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Carbohydrate Polymers 68 (2007) 561–567
Carbohydrate Polymers
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The effect of cross-linking of chitosan microspheres with genipin on protein release

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Received 6 May 2006; received in revised form 9 August 2006; accepted 31 October 2006
Available online 12 December 2006

Abstract

Genipin, a natural and non-toxic cross-linking reagent, was evaluated for its effects on the drug/protein release and swelling of chitosan microspheres. Chitosan microspheres, using albumin as a model protein, were prepared and cross-linked with 0.5 mM genipin for 4 to 16 h or for 4 h using 0.5 to 2.0 mM genipin. The degree of cross-linking, swelling and the release of albumin from the microspheres was determined by the ninhydrin assay, measuring change in mass between dry and wet spheres, and in 31-day elution tests, respectively. The degree of cross-linking increased up to maximum of 33% to 34% with up to 8 hour cross-linking time or with up to 1.0 mM genipin concentration. Additional cross-linking time or concentration did not significantly increase degree of cross-linking. Swelling ratios decreased significantly from 119.2% in the uncross-linked condition to 108.8% at 16 h cross-linking time. However, increasing the genipin concentration resulted in much smaller decreases in swelling. The release of albumin was reduced with as little as 4 h cross-linking time to 30.9% of uncross-linked microspheres for up to 24 days and by as much as 52.3–60.0% for up to 31 days with 8–16 h cross-linking time. Using genipin concentrations of 1.0 to 2.0 mM for 4 h, greatly reduced albumin release to only 12.4% to 27.1% on day 24. These data demonstrate that protein and drug delivery rates from chitosan microspheres may be controlled and extended by controlling the degree of cross-linking with genipin.

Keywords: Chitosan microsphere; Drug/protein delivery; Degree of cross-linking; Swelling ratio; Genipin

1. Introduction

Chitosan is a partially deacetylated polysaccharide obtained by alkaline treatment of chitin, one of the most abundant biopolymers in nature. Chitosan has been widely researched for biomedical applications such as wound healing, drug delivery systems, coatings and tissue engineering, as well as applications in food, cosmetics and agricultural industries (Bumgardner et al., 2003; Khor & Lim, 2003; Kumar, 2000; Martino, Sittinger, & Risbud, 2005; Senel & McClure, 2004). In drug delivery applications, chitosan has been used as a vehicle for drug, protein and gene delivery (Chen et al., 2004; Sinha et al., 2004). To increase the time frame for drug delivery, hydrophilic polymers such as chitosan need to be cross-linked (Jameela, Kumary, Lal, & Jayakrishnan, 1998). Several cross-linking reagents have been used for cross-linking chitosan such as glutaraldehyde, tripolyphosphate, ethylene glycol, diglycidyl ether and diisocyanate (Devi, Smitha, Sridhar, & Aminabhavi, 2005; Mi, Sung, Shyu, Su, & Peng, 2003; Neto, Dantas, Fonseca, & Pereira, 2005; Ngah, Ghani, & Kamari, 2005; Tomihata & Ikada, 1997). Dini et al. (Dini, Alexandridou, & Kiparissides, 2003) evaluated the cross-linking of chitosan microspheres containing hydrophilic drug, hydroquinone using glutaraldehyde. They reported that at 6, 12

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doi:10.1016/j.carbpol.2006.10.023

and 24.5 degrees of cross-linking, hydroquinone was released to 85%, 65% and 38% respectively as compared to uncross-linked microspheres after 300 min in PBS. Gupta and Jabrail (Gupta & Jabrail, 2006) also evaluated cross-linking of chitosan microspheres with glutaraldehyde as well as glyoxyal for the controlled delivery of centchroman, a non-steriodal contraceptive. In their study, 67.3% of the centchroman was rapidly released within 30 h from uncross-linked microspheres, while microspheres cross-linked with either 6% glutaraldehyde or 4% glyoxal significantly reduced initial 30–40 h burst release to 29.6% or 22.2%, respectively. Furthermore, they reported an extended and constant release of the drug from the cross-linked microspheres for an additional 60–70 h as compared to only 10 h for the uncross-linked microshperes. These studies demonstrated that drug release rates may be changed not only by the degree of cross-linking of the microspheres, but also by the type of cross-linker used. While these studies indicate promising results for controlling drug release from chitosan microspheres through cross-linking mechanisms, there are concerns over the toxicity of the cross-linking agents used, especially glutaraldehyde, which may impair the biocompatibility of the chitosan delivery system.

