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Genipin-crosslinked casein hydrogels for controlled drug delivery

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ABSTRACT

Recent advances in hydrogel technology have focused on finding more biocompatible, non-toxic materials intended for pharmaceutical and biomedical applications. In this study, naturally occurring genipin was used for the first time to crosslink casein protein in aqueous system for the formation of novel hydrogel materials. For aqueous 8.0 wt% casein solution, its gelation in the presence of genipin was investigated by time sweep rheometric measurements. With the increase of genipin amount from 2.5 to 10.0 mmol/L, the gelation time decreased from 119.8. to 18.5 min when the reaction temperature was kept to be 35 °C. With the increase of the reaction temperature from 35 to 50 °C, the gelation time decreased from 44.7 to 27.6 min when genipin concentration was kept to be 5.0 mmol/L. The apparent activation energy was determined to be 28.6 kJ/mol according to the Arrhenius equation. Moreover, the mechanical strength of the crosslinked casein hydrogel could be tuned by the amount of genipin. ^{13}C NMR analyses confirmed the crosslinking reaction between casein and genipin. For the resultant casein hydrogels, their swelling characteristics and in vitro release profiles of bovine serum albumin (BSA) were studied in simulated gastrointestinal tract conditions (pH 1.2 and pH 7.4). At pH 1.2, the swelling ratio of the hydrogel and the release amount of the entrapped BSA were relatively low. However, high amounts of the swelling and BSA release could be observed at pH 7.4. The release behavior could be related to various crosslinking and swelling degrees of the hydrogel networks formed by various amounts of genipin. It is suggested that the genipin-crosslinked casein hydrogel might be a suitable polymeric carrier for protein drug delivery in the intestine.

1. Introduction

Since the pioneering work of Wichterle and Lim (1960) in the early 1950s on the crosslinked three-dimensional polymers obtained by the copolymerization of 2-hydroxyethyl methacrylate (HEMA) with ethylene dimethacrylate (EDMA), polymeric hydrogels have been of great interest to biomaterial scientists for many years (Hoffman, 2002; Zhang et al., 2005a, b; Zhao et al., 2006). The important and influential work of Lim and Sun (1980) in 1980 demonstrated the successful application of alginate-based hydrogel for cell encapsulation. Later in the 1989, Yannas and co-workers (1989) incorporated collagen and shark cartilage into hydrogels for the use as artificial burn dressings. Up to now, the hydrogels based natural and synthetic polymers have shown their potential applications used as drug delivery systems (Lin and Metters, 2006; Hoffman, 2002; Zhang et al., 2005a, b), soft contact lenses (Ketelson et al., 2005; Alvarez-Lorenzo et al., 2006), tissue engineering matrices (Woerly, 1993; Lee and Mooney, 2001; Khademhosseini and Langer, 2007), wound dressings (Kokabi et al., 2007; Balakrishnan et al., 2005) and implants (Ashton et al., 2007; Liu et al., 2007). In particular, such materials are of special interest in controlled release applications because of their soft tissue biocompatibility, the ease with which the drugs are dispersed in the matrix, and the high degree of control achieved by selecting the physical and chemical properties of the polymer network (Lee and Yuk, 2007; Smolensky and Peppas, 2007; Park et al., 1993). Recent advances in hydrogel technology have focused on finding more biocompatible, non-toxic materials intended for pharmaceutical and biomedical applications.

In this work, a novel protein-based hydrogel for the controlled release of bovine serum albumin (BSA) has been prepared and characterized. For this purpose, naturally occurring genipin was used to crosslink casein, a phosphoprotein that precipitates from raw skim milk by acidification (Fox et al., 2000). It is known that genipin is found in traditional Chinese medicine and extracted from gardenia fruit (Akao et al., 1994). It is an effective naturally occurring crosslinking agent that can react with amino acids or proteins (Touyama et al., 1994; Yuan et al., 2007). Sung and co-workers (Sung et al., 1999a) have undertaken the investigation on the cytotoxicity, feasibility, and biocompatibility of genipin for tissue fixation, and

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found that genipin was 10,000 times less cytotoxic than commonly used glutaraldehyde. This finding encourages us to investigate the use of genipin for the formation of casein hydrogels with potential biomedical applications.

2. Materials and methods

2.1. Materials

Casein powder was purchased from Tongjian Bio-Tech Co. in Lanzhou (China). Genipin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BSA was purchased from Sigma (USA). Phosphate buffered saline (PBS) was provided by Guangzhou Chemical Company in China. All other chemicals and reagents used were of analytical grade.

2.2. Genipin-induced gelation and its characterization

Casein powder was firstly dispersed in deionized water, followed by adjusting pH value to about 8.0 with 2 mol/L sodium hydroxide solution. After heated at 50 °C for 30 min, the casein powder was dissolved completely in water. The as-obtained casein solution was then heated to a given temperature and held for 30 min. Then, a required amount of genipin from a 50 mmol/L stock solution was added. After mixing, the system was thoroughly stirred and set aside for a given time. The gelation process was monitored by the rheological method, and the viscoelastic parameters of the resulting hydrogels were measured by using an advanced rheometric extended system (ARES, TA Co.) in oscillatory mode with a Couette geometry (34 mm diameter). The dependence of gelation on temperature was investigated under thermostatic control. To ensure the rheological measurements within a linear viscoelastic region, a dynamic strain sweep was conducted prior to the frequency sweep, and the corresponding strain was determined to be 10%. For a comparative study, the rheological test was also carried out for aqueous 8.0 wt% casein solution in the absence of genipin. The crosslinking reaction between casein and genipin was confirmed by ^{13}C NMR spectrum analysis using a Bruker 400 MHz nuclear magnetic resonance (NMR) spectrometer.

