Carbohydrate Polymers 85 (2011) 674–680

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Genipin-cross-linked chitosan microspheres prepared by a water-in-oil emulsion solvent diffusion method for protein delivery

Jesada Karnchanajindanun, Mangkorn Srisa-ard, Yodthong Baimark*

Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahasarakham University, Mahasarakham 44150, Thailand

ARTICLE INFO

Article history:
Received 14 December 2010
Received in revised form 11 March 2011
Accepted 16 March 2011
Available online 25 March 2011

Keywords:
Chitosan
Cross-linking
Genipin
Emulsion solvent diffusion method
Microspheres
Protein delivery

ABSTRACT

Genipin-cross-linked chitosan microspheres containing model protein were prepared by a water-in-oil emulsion solvent diffusion method. Aqueous chitosan solution and ethyl acetate were used as water and oil phases, respectively. Bovine serum albumin (BSA) was used as a model protein. Chitosan solution was cross-linked with genipin before dissolving BSA and microsphere formation. Effects of BSA ratio and genipin-cross-linking on chitosan microsphere and BSA release characteristics were determined. BSA-loaded chitosan microspheres were spherical in shape. All BSA encapsulation efficiencies after rinsing the coated BSA were approximately 50–60%. The rinsing of coated BSA on the microspheres can reduce initial burst release effect. The cumulative release of BSA decreased as the BSA ratio decreased, and the genipin ratio and cross-linking time increased. This data indicated that protein release rate can be controlled by adjusting the protein ratio and the genipin-cross-linking.

1. Introduction

In the past few decades, appropriate polymeric matrices and techniques to prepare controlled protein drug release systems have been investigated. The polymeric matrix can protect protein drugs from proteolysis and antibody neutralization in the body (Lee & Yuk, 2007). A prolonged retention of protein drug activity *in vivo* could be obtained. For this purpose, chitosan microparticles have been widely prepared for use as protein delivery systems (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Amidi, Mastrobattista, Jiskoot, & Hennink, 2010; Gan & Wang, 2007; Sinha et al., 2004; Yuan et al., 2007).

Chitosan is a biocompatible and biodegradable natural polysaccharide that has been widely investigated for its potential in biomedical and pharmaceutical applications (Amidi et al., 2010; Kean & Thanou, 2010; Muzzarelli, 2010). Many methods have been reported for preparation of the chitosan microspheres (Agnihotri et al., 2004; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). However, a suitable method for fabricating the protein-loaded chitosan microspheres remains to be identified.

Genipin is a natural water-soluble bi-functional cross-linker. It is obtained from geniposide, a component of traditional Chinese medicine and is isolated from the fruits of the plant, *Gardenial jasminoides* Ellis. Genipin is a fully biocompatible reagent about 10,000 times less cytotoxic than glutaraldehyde (Nishi, Nakajima, & Ikada, 1995). A cross-linking reaction between chitosan and genipin has been proposed by Mi, Shyu, and Peng (2005). Chitosan device matrices have been successfully cross-linked with genipin (Muzzarelli, 2009).

In this paper, chitosan microspheres containing a model protein were prepared by the water-in-oil emulsion solvent diffusion method. The effects of protein loading content, BSA coated on microsphere surfaces and genipin-cross-linking on encapsulation efficiency and release behaviors of model protein were studied.

2. Materials and methods

2.1. Materials

Chitosan with a 100 kDa molecular weight and 85–90% degree of deacetylation was purchased from Seafresh Chitosan Lab Co., Ltd., (Thailand). Bovine serum albumin (BSA, fraction V, Acros Organics), ethyl acetate (AR, Lab Scan) and genipin (Challenge Bioproducts Co. Ltd., Taiwan) were used without further purification.

2.2. Preparation of BSA-loaded chitosan microspheres

A 0.5% (w/v) chitosan solution was prepared by using 2% (v/v) acetic acid solution as the solvent. BSA-loaded chitosan microspheres were prepared by the water-in-oil emulsion solvent diffusion method. BSA was dissolved in the chitosan solution before microsphere formation to load the BSA into the chitosan micro-

* Corresponding author. Tel.: +66 43 754246; fax: +66 43 754246.
E-mail address: yodthong.b@msu.ac.th (Y. Baimark).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.03.035

spheres. BSA/chitosan solution (0.5 mL) was added drop-wise into 100 mL of ethyl acetate with a stirring speed of 900 rpm for 1 h. The beaker was tightly sealed with aluminum foil to prevent evaporation of ethyl acetate during the emulsification–diffusion process. The chitosan microspheres suspended in ethyl acetate were recovered by centrifugation and dried in a vacuum oven at room temperature for 4 h. The BSA-free chitosan microspheres were also prepared with the same method for use as control. For cross-linked chitosan microspheres, the chitosan solution was cross-linked with genipin before dissolving BSA and fabricating microspheres. The effects of BSA/chitosan ratios (10, 25 and 50 wt%), genipin/chitosan ratios (5, 10 and 20 wt%) and cross-linking times (1.5, 3 and 6 h) were investigated.

