic therapy.[4–6]

Photodynamic therapy (PDT) is an endoscopic treatment of cancers based on the photosensitization of neoplasms following the administration of a photosensitizer prior to laser light-induced tissue destruction. It is an evolving modality for the treatment of carcinomas and sarcomas. It has been used to treat many types of cancers, including colon, bladder, lung, esophageal as well as head and neck cancers.[7–9] PDT requires both light and light sensitive agents (also called photosensitizers) and acts in an oxygen-rich environment[10]; that is to say, this therapy involves the appropriate photosensitizer and irradiation with light of a particular wavelength, thereby initiating tumor necrosis presumably through formation of singlet oxygen. Usually, hermotoporphrin and its derivatives are selected as the photosensitizer in PDT. Recent studies[5,11] show that R-PE and its subunits have a better absorbent character and less phototoxicity by sunlight irradiation when compared with hemotoporin derivatives, and so they will probably substitute the photfrin as new kinds of PDT photosensitizer. The traditional route of administration of the photosensitizer is i.v. injection and oral. As R-PE is a kind of protein, it is easily degraded by the enzymes in the body. Thus, the problem faced at present is the development of suitable protein delivery devices.[12]

In recent years, bioadhesive and biodegradable polymers have gained considerable interest as auxiliary

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agents for the administration of peptide and protein drugs.[13–15] Degradable polymers have the advantage of performing their function and then degrading, thus obviating the need for their surgical removal. Chitin, one of this kind of polymers, is the most abundant natural polysaccharide on earth after cellulose and can be obtained from the exoskeleton of marine crustaceans such as crabs, lobsters, shrimps, and krill.[16] Because of its excellent properties such as nontoxicity, biocompatibility, mucus adhesion, biodegradation, and the ability to control-release drug, chitosan has been developed for a variety of biomedical applications, including wound dressings[17] and drug delivery systems.[18] In the last decade it has been proven that chitosan is a useful excipient, and it can be formed into microspheres with many kinds of drugs[19,20] and vaccines.[21]

Several methods, including suspension crosslinking,[22] spray drying,[23] and ion tropic gelation,[24] have been employed to fabricate chitosan microspheres. Among them, the suspension crosslinking technique is the most widely used. It is prepared by mechanical stirring method first, and then the droplets are solidified with crosslinking agent. The introduction of a second ingredient may increase their versatility in terms of the adsorption and delivery of proteins and their susceptibility to interact with biological surface. Agarose, a neutral polysaccharide, is a component of the agar, which has a large range of applications, especially in the manufacture of foodstuff, pharmaceuticals, cosmetic products, and also in biological and medical research.[25] To date, few studies have attempted to investigate chitosan–agarose hybrid beads.

In this study, we prepared a kind of chitosan/chitosan–agarose hybrid porous beads and investigated their ability to adsorb and release R-PE *in vitro*. The effects of pH value, temperature, ionic strength, and R-PE concentration on loading efficiency and release behavior are discussed. A novel microsphere, which contained agarose (CS-AR MP), was prepared and the basic loading and releasing behavior for R-PE of this kind of new microspheres were also investigated.

EXPERIMENTAL

Materials

Chitosan from shrimp shell was purchased from Qingdao Haihui biotechnology Co. (Shandong, China), with the deacetylation degree of 90% and an M_v (viscosity–average molecular weight) of 830,000 Da. Glutaraldehyde was obtained from Sigma–Aldrich (Germany). Agarose was supplied by Shanghai Yito Enterprise Co. All other agents used were of analytical reagent grade.