Genipin (Fig. 1) is obtained from geniposide, a component of traditional chinese medicine and is isolated from the fruits of the plant, *Gardenial jasminoides* Ellis. This natural cross-linking reagent is reported to be less toxic than glutaraldehyde and ideal for clinical usage (Sung, Huang, Huang, & Tsai, 1999). Sung et al. (1999) compared the cytotoxicity of genipin to glutaraldehyde in vitro using 3T3 fibroblasts via the MTT assay. Their results demonstrated that genipin was about 10,000 times less cytotoxic than glutaraldehyde. Moreover, the colony forming assay showed that the proliferative capacity of cells after exposure to genipin was approximately 5000 times greater than cells exposed to glutaraldehyde. In an in vivo rodent model, Mi et al. (Mi, Tan, Liang, & Sung, 2002) reported that chitosan microspheres cross-linked with genipin histologically exhibited better biocompatibility and slower degradation rate than glutaraldehyde cross-linked microspheres. These studies strongly indicate that the compatibility of the genipin is superior to glutaraldehyde.

Genipin has been used to cross-link chitosan membranes to control swelling ratio and mechanical propertites (Chen et al., 2004; Jin, Song, & Hourston, 2004; Mi, Shyu, & Peng, 2005; Mi, Tan, Liang, Huang, & Sung, 2001).

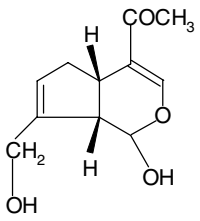

Fig. 1. Genipin chemical structure.

A novel pH-sensitive hydrogel of *N*,*O*-carboxymethyl chitosan (NOCC) and alginate cross-linked by genipin for protein drug delivery has been reported by Chen et al. (Chen et al., 2004). The swelling ratio and the amount of BSA released from the genipin-cross-linked NOCC/algine hydrogel were different at various pH. The amount of BSA released at pH 1.2 was relatively low (20%), while that released at pH 7.4 increased significantly (80%). These results clear suggest that the genipin-cross-linked NOCC/alginate hydrogel may be a suitable polymeric carrier for site-specific protein drug delivery in the intestine. However, studies on the release of drug or protein from chitosan microsphere cross-linked by genipin have not been reported. This is important because many drug delivery systems use microsphere forms as the basis of their delivery system.

In this study, bovine albumin as a model protein was mixed with chitosan to make microspheres cross-linked by genipin. The effect of cross-linking time and genipin concentration on swelling ratio, the degree of cross-linking and the elution of albumin from the microspheres was evaluated. Chitosan microspheres have shown much potential in minimally invasive applications for local delivery of therapeutic agents and in composites for combined tissue engineering and drug delivery systems (Chesnutt et al. 2006; Utturkar et al. 2006).

2. Materials and methods

2.1. Materials

Chitosan with 87.4% degree of deacetylation (DDA), 4.66×10^5 Dalton molecular weight, $2.46 \pm 0.02\%$ ash residual and 0.80 ± 0.13 mg/g protein content was purchased from Vanson HaloSource (Redman, WA). ImmunO bovine albumin fraction V (albumin) was purchased from MP Biomedical Inc. (Aurora, OH). Genipin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were reagent grade.