2.3. Morphology and sizes of BSA-loaded chitosan microspheres

The morphology of the chitosan microspheres was investigated by scanning electron microscopy (SEM) using a JEOL JSM-6460LV SEM. The microspheres were coated with gold for enhancing conductivity before scanning. Average particle size and standard deviation (SD) were measured from several SEM images by counting a minimum of 100 particles using smile view software (version 1.02).

2.4. BSA encapsulation efficiency of chitosan microspheres

The BSA loading content (LC) and encapsulation efficiency (EE) of non-cross-linked microspheres was measured by dissolving in a 2% (w/v) acetic aqueous solution for 48 h under stirring. The weight of loaded BSA in clear supernatant was determined using the Bradford protein assay compared to BSA standard calibration curve. The percentages of actual LC (LC_{actual}), theoretical LC ($LC_{theoretical}$) and EE of the non-cross-linked microspheres were calculated from Eqs. (1)–(3), respectively.

$$LC_{actual}\ (\%) = \frac{\text{weight of loaded BSA}}{\text{weight of BSA} - \text{loaded microspheres}} \times 100 \quad (1)$$

$$LC_{theoretical}\ (\%) = \frac{\text{weight of feed BSA}}{\text{weights of feed BSA} + \text{feed chitosan}} \times 100 \quad (2)$$

$$EE\ (\%) = \frac{LC_{actual}}{LC_{theoretical}} \times 100 \quad (3)$$

The cross-linked microspheres could not be completely dissolved in acetic aqueous solution. The LC of the cross-linked microspheres was determined by subtracting the amount of coated BSA from the total loaded BSA. For this purpose, the cross-linked chitosan microspheres were suspended in phosphate buffer (1 mM; pH 7.4) at 37 °C with gentle shaking for 5 min to dissolve the coated BSA from the microsphere surfaces. The coated BSA-free microspheres were separated from the supernatant by centrifugation at 10,000 rpm for 10 min. The amount of BSA in clear supernatant was measured using the Bradford protein assay. The percentage of LC_{actual} of the cross-linked microspheres was calculated from Eq. (4).

$$LC_{actual}\ (\%) = \frac{\text{weight of loaded BSA} - \text{weight of coated BSA}}{\text{weight of BSA} - \text{loaded microspheres}} \times 100 \quad (4)$$

where weight of loaded BSA in Eq. (4) is calculated on the basis of LC_{actual} of non-cross-linked microspheres with the same BSA/chitosan ratio.

2.5. In vitro BSA release tests

In vitro BSA release tests of the BSA-loaded chitosan microspheres with and without the coated BSA were performed in phosphate buffer (1 mM; pH 7.4) at 37 °C. The coated BSA-free chitosan microspheres were obtained by immersing in phosphate buffer for 5 min before centrifugation and freeze-drying. The BSA-loaded chitosan microspheres (~15 mg) were dispersed in 1 mL of buffer solution. The suspension was gently shaken. At the desired times, 0.2 mL of the buffer solution was withdrawn after centrifugation at 10,000 rpm for 10 min. Fresh buffer (0.2 mL) was returned to the container to maintain a constant volume. BSA content in the supernatant was determined using the Bradford protein assay. Cumulative BSA release was calculated from the ratio of cumulative mass of BSA released from microspheres at a given time and total loading amount of BSA in microspheres. *In vitro* BSA release tests were tested in triplicate ($n = 3$).

3. Results and discussion

3.1. Morphology and sizes

In this research, the BSA-loaded chitosan microspheres were prepared via a water-in-oil emulsion solvent diffusion method without any surfactants. Emulsion droplets of the aqueous BSA/chitosan solution solidified during diffusion out of water into the continuous oil phase, ethyl acetate. This method has been successfully used to prepare microparticles of hydrophilic biodegradable polymers by our research group previously, such as silk fibroin (Baimark, Srihanam, Srisuwan, & Phinyocheep, 2010; Imsombut, Srisuwan, Srihanam, & Baimark, 2010) and chitosan (Kotsaeng, Karnchanajindanun, & Baimark, 2010; Karnchanajindanun, Srisaard, Srihanam, & Baimark, 2010). Influences of processing parameters and genipin-cross-linking have been evaluated. The chitosan microspheres with and without genipin-cross-linking were spherical in shape. The microsphere matrices with and without cross-linking contained a porous structure and were complete covered with a continuous surface (Karnchanajindanun et al., 2010).