R-phycoerythin isolation

The R-phycoerythrin (R-PE) was extracted from the frozen *Polysiphonia urceolata* via a method described by Wang et al.[26] with a slight modification. The frozen *P. urceolata* was minced and kept at 4°C for 24 h to break the algal cell. Then the crude extract was centrifuged at 10,000 × *g* for 10 min at 4°C. Solid ammonium sulfate was then added to the supernatant to give a final concentration of 0.5 mol L^{-1}. The extract containing 0.5 mol L^{-1} ammonia sulfate was pumped into the column loaded with the STREAMLINE™ Phenyl as the expanded adsorbent upwardly. The adsorbent capturing the phycobiliproteins was washed upwardly with 0.5 mol L^{-1} ammonium sulfate to remove proteins loosely bound or unbound. Then 0.2, 0.1, and 0.05 mol L^{-1} of ammonia sulfate solutions were applied to the column at a speed of 5 mL min^{-1} successively to elute phycobiliproteins in the downward direction. All the eluates were collected and dialyzed against distilled water, and then pumped onto the ion-exchange column loaded with Q-Sepharose downwardly. After removing loosely bound or unbound proteins, 0.15 mol L^{-1} NaCl in 0.05 mol L^{-1} phosphate buffer (pH, 7.0) was pumped into the column to elute R-PE from the adsorbent till the effluent was clear. Then 0.2 and 0.4 mol L^{-1} NaCl in 0.05 mol L^{-1} phosphate buffer (pH, 7.0) were applied successively. The fractions whose purities (OD_{565}/OD_{280}) were more than 3.2 were collected and dialyzed against distilled water. Absorption spectrum was measured by a UV–vis spectrophotometer (Beckman DU 650). A fluorescence spectrometer F-4500 (HITACHI, Japan) was used to record fluorescence spectra of the purified R-PE. The purified R-PE was freeze-dried for later use.

Preparation of chitosan microspheres

Chitosan microspheres were prepared according to the suspension method mentioned in Ref. 22. A 20-mL 5% chitosan solution in 2% aqueous acetic acid was mixed with 20 mL of chain-extending agent—acetate ester, and the mixture was then added dropwise into 80 mL of toluene containing 2 mL of emulsifier (Span-80) in a 250-mL three-necked flask at room temperature. The suspension was stirred with a mechanical stirrer at 500 rpm for 30 min. Three milliliters of 50% glutaraldehyde was added into the suspension and the mixture was stirred for another 30 min at 40°C. Then, drops of 1 mol L^{-1} NaOH solution was gradually added to the flask until the pH of the mixture reached 9–10, and the stirring continued for a further 2 h at 70°C. Finally, the mixture of products was filtered and washed consecutively with acetone, ethanol, and

distilled water. The microspheres thus obtained were dried overnight in an oven at 60°C.

Preparation of chitosan–agarose microspheres

Chitosan solution was prepared as mentioned earlier and the temperature was raised to 60°C. A 5% agarose solution was obtained by dissolving 1 g of agarose into 20 mL of water under microwave heating for 1 min. The chitosan solution and the agarose solution were mixed together to obtain the water phase. Acetate ester (20 mL) was added as the chain-extending agent. The chemical crosslinking, washing, and drying processes and other experimental conditions were the same as those in the preparation of chitosan microspheres.

Characterization of chitosan microspheres

The morphological characterization of the chitosan microspheres was carried out with a scanning electron microscope (SEM, KYKY-2800B, China). About 10 mg of chitosan microspheres was dropped into a sample holder and placed in a vacuum oven at room temperature for 24 h to dry. The samples were coated with gold, and then scanning electron micrographs were obtained.

The Fourier transform infrared (FTIR) spectra were recorded as KBr disks on the Bio-Rad FTS 135 Fourier infrared spectrophotometer (USA). In a typical procedure, around 0.25 mg dry chitosan microspheres was thoroughly mixed with IR-grade KBr (0.1 g) (Merck) and pressed (10 ton) into tablet form, and the spectrum was then recorded.

R-PE loading

About 0.1 g of chitosan microsphere was suspended in 5 mL of R-PE aqueous PBS (phosphate-buffered solution). The suspension was kept at different temperatures of 4, 16, 20, 28°C for 6 h under shaking to load R-PE by adsorption. After a predetermined interval, the R-PE-loaded chitosan microspheres were separated and the amount of R-PE in the supernatant was determined using Beckman DU 650 UV–vis spectrophotometer via the absorbance at 565 nm. The loading efficiency (LE) for R-PE was calculated according to the following equation:

$$\mathrm{LE} = (C_0 - C_t)V/W \,(\text{mg R-PE/g microspheres}) \qquad (1)$$

In eq. (1), C_0 and C_t are the R-PE concentrations with respect to the R-PE solution before and after adsorption by chitosan microspheres. W is the dry mass of chitosan microspheres used in the adsorption experiments. The calibration curves and equations of absorbance versus R-PE concentrations up to 1.5 mg mL^{-1} in working condition were obtained by linear fit ($r^2 = 0.9992$). The effects of pH value, the ambient temperature, and the concentration of sodium chloride contained in PBS and R-PE concentration on LE were studied.