2.2. Preparation of microspheres

Plain chitosan microspheres were made using a solution of 3.5% chitosan in 2% acetic acid dripped via a syringe pump into NaOH: methanol: water (20:30:50 wt%) solution with stirring. Microspheres were rinsed with deionized water to pH <8 and then cross-linked in 0.5 mM genipin solution at room temperature for 4, 8 and 16 h or in 0.5, 1.0 and 2.0 mM genipin solutions for 4 h (approximate 0.28 g microspheres in 40 mL genipin solution). After cross-linking, microspheres were rinsed with DI water, dried at room temperature to remove majority of moisture before finally drying under vacuum for 4 h at 40 °C.

The chitosan microspheres containing albumin were made by adding 1 wt% albumin to the 3.5% chitosan in 2% acetic acid solution; the chitosan–albumin solution

was dripped via syringe pump into NaOH–methanol bath, and then cross-linked as described above.

2.3. Determination of the degree of cross-linking

The degree of cross-linking of the plain chitosan microspheres was determined by ninhydrin (NHN) assay (Mi et al., 2001). The assay determines the percentage of free amino groups remaining in the chitosan microspheres after cross-linking. Ninhydrin solution was prepared as follows: Solution A: 1.05 g citric acid, 10 mL (1.0 M) NaOH and 0.04 g $SnCl_2 \cdot 2H_2O$ were mixed, then deionized H_2O added until 25 mL; Solution B: 1 g ninhydrin was added to 25 mL ethylene glycol monomethyl ether. The two solutions A and B were combined and stirred for 45 min, then stored in dark bottle. For the assay, the microspheres were lyophilized for 24 h and then weighed. A 1.5 mg lyophilized sample was heated to 100 °C in water bath with 1 mL ninhydrin solution for 20 min. The solution was cooled down to room temperature, diluted with 5 mL 50% isopropanol, and then the optical absorbance of the solution at 570 nm was read with a spectrophometer (UV, Agilent 8453). The amount of free amino groups in the test sample, after heating with ninhydrin, is proportional to the optical absorbance of the solution. The concentration of free NH_2 groups in the sample is determined from a standard curve of glycine concentration vs absorbance. The concentration measured is divided by sample weight, and multiplied by the sample molecular weight to obtain the mole NH_2/mole sample. The degree of cross-linking of sample is then calculated following the equation:

$$\text{Degree of cross-linking} = \left[(\text{NHN reactive amine})_{\text{fresh}} - (\text{NHN reactive amine})_{\text{fixed}}\right] / \left(\text{NHN reactive amine}\right)_{\text{fresh}} \times 100$$

Where 'fresh' is the mole fraction of free NH_2 in non-cross-linked samples and 'fixed' is mole fraction of free NH_2 remaining in cross-linked samples. Three samples of each type of microspheres were evaluated.

2.4. Swelling ratio study

The swelling ratio of plain microspheres was determined by immersion in a phosphate buffered saline (PBS) (pH 7.4) at room temperature for 24 h with gentle shaking. Subsequently, the weight of the swollen microsphere (W_{sw}) was measured and the swelling ratio (E_{sw}) was calculated according to equation as follows:

$$E_{sw} = [(W_{sw} - W_0)/W_0] \times 100,$$

where E_{sw} is the swelling ratio of the microsphere, W_0 is microsphere initial dried weight and W_{sw} is the weight of the swollen microsphere. Swelling ratio was determined using tripicate samples of each type of plain microsphere at different cross-linking time or concentration.

2.5. Determination of initial albumin loading in chitosan microspheres

The chitosan albumin and plain chitosan microspheres were dissolved with 1 wt% acetic acid- 0.3 M sodium acetate buffer to make 5 mg/mL chitosan albumin and chitosan solution. Protein was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Using a standard curve of albumin in sodium acetate buffer vs absorbance, the amount of albumin per gram chitosan microsphere was then calculated. The plain chitosan microsphere solution was used as absorbance background control.

