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Genipin-cross-linked kappa-carrageenan/carboxymethyl cellulose beads and effects on beta-carotene release

Ida Idayu Muhamad*, Lim Shu Fen, Ng Hui Hui, Noor Anis Mustapha

Dept. of Bioprocess Engineering, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, Johor Bahru 81310, Johor, Malaysia

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ABSTRACT

Beads of kappa-carrageenan/sodium carboxymethylcellulose were prepared based on different blend formulations using genipin, a natural and non-toxic cross-linking reagent. Different genipin concentrations (0.5, 1.0, 1.5 mM) were used to study the effects on swelling ratio of the beads in different pH values under simulated gastrointestinal tract condition (pH 1.2 and 7.4). Results have shown that the cross-linked beads possess lower swelling ability in all pH conditions and swelling ratio decreases with increasing genipin concentration (95.24% in pH 1.2; 100% in pH 7.4 at 0.5 mM genipin; 76.2% in pH 1.2; 85.71% in pH 7.4 at 1.5 mM genipin). It was also found the beads released beta-carotene slower and lesser after being cross-linked. Microstructure study shows that cross-linked beads exhibited smoother surface and more spherical shape compared to the native beads. This indicates that cross-linking of genipin has enhanced the beads network stability and their structure to be applied as suitable hydrogel.

1. Introduction

Hydrogels are polymeric materials that have a three-dimensional network structure and can swell considerably in aqueous medium without dissolution. They can be prepared in a wide-variety of physical forms: foams, films, sheets, beads, powder, tubes and blocks (Graham, 1986). In response to stimuli such as pH, temperature, and presence of chemical species, solvent, light or pressure, hydrogels have the properties and ability to change their shapes or volumes (Gupta & Raghava, 2008). Qui and Park (2001) has reported that this environmental sensitive hydrogel has been studied for biomedical and pharmaceutical application in protecting drug and deliver it in response to the pH and temperature in human gastro-intestinal tract.

Recent advance in hydrogel technology have focused on finding more biocompatible, non-toxic material intended for pharmaceutical, biomedical or even in food application. Hydrogels formed from polysaccharides, such as carrageenan, are good candidates for drug release systems, owing to their nontoxicity and acceptance by regulating authorities and most importantly, to their easy gelling ability, thermo reversibility of the gel network and appropriate viscoelastic properties (Liu, Li, & Cai, 2006) that able to undergo harsh condition.

In order to increase the time frame and stability of hydrogel carrier for drug delivery, the polymeric material need to be cross-linked. Several cross-linking reagents have been used for cross-linking such as glutaraldehyde, tripolyphosphate, ethylene glycol, diglycidyl ether and diisocyanate. Genipin, a natural cross-linker is found to be 10000 times less toxicity than the common used cross linking agent, glutaraldehyde is utilized to cross-link the hydrogels with minimum cytotoxic effect. Studies have shown that cross-linked hydrogels exhibit different properties as compared to uncross-linked hydrogels (Yuan et al., 2007). Cross-linked hydrogels may have changes in their swelling properties, mechanical strength, degradation rate depending on the degree of cross linking and more other factors (Moffat & Marra, 2005). Muzzarelli (2009) reported the most important applications of genipin in conjunction with chitosan are the preparation of elastic and resistant gels such as the cartilage substitutes, the manufacture of drug carriers for controlled release, the encapsulation of biological products and living cells, and the medication of wounds in animals and humans. Genipin might replace glutaraldehyde with the advantages of stability and biocompatibility of the crosslinked products whose quality assessment and manipulation would be easier.

Therefore in this research, natural cross-linking agent—genipin is selected to study the effect of cross linking on the formulated kappa-carrageenan/carboxymethylcellulose hydrogel beads.

2. Experimental

2.1. Material

Sodium carboxymethyl cellulose (NaCMC) with average molecular weight of 250,000 was purchased from Acros Organic and κ-carrageenan (κC) was purchased from Sigma–Aldrich. Genipin

* Corresponding author. Tel.: +60 7 5535541; fax: +60 7 5581463.
E-mail address: idayu@fkkksa.utm.my (I.I. Muhamad).