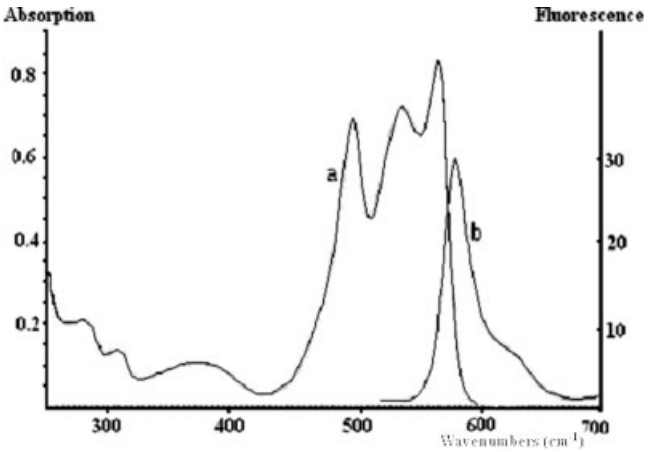

Figure 1 UV-absorption and fluorescence spectra of the purified R-PE solution: (a) UV-absorption spectra; (b) fluorescence spectra with an excitation wavelength at 498 nm.

In vitro R-PE release study

R-PE release from chitosan microspheres was studied in pH 7.4 PBS at 37°C using a shaking air bath (100 rpm). Accurately weighted chitosan microspheres loaded with R-PE were dispersed in 2 mL of release medium. The suspension (0.1 mL) was periodically removed and replaced by the same quantity of PBS. The amount of R-PE in the release medium was determined using Beckman DU 650 UV–vis spectrophotometer at 565 nm. The effects of various loading conditions of R-PE on release behavior and the influence of the presence of agarose on the release rate were studied.

RESULTS AND DISCUSSION

Isolation of R-PE

The absorption spectrum of R-PE is shown in Figure 1. This spectrum agreed well with the spectrum of purified R-PE published earlier.[26,27] Likewise, the ratio of A_{565}/A_{280} was larger than 3.5. The fluorescence emission maximum was at 580 nm with an excitation wavelength at 498 nm. This result was consistent with the earlier published data for the fluorescence spectrum of phycoerythrins.[27] We concluded that the purity of R-PE was up to the commonly accepted criterion.

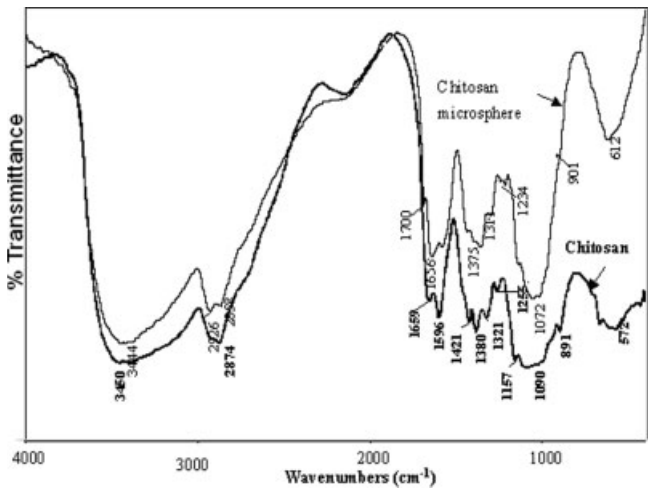

Figure 2 The FTIR spectra of chitosan and chitosan microspheres.

