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Nanoencapsulation of Protein Drug for Controlled Release

Kazi Farida Akhter, Jesse Zhu and Jin Zhang*

Department of Chemical and Biochemical Engineering, University of Western Ontario, Canada

Abstract

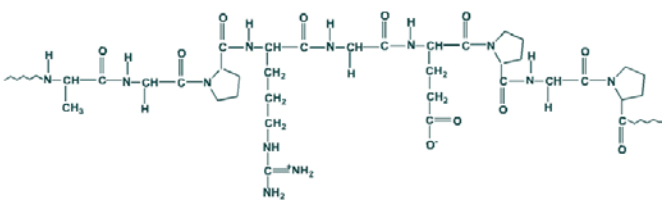
In this paper, gelatin nanoparticles (NPs) have been studied as a carrier to encapsulate protein drug for the controlled release with a prolonged manner. Bovine serum albumin (BSA), a hydrophilic protein drug, was loaded within gelatin NPs through an *in situ* two-step desolvation method. The average diameter of the NPs is estimated at 180 ± 10 nm by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Gelatin NPs show the mesoporous structure with average pore size of 2.82 nm. To confirm the encapsulation of BSA in gelatin NPs, fluorescent-labelled BSA was encapsulated in gelatin NPs, which were identified by confocal laser scanning microscopy (CLSM). The release kinetics of BSA from gelatin NPs in phosphate buffer saline (PBS) was studied through UV-Vis spectrometry. The release profile of BSA from nanoparticulate system could be monitored for 7 days. In addition, the rate of BSA release from gelatin NPs decreases with increasing the concentration of the cross-linker. The release profile of BSA from the gelatin NPs follows a diffusion-controlled release mechanism. Our results also indicate that the acidic condition can delay the release profile of protein from gelatin NPs.

Keywords: Gelatin nanoparticles; Protein releasing; Swelling; Nanoencapuslation

Introduction

Gelatin is produced by partially hydrolyzing of collagen from bovine bone and pig skin. It is a linear polymeric hydrogel, and is able to form helix structure because gelatin molecules have both acidic and basic functional groups [1]. The properties of gelatin depend on its manufacturing method (acidic or basic), the type and number of amino acids, and the molecular weight [2]. Type-A gelatin is obtained by acid treatment of collagen, and has an isoelectric point (Ip) between 7.0 and 9.0. Type-B gelatin, on the other hand, is obtained by alkaline hydrolysis of collagen, and has an Ip between 4.6 and 5.2. The primary structure of gelatin is the polypeptide chain made of specific 18 different amino acids as shown in the figure 1 [3]. Gelatin has been applied in food and pharmaceutical industry because it is a natural biopolymer and has unique gel-forming ability [2-4].

Quite recently, gelatin nanoparticles (NPs) have been used as a carrier for delivery of different drugs [5-7], genes [8-10], to lungs [11], to lymphocytes [12], and to leukemic cells [13]. Several techniques have been used to synthesize gelatin NPs, including desolvation technique, coacervation, and water-in-oil (w/o) emulsion. In case of w/o emulsion technique, a large amount of surfactant is required to produce the small-sized NPs, which needs a complicated post-process [4]. The coacervation method is a process of phase separation followed by cross-linking step, while, the non-homogeneous crosslinking occurs in this method and have unsatisfied loading efficiency. Here, we have applied a two-step desolvation method to *in situ* encapsulate hydrophilic protein, BSA, within gelatin NPs. In this paper, the release profiles of BSA-loaded gelatin NPs have been investigated as the function of pH value and crosslinker, respectively. In addition, we have studied the experimental and theoretical results to better understand the release mechanism of gelatin NPs acting as a drug carrier.



Chemical structure of gelatin

Figure 1: Chemical structure of gelatine.

Experiment Methods

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich.

Nanoencapsulation of BSA within gelatin NPs

Coester et al. reported a two-step desolvation method to produce gelatin NPs [2]. Here, this method was adapted to encapsulate BSA *in situ* within gelatin NPs. First gelatin was dissolved in deionic distilled water with the concentration of 5% (wt/vol). 25 ml of acetone was added into the gelatin solution with stirring for 20 min. Then, the precipitation was separated from the solution followed with re-dissolving in 25 ml acidic solution (pH=2.5). After stirring 10 min, BSA (0.0125 g) was added in the gelatin solution. 40 ml of acetone was then added dropwisely. The mixture was stirred for another 10 min. After then, the crosslinker, glutaraldehyde (25%), was added into the solution to form the stable gelatin nano-colloids. Different mounts of crosslinker were used, i.e. 175 µl, 250 µl, 350 µl, and 500 µl, respectively The solution was then kept stirring for 30 min. The solid gelatin particles were separated from solution through centrifuging at 5800 rpm for 10 min. The product was washed by water/acetone (70/30) in a water bath at 50°C, and washed three times by the sonication and centrifuging.