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was purchased from Challenge Bioproducts Co., Ltd. (Taiwan). All chemicals are used as received without further purification.

2.2. Preparation of beads

2.2.1. Control blend

Control blend of κ-carrageenan/NaCMC beads are formulated based on the κC:NaCMC ratio of 60:40, 70:30, 80:20 and 90:10. The hot carrageenan solution was blend with NaCMC solution according to the ratio as mentioned. The homogeneous beads were made by conventional dripping method into cold potassium chloride (KCl) hardening solution layered with cold rape seed oil. The solution was maintained at 10 °C for 5 h to let the beads harden. Beads then washed with diluted KCl solution to remove remaining oil layer. Finally, beads were dried in 37 °C oven overnight.

2.2.2. Cross-linking

The most suitable κC:NaCMC blend ratio was chosen to be cross-inked with three concentrations of genipin—0.5 mM, 1.0 mM and 1.5 mM. Genipin stock solutions were prepared by dissolving genipin powder in 10 percent of ethanol with continuous stirring. Genipin solution was then added to the blend of κC/NaCMC hot solution and dripped into KCl solution to form cross-linked beads. The formulated beads were then dried in oven at 37.5 °C for 24 h.

2.2.3. Swelling ratio measurement

The swelling ratio of native and cross-linked beads were determined by immersion in solution of pH 1.2 (0.1 N HCl) and pH 7.4 buffer solution. Subsequently, the diameter of the swollen bead (D_t) was examined under microscope and the swelling ratio was calculated according to the equation as follows:

$$\text{Swelling ratio}\,(\%) = \left[\frac{D_t - D_0}{D_0}\right] \times 100 \quad (1)$$

where D_t is the diameter of swollen beads at time t and D_0 is the initial diameter of dried beads. The experiment was performed in triplicate and represented as a mean value.

2.2.4. Immobilization of β-carotene in beads

0.5 mg/ml of β-carotene that dissolved in ethanol was added to the blend solution of κ-carrageenan/NaCMC. Different concentrations of genipin were then added to the hot solution to form cross-linked beta carotene loaded beads by using dripping method. All β-carotene loaded beads were kept in hardening solution for 30 min and release test was performed immediately after hardening.

2.2.5. Release study of β-carotene

The β-carotene release study of the beads from each cross-linked concentration was performed in the simulated gastrointestinal condition by the pH change method at 37 °C. One gram of beads was enclosed in the teabag and placed into beaker that contained 50 ml of the dissolution medium. The beaker was placed and incubated at 37 ± 2 °C water bath. The pH of the dissolution medium was kept at 0.1 N HCl for first 10 min, then changed to buffer solution pH 6.6 and finally pH 7.4 up to minutes 30. 5 ml of dissolution medium was withdrawn every 2 min and was then assayed by UV–vis spectrophotometer at 446 nm. All experiments were performed in triplicate. The amount of released β-carotene was calculated by interpolation from the β-carotene standard curve at 446 nm. A cumulative correction was made for the previously removed sample to determine the total β-carotene release.

2.2.6. Microstructure of hydrogel beads

The surface morphology of the native (uncross-linked), cross-linked bead was examined by using a Philips XL 40 scanning electron microscope operates at 25 kV accelerating voltage. In preparation of SEM examination, the samples were mounted on metal grids and coated by gold using gold sputter coater under vacuum before observation. The photomicrographs were taken at different magnifications.

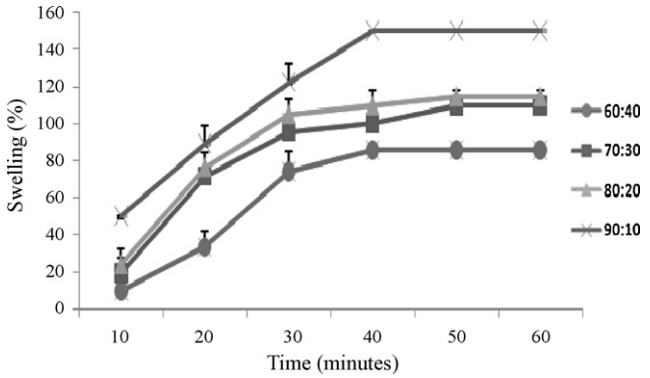

Fig. 1. Swelling ratio versus time for different blend ratios of beads in buffer solution pH 7.4.