2.3. Swelling ratio measurements

The swelling characteristics of the genipin-crosslinked casein hydrogels were determined by immersing dried samples to swell in 30 mL of a solution at pH 1.2 or a solution of pH 7.4 at 37 °C for 5 h, simulating gastrointestinal tract condition (Risbud et al., 2000). At specific time intervals, the samples were taken out from the swelling media and then weight of the hydrogel was measured. The swelling ratio of test hydrogel sample was calculated from the following equation:

$$\text{swelling ratio} = \frac{(W_t - W_0)}{W_0}$$

where W_t is the weight of the swollen test sample and W_0 is the weight of the dried test sample. All experiments were carried out in triplicate.

2.4. In vitro drug release

For the loading of BSA, a 2.0 mg/mL stock solution of BSA were prepared by dissolving BSA in phosphate-buffered solutions, respectively. Before loading BSA into the casein hydrogels, each freeze-dried hydrogel sample was washed with distilled water to remove unreacted genipin, and then placed into the BSA solution at 4 °C for 48 h. Thus, BSA was loaded into each hydrogel sample up to equilibrium, and the drug-loaded hydrogel samples were used for in vitro release study.

The drug release experiments were conducted by immersing the above drug-loaded hydrogel sample in a glass bottle filled with a 10 mL PBS (pH 1.2 or pH 7.4) at 37 °C. At a predetermined period of the in vitro release experiment, 1.0 mL aliquots of the buffer medium was removed from the glass tube and the concentration of the model drug in that aliquot was measured by using a UV spectrophotometer. A 1.0 mL fresh buffer solution was added back to the glass tube to maintain the same total solution volume. The percentage of cumulative amount of released BSA was determined from standard calibration curves. All release studies were carried out in triplicate.

3. Results and discussion

In order to investigate the gelation rate and extent of aqueous casein system in the presence of genipin, time sweep measurements were carried out at 35 °C using an advanced rheometric extended system, in which the storage modulus (G') and viscous modulus (G'') were monitored as a function of time. For a comparative study, aqueous casein solution without genipin was also investigated. Fig. 1 shows the time dependence of G' and G'' for aqueous 8.0 wt% casein solution and its systems crosslinked with 2.5, 5.0 or 10.0 mmol/L genipin, respectively. For aqueous casein system without genipin, a predominantly viscous behavior with the G'' value greater than the G' value over the entire time range studied was observed, as indicated in Fig. 2a. When genipin was used as the crosslinking agent, however, the corresponding systems showed a shift from a predominantly viscous liquid ($G'' > G'$) to a strongly viscoelastic solidlike material ($G' > G''$), as indicated in Fig. 1b–d. The time of the crossover from a viscous behavior to an elastic response could be regarded as the time for the onset of gelation (gelation time) (Winter and Chambon, 1986). From Fig. 1, the gelation time was determined to be 119.8 min in the case of 2.5 mmol/L genipin, 44.7 min in the case of 5.0 mmol/L genipin and 18.5 min in the case of 10.0 mmol/L genipin. As expected, the gelation time is inversely proportional to the genipin concentration. These results demonstrated that the used genipin could induce effectively the gelation of aqueous casein solution.

The effect of crosslinking temperature on the gelation of aqueous 8.0 wt% casein solution in the presence of 5.0 mmol/L genipin was also investigated, as shown in Fig. 2. When the temperature was kept to be 25 °C, the G'' was found to be larger than the G'' over the entire time range studied (Fig. 2a). In this case, the system was still in a liquid state, and the viscous properties dominated. As the temperature was kept to be 35 °C, however, a gel-like state could be observed, and the gelation time was determined to be 44.7 min (Fig. 2b). With the further increase of temperature, the gelation time was found to decrease to 40.6 min in the case of 40 °C (Fig. 2c), 30.6 min in the case of 45 °C (Fig. 2d) and 27.6 min in the case of 50 °C (Fig. 2e). These results show that an increase of the temperature is favorable for the gelation reaction. Silimar behavior was also reported by Sung et al. (1999b) for the preparation of gelatin-based hydrogels when formaldehyde, glutaraldehyde or carbodiimide was used as the crosslinking agent. It should be pointed out that a crosslinking temperature up to 50 °C does not result in the denaturation of the casein proptein. (Farrel et al., 2001) have confirmed that the secondary structure of casein was highly stable from 5 to 70 °C by circular dichroism and FTIR spectroscopy analyses. Therefore, the gelation of aqueous casein system in the presence of genipin could be also modulated by crosslinking temperature except for casein concentration. To explain this temperature-dependent gelation phenomenon, the existence of an energy barrier was hypothesized in the genipin-crosslinking casein