The morphology and size of chitosan microspheres were investigated from SEM images, as shown in Figs. 1 and 2. The BSA-loaded non-cross-linked microspheres [Fig. 1(b)–(d)] were nearly spherical in shape similar to the plain microspheres [Fig. 1(a)]. This suggests that BSA loading did not affect microsphere shape. The BSA-loaded genipin-cross-linked chitosan microspheres with different genipin/chitosan ratios and cross-linking times were also nearly spherical in shape, as shown in Fig. 1(e)–(h).

The microphere surfaces were observed from the expanded SEM images. The microspheres with the highest feed BSA loading (50 wt% BSA/chitosan) had a smoother surface than the plain microspheres and the lower feed BSA/chitosan ratios, as shown in Fig. 2(a)–(d). However, the surfaces of BSA-loaded cross-linked microspheres were rougher than those of non-cross-linked microspheres [Fig. 2(e)–(h)]. The microsphere matrices were investigated through their broken surfaces, examples of which are shown in Fig. 3. It can be seen that the non-cross-linked microsphere matrices with and without BSA entrapment [Fig. 3(a) and (b)] showed a similar porous structure, resembling a sponge. The porous structure may be created during diffusion out of water from emulsion droplets to continuous phase and chitosan solidification process. The cross-linked microsphere matrices were denser than the non-cross-linked [Fig. 3(c) and (d)].

The average particle sizes obtained from several SEM images are summarized in Table 1. Average particle sizes varied between 80 and 100 μm. However, there were no significant differences between the average particle sizes from BSA entrapment and genipin-cross-linking.