2.6. The elution test of protein

The elution of albumin was characterized by placing groups of 0.2 g chitosan microspheres with albumin ($n = 3$) in 2.5 mL PBS at 37 °C with gentle shaking. At day 1, 2, 3, 7, 17, 24 and 31, the PBS solution was changed and the amount of protein in the eluate was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The protein detection limit of the BCA assay is 25 μg/mL. Data were plotted as accumulated amounts of μg albumin/g chitosan over time. Four specimen of each type of cross-linked microspheres were tested in elution.

2.7. Statistical analysis

Analysis of variance (ANOVA) was performed to determine statistical differences in degree of cross-linking, swelling and protein release due to the genipin treatments. If significant differences were indicated ($p < .05$), Student–Newman–Keuls and Tukeys tests were performed.

3. Results and discussion

3.1. Chitosan microspheres

Chitosan microspheres made using the syringe pump and re-precipitation method exhibited uniform spherical shape. Diameter of the microspheres was 0.8 to 0.9 mm as shown in Fig. 2(a). This size microsphere was used since it has shown promise in making 3D structures for bone tissue engineering and in composites for the controlled delivery of antibiotics and growth factors (Chesnutt et al., 2006; Utturkar et al., 2006).

3.2. Cross-linking degree

In this study, genipin, a natural and non-toxic cross-linking reagent, was used to cross-link chitosan. It was observed that the color of the genipin cross-linked chitosan microcrospheres turned dark-bluish (Fig. 2b). The color was deepened with the increase of genipin concentration or cross-linking time. Bluish color attributed to double bonds in the genipin cross-linking molecules.

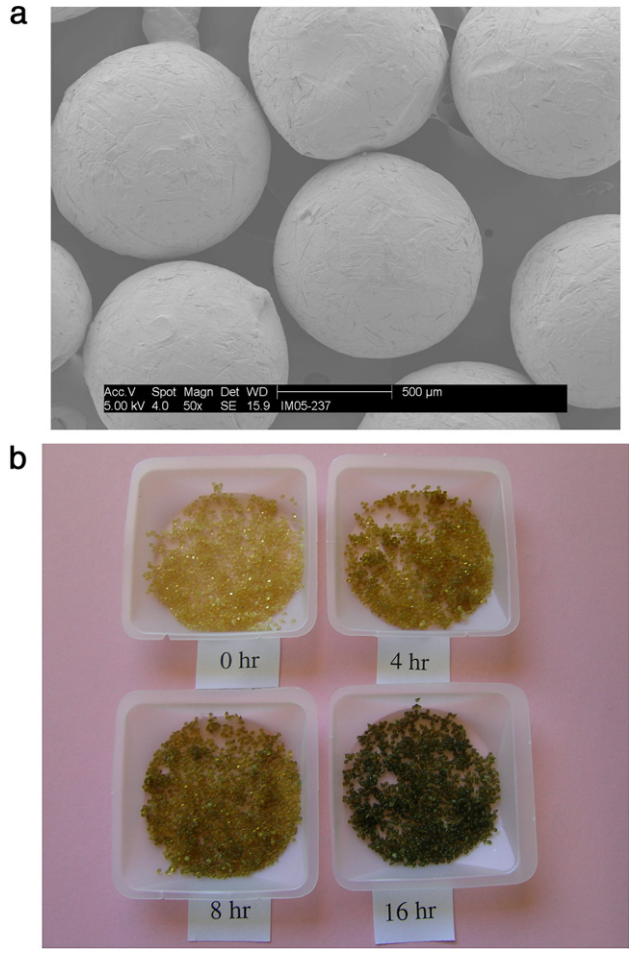

Fig. 2. (a). SEM image (50×) of chitosan microspheres. (b) Photomicrograph of chitosan microspheres cross-linked with genipin after 0, 4, 8 and 16 h. Microspheres exhibit an increase in bluish color with increasing cross-linking time due to the double bonds of the genipin cross-linking molecule.