3. Results and discussion

3.1. Preparation of beads

κ-Carrageenan/NaCMC beads were formulated based on the κC:NaCMC ratio of 60:40, 70:30, 80:20 and 90:10. Significantly spherical beads with homogeneous surface were produced and the diameter of the beads formed is approximately 0.016–0.018 mm. The wet beads were then dried in oven (37.5 °C) overnight before the swelling test. Swelling ratio measurement was done to determine the suitable blend ratio for further study.

3.2. Swelling ratio measurement

3.2.1. Control blend

Figs. 1 and 2 show the swelling trend for 60:40, 70:30, 80:20 and 90:10 blends when immersed in pH 7.4 buffer solution and pH 1.2 acidic medium for 60 min respectively. Dried bead with initial diameter of 0.007 mm start to swell once immersed in the medium and have reached the equilibrium of swelling after 40 min immersion in both medium. From both Figs. 1 and 2, the swelling ratio of κC/NaCMC beads displayed a systematic trend in accordance with weight fraction, in which the degree of swelling increases with the ratio of carrageenan. As in 60:40 blends, the beads contain highest weight fraction of NaCMC, therefore the swelling in both medium is the lowest. From the plot of swelling ratio versus time for both

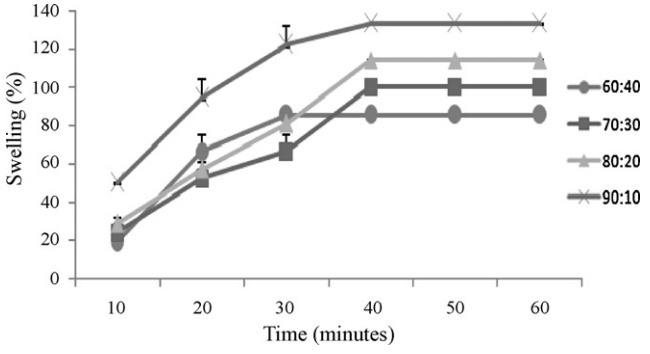

Fig. 2. Swelling ratio over time for different blend ratios of bead in 0.1 N HCl (pH 1.2).

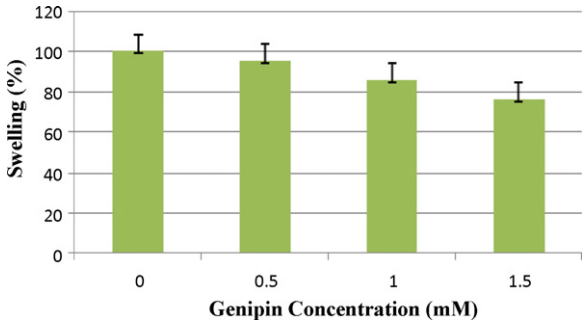

Fig. 3. Comparison between swelling ratio of native beads and beads cross-linked with different concentrations of genipin in pH 1.2 medium (0.1 N HCl) after equilibrium state.

pH, the 90:10 blend beads swell the fastest among all, followed by 80:20, 70:30 and the slowest 60:40.

NaCMC is considered as in weakly negative while carrageenan is in negative states. NaCMC is a weak polyelectrolyte behaves almost neutral polymer as in buffer solution pH 7.4. Therefore, cross-linking point among polymer was coming from the sulfonate group (SO^{3-}) in carrageenan. As the weight fraction of carrageenan increases in the blend, i.e. in 90:10 ratios, the counterions in the solution (SO^{3-}) also increase. The increases of SO^{3-} ion contribute to stronger electrostatic repulsion between the SO^{3-} groups and therefore the swelling of the polymer also increases (Mistsumata, Suemitsu, Fujii, Fujii, & Taniguchi, 2003). Besides, more SO^{3-} ion causes the osmotic pressure to increase and finally the degree of swelling increases as well.

