Reinforcement of Chitosan Nanoparticles Obtained by an Ionic Cross-linking Process

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ABSTRACT

The reduction in burst release of a new drug nanocarrier system was achieved by ionic gelation of nanoparticles made of chitosan and tripolyphosphate. Capability of calcium alginate for reinforcement of the chitosan-tripolyphosphate nanoparticle matrix was studied. Bovine serum albumin (BSA) was loaded into nanoparticles as a model drug. The final particle size, polydispersity index, entrapment efficiency and the release rate of BSA were optimized in relation to variations in parameters such as sodium alginate concentration, theoretical loading of BSA and degree of deacetylation of chitosan. It was found that increases in sodium alginate concentration resulted in smaller particle size, higher polydispersity index, lower entrapment efficiency and lower release rate of BSA. The same parametric changes were observed for the theoretical loading of BSA, with the exception of drug release which was followed by higher rate. Nanoparticles of chitosan-tripolyphosphate with a high degree of deacetylation led to production of smaller particle size, higher polydispersity index and entrapment efficiency, and a faster drug-release profile. The experimental data of this study revealed that burst release decreased significantly in reinforced chitosan-tripolyphosphate nanoparticles.

INTRODUCTION

Application of chitosan, a cationic polysaccharide, has been extensively investigated in various fields of applications, such as biotechnology [1], as a pharmaceutical excipient in oral drug formulations [2], waste water treatment [3], cosmetics [4] and food science [5]. These broad fields of applications are due to chitosan's unique set of properties. Favourable biological properties [6], low toxicity [7], biodegradability [8], proper mucoadhesive properties [9], absorption enhancer across intestinal epithelium and mucosal sites for drugs, peptides and proteins [10], metal complexation [11], antimicrobial activity [12], good homostatic properties [13], and acceptable anti-cancerous properties [14] are the most important set of properties. Among all applications of chitosan drug delivery systems are widely recognized as a promising research field [15]. Tablets, microspheres, microgels and nanoparticles are the different forms...
of chitosan-based drug delivery devices that have been studied. In the past decade, chitosan nanoparticles have been extensively investigated because they can control drug release rate, prolong the duration of therapeutic effectiveness, and deliver drugs to their specific sites in the body [16]. Chitosan nanoparticles exhibit superior activity that can be attributed to their small and quantum size effect [17].

Common methods to prepare chitosan nanoparticles are ionic gelation, coacervation or precipitation, emulsion-droplet coalescence, reverse micellar, and self-assembly chemical modification [14]. The ionic gelation process is commonly used to prepare chitosan nanoparticles because it is a very simple and mild method [18]. This process can be performed either by chemical or physical cross-linking. It is worth noting that chitosan has a high density of amine groups in its backbone and the amine groups are protonized to form -NH$_3^+$ in acidic solution. These positively charged groups in chitosan can be chemically cross-linked with dialdehydes such as glutaraldehyde [19] and ethylene glycol diglycidyl ether [20], or physically cross-linked with multivalent anions derived from sodium tripolyphosphate (TPP) [21], citrate [22,23] and sulphate [24]. Both glutaraldehyde and ethylene glycol diglycidyl ether are toxic and can cause irritation to mucosal membranes [25]. Physically cross-linked chitosan gels have been used in drug delivery systems due to their enhanced biocompatibility over chemically cross-linked chitosan [26].

Non-toxicity and quick gelling ability of TPP are the important properties that make it a favourable cross-linker for ionic gelation of chitosan [14]. In addition, TPP has been also recognized as an acceptable food additive by the US Food and Drug Administration [27]. Moreover, the process of ionic gelation of chitosan with TPP as a cross-linker is feasible for the scale-up of entrapment in a particle processing operation [28]. Chitosan nanoparticles prepared by TPP as an anionic cross-linker are homogeneous, and possess positive surface charge that make them suitable for mucosal adhesion applications [14].

The properties of ionically cross-linked chitosan are influenced by electrostatic interactions between the anionic cross-linker and chitosan. This interaction depends on the variables such as: anionic molecular structure, its charge density and molecular concentration, pH of chitosan solution, and physical properties of chitosan, i.e., molecular weight and degree of deacetylation (DDA) [25]. Several studies have investigated the effect of these variables on the properties of ionically produced chitosan, its drug entrapment efficiency and drug release behaviour [25].