As shown in Fig. 3(a), under the same genipin concentration (0.5 mM), the cross-linking degree increased with increasing cross-linking time. The cross-linking degree significantly increased from 4 to 8 h cross-linking time ($p < .05$), but not from 8 to 16 h. Since the degree of cross-linking changed significantly from 4 to 8 h at 0.5 mM genipin concentration, 4 h cross-linking time was selected in order to observe the effect of genipin concentration on degree of cross-linking. It is also shown in Fig. 3(b) that the cross-linking degree increased with increasing genipin concentration at the 4 hours. The cross-linking degree was significantly increased when genipin concentration increased from 0.5 to 1.0 mM ($p < .05$), but not from 1.0 to 2.0 mM genipin solution. The reaction mechanism of chitosan with genipin has been studied (Mi et al., 2005). It was found that genipin may undertake a ring-opening reaction to form an intermediate aldehyde group due to the nucleophillic attack by the amino groups in chitosan. The genipin molecules reacting with a nucleophilic reagent such as chitosan may further undergo polymerization to form a cyclic cross-linking structure. The maximum degree of cross-linking of 33–34% did not increase much after 8 h cross-linking at 0.5 mM genipin solution or when genipin concentration increased from 1.0 to 2.0 mM. It is hypothesized that this is due to the outer layers of microspheres being cross-linked, thereby limiting cross-linking of inner layers. Examination of the cross-section of microspheres at 0 and 16 h cross-linking times via stereo zoom microscopy (Spot RT Color camera and software V 3.0, DIAGNOSTIC Instruments Inc., Sterling Heights, MI) revealed that the un-cross-linked microsphere (Fig. 4(a)) was transparent, while microsphere cross-linked for 16 h (Fig. 4(b)) showed the dark-bluish color only on the outer edges (arrows indicated). These observations support the hypothesis that cross-linking occurred only in the out layers of the microspheres. Mi et al. (Mi et al., 2001) used 0.5 mM genipin solution for 6 hours to cross-link chitosan film and achieved only 9.9% degree of cross-linking. This is reasonable since microspheres have more surface area to be cross-linked than films. Nevertheless these data demonstrate that chitosan microspheres is cross-linked by genipin and the degree of cross-linking is increased with increasing concentration of genipin or cross-linking time.

3.3. Swelling ratio

Chitosan is readily hydrated in water since it contains hydroxyl and amino groups. After cross-linking, the hydro-

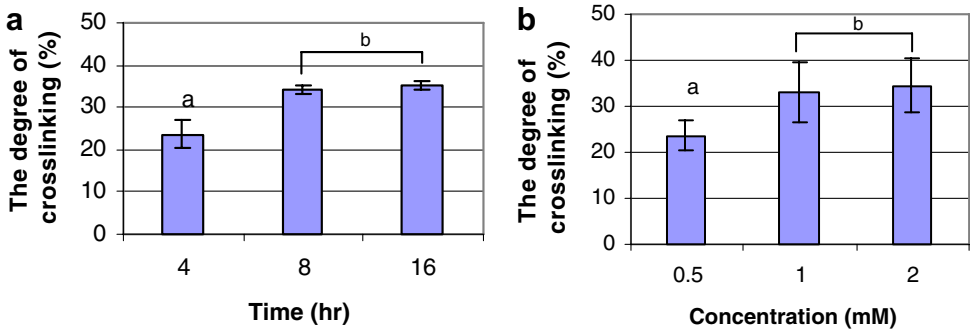

Fig. 3. (a) The degree of cross-linking chitosan microsphere by genipin of fixed concentration (0.5 mM) but at different times (4, 8 and 16 h); (b) fixed time (4 h) but different concentration (0.5, 1.0 and 2.0 mM). Cross-linking increased significantly from 4 to 8 h and from 0.5 to 1.0 mM concentrations. Addition time or increasing concentration did not increase degree of cross-linking ($P < 0.05$). Bars link statistically similar group. Letters indicate statistically different group.

