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Development of Ultrasmall Chitosan/Succinyl β-Cyclodextrin Nanoparticles as a Sustained Protein-Delivery System

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ABSTRACT: In this article, we introduce a new method for preparing ultrasmall chitosan (CS)/succinyl β-cyclodextrin (SCD) nanoparticles (NPs) intended for loading bovine serum albumin (BSA) as a model protein. The proposed method is based on the complex coacervation technique followed by ionotropic gelation with tripolyphosphate. SCD, an anionic derivative of cyclodextrin, was synthesized and used in CS-based NPs to enhance the entrapment efficiency of BSA. The results show that with this approach, ultrasmall, compact, and neutralized NPs with a mean particle size near 30 nm were obtained. A high degree of protein entrapment in the NPs led to a significant improvement in the BSA release profile with a low initial burst release (ca. 3% w/v of the initially loaded BSA) and a sustained release over time. This enabled a suitable nanocarrier for long-term protein delivery (30% release over 120 h). © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2013

KEYWORDS: biodegradable; biopolymers; drug-delivery systems

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INTRODUCTION

Chitosan (CS), which is mainly obtained by the alkaline deacetylation of chitin, is a linear polysaccharide composed of randomly distributed D-glycosamine and *N*-acetyl glycosamine units.[1] CS has attracted much interest in a wide range of applications, including enzyme immobilization,[2] tissue engineering,[3] surgery adhesion bandages,[4] antigen and drug-delivery systems,[5,6] and other environmental, biomedical, and pharmaceutical applications. CS owes this to its versatile outstanding characteristics, such as its bioavailability, low toxicity, biocompatibility, biodegradability, low immunogenicity, and high charge density.[7] Recently, a large number of studies have been conducted with regard to applications of CS nanoparticles (NPs) in gene/drug delivery through various administration routes, including oral, bucal, nasal, transdermal, parenteral, vaginal, cervical, intrauterine, and rectal administration.[7–11] Benefitting from precise targeting at the cellular level, CS-based microparticles and NP-delivery systems provide effective control of gene and drug-release profiles.[12]

CS modification is necessary to render them soluble in common solvents to use them to the fullest extent.[13,14] The use of modified CS particles as peptides, polypeptides, and protein drug carriers has been frequently reported.[15,16] CS-coated alginate microspheres were chosen as carriers for a model protein, hemoglobin, with regard to the nontoxic properties of CS.[17]

Cyclodextrins (CDs) are cyclic oligosaccharides with a hydrophilic exterior surface. Their free hydroxyl groups on the outer surface[18] and their hydrophobic interior cavity make them suitable candidates for forming partial inclusion complexes with macromolecular drugs and so enable more stable drugs.[19]

CDs are widely used in biorelated applications as host molecules because of their sustainability.[20] CDs, as drug carriers, offer a series of favorable properties, including the reduction of undesired side effects and the solubilization and stabilization improvement of drug-delivery systems. Chemical modification has been applied to CDs to alter their solubility, complex formation capacities, and introduce functional groups.[21]

The complex formation between anionic modified β-CD and CS derivatives results in inclusion combination and size specificity, along with enhanced transport properties. These modified β-CD molecules possess controlled release ability of the CS derivatives as drug carriers.[17,19,22,23] Because of the superior properties of these formulations, β-CD/CS complexed NPs have gained a large interest for drug/gene delivery.[24]

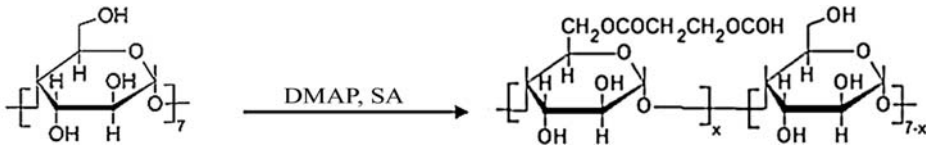

Figure 1. Reaction scheme for the synthesis of SCD.