Preparation of chitosan microspheres

Figure 2 shows the FTIR spectra of chitosan and chitosan microspheres. The peaks at about 3450 cm^{-1} observed in the spectra correspond to stretching vibrations of hydroxyl group. The peak observed at 1659 cm^{-1} in the spectrum of chitosan can be ascribed to the amino group ($—NH_2$ deformation). This band disappears in the spectrum of chitosan microsphere and is replaced by a new band centered at 1656 cm^{-1} corresponding to the stretching vibrations of C=N bond. This peak indicates the formation of Schiff's base as a result of the reaction between carbonyl group of glutaraldehyde and amine group of chitosan chains.[28] Furthermore, the spectra also exhibit absorption band in the region from 2980 to 2830 cm^{-1}, which are due to the C—H stretching vibration of the polymer backbone. The stretch vibrations of C—O are found at 1090 and 1157 cm^{-1}. The little peak at 1700 cm^{-1}in the spectrum of chitosan microsphere shows the existence of impendent aldehydic group in chitosan microspheres.

The FTIR spectra of chitosan, agarose, and chitosan–agarose microspheres are shown in Figure 3. The spectral feature at $\sim$ 890 cm^{-1} is mainly associated with the C—H bending at the anomeric carbon in β-galactose residues.[29] The presence of a strong band in the FTIR spectra at 930 cm^{-1} is indicative of the occurrence of 3,6-anhydro-D-galactose.[30]

Morphology of chitosan microspheres

The chitosan molecule chains have abundant free amino groups, which can interact with glutaraldehyde to form chitosan microspheres. During the crosslinking and hardening process, acetate ester was extruded from the microspheres, which resulted in abundant holes left in the positions where acetate ester was present. Scanning electron micrographs of natural chitosan polymer, agarose, chitosan microspheres, and chitosan–agarose microspheres are shown in Figure 4. As can be seen, natural chitosan polymer and agarose formed networks and appeared to be slightly wrinkled, whereas chitosan microspheres and chitosan–agarose microspheres prepared by crosslinking with glutaraldehyde had a well-shaped spherical form with porous surface. Furthermore, the pores in the chitosan microspheres are larger than those in the chitosan–agarose microspheres.

R-PE loading onto chitosan microspheres

Drugs can usually be encapsulated in microspheres by dispersing them in the chitosan aqueous phase during the preparation of W/O (water/oil) suspension. However, as R-PE is a kind of protein with amino group, it will be crosslinked inside the microspheres when the chitosan microspheres were solidified by crosslinking method. Thus, in this study, the porous biodegradable chitosan microspheres were fabricated first and then used as vehicles for the controlled release of R-PE; that is, R-PE was immobilized in porous chitosan microspheres by being

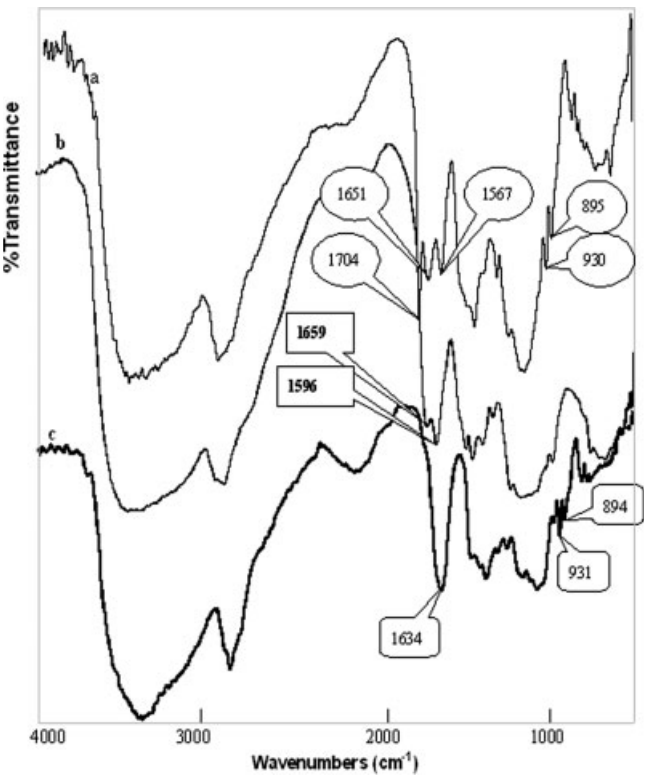

Figure 3 The FTIR spectra of chitosan (a), agarose (b), and chitosan–agarose microspheres (c).