Advantages of protein delivery systems based on chitosan-TPP nanoparticles are reported in many research works performed in the past few years [29]. A shortcoming of chitosan-based nanoparticles as protein release system is that such particles release 30-70% of the protein within 3-6 h placed in release environment. This is due to the mechanism of burst release that has been considered as a slow release in many studies [30].

Burst release management is a major challenge in the development of drug delivery systems because it may lead to inefficient delivery and significant toxicity hazards. The degree of burst release generally depends upon the nature of the polymer, drug molecular structure and its molecular weight, polymer/drug ratio, relative affinities of the drug and polymer and the aqueous phase [31].

Many methods, including blending with other polymers, grafting with monomers, varying the chemistry of the polymer [32], adding excipients into polymer phase [33], employing new polymers [34], encapsulating particulate forms of the drugs into microparticles, preparation of novel biopolymer/inorganic material composites [35] and spray/freezing [36] have been applied to improve the burst release properties of the polymeric carriers.

Polymer coating is another burst release control method. Tavakol et al. [37] have studied this method for N,O-carboxymethyl chitosan (NOCC) beads coated with chitosan. They produced beads of NOCC and alginate with ionotropic gelation method and then, coated the beads with chitosan. The effect of coating and drying methods on the swelling and release behaviour of the prepared beads has been investigated. The results of this work indicate that burst release has not been observed in chitosan coated beads. Using additional barrier layers is
naturally an efficient method to manipulate drug release profiles and reduce the burst release, though it is inherently an expensive method due to the additional materials employed and difficulties associated with controlling the barriers quality. Chen et al. [30] have reported nanoparticles based on $N$-trimethyl chitosan chloride (TMC) and TPP. They used BSA as a model protein entrapped into the particles with sodium alginate as a modifier of TMC nanoparticles. They showed that burst release of BSA can be reduced from 60% to 45%.

The main objective of this work was to reduce the burst release of BSA-loaded chitosan nanoparticles prepared by a one-step simple method. Very few studies have focused on high loadings (10-15%) of proteins in micro/nanospheres with low burst release. Due to the nature of BSA, an optimum BSA delivery system must undergo burst release as low as possible while keeping drug loading as high as possible. The design of an optimum system is the main objective in this study. Comparison of the release test results of the current work with other similar works clearly shows higher efficiency of BSA-loaded chitosan nanoparticles by the method developed in this study. While this work and the study carried out by Chen et al. [30] are both using ionic gelation method, two important differences are to be taken into account. Chen et al. [30] used $N$-trimethyl chitosan as a polymeric carrier without any ionotropic cross-linker. In this work chitosan is used as a polymeric carrier and CaCl$_2$ as an ionotropic cross-linker to further reinforce the chitosan/alginate composite matrix. In order to decrease burst release, chitosan nanoparticles with two different degrees of deacetylation were modified using calcium alginate, and the effects of sodium alginate concentration on particle size, entrapment efficiency and BSA release profile were investigated.

**EXPERIMENTAL**

**Materials**

Medium molecular weight chitosan (Fluka, The Netherlands) was used as an encapsulating polymer. According to its specification analysis, the molecular weight was 400 kDa. The degree of deacetylation was about 83% as determined by potentiometric titration. Alginic acid as its sodium salt (medium viscosity, 3500 cps, 2% (w/v) aqueous solution at 25°C from Sigma-Aldrich, The Netherlands) was used as a reinforcing polymeric agent. Sodium tripolyphosphate as ionotropic cross-linker was supplied by Sigma-Aldrich. Calcium chloride was provided by Merck Chemical Co., Germany, and used as ionotropic cross-linking agent. As a model protein molecule for entrapment and control release studies, BSA was obtained from Merck Chemical Co., Germany. The other chemicals were analytical grade reagents and used as received.