***Corresponding author:** Jin Zhang, Department of Chemical and Biochemical Engineering, University of Western Ontario, London, Ontario N6A 5B9, Canada, Tel: 519-661-2111; E-mail: jzhang@eng.uwo.ca

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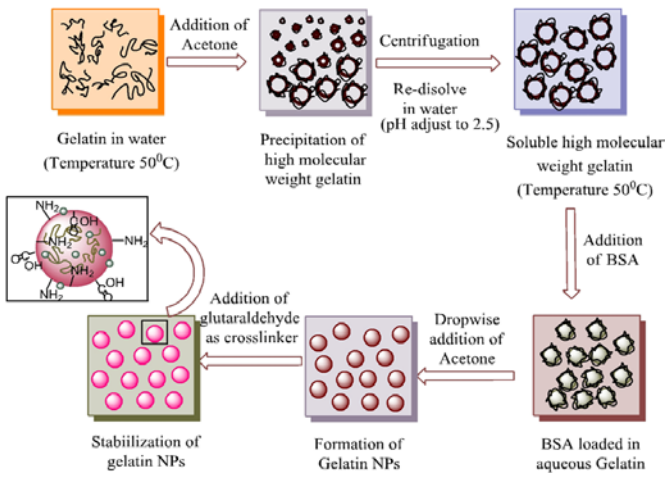


Schematic illustration of BSA loaded gelatin NPs produced by two-step disolvation.

Figure 2: Schematic illustration of BSA loaded gelatin NPs produced by two-step desolvation.

Finally, the NPs were freeze-dried for overnight and stored as powder. The process is described schematically in Figure 2.

Materials characterization

Scanning electron microscopy (Hitachi 3400-N) and Transmission electron microscopy (TEM- Philips CM 10) were used to study the particle size, morphology, and microstructure of the gelatin NPs. To identify the location of BSA in gelatin NPs, the FITC - labelled BSA was encapsulated in gelatin NPs, which was studied by using a Zeiss (LSM 510 Duo) Confocal laser scanning microscopy (CLSM) with the excitation wavelength (λ_{ex}) at 490 nm. The FTIR spectra of gelatin NPs were recorded on a FTIR spectrophotometer (BRUKER, VECTOR 22). The average pore size of the gelatin NPs were estimated by the Brunauer-Emmett-Teller (Micromertics ASAP 2010 BET).

Release kinetics

The releasing profiles of BSA from gelatin NPs were monitored by the UV- Vis spectrophotometer (Model: Agilent 8453). The standard curve was plotted to represent the correlation of the concentration of BSA and the corresponding intensity of the absorbance in the UV-Vis spectra. Different amounts of glutaraldehyde (25%), i.e. 175, 250, 350, and 500 µl, were added, respectively, in the reaction to harden the gelatin NPs in the synthesis process. On the other hand, PBS with pH value at 1, 4.7, and 7.5 were applied, respectively, to study the releasing kinetics of the BSA encapsulated within gelatin NPs. The release sample was collected at 0.5 h, 1 h, 3 h, 6 h, and 12 h on the first day followed by sampling at every 24 h interval for 7 days. All the points for each time interval were measured triplicates.

Swelling characteristics

Because of the strong absorption of water, gelatine is able to swell in the aqueous solution. The effects of the amount of crosslinker, and the pH value on the swelling behaviour of the gelatin NPs were investigated to further find out the mechanism of the releasing kinetics. The swelling ratios of the samples were calculated based on the equation below;

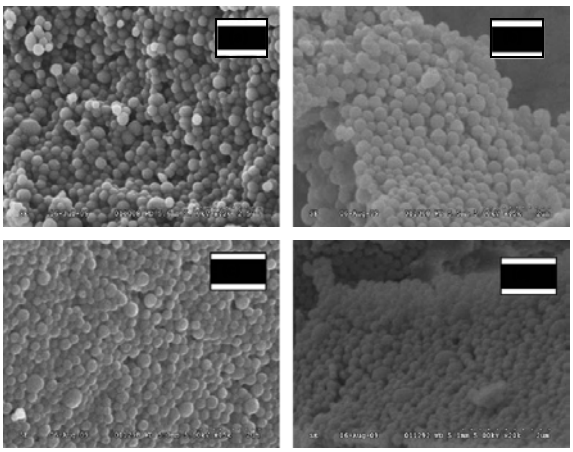
$$Swelling\ ratio\,(\%) = \frac{W_s - W_d}{W_d} \times 100\% \qquad (1)$$

where, W_s and W_d are the weights of swollen and dried spherical NPs, respectively. Each experiment was carried out in triplicate.