In order to know the respond of hydrogel swelling on pH, the swelling of beads was tested in pH 1.2 and pH 7.4 medium. By comparing the swelling ratio of beads in both medium, most blend ratio of beads exhibit better swelling in pH 7.4 medium than acidic medium. In blend ratio of 70:30 beads, the swelling degree of bead after equilibrium state is 109% and 100% respectively in pH 7.4 medium and acidic medium. At low pH, the carboxylate COONa changes to acid form COOH, therefore most carboxymethyl groups are in the form of COOH that is less ionized. Table 1 shows the comparison of beads characteristics for the different blend ratio beads. As the pH increases, the carboxylic groups become ionized, and the resulting repulsion in the network will cause the beads to swell. Collapsing is observed in the 60:40 blend ratio beads (in acidic medium) due to hydrogen bond formation, where the beads dissolved in the medium. This happened when the 60:40 blend beads were immersed in pH 1.2 medium longer than 60 min.

Consequently, beads with blend ratio of 70:30 were chosen for further investigation in cross-linking with genipin. Beads in this ratio have not much different in degree of swelling as compared to the blend ratio 80:20 beads. Although beads with blend ratio 80:20 and 90:10 have better swelling degree than beads with 70:30 ratio, they were not suitable for the formation of beads as it did not produce spherical beads. According to Sankalia, Mashru, Sankalia, and Sutrariya (2006), higher carrageenan concentrations in the ratio did not produce spherical beads, probably due to the high viscosity of the dripping solution. In addition, increase in dripping solution viscosity will lead to formation of larger beads that is less suitable for nutraceutical study. Beads with blend ratio 60:40 were not chosen as its gel structure is not strong and might dissolve in the medium during study.

3.2.2. Cross-linking

The most suitable blend ratio—70:30 blends were cross-linked with different genipin concentrations: 0.5 mM, 1.0 mM and 1.5 mM to study the effect of cross-linker concentration on swelling. One of the cross-linking effects can be obviously seen from Figs. 3 and 4, where all the beads have shown a decrease in swelling ratio after crosslinked with genipin. From observation in both mediums, beads sample cross-linked with lowest concentration of genipin linked to the highest percentage increase in its swelling diameter. It can be seen from both Figs. 3 and 4, beads crosslinked with 0.5 mM genipin have the swelling of approximately 95.24% and 100% in pH 1.2 and pH 7.4 medium respectively. In contrast, beads crosslinked with highest concentration of genipin (1.5 mM) show least percent increase in diameter, approximately 76.2% in pH 1.2 medium and 85.71% in pH 7.4 medium. Native beads without cross-linking have the highest swelling in both medium. This may due to the fact that high concentration of genipin could result in great extent of chemical crosslinking of the κC/NaCMC chains. This restricts the mobility and hydration of the macromolecular chain in the beads and lead to less swelling in diameter. For each crosslinked beads sample investigated, the swelling ratio in pH 1.2 medium was lower than the swelling ratio in a pH 7.4 medium. This maybe attribute to the formation of hydrogen bond between the carboxymethyl cellulose due to the existence of carboxylic group (–COOH) at low pH. At pH 7.4, the carboxylic acid groups on the crosslinked beads became progressively ionized (–COO–). So the resultant beads swelled more significantly due to a large swelling force induced by the electrostatic repulsion between the ionized groups (Song, Li, Yang, & Li, 2009).

Mi, Sung, Shyu, Su, and Peng (2003) who synthesized novel chitosan gel beads by concurrent ionic and covalent crosslinking mechanisms involving tripolyphosphate (TPP) and genipin reported that the co-crosslinking depends on pH where the covalent crosslinking dominates at pH values 7.0 and 9.0, whilst ionic crosslinking dominates at pH 1.0, 3.0 and 5.0. There are evident effects on the swelling and the enzymatic degradation of genipin crosslinked chitosan derivatives. Chen et al. (2004) studied on N,O-carboxymethyl chitosan/alginate hydrogel crosslinked by genipin reported that the swelling ratio was higher at high pH than at low pH.