In this study, new hybrid polysaccharide nanocarriers, based on CS combined with succinyl β-cyclodextrin (SCD), were prepared; and their drug loading efficiency (LE), release profile, and morphological characteristics were compared with CS NPs loaded with bovine serum albumin (BSA). BSA has been frequently reported as a calibration standard or model protein for the study of protein-delivery systems.[25]

Among various methods for preparation of CS NPs (ionotropic gelation, coacervation, emulsion–droplet coalescence, etc.), the ionotropic gelation process is the most widely used because of its simplicity.[26,27] One challenging issue in the preparation of CS NPs is the development of *small* monodispersed CS nanocarriers with a mean size below 100 nm.[23,28–30] In this study, we used a combination of ionotropic gelation and complex coacervation methods to prepare compact CS/SCD NPs with reduced size and improved encapsulation efficacy and release profiles.

EXPERIMENTAL

Materials

CS (low molecular weight, 75–85% deacetylated), β-CD, 4-(*N*,*N*′-dimethyl amino) pyridine (DMAP), BSA, succinic anhydride (SA), pyridine, and anhydrous pyridine were purchased from Aldrich and used as received. Sodium tripolyphosphate (TPP) and isopropyl alcohol were obtained from Merck (Germany). All of the other reagents used were analytical grade.

Synthesis of CS/SCD

SCD was prepared according to a method previously reported by Constantin and Funduenu[31] (Figure 1). First, 4 g of SA was dissolved in 6 mL of pyridine. The prepared solution was added to 10 mL of anhydrous pyridine containing 2 g of β-CD and then mixed together (stirrer speed = 100 rpm) at 45°C. Then, 100 mg of DMAP was added to the mixture; this was followed by stirring under the same conditions for 6 h. The prepared SCD was precipitated by 20 mL of isopropyl alcohol and washed again with an excess 5 mL of isopropyl alcohol and subsequently with 5 mL of acetone. It was then freeze-dried and stored at room temperature.

^{1}H-NMR Analysis

The ^{1}H-NMR spectrum was recorded in D_2O and hexadeuterated dimethyl sulfoxide solvents, respectively, with a Varian Mercury Plus 400/VARIAN VXR 200 NMR instrument. The chemical shifts were quoted in parts per million from tetramethylsilane.

Preparation of BSA-Loaded NPs

Free and BSA-loaded CS- or CS/SCD-based NPs were prepared according to different procedures and are presented in Table I. For the preparation of free and loaded CS NPs, a modified ionotopic gelation method was used. A CS solution of 0.05% w/v was prepared by the dissolution of CS in an acetic acid solution (pH 5.0). A volume of 10 mL of a BSA aqueous solution (0.1% w/v) was mixed with 100 mL of the CS solution at room temperature, and the mixture was shaken (200 rpm) for 24 h. Then, BSA-loaded CS NPs were prepared by the addition of 10 mL of a TPP aqueous solution (0.1% w/v, pH 5.0) to 55 mL of the CS–BSA solution, and the mixture was ultrasonicated (ultrasonication power = 45 W) for 60 min.

The preparation of the BSA-loaded CS/SCD(1) NPs was carried out with a complex coacervation method. SCD solutions of 0.2% w/v were prepared by the dissolution of the respective SCD in deionized water. To load BSA, 10 mL of a BSA aqueous solution (0.1% w/v) was mixed with 15 mL of the SCD solution at room temperature for 24 h with shaking (200 rpm). Then, the solution was added to 4 mL of the prepared CS solution and was mixed with stirring (200 rpm) for 2 h.

For the BSA-loaded CS/SCD(2) NPs, TPP was used in a formulation other than CS and SCD. Preparation of the NPs was performed by a combination of both a complex coacervation technique and modified ionotropic gelation method. A volume of 10 mL of a BSA aqueous solution (0.1% w/v) was mixed with 10 mL of the SCD solution (0.2% w/v) at room

Table I. Codes, Formulations, and Preparation MethodS for the Studied NPs

Sample code	SCD/CS (w/w)	TPP/CS (w/w)	BSA/(CS + SCD) (w/w)	Preparation method
Free CS	—	2/9	—	Ionotropic gelation
BSA–CS	—	2/9	1/5	Ionotropic gelation
Free CS/SCD(1)	1.5/1	—	—	Complex coacervation
BSA–CS/SCD(1)	1.5/1	—	1/5	Complex coacervation
Free CS/SCD(2)	1/1.5	1/5	—	Ionotropic gelation/complex coacervation
BSA–CS/SCD(2)	1/1.5	1/5	1/5	Ionotropic gelation/complex cacervation