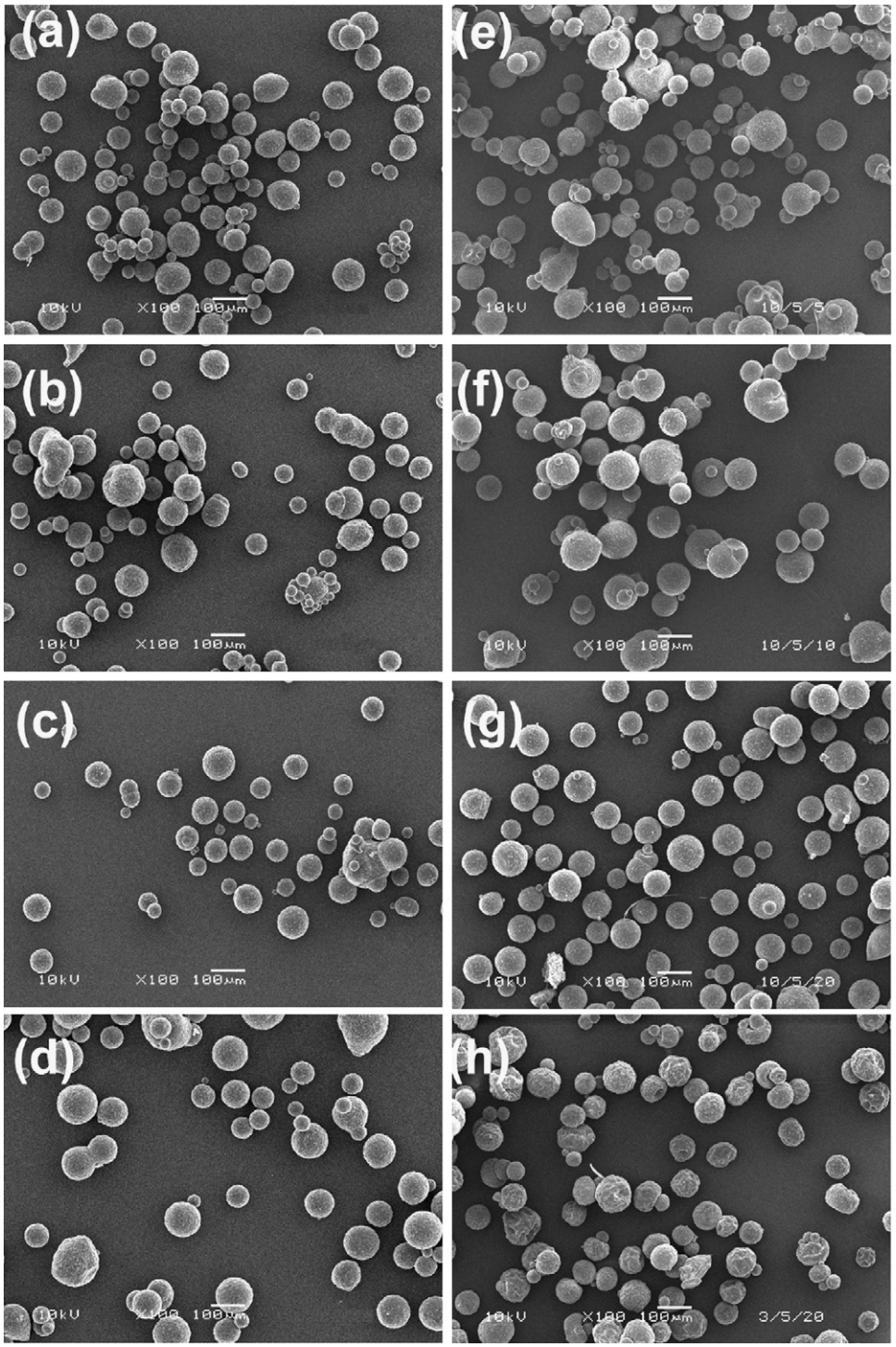

Fig. 1. SEM images of non-cross-linked microspheres loaded with (a) 0, (b) 10, (c) 25 and (d) 50 wt% BSA/chitosan, BSA/chitosan (50 wt%) microspheres cross-linked with (e) 5, (f) 10 and (g) 20 wt% genipin/chitosan for 6 h cross-linking time, and (h) BSA/chitosan (50 wt%) microspheres cross-linked with 20 wt% genipin/chitosan for 3 h cross-linking time. All bars = 100 μm.

3.2. BSA encapsulation efficiency

In our previous study, the BSA could solidify as microparticles via this W/O emulsion solvent diffusion method with approximately 100% yield. Therefore, the microspheres prepared from the BSA/chitosan blend solution through this method are the BSA/chitosan blend matrices. This estimates that the chitosan microspheres with different BSA loading contents could be prepared by adjusting the feed BSA ratio.

The percentage of BSA actual loading content (LC_{actual}) of non-cross-linked microspheres before rinsing the coated BSA were 7.6%, 18.2% and 29.3% for 10%, 25% and 50% BSA/chitosan microspheres, respectively. After the coated BSA was rinsed, the LC_{actual} was reduced to 5.2%, 11% and 18% for microspheres loaded with 10%, 25% and 50% BSA/chitosan ratios, respectively.

The LC_{actual} of the cross-linked chitosan microspheres was determined by an indirect method. The BSA coated on cross-linked microsphere surfaces was dissolved. The BSA content in clear