3.3. Immobilization of β-carotene in beads

Immobilization of β-carotene in beads crosslinked with different genipin concentrations was then carried out to investigate their characteristics. The beads with successful immobilization of β-carotene are easy to be recognize as it turned into transparent orange in color beads compared to the original transparent beads. Higher concentration of genipin cross-linked beads has darker orange color and the beads are more elastic compared to the lower concentration of genipin cross-linked beads. Moura, Figueiredo, and Gil (2007) also concluded that under physiological conditions the viscoelastic features of a chitosan solution and its gelling ability could be tuned by changing the genipin concentration (very low in all cases); relatively strong elastic gels were obtained.

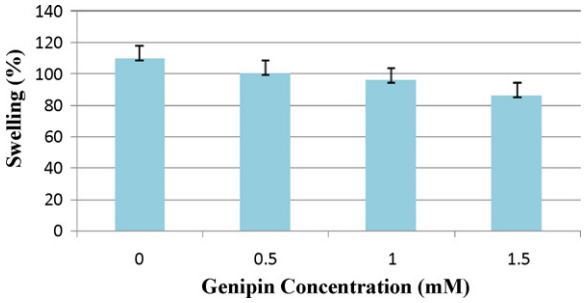

Fig. 4. Comparison between swelling ratio of native beads and beads crosslinked with different concentrations of genipin in buffer solution pH 7.4 after equilibrium state.

Table 1
Comparison of bead characteristics for the different blend ratio beads.

Blend ratio (κC:NaCMC)	Swelling ratio (%)		Shape (spherical)	Beads strength
	pH 7.4	pH 1.2		
60:40	85.71	85.71	Yes	Poor (dissolved in swelling medium)
70:30	109.52	100.00	Yes	Good
80:20	114.29	114.29	No	Too viscous
90:10	150.00	133.33	No	Too viscous

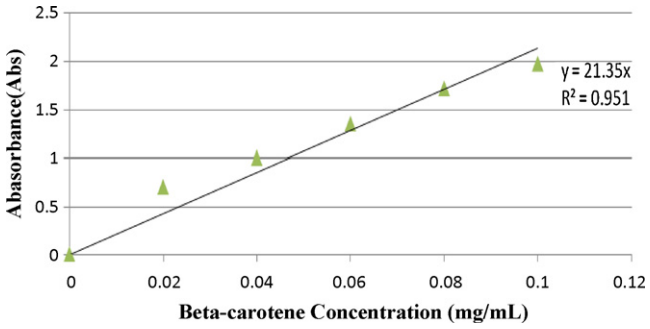

Fig. 5. Standard curve of β-carotene.

3.4. Release of β-carotene

In this section, the ability of the beads to hold β-carotene was investigated by determining the release of β-carotene from each type of beads. To determine the concentration of β-carotene released, standard curve representing the absorbance at wavelength of 446 nm of different concentrations of β-carotene was constructed as shown in Fig. 5. The relation correlating this curve is calculated using Eq. (2) (from β-carotene standard curve):

$$\text{Absorbance} = \text{concentration (mg/mL)} \times 21.355 \quad (2)$$

From this relation, a concentration of unknown sample can be determined. The accumulated release of β-carotene was carried out as a function of time at a pH change system in order to simulate the gastrointestinal tract (GIT) conditions. The release rate of 1 g native and crosslinked κC/NaCMC beads was measured in 30 min time.

From the accumulated release profile of β-carotene as shown in Fig. 6, the release of β-carotene from the native beads was faster compared to the release from crosslinked beads. At the first 2 min immersion in pH 1.2 medium, the release of β-carotene from native beads was 7% while for 0.5 mM crosslinked beads was only 4%. The amount of β-carotene released from the crosslinked beads was found to be less than that from the native beads. The lower the concentration of the genipin used, the larger amount of β-carotene was released. For lower concentration of cross-linker used, the cross-linking degree will decrease. According to Bachtsi and Kiparissides (1995), 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. Meanwhile, an increase of the degree of polymer cross-linking increases the polymer density and therefore decreases the available free space for drug diffusion which results in a decrease in drug release rates.