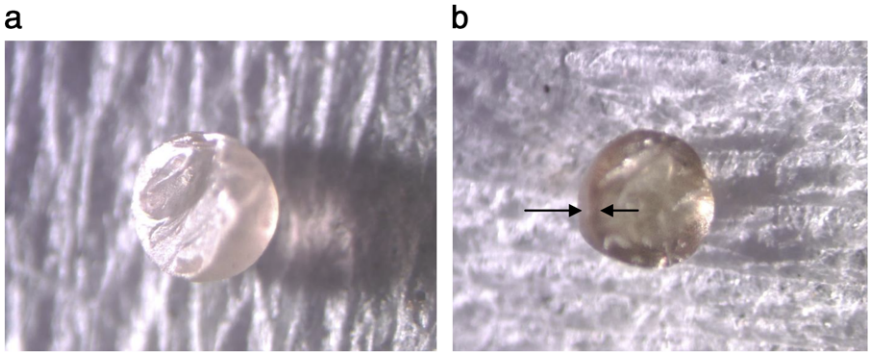

Fig. 4. The images (4× magnification) of cross-section of (a) plain un-cross-linked chitosan microspheres and (b) 16 h cross-linked chitosan microspheres under stereo microscopy. Plain un-cross-linked chitsoan microspheres remained clear and colorless while a distinct zone of dark color is apparent on the outer edge of cross-linked microspheres (arrows highlight the colored zone) indicating that only the outer layers of the microspheres were cross-linked.

philicity will be changed. It is shown in Fig. 5(a) that the swelling ratios of chitosan microspheres significantly decreased with increasing cross-linking time. The swelling ratio did not change much when using different genipin concentrations (Fig. 5(b)). These results are similar to a previous report in which swelling of films decreased with increasing genipin concentrations up to 2.5 mM but not at higher concentration (Mi et al., 2001). The reason higher concentration of genipin do not decrease swelling of microspheres more may be that the genipin could react and cross-link only with the outer layers. For microspheres reacted with different genipin concentrations, swelling ratio did not change much due to its short reaction time. The cross-linking can change the chitosan microsphere swelling ratio. It is known that, as the degree of polymer cross-linking decreases, the density of the polymer network also decreases. Consequently, as the available free space for drug diffusion increases, the rate of drug release also increases (Bachtsi & Kiparissides, 1995). On the contrary, an increase of the degree of polymer cross-linking increases polymer density and decreases available free space for drug diffusion which results in a decrease in drug release rates.

3.4. Elution test of chitosan microsphere with albumin

The initial amount of albumin trapped in chitosan microspheres was 4.79 ± 2.90 μg/mg chitosan. The results of elution test of chitosan microsphere with albumin cross-linked with genipin were shown in Figs. 6 and 7. From Fig. 6, albumin release was less from cross-linked microspheres than that from the non-cross-linked microspheres over the entire 31 day test period. After one day, the amount of the released albumin from plain chitosan microspheres was more than twice that of 4 h cross-linked microspheres. The amount of albumin released from microspheres decreased with increasing cross-linking time. After 31 days, 75%, 63%, 45% and 39% of the albumin was released from microsphere cross-linked for 0, 4, 8 and 16 h, respectively (Table 1) based initial loading values. It can be found that the microspheres with a similar degree of cross-linking (4 h at 1.0 mM genipin vs. 4 h at 2.0 mM genipin) result in a different amount of albumin released. The reason may be that at the higher concentration, more genipen was available to react with the NH_2 groups of albumin as well as those of the chitosan. This additional reaction would result in lower amounts of albumin release since the albumin molecules may have been cross-linked with each other and or to the chitosan polymer.