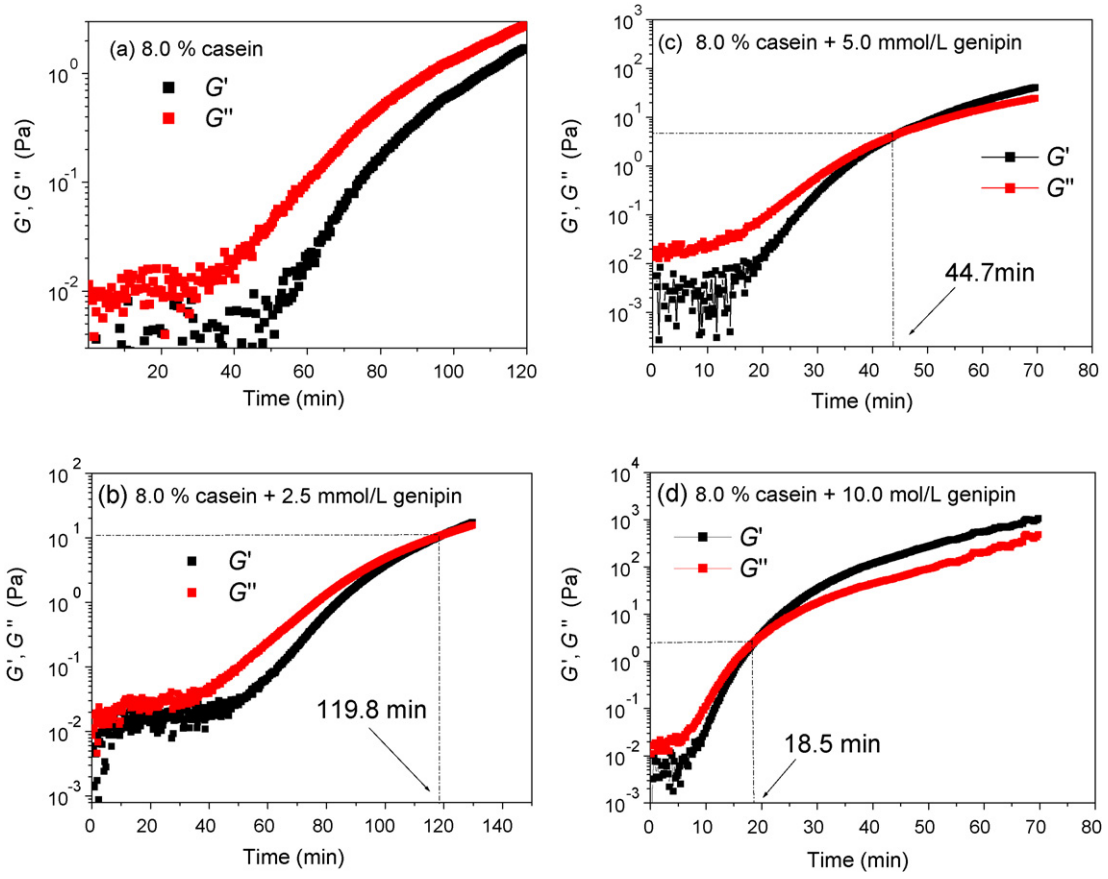

Fig. 1. Effect of genipin amount on the gelation of aqueous 8.0% casein solution. Test conditions: frequency, 1.0 rad/s; strain, 10.0%; temperature, 35 °C.

system. The energy barrier, named as apparent activation energy (E_a), was calculated according to Arrhenius equation (Song and Zhang, 2008):

$$\ln t_{gel} = A + \frac{E_a}{RT} \quad (1)$$

where t_{gel} is the gelation time determined, T is temperature in Kelvin, R is the universal gas constant (8.314 J mol^{-1} K^{-1}), and A is a constant. Fig. 3 shows the semilogarithmic plot of the gealtion time as a function of inverse reaction temperature. The linear relationship with the determination coefficient of 0.980 suggested that the temperature effect on the gelation time could be described by Eq. (1). From the slope of linear plot, the E_a value for the system was calculated to be 28.6 kJ/mol, which was lower than those obtained for the gelation process of other crosslinking systems reported, such as cold-set casein gel system (more than 100 kJ/mol) (Zhong and Daubert, 2004), heat induced casein gel system (40.0 kJ/mol) (Panouille et al., 2004), and genipin-crosslinked gelatin system (63.2 kJ/mol) (Nickerson et al., 2006).

Further investigation was carried out to understand the effect of genipin amount on the gel strength of the crosslinked casein system. For this purpose, each hydrogel sample was subjected to a frequency sweep from 0.01 to 100 rad/s. Fig. 4 presents the plots of the storage modulus versus oscillatory frequency. It was found that the storage modulus of the hydrogel sample increased with the increase of genipin amount in the frequency range investigated. Moreover, the dependence of the storage modulus on the frequency became weaker when the genipin amount increased, which shows that the increase of genipin amount could induce the enhancement in the stability of the crosslinked network. These facts implied that the mechanical property or strength of the crosslinked casein hydrogel could be tuned by the amount of genipin.