It is also noticed that the release of β-carotene from beads in acidic medium was slower compared to buffer solution pH 7.4 and buffer solution pH 6.0. This can be observed from the accumulated release profile where β-carotene concentration from $t = 0$ to $t = 10$ min (when immersed in acidic medium) was low and increase fast after $t = 10$ min (immersed in pH 6.0 medium) and fastest after $t = 20$ min (when immersed in pH 7.4 medium). This can be explained by the swelling behavior of the beads in which the beads swell better in buffer solution pH 7.4 as compared to acidic condition. Therefore the medium could easily diffuse into the beads and hence a faster and higher amount of β-carotene was released. In short, native beads have the highest release rate if compared to cross-linked beads. This means that cross-linking has changed the mechanical characteristics and properties of the beads as it is now able to hold the content in it for longer period. This agrees with Chen et al. (2004) who found that the amount of albumin released from their genipin-crosslinked NOCC/alginate hydrogel at pH 1.2 was relatively low (20%), while at pH 7.4 it increased significantly (80%). Another substituted chitosan, the carboxymethyl-hexanoyl chitosan amphiphatic hydrogel with excellent water-absorption and water-retention capacity under neutral conditions was crosslinked with genipin and then employed as a carrier for delivering ibuprofen and other amphiphatic agents (Liu et al., 2006).

3.5. Microstructure of hydrogel beads

The observation of shape and surface topography of the native and crosslinked beads was done by scanning electron microscope. Fig. 7(A)–(D) shows the micrograph of native, cross linked beads with genipin concentration of 0.5, 1.0, and 1.5 mM respectively

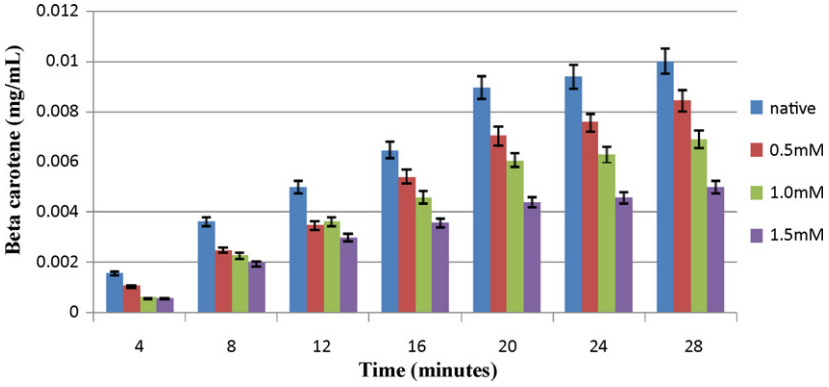

Fig. 6. Accumulate release profile of β-carotene under in vitro release condition (pH 1.2, followed by pH 6.6 and pH 7.4 medium).