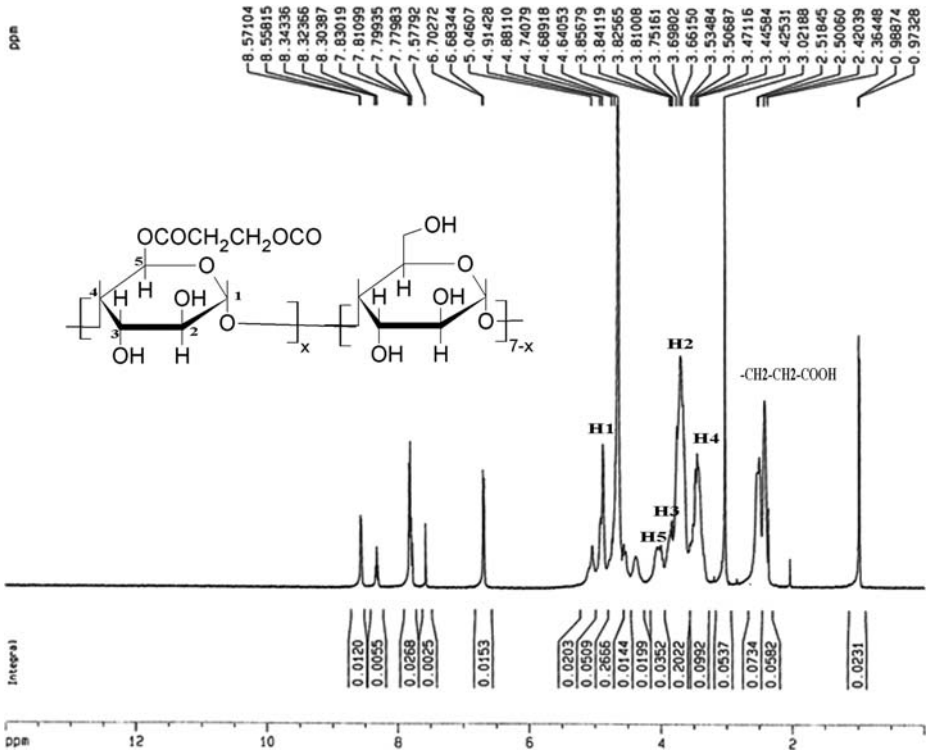

Figure 2. [1]H-NMR spectrum of SCD.

temperature and was shaken (200 rpm) for 24 h. The SCD/BSA solution was added to 60 mL of the prepared CS solution and was mixed by stirring (200 rpm) for 2 h. While the BSA-loaded CS/SCD was forming, 6 mL of TPP aqueous solution (0.1% w/v, pH 5.0) was added to the solution, and the mixture was ultrasonicated (ultrasonication power = 45 W) for 60 min. After the formation of NPs, the prepared suspensions were centrifuged (180 g, 30 N) for 10 min to remove free, unloaded BSA and unreacted TPP. The LE of BSA was calculated according the following equation:

$$\text{LE} = \frac{\text{Total amount of BSA-Free BSA}}{\text{Total amount of BSA}} \times 100\%$$

Then, the NPs were freezedried (MODULYOD freeze dryer, Thermoelectron Corp.). The free NPs were prepared in a similar manner but without BSA.

Morphology Study, ζ Potential, and Size Measurement

A Philips XL30 scanning electron microscope at an accelerating voltage of 15 kV was used to examine the morphology of the free and BSA-loaded NPs. All of the samples were previously sputter-coated with an ultrathin layer of gold. The size measurement and ζ-potential of the NPs were probed by a Zetasizer Nano-ZS-90 (Malvern Instruments) in a 0.1 m*M* KCl solution (pH 7.4) at 25°C under automatic mode.

In Vitro Release Studies

To study the *in vitro* release profile of BSA from the loaded NPs, 2 mg of BSA-loaded NPs were suspended in a microtube containing 2 mL of phosphate-buffered saline (pH 7.4) as the release medium in a thermostatic shaker maintaining a constant temperature (37°C) under stirring (300 rpm). At predetermined intervals, the suspension was centrifuged (180 g, 30 N) for 2 min, and 300-μL samples from the supernatant were removed and replaced with the same volume of fresh phosphate-buffered saline medium. Because BSA showed a maximum absorption wavelength at 280 nm in the ultraviolet–visible spectra, the study of the amount of released BSA was carried out by a UV spectrophotometer at 280 nm in triplicate for each sample.