By reacting casein with genipin in aqueous system, we obtained dark bluish casein hydrogels. It was known (Touyama et al., 1994) that genipin could form blue pigments upon spontaneous reaction with amino groups. Therefore, we conjecture that the appearance of the dark bluish colour may result from the chemical crosslinking of genipin with the amino groups on the casein macromolecular chains. Fig. 5 illustrates the possible mechanism for the reaction of casein with genipin. To confirm this crosslinking reaction, ^{13}C NMR analyses were carried out for casein and genipin-crosslinked casein. From the ^{13}C NMR spectra shown in Fig. 6, it was found that the NMR signal of genipin-crosslinked casein at 40 ppm is different from that of casein, which can be attributed to the reaction between amino groups of lysine residue on casein and genipin.

The casein hydrogels crosslinked with genipin of different concentrations were allowed to swell in 30 ml of a pH 1.2 or 7.4 solution at 37 °C. Fig. 7 gives the swelling kinetic curves of the casein hydrogels in these two pH media. For each casein hydrogel sample investigated, the swelling ratio in a pH 1.2 medium was lower than the swelling ratio in a pH 7.4 medium. In the low pH medium, a lower swelling ratio of the genipin-crosslinked casein hydrogel may be attributed to the formation of hydrogen bonds between casein due to the existence of the carboxylic acid groups (–COOH) and hydroxyl groups (–OH). At pH 7.4, the carboxylic acid groups on the genipin- crosslinked casein hydrogel became progressively ionized (–COO$^-$). In this case, the resultant casein hydrogel swelled more significantly due to a large swelling force induced by the electrostatic repulsion between the ionized acid groups. In addition, the swelling ratio of the casein hydrogel obtained by a high concentration of genipin (10.0 mmol/L) is lower than that of the casein hydrogel obtained by a lower concentration of genipin (2.5 or 5.0 mmol/L) at both pH 1.2 and 7.4. This may be due to the fact that a

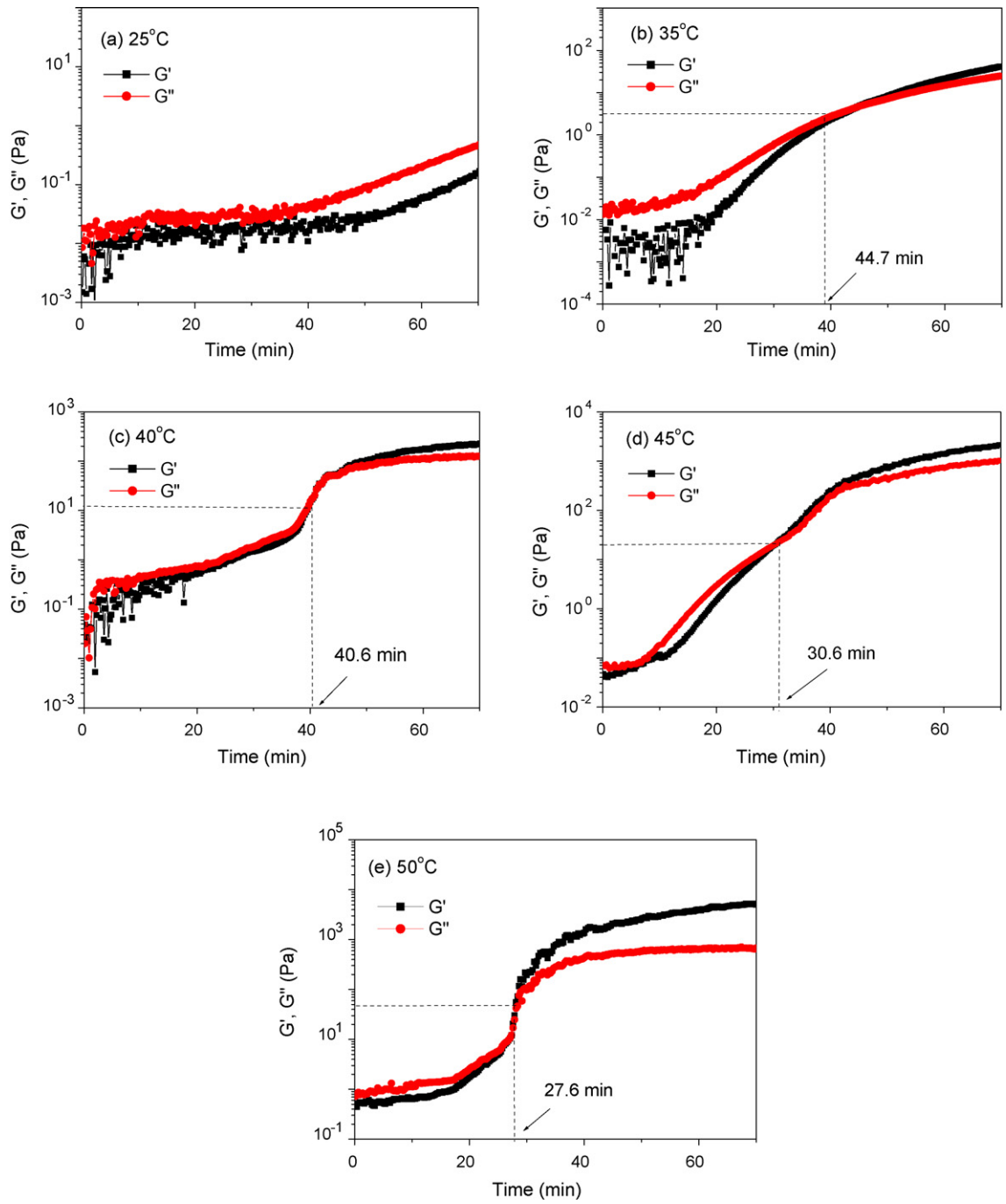

Fig. 2. Effect of the crosslinking temperature on the gelation of aqueous 8.0% casein solution in the presence of 5.0 mmol/L genipin. Test conditions: frequency, 1.0 rad/s; strain, 10.0%.