Results and Discussion

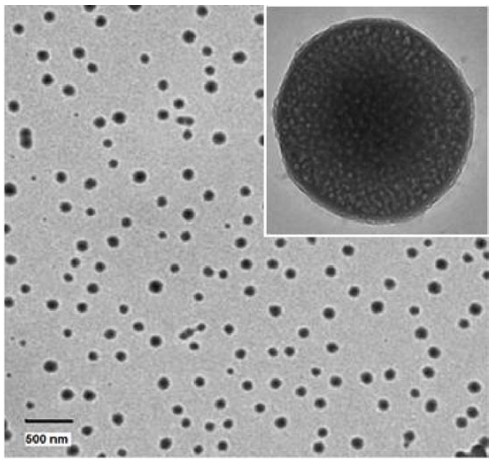
Materials characterization

As the isoelectric point (Ip) of gelatin is 6, the formed nanoparticles are positively charged in a solution with pH=2.5. So, the electrostatic repulsion prevents the polymer chains from undesired aggregation. In the process, acetone keeps the gelatin from hydration. In addition, the amount of crosslinker glutaldehyde, may affect the size and morphologies of the formed NPs. Figure 3 (a-d) show the SEM micrographs of BSA loaded gelatin NPs with different amount of glutaraldehyde, 175 µl, 250 µl, 350 µl, and 500 µl, respectively. The SEM micrographs clearly exhibit a difference in the morphology of NPs. The gelatin NPs have the most narrow size distribution when the amount of glutaraldehyde is 175 µl as shown in Figure 3a. The corresponding TEM micrograph of the gelatin NPs is shown in Figure 4. It is estimated that the diameter of the gelatin NPs is around 180 ± 10 nm. It is clear that the NPs are spherical and have large porous structure, i.e.



SEM micrographs of BSA loaded gelatin NPs with different amount of glutaraldehyde as crosslinker, (a) 175µl, (b) 250µl, (c) 350µl, and (d) 500µl.

Figure 3: SEM micrographs of BSA loaded gelatin NPs with different amount of glutaraldehyde as crosslinker, (a) 175µl, (b) 250µl, (c) 350µl, and (d) 500µl.



TEM micrograph of gelatin nanoparticles

Figure 4: TEM micrograph of gelatin nanoparticles.

mesoporous structure. The dried gelatin NPs were investigated by the BET. It indicates that the average pore size is about 2.82 nm for dried gelatin NPs.

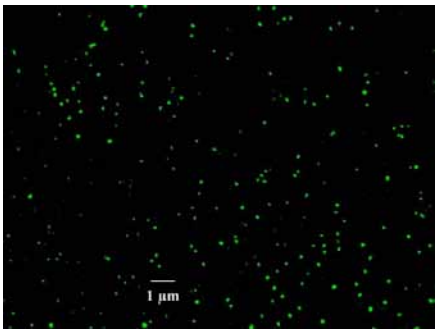
To further identify the encapsulation of BSA within gelatin NPs, fluorescein isothiocyanate (FITC)-labelled BSA was loaded in the gelatin by using the same process. Figure 5 is the dark field of fluorescent image. It shows that BSA is homogeneously located with the gelatin NPs.

Release profiles of BSA from gelatin nps

The release profiles of BSA from gelatin NPs were measured by using UV-Vis spectrophotometer. First the standard curve of the concentration of BSA in the range of 10 mg/ml to 400 mg/ml is plotted as shown in Figure 6. The BSA has the maximum absorption centering at 280 nm in the UV-Vis spectra. The relationship between the concentration (*C*) and the intensity of the absorbance (I) is able to be described as the Equation 2.