RESULTS AND DISCUSSION

[1]H-NMR Analysis

The [1]H-NMR spectrum of SCD is presented in Figure 2. The clear peak situated at 2.4–2.6 ppm, related to the succinyl group, confirmed the grafting of the succinyl group on the β-CD.[28]

Morphology, ζ Potential, and Size of the Prepared NPs

Figure 3 represents the scanning electron microscopy (SEM) images of the free NPs prepared through different procedures. As shown, all of the formulations led to small NPs with a mean size below 100 nm. The CS/SCD(1) NPs demonstrated more spherical shapes compared to the other prepared NPs. Table II presents the mean particle size of the free and BSA-loaded NPs. The tabulated results demonstrate that the NPs produced by the ionotropic gelation method were much smaller than those prepared with a mixture of CS and SCD. The small and linear TPP molecules, because of their active anionic nature, readily penetrated and interacted with the cationic molecules of CS.[32] This characteristic resulted in more compact and smaller NPs in comparison with those prepared with SCD.

According to a previous report, in the case of the CS/SCD(1) sample, insufficient negative charges of SCD might not be able

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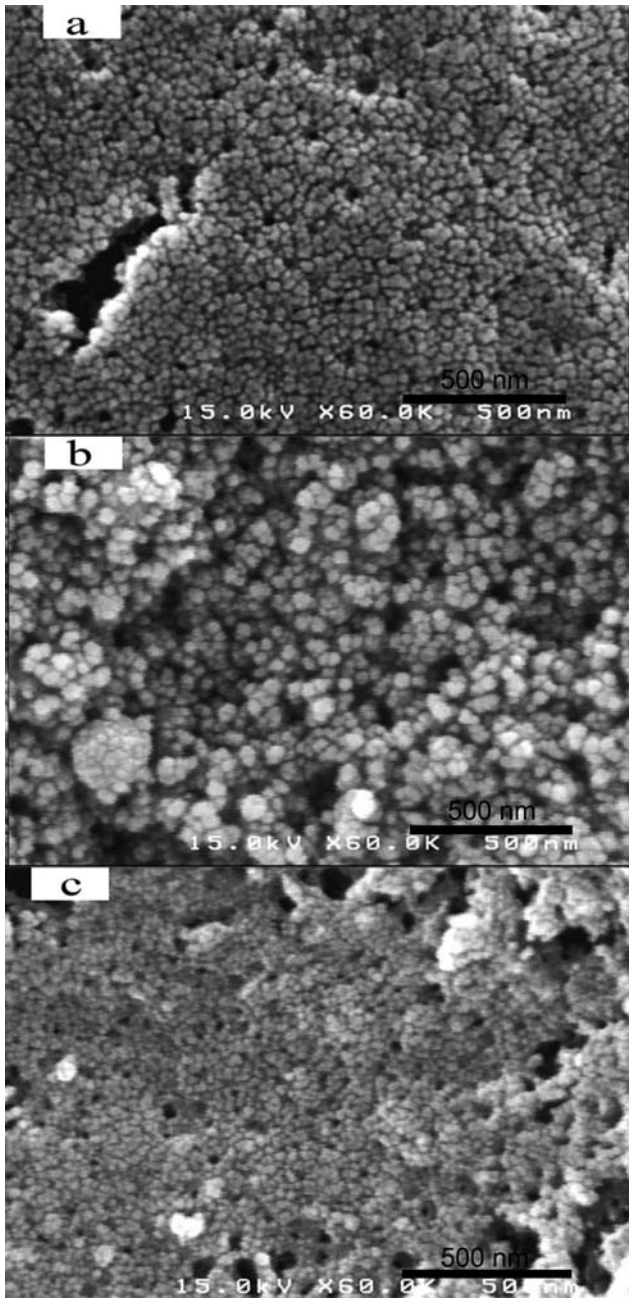

Figure 3. SEM microphotographs of the unloaded NPs: (a) CS, (b) CS/SCD(1), and (c) CS/SCD(2).