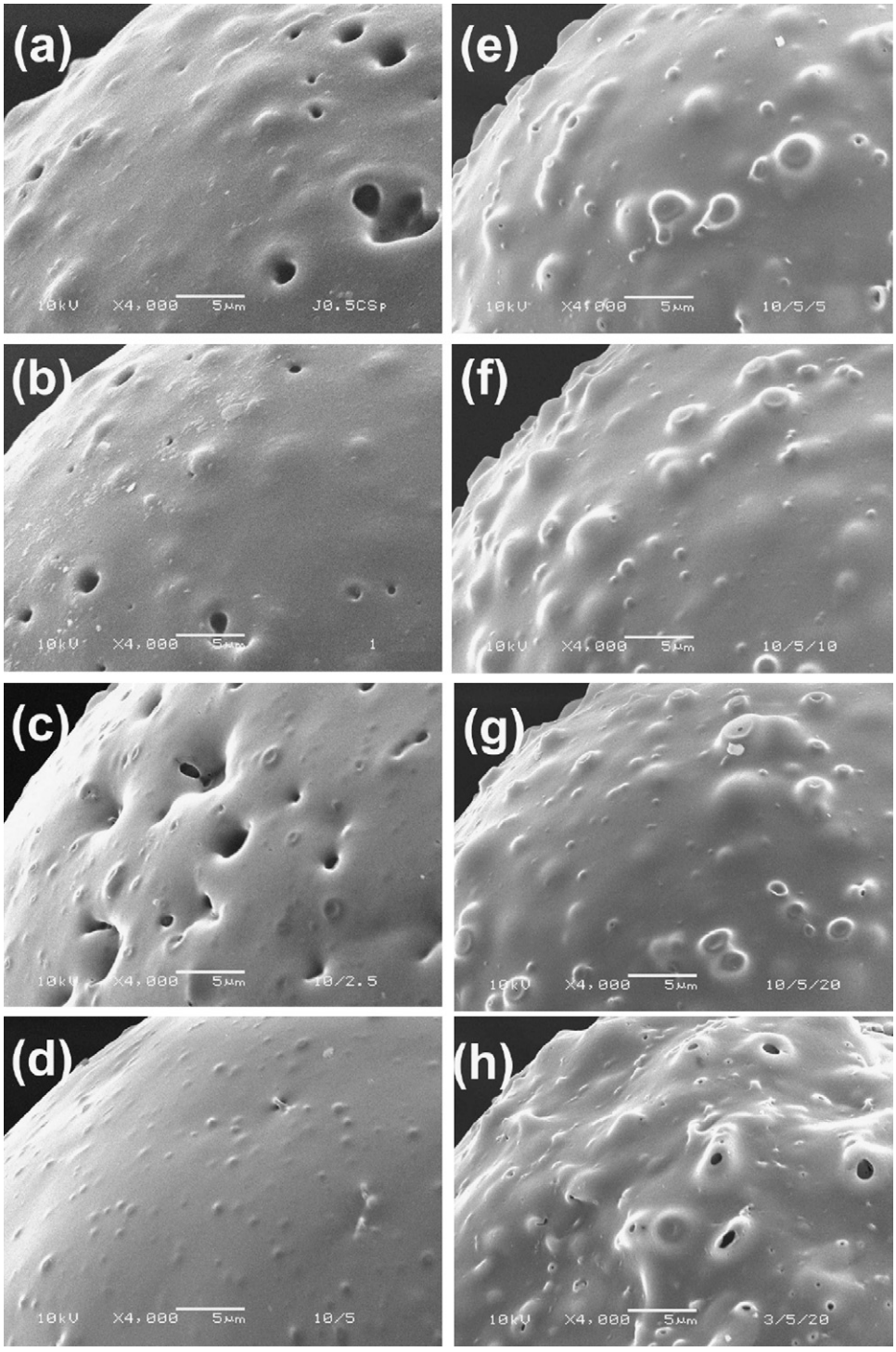

Fig. 2. SEM images of surfaces of non-cross-linked microspheres loaded with (a) 0, (b) 10, (c) 25 and (d) 50 wt% BSA/chitosan, BSA/chitosan (50 wt%) microspheres cross-linked with (e) 5, (f) 10 and (g) 20 wt% genipin/chitosan for 6 h cross-linking time, and (h) BSA/chitosan (50 wt%) microspheres cross-linked with 20 wt% genipin/chitosan for 3 h cross-linking time. All bars = 5 μm.

supernatant was determined. The surfaces of BSA-encapsulated chitosan microspheres, after dissolving the coated BSA are shown in Fig. 4 for the non-cross-linked microspheres prepared using 10 and 50 wt% BSA/chitosan ratios. The surface roughness increased slightly when the coated BSA was dissolved out. However, the particles were still nearly spherical in shape. The $\%LC_{actual}$ and %EE values are reported in Table 1. The $\%LC_{actual}$ increased steadily with the feed BSA ratio. Significant differences between the LC_{actual} values with regard to the genipin/chitosan ratio and the cross-linking time used for cross-linking were not observed. The LC_{actual} of both non-cross-linked and cross-linked microspheres are similar (18–19%) for the 50 wt% feed BSA/chitosan ratio (Table 1). All EE are in the range of 52–59%.

3.3. *In vitro* BSA release

The BSA release profiles were plotted between cumulative release of BSA and release time. Fig. 5 shows cumulative release