From Fig. 7, the release of albumin was not detected from day 1 to day 17 for chitosan–albumin microspheres cross-linked with 1.0 and 2.0 mM genipin. Albumin may have been released but was below detection limit of the BCA assay. Albumin was detected after 24 days. The reason for this may be the genipin reacted not only with

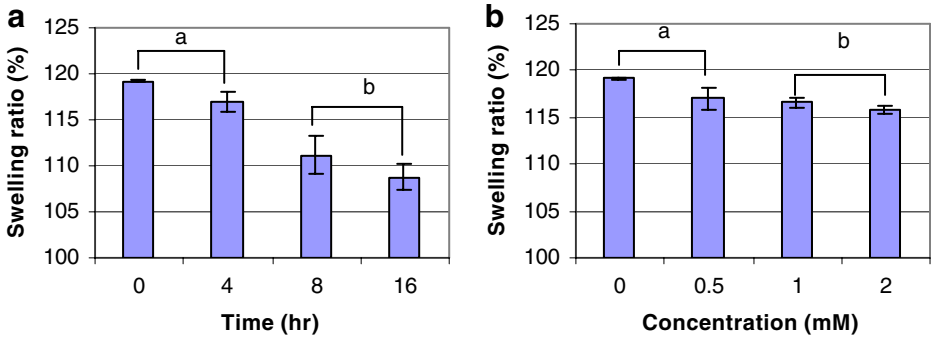

Fig. 5. (a) Swelling ratio of the chitosan microsphere after cross-linking by genipin at fixed concentration (0.5 mM) but at different time (4, 8 and 16 h) and (b) at fixed time (4 h) but different concentration (0.5, 1.0 and 2.0 mM). Swelling decreased with increasing cross-linking time, but effect of concentration was less ($P < 0.05$). Bars link statistically similar group. Letters indicate statistically different group.

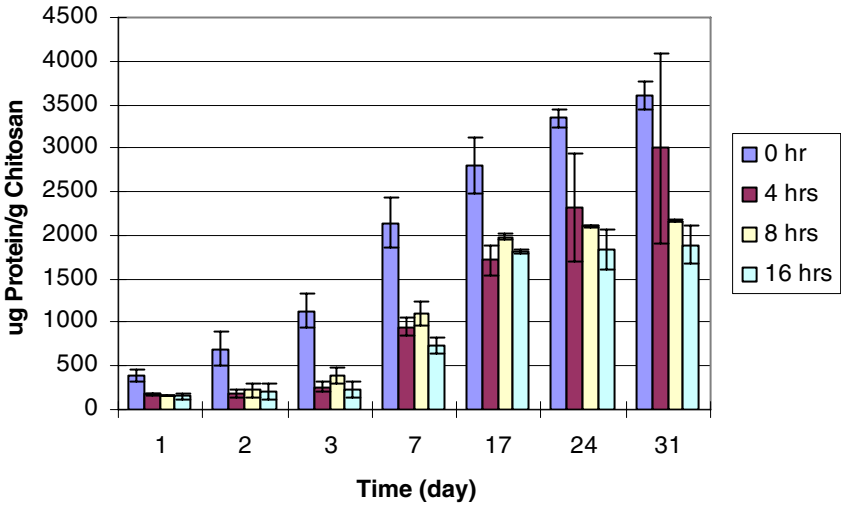

Fig. 6. The accumulated release profile of albumin from chitosan–albumin microspheres after cross-linking with 0.5 mM genipin for 4, 8 and 16 h. On day 31, albumin release from 0.5 mM genipin cross-linked 16 h microspheres were significantly different from that of plain microspheres ($p < 0.05$). Differences in albumin release from 0.5 mM genipin cross-linked for 4, 8 and 0 h were not significant.