**Purification of Chitosan**

The chitosan was dissolved in 2% (v/v) acetic acid solution which was filtered to remove the residues of insoluble particles. A two-molar solution of NaOH was added to the filtrate to obtain purified chitosan in the form white precipitate. The precipitated chitosan was washed thoroughly using double distilled water, and then dried at 40°C for 48 h. Further deacetylation of chitosan occurred during the purification process.

**Determination of Chitosan Degree of Deacetylation**

The degree of deacetylation numerical values of the unpurified and purified chitosan samples were determined by potentiometric titration of chitosan (25 mL of 0.2 M HCl) against 0.1 M NaOH using the following equation [38]:

$$DDA (\%) = \frac{\Delta V \times C_{NaOH} \times 10^{-3} \times 16}{M_{Chitosan} \times 0.0994}$$

where, $\Delta V$ is the volume of alkali consumed in the titration of amino groups present in the chitosan samples, $C_{NaOH}$ is the concentration of sodium hydroxide used for titration, $M_{Chitosan}$ is the mass of chitosan sample, and 16 and 0.0994 are the molecular weight and theoretical molar mass of the amino groups, respectively.

**Preparation of Chitosan Nanoparticles**

Chitosan nanoparticles were prepared on the basis of ionic gelation of chitosan in presence of TPP. Unpurified and purified medium molecular weight chitosan were dissolved in 1% (v/v) acetic acid solution at the final concentration of 0.5% (w/v). Two different concentrations of 20% and 50% (w/w) BSA, based on chitosan and alginate, were added each into
a separate chitosan solution prior to nanoparticles formation. The pH of chitosan-BSA solution was adjusted to 5.9 by 2 N NaOH solution. Then, 10 mL of TPP solution (0.5% w/v) prepared in double distilled water was added into the chitosan-BSA solution (50 mL) through an insulin syringe needle at the speed of 60 mL/h under magnetic stirring at room temperature. The pH of chitosan-BSA solution reached 5.5 after adding TPP solution. The procedure described so far results in producing unreinforced chitosan nanoparticles.

Reinforced chitosan nanoparticles were prepared by the same procedure, except that various amounts of sodium alginate (10% and 20% w/v) were dissolved in the TPP solution before adding the chitosan-BSA solution. Chitosan nanoparticles were formed upon adding the TPP solution to chitosan-BSA solution and mixing. After ten minutes of mixing these two solutions, 0.9 g calcium chloride was added to the suspension which was stirred for 20 min. Finally, the nanoparticles were isolated by centrifugation at 6000 rpm for 45 min. The supernatant was stored for further analysis. The precipitate was suspended in double distilled water, centrifuged again and dried at 37°C. The dried chitosan nanoparticles were suspended in milli-Q distilled water for characterization tests where appropriate; or directly used for in vitro release experiments.

Formulations of all chitosan-TPP samples that were used to investigate the effect of BSA loading, alginate concentration, and degree of deacetylation on nanoparticles and in vitro release profiles are presented in Table 1.

**Characterization Tests of Nanoparticles**

The chemical interactions between chitosan, alginate, TPP and BSA were established through FTIR spectrometry. FTIR Spectra of chitosan, TPP and BSA prepared nanoparticles were recorded on a 460 Plus Jasco Inc FTIR spectrophotometer (Maryland, USA) within KBr pellets. The instrument was operated with resolution of 4 cm\(^{-1}\) and frequency range of 400-4000 cm\(^{-1}\).

The mean particle size and size distribution of nanoparticles were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS-ZEN 1600 (Malvern Instruments Co., UK). The analysis was carried out at a scattering angle of 90° at a temperature of 25°C using nanoparticles dispersed in de-ionized distilled water. Every sample measurement was repeated 10 times. Particle size distribution of the nanoparticles is reported as a polydispersity index (PDI). The morphology of dried nanoparticles was observed by transmission electron microscopy (TEM). For TEM analysis, about 3 mL ethanol was added to the prepared chitosan nanoparticles, and dispersed in Sonicator (Power Sonic 405, Hwashin Technology Co., Korea) for 5 min, and one droplet of the nanoparticles solution was dripped onto copper grids. The samples were dried by natural air flow and then analyzed using TEM without any further treatment or coating.