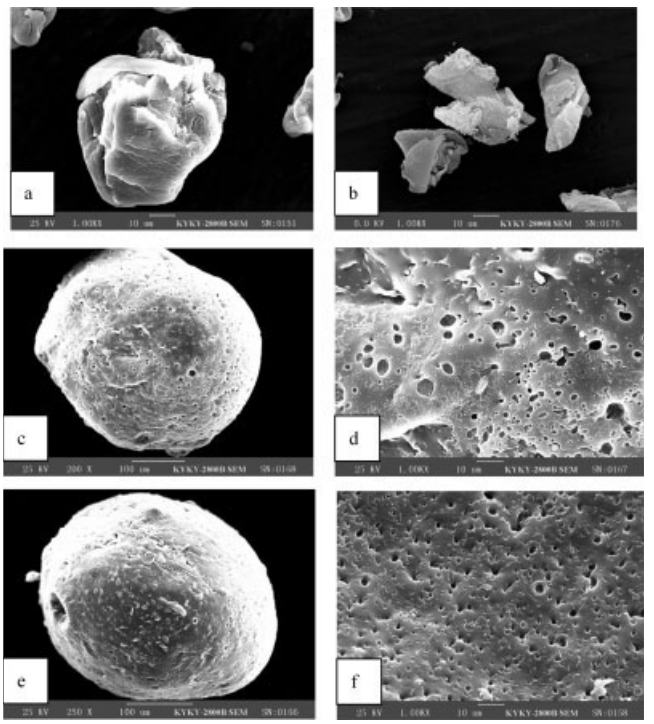

Figure 4 Scanning electron micrographs of chitosan, agarose, chitosan microspheres, and chitosan–agarose microspheres prepared by suspension crosslinking technique: (a) chitosan, (b) agarose, (c) chitosan microspheres, (d) surface of chitosan microspheres, (e) chitosan–agarose microspheres, and (f) surface of chitosan–agarose microspheres.

adsorbed into their pores instead of traditional gel entrapment methods.

Herein the chitosan microspheres were used to load R-PE in medium with different pH values, different R-PE concentrations, different ambient temperatures, and various ionic strengths (obtained by different concentrations of sodium chloride in PBS).

Figure 5 shows that the pH of liquid phase has an important effect on R-PE adsorption. The maximum R-PE adsorption is found at pH 3.59, which is close to the isoelectric point (IEP) of R-PE (pI 3.56).[31] Similar phenomena have also been observed by other researchers.[32,33] The reasons maybe as follows. First, the net electrostatic repulsion interaction between R-PE molecules and the microspheres is the smallest at R-PE IEP (pH, 3.56); thus the R-PE molecules are more readily adsorbed onto the microspheres. Second, the R-PE molecules may aggregate more easily because of hydrophobic interaction and reduced intramolecular and intermolecular electrostatic repulsion interactions at IEP. This also leads to stronger adsorption. Furthermore, the change of pH can cause the changes in secondary structure and conformation of R-PE, which also influences adsorption. Figure 6 shows that for a certain concentration (0.25 mg mL^{-1}) the R-PE absorbance is pH dependant. This difference

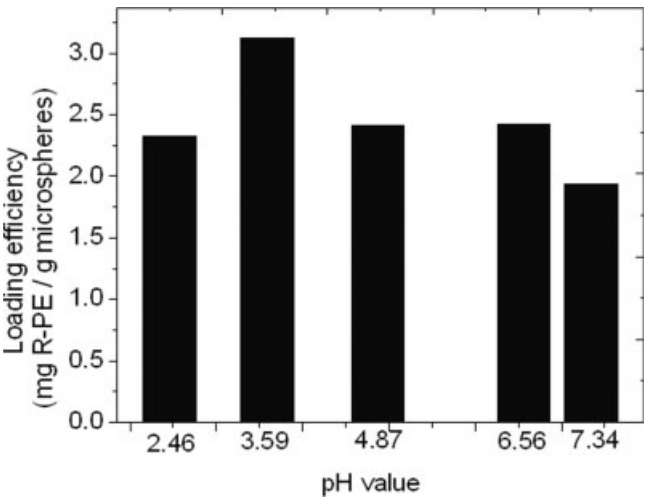

Figure 5 Effect of pH value on the R-PE loading efficiency onto chitosan microspheres.