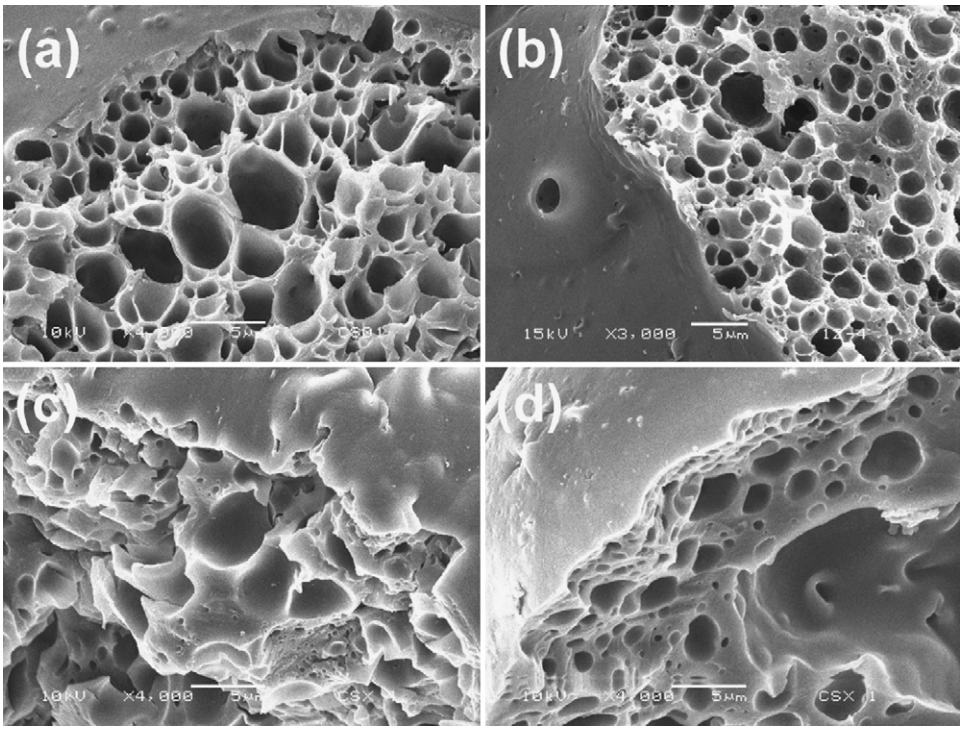

Fig. 3. SEM images of matrices of non-cross-linked microspheres loaded with (a) 0 and (b) 50 wt% BSA/chitosan, and BSA/chitosan (50 wt%) microspheres cross-linked with 20 wt% genipin/chitosan for (c) 3 and (d) 6 h cross-linking times. All bars = 5 μm.

Table 1
Average sizes and BSA loading of chitosan microspheres.

Chitosan microspheres			Average size[a] (μm) ± SD	LC_{actual}[b] (%)	EE[c] (%)
BSA/chitosan ratio (wt%)	Genipin/chito san ratio (wt%)	Cross-linking time (h)			
–	–	–	85 ± 12	–	–
10	–	–	90 ± 16	5.2	59
25	–	–	97 ± 18	11.0	54
50	–	–	98 ± 16	18.0	55
50	5	6	86 ± 17	18.5	56
50	10	6	94 ± 21	17.9	54
50	20	6	92 ± 15	17.2	52
50	20	1.5	81 ± 14	18.8	56
50	20	3	85 ± 14	18.1	54

[a] Determined from several SEM micrographs.
[b] BSA actual loading content calculated from Eqs. (1) and (4) for the non-cross-linked and cross-linked microspheres, respectively.
[c] BSA encapsulation efficiency calculated from Eq. (3).

of BSA from the non-cross-linked microspheres without rinsing the coated BSA. An initial burst release effect was observed during the first hour of drug release followed by slow release. There was a 29%, 36% and 49% release of BSA in the first hour for 10, 25 and 50 wt% BSA/chitosan microspheres, respectively.

The coated BSA-free chitosan microspheres were obtained by dispersing the BSA-loaded chitosan microspheres in phosphate buffer before centrifugation and freeze-drying. The effects of BSA loading content, genipin ratio and cross-linking time on the BSA release behaviors from the coated BSA-free microspheres are

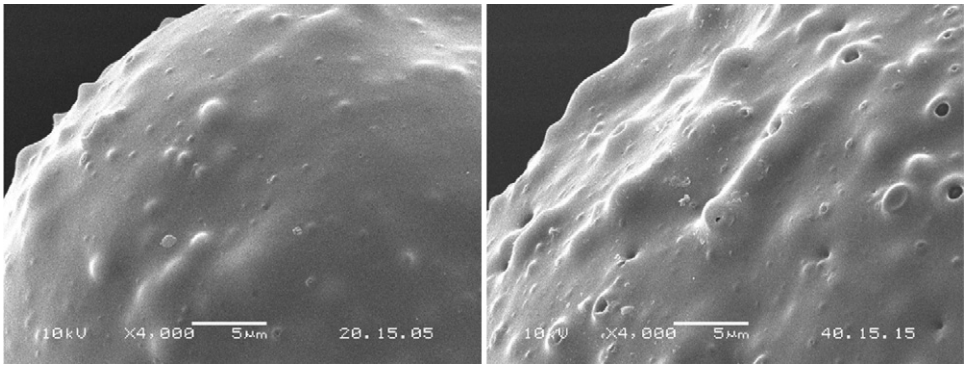

Fig. 4. SEM images of surfaces of non-cross-linked chitosan microspheres loaded with (left) 10 and (right) 50 wt% BSA/chitosan after rinsing the coated BSA. All bars = 5 μm.