To determine the entrapment efficiency (EE) of BSA into nanoparticles, the amount of free BSA in supernatant was analyzed with UV-Vis spectrophotometer at 595 nm using the Bradford protein assay. The supernatant of non-loaded chitosan (DDA: 83.5% or 96.4%) nanoparticle suspension was used as a blank. The BSA entrapment efficiency (EE) was calculated using the following equation:

\[
EE (%) = \frac{[\text{total BSA added} - \text{free BSA in supernatant}]}{\text{total BSA added}} \times 100
\]  

**Table 1. Formulations of the prepared chitosan-TPP samples loaded with BSA.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alginate concentration (w/v%)</th>
<th>Theoretical loading (w/w%)</th>
<th>DDA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.0</td>
<td>20</td>
<td>83.54</td>
</tr>
<tr>
<td>II</td>
<td>0.1</td>
<td>20</td>
<td>83.54</td>
</tr>
<tr>
<td>III</td>
<td>0.2</td>
<td>20</td>
<td>83.54</td>
</tr>
<tr>
<td>IV</td>
<td>0.0</td>
<td>50</td>
<td>83.54</td>
</tr>
<tr>
<td>V</td>
<td>0.1</td>
<td>50</td>
<td>83.54</td>
</tr>
<tr>
<td>VI</td>
<td>0.2</td>
<td>50</td>
<td>83.54</td>
</tr>
<tr>
<td>VII</td>
<td>0.0</td>
<td>20</td>
<td>96.38</td>
</tr>
<tr>
<td>VIII</td>
<td>0.1</td>
<td>20</td>
<td>96.38</td>
</tr>
<tr>
<td>IX</td>
<td>0.2</td>
<td>20</td>
<td>96.38</td>
</tr>
<tr>
<td>X</td>
<td>0.0</td>
<td>50</td>
<td>96.38</td>
</tr>
<tr>
<td>XI</td>
<td>0.1</td>
<td>50</td>
<td>96.38</td>
</tr>
<tr>
<td>XII</td>
<td>0.2</td>
<td>50</td>
<td>96.38</td>
</tr>
</tbody>
</table>
In Vitro Drug Release

The dried BSA-loaded nanoparticles were resuspended in 25 mL of 0.1 M phosphate buffer solution (PBS, pH 7.4), then incubated at 37°C while stirred at 100 rpm. At specific time intervals, 0.5 mL of the sample was removed and replaced with fresh PBS. The withdrawn samples were centrifuged at 6000 rpm for 5 min. The BSA released from the nanoparticles and present in the supernatant was measured by Bradford protein assay. The blank sample consisted of non-loaded chitosan nanoparticles resuspended in PBS. Triplicate samples were analyzed for each measurement.

RESULTS AND DISCUSSION

Chitosan TPP nanoparticles are formed by ionic cross-linking (ionic gelation) of oppositely charged ions of chitosan and TPP. However, the preparation of reinforced chitosan-TPP nanoparticles has been attributed to the three following phenomena: (a) ionic gelation technique between positively charged chitosan and negatively charged TPP and BSA, (b) electrostatic interaction between the positively charged -NH$_3^+$ of chitosan and negatively charged -COO$^-$ of alginate, and (c) ionotropic gelation process between negatively charged alginate and calcium ion (Ca$^{+2}$) [39]. In this study, calcium ion is used as a cross-linking agent to strengthen and stabilize the particles.

The ratio between chitosan and TPP is critical and does control the size and PDI of the nanoparticles. It is shown that by increasing the chitosan/TPP ratio, smaller nanoparticles can be produced [16]. TPP is a polyfunctional cross-linking agent and can create five ionic cross-linking points with amino groups of chitosan. Thus, in this study the ratio of 5/1 (w/w) was selected as a suitable chitosan/TPP ratio [16].

The ionic interaction between chitosan and TPP is dependent on the solution pH. This is due to the variation in ionization degree of chitosan and TPP. Other researchers [25] showed that a pH range 3-6 was a suitable range for ionic gelation of chitosan with TPP. The pH of 5.9 is selected in the present work because BSA (PI = 4.8) is negatively charged at this pH and electrostatic interaction between the negatively charged BSA and positively charged chitosan causes greater entrapment of BSA into the nanoparticles.