to neutralize positive charges of CS and so might cause less efficient crosslinking.[29] The electrostatic repulsion between the remaining ionized amine groups of CS NPs and the bulky ring structure of SCD resulted in a more expanded structure and larger particles.[17] The simultaneous use of TPP and SCD resulted in the formation of the smallest and most compact and neutralized NPs. This surprisingly unexpected observation might have been related to the different functions of TPP and SCD as physical crosslinkers in the NP formulation. TPP might have neutralized the positive charge of the CS and SCD molecules in an efficient manner because of its modest neutralizing effect. So, in the preparation procedure, the mean particle size

Table II. Characteristics of the NPs with and without BSA

Sample code	Particle size: Mean ± Standard deviation (nm)	ζ potential: Mean ± Standard deviation (mV)	BSA LE (%)
Free CS	34.4 ± 4.5	27.8 ± 2.2	—
BSA-CS	37.4 ± 4.2	22.4 ± 2.1	64.4 ± 2.3
Free CS/SCD(1)	52.6 ± 5.4	32.8 ± 4.3	—
BSA-CS/SCD(1)	61.4 ± 3.6	29.4 ± 3.8	80.2 ± 1.9
Free CS/SCD(2)	28.7 ± 1.8	19.3 ± 1.4	—
BSA CS/SCD(2)	31.4 ± 1.1	18.7 ± 3.3	77.2 ± 2.5

decreased remarkably. The ζ potential value of the NPs decreased with the addition of TPP to the formulation and demonstrated a drop off in the number of free amino groups on the NP surface.[29]

It has been shown before that linear water-soluble polymers partially penetrate into the inner cavity of CD to form inclusion complexes with a necklacelike supramolecular structure.[18,19,32–34] This phenomenon possibly provides more compact NPs, even in the presence of the bulky structure of CD. However, the nice spherical shape of the CS/SCD(1) NPs might have been due to the role of CD in preparation procedure of the CS NPs. The localized cationic CS molecules on the NP surface in comparison with the NP core[35] minimized the surface area with a smoother and more spherical shape to reach a thermodynamically stable level. A remarkable increase in the ζ potential values of both the free and BSA-loaded CS/SCD(1), as presented in Table II, confirmed this. In the case of CS/SCD(2), the addition of TPP led to a slight decrease in the ζ potential value and caused less spherical NPs.

All of the prepared free NP mean sizes were below 60 nm with a narrow size distribution; this was achieved by optimization of the preparation parameters, including CS concentration, CS/TPP ratio,[27] amount of incorporated CD,[23] CS solution, pH, and temperature.[28] In addition, the opposite charges of CS and BSA caused the spontaneous formation of particles.[24] This factor, together with the aforementioned parameters, resulted in compact NPs.

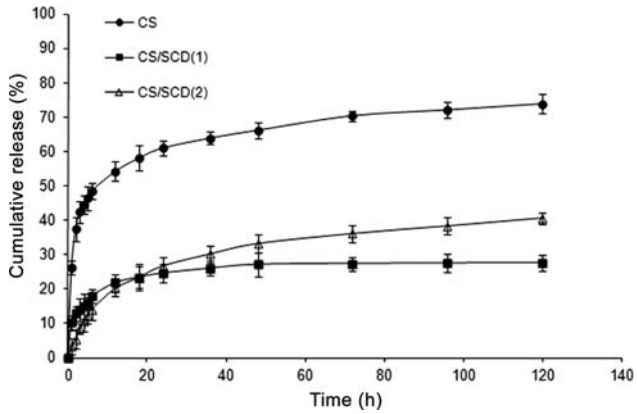

Figure 4. Cumulative BSA release profile from the NPs ($n = 3$).

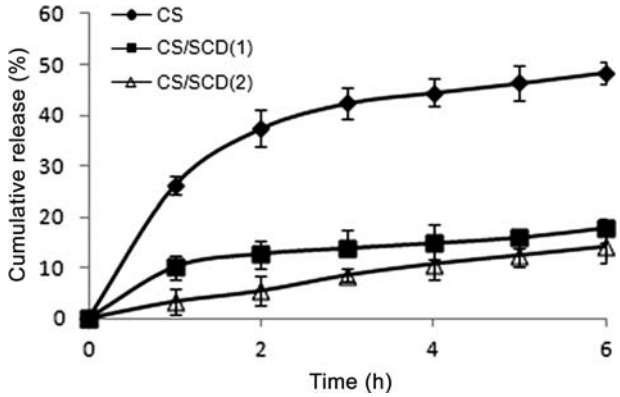

Figure 5. Cumulative BSA release profile from the NPs in the first 6 h (n = 3).