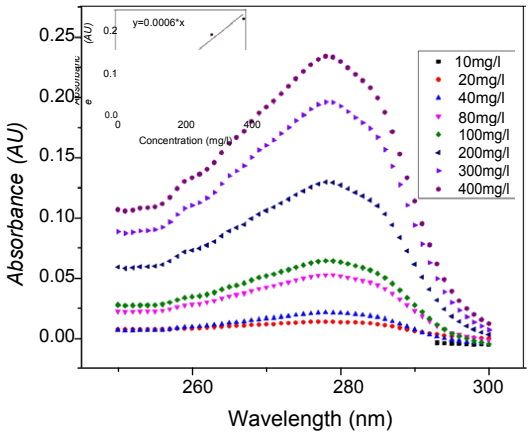
$$I = 0.0006 \times C \tag{2}$$

The release profile of BSA from gelatin NPs with the most narrow size distribution is further studied in PBS with different pH values.



Laser Confocal Scanning Microscopic (LCSM) image of FITC-BSA loaded gelatin NPs.

Figure 5: Confocal Laser Scanning Micrograph of FITC-BSA-loaded gelatin NPs.



UV-Vis absorption spectra of standard BSA

Figure 6: UV-Vis absorption spectra of standard BSA.

The release profiles of BSA from gelatin NPs in PBS solution, with different pH values, i.e. 7.4, 4.5, and 1.0, respectively, were further studied. The release was continuously monitored for 7 days as shown in Figure 7 and 8. At pH=7.4, over 80% of BSA is released when the release time (t) is less than 80 hrs. The initial 'burst effect' is observed in the first 8 hours, which is the typical diffusion – controlled polymer drug systems. The encapsulated BSA is continuously released with a lower release rate when 20 hrs<t<80 hrs. When t>80 hrs, the release rate is quite slow, and get to the saturation when *t* is approaching to 120 hrs. In addition, it indicates that release rate increases with increasing pH value from 1.0 to 7.4. It is caused by the presence of acidic pendant group, which makes gelatin hardly dissociate in a lower pH solution [14].

Swelling behaviors of gelatin NPs

The swelling behaviors of gelatin NPs were further studied at pH=1.0, 4.5, and 7.4, respectively. It is found that gelatin NPs show strong pH-dependent swelling behaviors. At pH=1.0, the swelling rate is a considerable decrease with comparison of that of gelatin NPs in a higher pH solution, i.e. pH=7.4.The phenomenon may be attributed to the electrically neutral NPs when pH value closes to its Ip. Whereas, the negative change of NPs network at acidic condition could have electrically repulsive interaction with water. The substantial swelling of gelatin NPs at a larger pH value, i.e. pH=7.4, may lead to quick release profile of the drug.

Consequently, the mechanism of slow release of hydrophilic protein from gelatin NPs in acidic condition may involve the following aspects: (1) water permeation through the hydrogel matrix and absorption by the gelatin NPs, (2) gelatin NPs swelling, (3) diffusion of BSA molecules through the swollen gelatin NPs.

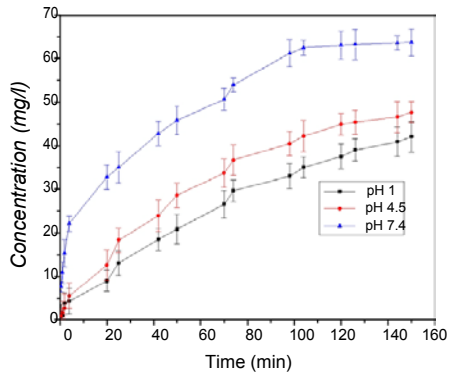
Release mechanism

The controlled release of protein drug in nanospheres was estimated following the model developed by Batycky, R.P. and co-workers [15]. According to the model, drug is released from the nanosphere in a Fickian manner, characterized by an effective drug diffusivity (D_d).

Therefore, following the induction time Fickian diffusion of drug through the pore of hydrogel allows the mass of drug remaining in the nanoparticle to be determined as follows:

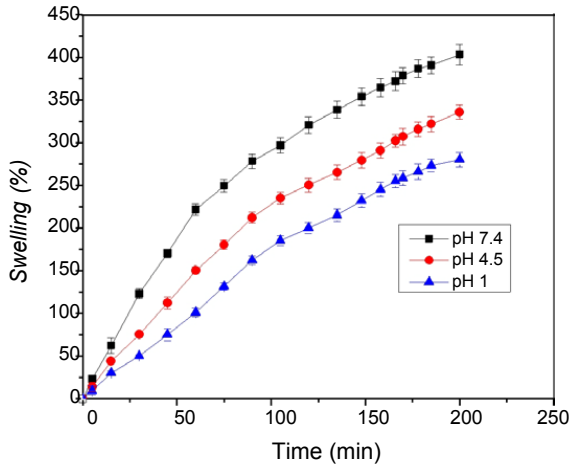
$$\frac{m_d(t)}{m_d(0)} = 1 - \phi_d^{burst}(1 - e^{-k_d t}) - (1 - \phi_d^{burst})\left(1 - \frac{6}{\pi^2}\sum_{j=1}^{\infty}\frac{e^{-j^2\pi^2\bar{D}_d(t-t_d)/r_0^2}}{j^2}\right) \tag{3}$$