FTIR was used to confirm the incorporation of calcium alginate into the chitosan matrix and loading of BSA in the prepared nanoparticles. The FTIR spectra of chitosan, alginate, BSA, BSA-loaded chitosan nanoparticle (sample IV) and BSA-loaded chitosan-alginate nanoparticles (sample VI) are presented in Figure 1.

Three characteristics absorption bands observed in chitosan (spectrum a in Figure 1), at 3413, 1672 and 1317 cm$^{-1}$, as in the given order are due to the N-H, amide I and amide III groups present in chitosan. According to Yuan et al. [40] the peak of 3413 cm$^{-1}$ corresponds to stretching vibration of N-H in pure chitosan. It is noticeable that this peak has shifted to lower wavenumbers centered at 3395 cm$^{-1}$ and it is broader and stronger in chitosan particles (samples IV and VI, spectra d and e in Figure 1). This is an evidence of molecular interaction between these...
groups and sodium tripolyphosphate and also due to hydrogen bonding involvement. Primary amines also show sharp peak between 3500 and 3400 cm\(^{-1}\) which could be attributed to the asymmetric and symmetric stretching of the N-H bonds [40]. The peak in pure chitosan and chitosan particles appears broad in this region due to the contribution of O-H stretching peaks and hydrogen bonding [41].

FTIR Spectra of BSA showed peaks around 1655, 1534 and 3309 cm\(^{-1}\), reflecting the acetylamino I, acetylamino II and (NH\(_2\)) groups, respectively [42]. Acetylamino I at 1655 cm\(^{-1}\) and acetylamino II at 1534 cm\(^{-1}\) in BSA (spectrum c in Figure 1) overlapped 1672 cm\(^{-1}\) of amide I and 1597 cm\(^{-1}\) in chitosan, so more intensive peaks appeared for the BSA-loaded chitosan nanoparticles (sample IV, spectrum d in Figure 1).

The characteristic peaks of sodium alginate included O-H at 3397 cm\(^{-1}\), COO- (asymmetric) at 1617, COO- (symmetric) at 1429 and 1028 cm\(^{-1}\) for C-O-C stretching. For the BSA-loaded chitosan nanoparticle of sample IV, the stretching vibration of -OH and -NH\(_2\) at 3392 cm\(^{-1}\) became broader. Spectrum e in Figure 1 is an indication of intramolecular and intermolecular hydrogen bonds which were formed and enhanced between chitosan and alginate molecules [43].

The mean particle size, PDI and EE of the prepared samples are presented in Table 2. PDI is a measure of homogeneity in dispersed systems and ranges from 0 to 1. Homogeneous dispersion has PDI value close to zero while PDI values greater than 0.3 suggest high heterogeneity [44]. Except the three samples (samples I, IV and XII) other prepared nanoparticles have PDI values lower than 0.3 (Table 2).

Morphological studies of nanoparticles were conducted by TEM. The TEM images such as those presented in Figure 2 indicate that nanoparticles are

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean particle size (nm)</th>
<th>PDI</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>396</td>
<td>0.162</td>
<td>70.54</td>
</tr>
<tr>
<td>II</td>
<td>116</td>
<td>0.572</td>
<td>71.55</td>
</tr>
<tr>
<td>III</td>
<td>459</td>
<td>0.188</td>
<td>75.14</td>
</tr>
<tr>
<td>IV</td>
<td>615</td>
<td>0.179</td>
<td>60.97</td>
</tr>
<tr>
<td>V</td>
<td>255</td>
<td>0.201</td>
<td>59.48</td>
</tr>
<tr>
<td>VI</td>
<td>459</td>
<td>0.226</td>
<td>55.4</td>
</tr>
<tr>
<td>VII</td>
<td>295</td>
<td>0.075</td>
<td>84.55</td>
</tr>
<tr>
<td>VIII</td>
<td>255</td>
<td>0.123</td>
<td>75.21</td>
</tr>
<tr>
<td>IX</td>
<td>190</td>
<td>0.631</td>
<td>71.12</td>
</tr>
<tr>
<td>X</td>
<td>459</td>
<td>0.274</td>
<td>80.74</td>
</tr>
<tr>
<td>XI</td>
<td>220</td>
<td>0.261</td>
<td>69.16</td>
</tr>
<tr>
<td>XII</td>
<td>122</td>
<td>0.523</td>
<td>68.56</td>
</tr>
</tbody>
</table>