in absorbance reflects the changes in secondary structure and conformation of R-PE. In addition, the highest absorbance at IEP of R-PE also reflects the major aggregation of R-PE.

Figure 7 shows the R-PE LE as a function of R-PE concentration in solution. When R-PE concentration was below 1.25 mg mL^{-1}, the LE enhanced with increase in R-PE concentration. However, little change was observed when R-PE concentration was higher than 1.25 mg mL^{-1}, which indicated that there existed a saturated adsorption.

Figure 8 shows the effect of temperature on the R-PE LE onto chitosan microspheres. The R-PE LE increased with the increasing of the temperature. This could be ascribed to the stronger intermolecular function caused by the higher temperature.

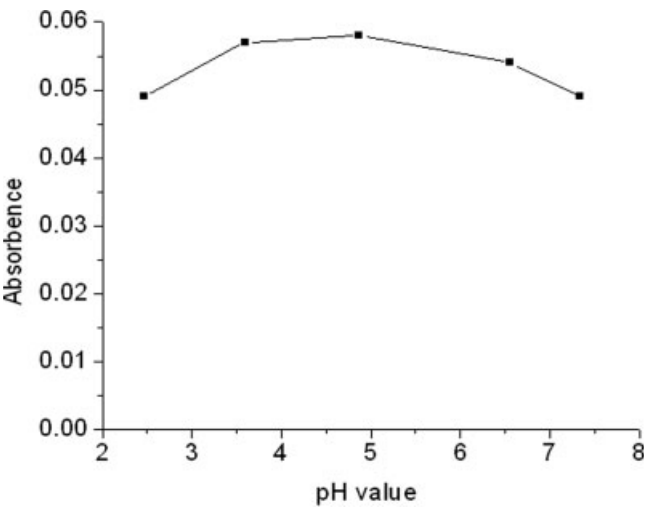

Figure 6 Influence of pH value upon the absorbance of the R-PE at 565 nm at the concentration of 0.25 mg mL^{-1}.

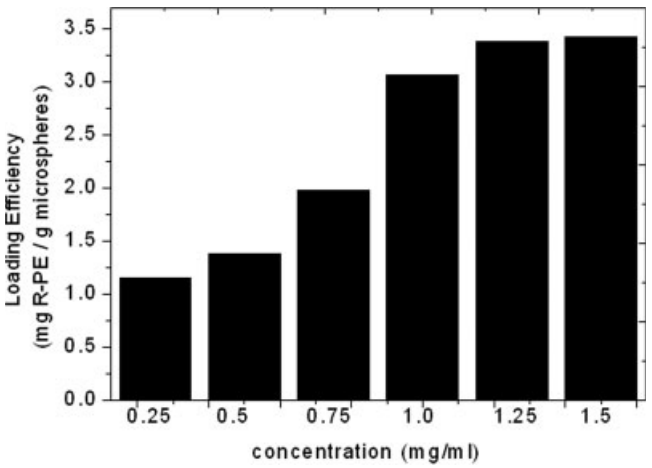

Figure 7 Effect of R-PE concentration on the R-PE loading efficiency.