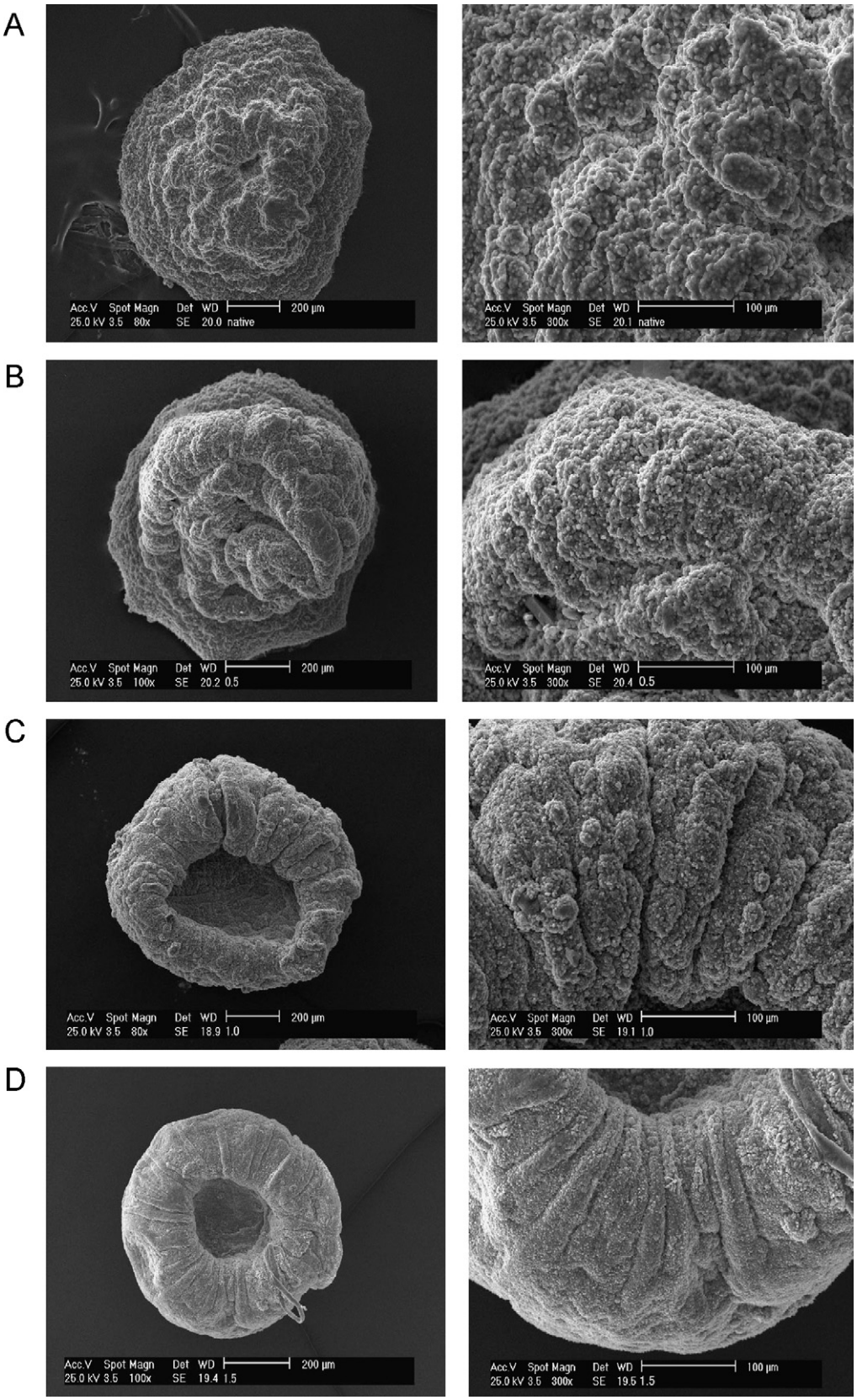

Fig. 7. Scanning electron micrograph (100×) and (300×), (A) native bead, (B) 0.5 mM genipin crosslinked, (C) 1.0 mM genipin crosslinked, and (D) 1.5 mM genipin crosslinked.

at magnification of 100× and 300×. From the micrograph, it can be observed that the shapes of the beads were not completely spherical and the surface was rough and folded. These phenomena are due to the beads shrunk during the drying process. Fig. 7(A) shows that the surface of the native beads was irregular and rough moon-like surface. Meanwhile, as for Fig. 7(B)–(D), the surface of cross linked beads exhibited more spherical shape and smoother as the cross-linker concentration increases. The change in the surface

topography might be due to the change in molecular arrangement after crosslinked with genipin. In view of this more stable behavior the formulations were deemed suitable for encapsulation of cells and bioactive compounds. This phenomenon genipin crosslinked polymers results in highly swelling microgels with a variation in volume of more than 100% between shrunken state and swollen state in dilute solutions which can be of tremendous interest for targeted release of the microgels contents in the gastrointestinal tract.