Figure 2. TEM Photographs of samples: (a) IV and (b) VI.
not spherical in shape. The size of nanoparticles based on TEM was smaller than the size determined by DLS. This can be attributed to the dehydration of the nanoparticles in the process of sample preparation required for TEM. It is worth noting that DLS gives the size of swollen particles. Swelling ratio of some selected samples of the nanoparticles was measured according to the method proposed by Denkbas et al. [45].

About 0.050 g of the nanoparticles was inserted into a tube with a diameter of 5 mm with a height of 100 mm. The level of the beads in the tube was marked before filling it with 1 mL of distilled water and the tube was left for 24 h. After 24 h, the height of the beads in the solution was measured. The percentage of swelling was calculated based on the following equation:

\[ S = \frac{h_t - h_o}{h_o} \times 100 \]  

where \( S \) is the percentage of swelling, \( h_t \) is the height of swollen beads (cm) at specific time \( t \) and \( h_o \) is the initial height of the beads (cm).

The obtained results for samples IV, V and VI based on three independent measurements for each sample were 119.3% ± 20.3, 223% ± 67 and 146.3% ± 18.8, respectively.

**Effect of Alginate Concentration on Physicochemical Properties of Nanoparticles**

As can be seen in Table 2, mean particle size decreases as sodium alginate concentration increases. By contrast, PDI of the prepared nanoparticles increases as more sodium alginate was added to the gelation system. In ionic gelation process, TPP as a cross-linking agent forms hydrogen bond with free amine groups of BSA and chitosan. Sodium alginate competes with TPP to form electrostatic complex with chitosan and BSA. While smaller mean particle size can be attributed to strong inter-chain reactions between chitosan and alginate, as more sodium alginate causes local aggregation and increases PDI [30]. In other words, the reduction in particle size determined by DLS at swollen state in presence of calcium alginate in the network is due to higher degree of cross-linking of the whole network. Higher concentration of sodium alginate results in reduced entrapment efficiency (Table 2). This may be due to competition of anionic alginate and BSA to form interchain bonds with cationic chitosan.

**BSA Loading in Relation to the Physicochemical Properties of Nanoparticles**

The general pattern which can be deduced from the experimental data given in Table 2 is that, lowering theoretical loading of BSA leads to formation of smaller nanoparticles. BSA loading of nanoparticles is based on two different phenomena such as entrapment into particles and adsorption on the particle surface. As mentioned earlier, BSA molecules are negatively charged under the solution conditions used in this study. BSA entrapment into particles is due to interactions between oppositely charged chitosan and BSA, and formation of hydrogen bonds between the TPP and BSA. Additional adsorption of BSA on particle surface may also occur [14] that leads to higher mean particle size. PDI of the prepared nanoparticles increases with theoretical BSA of higher loading. Also, the increase of the particle size and PDI by increased BSA can be attributed to the increased viscosity of the chitosan/BSA solution and ionic interaction, respectively. Few data from Table 2 regarding the effect of BSA loading on entrapment efficiency of nanoparticles are presented in Figure 3. Sample preparation for samples I and IV were the same except for the theoretical BSA loading that was higher for sample IV compared to sample I. The same sample preparation was applied to sample pair VIII and XI. It can be
concluded from Figure 3 that higher theoretical BSA loading results in lowering entrapment efficiency. It is worth noting that data from Table 2 other than those presented in Figure 3 also support this conclusion. The decrease of entrapment efficiency with initially increased drug content is due to increased chemical potential of the drug in chitosan/BSA solution. This conclusion is in agreement with reported studies such as the one carried out by Xu et al. [46] and it is in contrast to other research reports attesting the opposite effect of drug loading on entrapment efficiency [14].

**DDA in Relation to the Physicochemical Properties of Nanoparticles**

The effects of degree of deacetylation on particle size and entrapment efficiency of BSA were investigated and the obtained experimental data are presented in Table 2. The data show that samples which were prepared with higher DDA chitosan (purified chitosan, DDA 96.4%) have smaller mean particle size than those prepared with unpurified chitosan (DDA 83.5%).