high concentration of genipin could result in a great extent of chemical crosslinking of the casein chains, which restricts the mobility and hydration of the macromolecular chains in the casein hydrogel. To further understand the swelling mechanism, the widely used power law (Baker, 1987) was used to fit the time-dependent swelling ratio data:

$$q = k_1 t^n \quad (2)$$

where q is the swelling ratio, t is the time, and k_1 and n are constants. When the exponential $n = 0.5$, solvent diffusion follows Fichian diffusion. When $n = 1.0$, the diffusion is case II diffusion, and when $0.5 < n < 1.0$, the diffusion is a combination of Fichian and case II diffusion and is usually called as anomalous diffusion. As a result, we found that Eq. (2) could not be used to describe the swelling kinetics of the casein hydrogels in the pH 1.2 medium due to fast swelling equilibrium. In contrast, the swelling kinetic curves of the casein hydrogels in the pH 7.4 medium could be fitted by the power law with good determination coefficients. In this case, the n value was determined to be 0.94 for the hydrogel with 2.5 mmol/L genipin, 0.92 for the hydrogel with 5.0 mmol/L genipin, and 0.74 for the hydrogel with 10.0 mmol/L genipin, respectively. These results indicate that water uptake in the crosslinked casein hydrogel at pH 7.4 follows an anomalous diffusion mechanism.

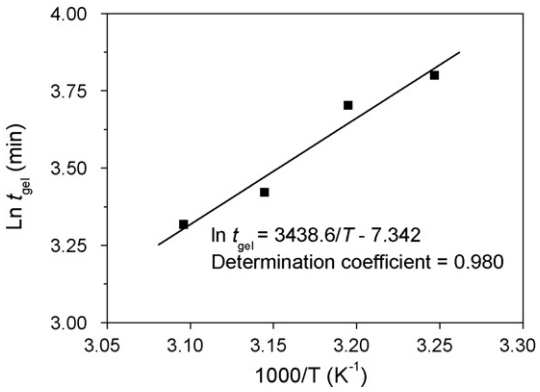

Fig. 3. Arrhenius plot for the temperature dependence of the gelation time (t_{gel}). The line represents a linear fitting for the data points.

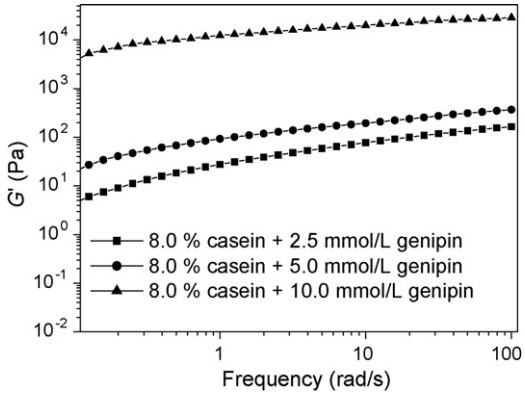

Fig. 4. The effect of genipin amount on the hydrogel strength of the crosslinked casein system. Test conditions: strain, 10.0%; temperature, 35 °C.

Fig. 8 shows the in vitro release profiles of BSA from the genipin-crosslinked casein hydrogels in simulated gastrointestinal tract conditions (37 °C). As seen, the amount of BSA released at acidic condition (pH 1.2) was relatively low. During the first 5 h period, for example, the cumulative BSA release was found to be 51.8% for the casein hydrogel crosslinked with 2.5 mmol/L genipin,

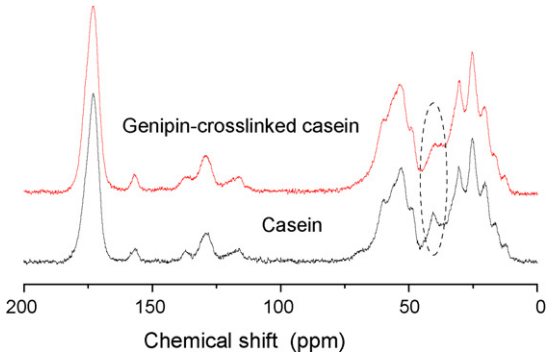

Fig. 6. The ^{13}C NMR spectra of casein and genipin-crosslinked casein.