where, $m_d(t)$ is the mass of drug present in nanosphere at time *t*, $m_d(0)$ the initially mass of drug, $\phi_d$burst the mass fraction of drug involved in the initial burst, k_d the drug desorption rate constant, j the number of different types monomer subunits, D_d the effective drug diffusivity, t the release time, t_d the drug induction time, and r_0 the initial nanoparticle radius. Figures 9 and 10 show the comparison of experimental and the theoretical value of the fractional release of BSA from gelatin nanoparticulate system with different amount of crosslinker. It is revealed that the diffusion model is fit for the experimental data. Good agreement between theory and experiment may suggest the validity of the presented model equation (Equation 3) to the delivery system of gelatin NPs. In Figure 9, the drug diffusion coefficient is decreased with increasing the cross-linker due to the steric hindrance provided by polymer chains within the cross-linked networks. In addition, the diffusion coefficient is maximum with the pH 7.4 whereas it is minimum with pH=1 as shown in Figure 10.



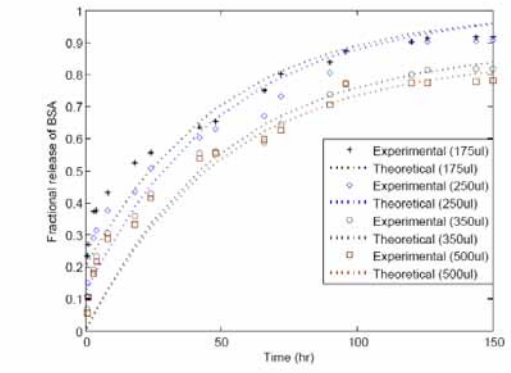
Release profile of BSA encapsulated in gelatin NPs at different pH values

Figure 7: Release profiles of BSA encapsulated in gelatin NPs at different pH values.



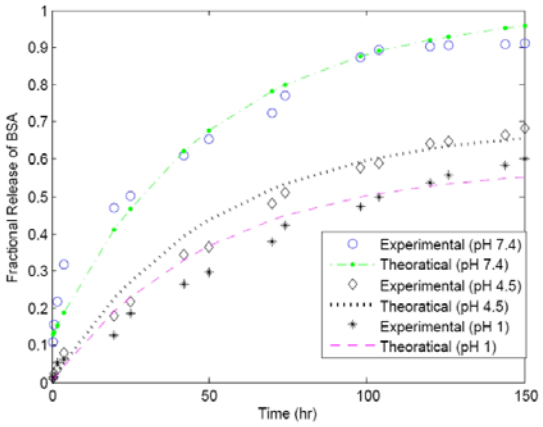
Swelling behaviors of gelatin NPs at different pH values

Figure 8: Swelling behaviors of gelatin NPs at different pH values.



Model fitting on the release profiles of BSA from gelatin NPs with different amount of crosslinker in PBS solution at pH =7.4.

Figure 9: Model fitting on the release profiles of BSA from gelatin NPs with different amount of crosslinker in PBS solution at pH =7.4.



Model fitting on release of BSA from gelatin NPs in different pH solutions.

Figure 10: Model fitting on release of BSA from gelatin NPs in different pH solutions.

Conclusion

A biodegradable nanosystem, gelatin NPs, has been developed as an efficient carrier for the delivery of hydrophilic protein drugs. The average diameter of gelatin NPs is approximately 180 ± 10 nm. The encapsulated protein drug, BSA, is located inside the gelatin NPs homogeneously. The releasing profile has been monitored for 7 days. The effects of crosslinking and pH values on the release profile of protein drug from the nanosytems have been investigated. The experimental results revealed that the release rate of BSA from gelatin NPs decreases with increasing the amount of crosslinker. In addition, the release rate decreases at acidic condition, which is able to be explained by the pH-dependant swelling behavior of the hydrogel NPs. The experimental results also revealed that the release profile of BSA from the gelatin nanoparticulate follows a diffusion-controlled release mechanism. All the studies conclude that the gelatin NPs are able to encapsulate water-soluble protein drug for the prolonged drug release in a controlled manner.

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