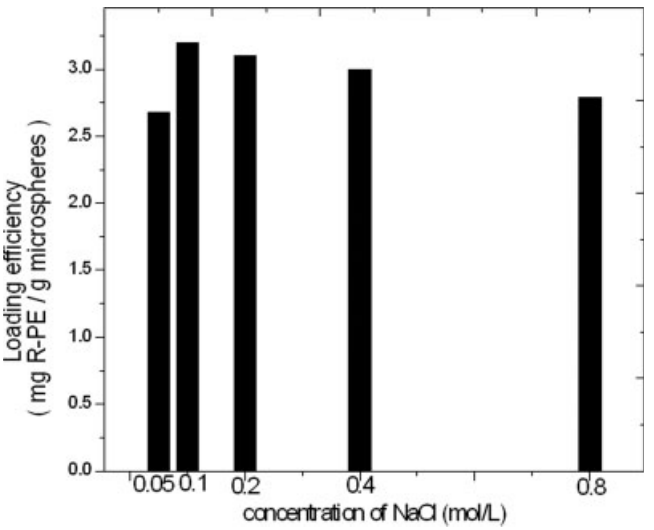

Figure 9 Effect of ionic strength on the R-PE loading efficiency onto chitosan microspheres.

As for the effect of ionic strength (Fig. 9), the capacity of the adsorption was slightly increased with the increasing concentration of sodium chloride and then decreased. The adsorption of R-PE in pH = 6.58 buffer containing NaCl at different concentrations is shown in Figure 9. An increase in R-PE adsorption is obtained with the increase in NaCl concentration from 0.05 to 0.1 mol L^{-1}; upon increase of the NaCl concentration above 0.1 mol L^{-1}, we can observe the decreased adsorption of R-PE. This indicates that the adsorption of R-PE is a charge control process. And the change in the R-PE conformation and local charge in chitosan microspheres may be responsible for the decrease in the adsorption amount. The increased adsorption of R-PE upon the NaCl concentration below 0.1 mol L^{-1} might be due to the construction of R-PE.[34]

The R-PE LE of chitosan microspheres decreased upon the addition of agarose in chitosan solution (Fig. 10). The lager pores in chitosan microspheres, compared with those in chitosan–agarose microspheres, should be responsible for this phenomenon. Another reason might be the intermolecular hydrogen bonds formed between the electronegative oxygen atom of agarose and the amino groups of chitosan in gel system as reported in the chitosan nanoparticles with the addition of PEG.[35] The acid group of R-PE and oxygen atom of agarose may compete in their action with chitosan amino groups,

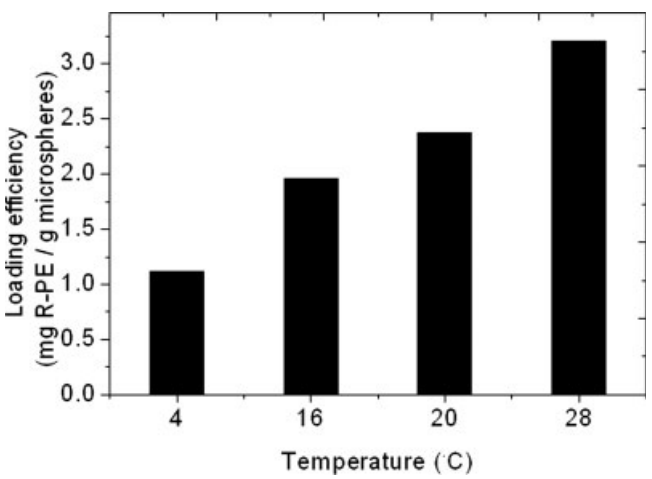

Figure 8 Effect of temperature on the R-PE loading efficiency onto chitosan microspheres.

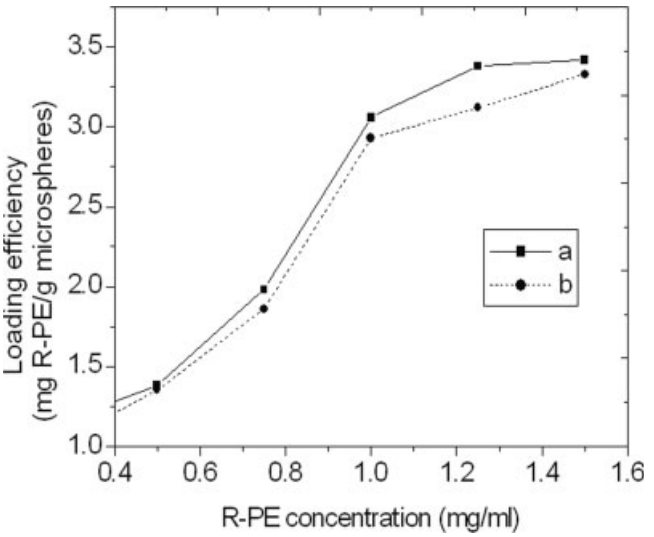

Figure 10 Influence of agarose presence in the chitosan microspheres: (a) CS-MP; (b) CS-AG MP.