39.9% for the casein hydrogel crosslinked with 5.0 mmol/L genipin and 34.7% for the casein hydrogel crosslinked with 10.0 mmol/L genipin, respectively. This was probably related to the low swelling degree of the genipin-crosslinked casein hydrogel in a pH 1.2 medium (Fig. 7a). In the case of pH 7.4, the cumulative BSA release increased significantly because the swelling of the casein hydrogel network increased considerably (Fig. 7b). After the first 5 h release at pH 7.4, the cumulative BSA release was found to be 98.5% for the casein hydrogel crosslinked with 2.5 mmol/L genipin, 68.7% for the casein hydrogel crosslinked with 5.0 mmol/L genipin and 60.1% for the casein hydrogel crosslinked with 10.0 mmol/L genipin, respectively. In the same pH medium, the cumulative BSA release from the casein hydrogel was observed to decrease with the increase of the genipin concentration used for its preparation. This phenomenon is similar to the aforementioned effect of genipin amount on the swelling ratio of the casein hydrogel at both pH 1.2 and 7.4. As a further investigation, the following semiempirical equation (Franson and Peppas, 1983) was used to analyze the drug release behavior of BSA from the genipin-crosslinked casein hydrogels:

$$\frac{M_t}{M_\infty} = k_2 t^m \quad (\text{for } M_t/M_\infty \leq 0.6) \tag{3}$$

where M_t/M_∞ is the fractional BSA release, M_t is the concentration of BSA released at time t, M_∞ is the concentration of BSA released at equilibrium, k is a constant relating to the proper-

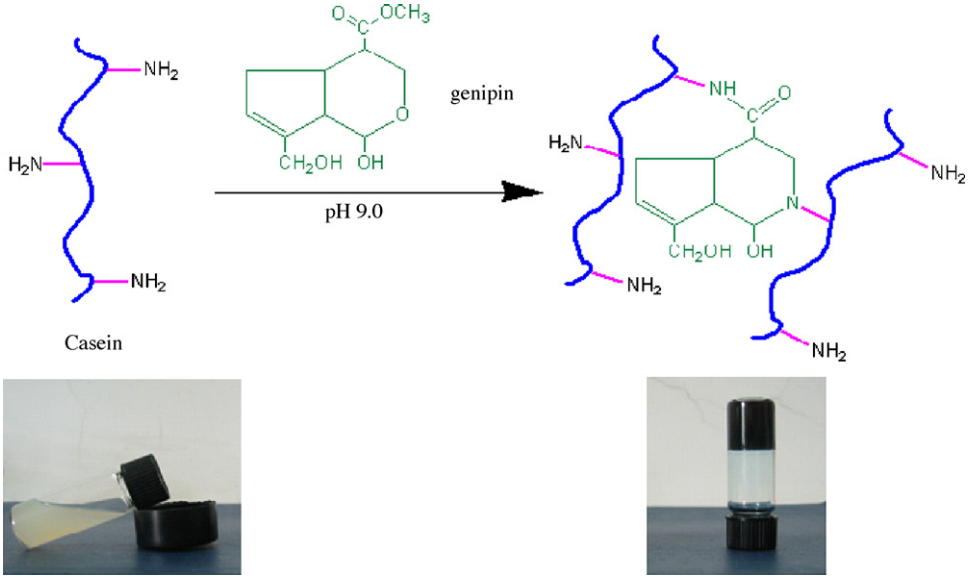

Fig. 5. A possible crosslinking mechanism for the reaction of casein with genipin in aqueous system.

Table 1
Release characteristics of BSA from the genipin-crosslinked casein hydrogels.

Release matrix	pH	n	k	R^2	Transport mechanism
Casein hydrogel with 2.5 mmol/L genipin	1.2	0.68	6.46×10^{-4}	0.98	Case III
	7.4	0.65	2.57×10^{-3}	0.97	Case III
Casein hydrogel with 5.0 mmol/L genipin	1.2	0.66	6.03×10^{-4}	0.92	Case III
	7.4	0.59	3.02×10^{-3}	0.99	Case III
Casein hydrogel with 10.0 mmol/L genipin	1.2	0.31	1.51×10^{-2}	0.91	Pseudo-Fickian
	7.4	0.72	5.50×10^{-4}	0.98	Case III

ties of the matrix and the drug, and m is the release exponent that depends on the transport mechanism and the geometry of the device. According to this classification, there are four distinguishable modes of diffusion: (i) the value of $m = 0.5$ suggests Fickian or Case I transport behaviour in which the relaxation coefficient is negligible during transient sorption; (ii) the value of $m = 1$ refers to a non-Fickian or Case II mode of transport where the morphological changes are abrupt; (iii) if $0.5 < n < 1$, the transport process is anomalous, corresponding to Case III, and the structural relaxation is comparable to diffusion; (iv) a value of $m < 0.5$ indicates a pseudo-Fickian behaviour of diffusion where sorption curves resemble Fickian curves, but the approach to final equilibrium is very slow. By plotting $\log(M_t/M_\infty)$ versus $\log(t)$, the m and k values as well as the corresponding determination coefficients (R^2) were obtained, as listed in Table 1. For the BSA-loaded casein hydrogel obtained in the presence of 10.0 mmol/L genipin, the m value was found to be o.31 at pH 1.2, showing a pseudo-Fickian diffusion behaviour. This might be attributed to the dense network structure and low swelling ratio, which hindered the diffusion of high molar mass BSA ($M_w = 67{,}000$ g/mol) and thus prolonged the final equilibrium process. Except for this case, the