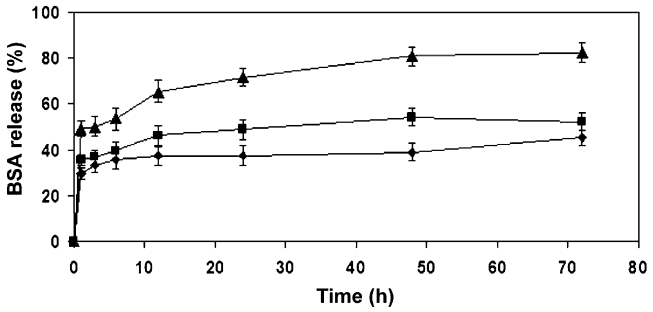

Fig. 5. Effect of (♦) 10, (■) 25 and (▲) 50 wt% BSA/chitosan on BSA release from non-cross-linked chitosan microspheres.

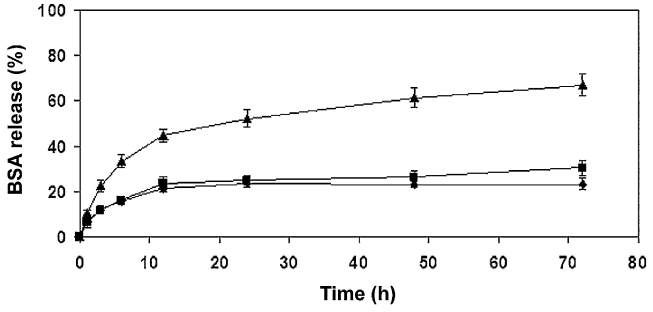

Fig. 6. Effect of (♦) 10, (■) 25 and (▲) 50 wt% BSA/chitosan on BSA release from coated BSA-free non-cross-linked chitosan microspheres.

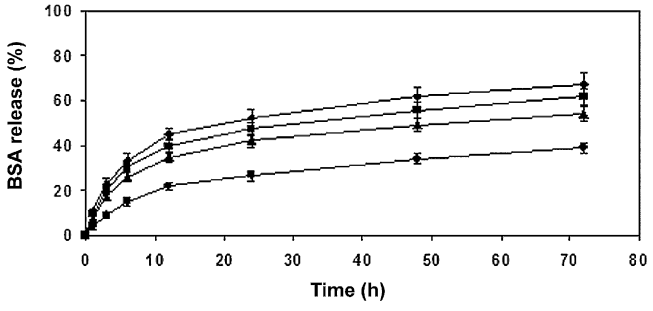

Fig. 7. Effect of (♦) 0, (■) 5, (▲) 10 and (•) 20 wt% genipin/chitosan on BSA release from coated BSA-free chitosan microspheres for 50 wt% BSA/chitosan and 6 h cross-linking time.

presented in Figs. 6–8, respectively. The coated BSA-free non-cross-linked microspheres in Fig. 6 showed lower initial burst release effect than those of the microspheres containing coated BSA in Fig. 5 for the same feed BSA/chitosan ratio. The results suggested that the initial burst release in Fig. 5 occurred due to immediate release of the coated BSA. It should be noted that the non-cross-linked microspheres loaded with 10 and 25 wt% BSA/chitosan ratios showed similar BSA release profiles. Whereas, the microspheres with 50 wt% BSA/chitosan ratio provided the fastest BSA release rate (Fig. 6). This may be explained by the extremely high amount of feed BSA (50 wt%) which could affect the properties of the chitosan polymeric network, thus affecting the diffusion barrier.

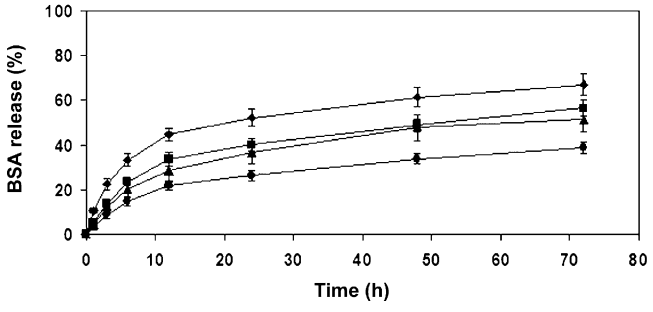

Fig. 8. Effect of (■) 1.5, (▲) 3 and (•) 6 h cross-linking times on BSA release from coated BSA-free chitosan microspheres for 20 wt% genipin/chitosan and 50 wt% BSA/chitosan compared with (♦) non-cross-linked microspheres.