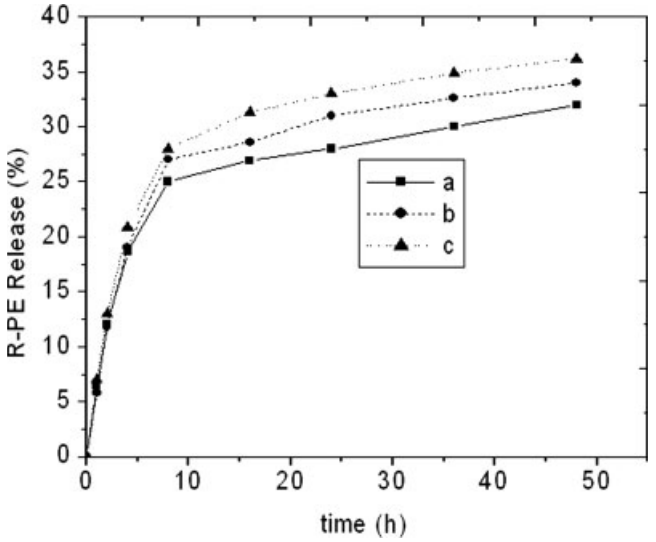

Figure 11 The release profile of chitosan microspheres and chitosan–agarose microspheres: (a) chitosan microspheres (LE, 1.98 mg R-PE/g microsphere), (b) chitosan–agarose microspheres (LE, 1.86 mg R-PE/g microsphere), and (c) chitosan microspheres (LE, 3.38 mg *R*-PE/g microsphere).

and so the possibilities of an interaction between the R-PE and the chitosan were reduced.

In vitro release profiles of R-PE

The R-PE *in vitro* release behavior of chitosan and chitosan–agarose porous microspheres is shown in Figure 11. The release profiles of chitosan and chitosan–agarose porous microspheres are similar. They all exhibit a burst release less than 30% in the first 10 h, and then slow release at a constant but a different rate. This indicates that the chitosan and chitosan–agarose porous microspheres both have the abilities to release R-PE in a sustained manner.

R-PE loading capacity is an important factor, as for release property of microspheres, the release rate is usually drug concentration gradient driven. Higher loading capacity leads to a wider concentration gap between the polymeric microspheres and the release medium, and causes a higher diffusion rate. As shown in Figure 11, R-PE release rate was influenced by the amount of R-PE entrapped; the high loading capacity provided a fast release rate. Different release rate between the chitosan microspheres with 1.98 and 3.38 mg R-PE/g microspheres of LE is attributed to R-PE concentration gradient [Figs. 11(a) and 11(c)]. The introduction of agarose chains with the chitosan molecules hinders the packed and rigid bonding between chitosan and R-PE; furthermore, the agarose cannot crosslink with glutaraldehyde. Relatively loose structure of microspheres contained R-PE, resulting in a high rate of R-PE release.

CONCLUSIONS

In this article, a hydrophilic natural polymer chitosan and agarose were used to form macroporous microspheres. The morphology of these microspheres and the raw materials are evaluated by a SEM. The chitosan microspheres were used to load R-PE by adsorption method. The R-PE LE was highest when pH value was 3.59 (close to its IEP). When R-PE concentration was below 1.25 mg mL^{-1}, the LE increased with enhancing of R-PE concentration. In addition, the R-PE LE was enhanced with the increasing temperature, and was little influenced by the ionic strength. The presence of agarose in the microspheres can somewhat slow down the loading capacity and accelerate the release rate of R-PE. Changing R-PE concentration and adding agarose to the chitosan microspheres can adjust the release rate of R-PE someway.

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