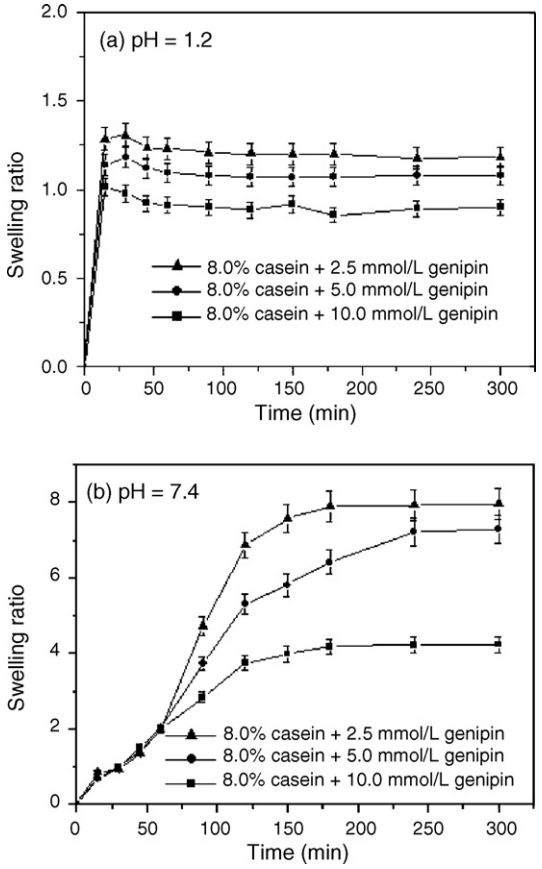

Fig. 7. Swelling characteristics of genipin-crosslinked casein hydrogels at (a) pH 1.2 and (b) pH 7.4 (37 °C).

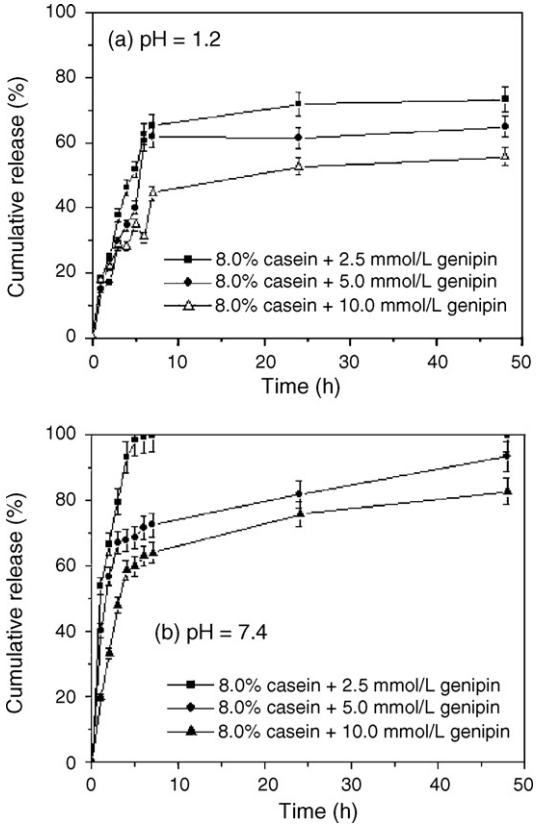

Fig. 8. The in vitro release profiles of BSA from the genipin-crosslinked casein hydrogels at (a) pH 1.2 and (b) pH 7.4 (37 °C).

m values of the system in all other cases were found to be in the range from 0.6 to 0.8, showing an anomalous diffusion release mechanism.

4. Conclusions

The naturally occurring genipin was used for the first time to crosslink aqueous 8.0% casein solution, resulting in a novel hydrogel material. With the increase of genipin amount, the gelation time decreased while the hydrogel strength enhanced. Additionally, the crosslinking temperature was found to have an important influence on the gelation. Depending on genipin amount, the resultant casein hydrogels have various swelling and drug release characteristics in simulated gastrointestinal tract conditions. The results suggest that the genipin-crosslinked casein hydrogel may be used as a suitable carrier for protein drug delivery.

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References

Akao, T., Kobashi, K., Aburada, M., 1994. Enzymic studies on the animal and intestinal bacterial metabolism of geniposide. Biol. Pharm. Bull. 17, 1573–1579.

Alvarez-Lorenzo, C., Yañez, F., Barreiro-Iglesias, R., Concheiro, A., 2006. Imprinted soft contact lenses as norfloxacin delivery systems. J. Control. Rel. 113, 236–244.

Ashton, R.S., Banerjee, A., Punyani, S., Schaffer, D.V., Kane, R.S., 2007. Scaffolds based on degradable alginate hydrogels and poly(lactide-co-glycolide) microspheres for stem cell culture. Biomaterials 28, 5518–5525.

Balakrishnan, B., Mohanty, M., Umashankar, P.R., Jayakrishnan, A., 2005. Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin. Biomaterials 26, 6335–6342.

Farrel Jr., H.M., Wichham, E.D., Unruh, J.J., Qi, P.X., Hoagland, P.D., 2001. Secondary structural studies of bovine caseins: temperature dependence of β-casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization. Food Hydrocolloids 15, 341–354.

Fox, P.F., Guinee, T.P., Cogan, T.M., McSweeney, P.H., 2000. Fundamentals of Cheese Science. Aspen Publishers, Inc., Gaithersburg, Maryland.

Franson, N.M., Peppas, N.A., 1983. Influence of copolymer composition on non-Fickian water transport through glassy copolymers. J. Appl. Polym. Sci. 28, 1299–1310.