As shown in Fig. 7, total cumulative release of BSA at 72 h decreased from 67% to 39% when genipin ratio was increased from 0 to 20 wt%. The total cumulative release of BSA at 72 h also decreased from 57% to 39% when the cross-linking time was increased from 1.5 to 6 h as shown in Fig. 8. This could be explained due to the network or cross-linked structures of chitosan molecules obtained from genipin-cross-linking which can reduce the release of BSA from chitosan microsphere matrices. The swelling ratio of chitosan microspheres decreased when the degree of cross-linking increased (Yuan et al., 2007). From our previous work, the %dissolution decreased significantly as the genipin ratio and cross-linking time increased (Karnchanajindanun et al., 2010). The results of BSA releasing demonstrated that BSA release from chitosan microspheres was controlled by BSA loading content, genipin ratio and cross-linking time.

4. Conclusion

This study has demonstrated that it was possible to prepare the BSA-loaded chitosan microspheres by using a single W/O emulsion solvent diffusion method. We underlined the variation of the BSA loading and *in vitro* release behavior. The influences of BSA content and genipin-cross-linking were studied. The resultant microspheres exhibited sustained release profiles over an extended period. The initial burst release effect can be reduced by rinsing the coated BSA. The cumulative BSA release decreased steadily as BSA loading content decreased and genipin ratio and cross-linking time increased. This approach to controlling the BSA release behavior from chitosan microspheres could be useful for the tailoring of protein drug delivery systems.

Acknowledgements

This work was supported by Mahasarakham University (fiscal year 2011), The National Metal and Materials Technology Center (MTEC), National Science and Technology Development Agency (NSTDA), Ministry of Science and Technology, Thailand (MT-B-52-BMD-68-180-G) and The Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education, Thailand.

References

Agnihotri, S. A., Mallikarjuna, N. N., & Aminabhavi, T. M. (2004). Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *Journal of Controlled Release*, *100*, 5–28.

Amidi, M., Mastrobattista, E., Jiskoot, W., & Hennink, W. E. (2010). Chitosan-based delivery systems for protein therapeutics and antigens. *Advanced Drug Delivery Reviews*, *62*, 59–82.

Baimark, Y., Srihanam, P., Srisuwan, Y., & Phinyocheep, P. (2010). Preparation of porous silk fibroin microparticles by a water-in-oil emulsification–diffusion method. *Journal of Applied Polymer Science*, *118*, 1127–1133.

Gan, Q., & Wang, T. (2007). Chitosan nanoparticle as protein delivery carrier-systematic examination of fabrication conditions for efficient loading and release. *Colloids and Surfaces B: Biointerfaces*, *59*, 24–34.

Imsombut, T., Srisuwan, Y., Srihanam, P., & Baimark, Y. (2010). Genipin-cross-linked silk fibroin microspheres prepared by the simple water-in-oil emulsion solvent diffusion method. *Powder Technology*, *203*, 603–608.

Karnchanajindanun, J., Srisa-ard, M., Srihanam, P., & Baimark, Y. (2010). Preparation and characterization of genipin-cross-linked chitosan microparticles prepared by water-in-oil emulsion solvent diffusion method. *Natural Science*, *2*, 1061–1065.

Kean, T., & Thanou, M. (2010). Biodegradation, biodistribution and toxicity of chitosan. *Advanced Drug Delivery Reviews*, *62*, 3–11.

Kotsaeng, N., Karnchanajindanun, J., & Baimark, Y. (2010). Chitosan microparticles prepared by the simple emulsification–diffusion method. *Particulate Science and Technology*, *28*, 369–378.

Lee, K. Y., & Yuk, S. H. (2007). Polymeric protein delivery systems. *Progress in Polymer Science*, *32*, 669–697.

Mi, F. –L., Shyu, S. S., & Peng, C.-K. (2005). Characterization of ring-opening polymerization of genipin and pH-dependent cross-linking reactions between chitosan and genipin. *Journal of Polymer Science Part A: Polymer Chemistry*, *43*, 1985–2000.

Muzzarelli, R. A. A. (2009). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*, 1–9.

Muzzarelli, R. A. A. (2010). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*, 167–182.

Nishi, C., Nakajima, N., & Ikada, Y. (1995). In vivo evaluation of cytotoxicity of diepoxy compounds used for biomaterial modification. *Journal of Biomedical Material Research*, *29*, 829–834.

Ravi Kumar, M. N. V., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews*, *104*, 6017–6084.

Sinha, V. R., Singla, A. K., Wadhawan, S., Kaushik, R., Kumria, R., Bansal, K., et al. (2004). Chitosan microspheres as a potential carrier for drugs. *International Journal of Pharmaceutics*, *274*, 1–33.

Yuan, Y., Chesnutt, B. M., Utturkar, G., Haggard, W. O., Yang, Y., Ong, J. L., et al. (2007). The effect of cross-linking of chitosan microspheres with genipin on protein release. *Carbohydrate Polymers*, *68*, 561–567.