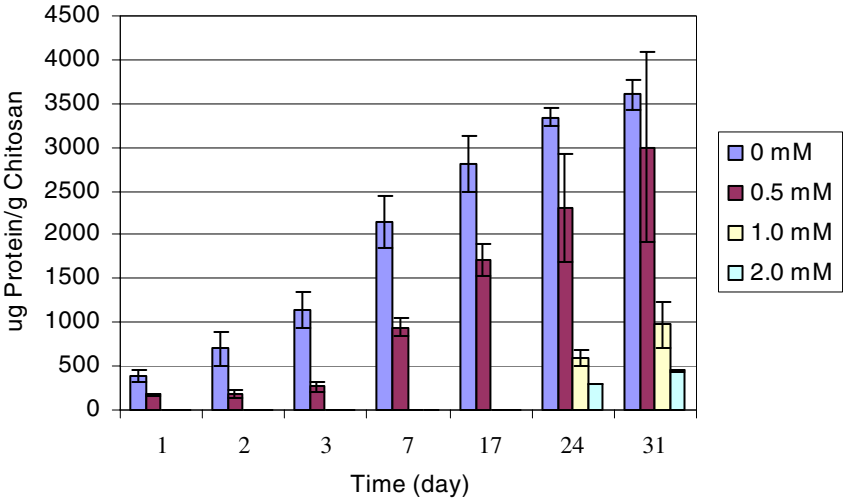

Fig. 7. The accumulated release profile of albumin from chitosan–albumin microspheres after cross-linking with 0.5, 1.0 and 2.0 mM genipin for 4 h. On day 31, albumin release from 2.0 mM genipin cross-linked microspheres was significantly lower than the other cross-linked and un-cross-linked microspheres ($p < 0.05$).

Table 1
Total albumin released after 31 days in PBS from chitosan microspheres cross-linked to different degrees

Cross-link treatment of microspheres	Degree of cross-linking (%) ($n = 3$)	Total albumin released (%) ($n = 3$)*
Plain no treatment	0	75.4 ± 3.4
4 h at 0.5 mM genipin	23.6 ± 3.4	62.7 ± 22.7
8 h at 0.5 mM genipin	34.3 ± 0.9	45.2 ± 0.1
16 h at 0.5 mM genipin	35.4 ± 1.1	39.4 ± 4.6
4 h at 1.0 mM genipin	33.0 ± 6.6	20.4 ± 5.4
4 h at 2.0 mM genipin	34.6 ± 6.0	9.3 ± 0.4

For elution vs cross-linking time, only 0 h vs 16 h was significantly different ($p < 0.05$); For elution vs genipin concentration, 0 mM vs 2 mM, 0.5 mM vs 2 mM and 1 mM vs 2 mM were all significantly different ($p < 0.05$).

chitosan but also the protein as previously described. Using the more concentrated genipin solutions most likely resulted in consumption of the albumin in the outer zones of the microspheres, thus resulting in low or no albumin release during the initial 17 days. These results, demonstrate that genipen cross-linking may delay and extend drug or protein delivery.

While the results of this study demonstrated that swelling of chitosan microspheres and protein or drug release rate may be controlled by the degree of cross-linking with genipin, there are several limits and additional questions raised. First, studies are needed to verify that the protein released from genipin cross-linked microspheres is not altered and remains functional. This may be done through the use of ELISA type assays which use antibodies to

detect-specific protein structures/conformation. The loss of specific protein conformations due to cross-linking may result in loss of protein functionality. This is important since genipen may also react with the amino groups of proteins thereby altering their conformation or tying them to the chitosan molecule. This issue is also faced by other cross-linking agents such as glutaraldehyde. Second, the release of albumin should be continued until 100% release is achieved. This is important particularly for antibiotics since continued low level exposures can lead to antibiotic resistance bacterial species. Additionally, the effect of the degree of cross-linking on chitosan microsphere degradation needs to be determined since the microspheres should remain biodegradable.

4. Conclusions

In this work, the degree of cross-linking and swelling ratio of chitosan microsphere cross-linked with genipin, a natural and non-toxic cross-linking reagent, and elution of albumin as a model protein were determined. The degree of cross-linking of chitosan microsphere increased with cross-linking time or genipin concentration. The swelling ratio decreased with increase cross-linking time or genipin concentration. The chitosan microspheres with albumin cross-linked with genipin released albumin more slowly than non-cross-linked microspheres. This slower release rate may be beneficial to extending the time frame for drug or protein delivery. These data suggest that protein or drug release rates and the swelling ratio of chitosan microsphere may be controlled by the degree of cross-linking.

Acknowledgements

This work was supported in part by the Biomaterials Applications of Memphis (BAM) research group at the University of Memphis–University of Tennessee Health Science Center at Memphis.

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