Hoffman, A.S., 2002. Hydrogels for biomedical applications. Adv. Drug Deliv. Rev. 43, 3–12.

Ketelson, H.A., Meadows, D.L., Stone, R.P., 2005. Dynamic wettability properties of a soft contact lens hydrogel. Colloids Surf. B 40, 1–9.

Khademhosseini, A., Langer, R., 2007. Microengineered hydrogels for tissue engineering. Biomaterials 28, 5087–5092.

Kokabi, M., Sirousazar, M., Hassan, Z.M., 2007. PVA–clay nanocomposite hydrogels for wound dressing. Eur. Polym. J. 43, 773–781.

Lee, K.Y., Mooney, D.J., 2001. Hydrogels for tissue engineering. Chem. Rev. 101, 1869–1879.

Lee, K.Y., Yuk, S.H., 2007. Polymeric protein delivery systems. Prog. Polym. Sci. 32, 669–697.

Lim, F., Sun, A.M., 1980. Microencapsulated islets as bioartificial pancreas. Science 210, 908–910.

Lin, C.C., Metters, A.T., 2006. Hydrogels in controlled release formulations: network design and mathematical modeling. Adv. Drug Deliv. Rev. 58, 1379–1408.

Liu, D.Z., Sheu, M.T., Chen, C.H., Yang, Y.R., Ho, H.O., 2007. Release characteristics of lidocaine from local implant of polyanionic and polycationic hydrogels. J. Control. Rel. 118, 333–339.

Nickerson, M.T., Patel, J., Heyd, D.V., Rousseau, D., Paulson, A.T., 2006. Kinetic and mechanistic considerations in the gelation of genipin-crosslinked gelatin. Int. J Biol. Macromol. 39, 298–302.

Panouille, M., Nicolai, T., Durand, D., 2004. Heat induced aggregation and gelation of casein submicelles. Inter. Dairy J. 14, 297–303.

Park. K., Shalaby, W. S. W., Park, H., 1993. Biodegradable Hydrogels for Drug Delivery. Technomic, Lancaster, PA.

Risbud, M.V., Hardikar, A.A., Bhat, S.V., Bhonde, R.R., 2000. pH-sensitive freeze-dried chitosan–poly(vinyl pyrrolidone) hydrogels as controlled release system for antibiotic delivery. J. Control. Rel. 68, 23–30.

Smolensky, M.H., Peppas, N.A., 2007. Chronobiology, drug delivery, and chronotherapeutics. Adv. Drug Deliv. Rev. 59, 828–851.

Song, F., Zhang, L.M., 2008. Enzyme-catalyzed formation and structure characteristics of a protein-based hydrogel. J. Phys. Chem. B 112, 13749–13755.

Sung, H.W., Huang, D.M., Chang, W.H., Huang, R.N., Hsu, J.C., 1999a. Evaluation of gelatin hydrogel crosslinked with various crosslinking agents as bioadhesives: in vitro study. J. Biomed. Mater. Res. 46, 520–530.

Sung, H.W., Huang, R.N., Huang, L.H., Tsai, C.C., 1999b. In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. J. Biomat. Sci. Polym. Ed. 10, 63–69.

Touyama, R., Takeda, Y., Inoue, K., Kawamura, I., Yatsuzuka, M., Ikumoto, T., Shingu, T., Yokoi, T., Inouye, H., 1994. Studies on the blue pigments produced from genipin and methylamine: I. Structures of the brownish-red pigments, intermediates leading to the blue pigments. Chem. Pharm. Bull. 42, 668–678.

Wichterle, O., Lim, D., 1960. Hydrophilic gels in biologic use. Nature 185, 117–123.

Winter, H.H., Chambon, F., 1986. Analysis of linear viscoelasticity of a crosslinking polymer at the gel point. J. Rheol. 30, 367–378.

Woerly, S., 1993. Hydrogels for neural tissue reconstruction and transplantation. Biomaterials 14, 1056–1058.

Yannas, I.V., Lee, E., Orgill, D.P., 1989. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc. Natl. Acad. Sci. U.S.A. 86, 933–937.

Yuan, Y., Chesnutt, B.M., Utturkar, G., Haggard, W.O, Yang, Y., Ong, J.L., Bumgardner, J.D., 2007. The effect of cross-linking of chitosan microspheres with genipin on protein release. Carbohydr. Polym. 68, 561–569.

Zhang, L.M., Wang, G.H., Lu, H.W., Yang, C., Yan, L., 2005a. A new class of starch-based hydrogels incorporating acrylamide and vinyl pyrrolidone: effects of reaction variables on water sorption behavior. J. Bioact. Compat. Polym. 20, 491–501.

Zhang, L.M., Yang, C., Yan, L., 2005b. Perspectives on strategies to fabricate starch-based hydrogels with potential biomedical applications. J. Bioact. Compat. Polym. 20, 297–314.

Zhao, S.P., Zhang, L.M., Ma, D., 2006. New semi-interpenetrating network hydrogels: synthesis, characterization and properties. Macromol. Biosci. 6, 445–451.

Zhong, Q., Daubert, C.R., 2004. Kinetics of rennet casein gelation at different cooling rates. J. Colloid Interface Sci. 279, 88–94.