LE of Drug–CS NPs

In all of the prepared BSA-loaded samples, the pH optimization of the polymer solution resulted in a high degree of LE, as shown clearly in Table II.

With SCD in the formulation of the prepared NPs, which benefitted from a hydrophobic cavity and a sufficiently hydrophilic exterior,[18,24] an obvious enhancement in the entrapment efficiency was observed. Highly entrapped BSA in SCD, together with the inclusion of SCD molecules into the NP core, increased the BSA entrapment efficiency. The hydrophobic part of the protein molecules, which penetrated into the hydrophobic CD cavity, resulted in a higher LE. This observation was attributed to the formation of noncovalent inclusion complexes.[36–39] In conclusion, the use of CDs increased the drug-loading capacity.[40] The use of both TPP and SCD led desirably to more compact NPs; however, it had an insignificant effect on the BSA LE; this was due to the lower amount of SCD as a protein entrapping agent in this formulation.

In Vitro Release of BSA

As presented in Figure 4, the samples, including SCD, displayed more sustained release profiles. The partial inclusion of BSA macromolecules with SCD via BSA hydrophobic side chains resulted in more stable complexes[39] and could be considered as the main effective factor in improving the BSA release profile. Furthermore, it has been recently reported that a majority of CD anionic derivatives are usually integrated into the CS-based NP core.[23,35,36] Because of this fact, a large portion of BSA molecules in both the CS/SCD(1) and CS/SCD(2) samples were located in the NPs core. This phenomenon successively *delayed the BSA diffusion from the NPs* to the media.[34] The fast release profile of BSA in the CS NPs might have been related to the typical feature of CS NPs.[41] The initial rapid release stage reached a plateau in a short time period.

As simple diffusion through the CS network followed by polymer degradation was considered to be the main reason for protein release,[41] the observed high degree of mobility and protein release might have been due to the presence of small linear molecules, such as TPP, which provided a high structural mobility for the system.

Interestingly, the CS/SCD(2) sample represented a minimum BSA burst release, which is depicted in Figure 5. As stated previously, before we added TPP, a large number of CS chains were integrated with positive charges on the NP surface. The addition of TPP additionally supported BSA on the NP surface and resulted in a delayed release, along with a minimized burst release. This improved the sustained release from the CS/CD NPs compared to that of CS NPs reported before.[42] Zhang et al.[43] have reported that the insulin release in intestinal fluid decreased remarkably with the use of CDs because they occupied the major part of insulin in the NP core. The combination of CS and anionic derivatives of CD with proteins such as insulin has been known as an effective approach for enhancing proteins adsorption.[44] In another study, it was observed that sulfobutyl ether-7-β-cyclodextrin modified CS NPs exhibited slow release profiles with around 20% of dalargin released over 72 h.[18] The stabilizing role of the CDs caused such a prolonged release,[44] wherein the anionic derivatives of CD interacted with the cationic molecules of CS, and thus, stabilized the complex formation with protein.[45–47] However, as presented in Figure 4, because of the lower incorporated SCD amount in CS/SCD(2), the supportive role of SCD in CS/SCD(1) was more significant in comparison with that of CS/SCD(2). The plateau, which was reached at about 28% of the total loaded BSA, might be considered as a direct consequence of the larger amount of supportive SCD molecules.

CONCLUSIONS

In this study, NPs, including CS and an anionic derivative of CD, SCD, were prepared and characterized *in vitro* to study BSA release from the prepared nanocarriers, as a simple model for drug delivery. The obtained results show that with appropriate amounts of TPP and SCD in the preparation of CS-based NPs, together with the combination of the complex coacervation and ionotropic gelation methods under preadjusted conditions, we fine-tuned the physicochemical characteristics of the CS NPs with a low initial burst release (ca. 3% w/v of the initially loaded BSA). This strategy also enhanced the BSA release profile, which showed a 30% w/v release over 120 h. This formulation and preparation technique could be extended to increase the robustness of other protein-/gene-delivery systems to reach a minimum burst release and more enhanced release profile.

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