4. Conclusion

In this study, the most suitable blend ratio of 70:30 was chosen to further study the effect of different concentrations of genipin cross-linking on several beads' properties. All the beads have achieved higher swelling in buffer solution at pH 7.4 than that in acidic medium, pH 1.2. This is due to the change in ionic structure of the polymer when expose in these medium. The swelling ratio of the beads was much lower if compared to the non-crosslinked beads. It decreases with the increase in the concentration of cross-linker used, in which beads crosslinked with 1.5 mM genipin has the lowest swelling among all. The release of β-carotene from the beads under simulated gastrointestinal tract conditions was studied. The β-carotene loaded beads crosslinked with genipin released β-carotene slower and lesser than native beads. This slower release may be beneficial to extending the time frame for drug or protein delivery if the bead is used in nutraceuticals. The release of β-carotene from beads decreases with the increase in cross-linker concentration. It is suggested that the β-carotene release rate is proportional to the swelling ability of the beads. Lastly, the surface morphology and shape for the native and crosslinked beads were studied. Images produced have shown that the surface topography of the crosslinked beads were smoother as compared to native beads. Native beads was irregular in shape and have rough moon-like surface while the shape of the crosslinked beads have shown to be more spherical as the concentration of cross-linker increase.

In conclusion, cross-linking of genipin on the beads enhances the beads network stability and their structure. Depending on the genipin amount used, the κC/NaCMC beads have various swelling and release characteristics in simulated gastrointestinal tract conditions. The results suggest that the genipin-crosslinked κC/NaCMC beads may be used as a suitable carrier for nutraceuticals.

Acknowledgements

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References

Bachtsi, A., & Kiparissides, C. (1995). An experimental investigation of enzyme release from polyvinyl alcohol cross-linked microsphere. *Journal of Microencapsulation*, *12*, 23–35.

Chen, S. C., Wu, Y. C., Mi, F. L., Lin, Y. H., Yu, L. C., & Sung, H. W. (2004). A novel pH sensitive hydrogel composed of N, O-carboxymethyl chitosan and alginate crosslinked by genipin for protein drug delivery. *Journal of Controlled Release*, *96*, 285–300.

Graham, N. B. (1986). Hydrogel in controlled drug delivery. In E. Piskin, & A. S. Hoffman (Eds.), *Polymeric material* (p. 170). Netherlands: Martinus Nijhoff Publishers.

Gupta, M. N., & Raghava,. S. (2008). *Natural-based polymers for biomedical applications*. Cornwall, England: TJ International Limited., pp. 129–130, 140–142.

Liu, J., Li, L., & Cai, Y. (2006). Immobilization of campthothecin with surfactant into hydrogel for controlled drug release. *European Polymer Journal*, *42*, 1767–1774.

Mi, F. L., Sung, H. W., Shyu, S. S., Su, C. C., & Peng, C. K. (2003). Synthesis and characterization of biodegradable TPP/genipin co-crosslinked chitosan gel beads. *Polymer*, *44*, 6521–6530.

Mistsumata, T., Suemitsu, Y., Fujii, K., Fujii, T., & Taniguchi, T. (2003). pH respond of chitosan, κ-carrageenan, carboxymethyl cellulose sodium salt complex hydrogels. *Polymer*, *44*, 7103–7111.

Moffat, K. L., & Marra, K. G. (2005). Biodegradable poly (ethylene glycol) hydrogel crosslinked with Genipin for tissue engineering application. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *71B*(1), 181–187.

Moura, M. J., Figueiredo, M. M., & Gil, M. H. (2007). Rheological study of genipin crosslinked chitosan hydrogels. *Biomacromolecules*, *8*(12), 3823–3829.

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

Qui, Y., & Park, K. (2001). Environment-sensitive hydrogels for drug delivery. *Advanced Drug Delivery Reviews*, *53*, 321–339.

Sankalia, M. G., Mashru, R. C., Sankalia, J. M., & Sutrariya, V. B. (2006). Stability improvement of alpha-amylase entrapped in kappa-carrageenan beads: Physiochemical characterization and optimization using composite index. *International Journal of Pharmaceutical*, *312*, 1–14.

Song, F., Li, M. Z., Yang, C., & Li, Y. (2009). Genipin-crosslinked casein hydrogels for controlled drug delivery. *International Journal of Pharmaceutics*, *373*, 41–47.

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.