In this study, purification process was conducted by NaOH. It is anticipated that further deacetylation of chitosan molecules may have occurred under strong caustic condition. This means that more amine groups are presented in purified chitosan of high DDA, which can be transformed into -NH$_3^+$ at acidic solution. The ionic gelation process is dependent on the number of positively charged groups (-NH$_3^+$) left on the dissolved chitosan molecules. Due to sufficient positively charged groups, the process of nanoparticles production occurs more easily for chitosan with high DDA leading to smaller particles [47].

Entrapment of negatively charged BSA molecules is also occurring more efficiently in the presence of positively charged amine groups. Sample preparation for samples I and VII was the same except for the DDA that was higher for sample VII compared to sample I. The same sample preparation is applicable to sample pairs II-VIII, III-IX, IV-X, V-XI and VI-XII. The presented data in Table 2 for these pairs reveal that entrapment efficiency of BSA increases at higher DDA.

**In Vitro Release Study**

Experimental data on BSA release studies are presented in Figure 4. The data show that both BSA release rate and especially burst release are reduced significantly with higher sodium alginate concentration used in preparation of nanoparticles. Sustained drug release from the nanoparticles is important in many fields of applications. BSA release follows a biphasic pattern, characterized by initial burst release followed by a period of slower release. According to the presented data in Figure 4, fractional BSA released for sample IV (chitosan-TPP nanoparticles without alginate) and sample VI (reinforced chitosan-TPP with alginate) in 30 min are 10.5% and 3.5%, respectively. Also, these quantities in 6 h reach 14% and 11.8% for samples IV and VI, respectively. In addition, fractional drug released for sample IV is 35.7% at 98 h while this quantity for sample VI is 26.8% at 98 h. These results prove that reinforcement of chitosan-TPP nanoparticles with CaCl$_2$ can reduce both burst release and release rate of BSA.

Reinforcement of chitosan-TPP matrix with calcium alginate may be considered the cause for lower burst effect and slower release rate. Modification of N-trimethyl chitosan chloride (TMC)-TPP matrix by sodium alginate studied by Chen et al. [30] also showed that sodium alginate demonstrates the same result on burst effect and release rate as calcium alginate in this study. The amine groups in chitosan interact with TPP through ionic bonding. The interaction between chitosan matrix and alginate and ionotropic gelation of sodium alginate with CaCl$_2$ in reinforced nanoparticles may...
also contribute to enhanced cross-linking density of the matrix. Increased cross-linking density may lead to lower diffusion of BSA from the reinforced matrix which consequently decreases the burst release and rate of release. To investigate the effect of sodium alginate on drug release profile, without its conversion to calcium alginate network, a new sample designated as XIII, was prepared. Preparation of samples XIII and VI were the same except that CaCl₂ was not used in preparation of sample XIII. The release profile of this sample is shown in Figure 4. As can be seen, the burst release and release rate of BSA is lower in sample VI compared to sample XIII.

Fractional BSA release profiles for two theoretical BSA loadings, 20% and 50% corresponding to loading capacity of 4.13% ± 0.04 and 9.75% ± 0.32, are presented in Figure 5. Release profiles show that when loading capacity of BSA increases from 4.13% ± 0.04 to 9.75% ± 0.32, total BSA release at 6 h increases from 7.5% to 15.8%. The nanoparticles prepared with higher loading capacity of BSA have greater overall release. At higher BSA loading, BSA binding on chitosan molecule is weaker, and some of the loaded BSA molecules are adsorbed onto the surface of particles. Weak binding and adsorption of BSA on the particle surfaces leads to higher release rate. This result is in agreement with reports by other researchers [14].

Effect of DDA on the release profile is shown in Figure 6. The results show that BSA release rate and burst release increase with higher DDA. As it was stated above, higher DDA causes significant reduction in mean particle size, thus increasing the ratio of surface area to volume of nanoparticles, that consequently increases the release rate and burst release.

CONCLUSION

Reinforced chitosan-TPP nanoparticles loaded with BSA were prepared by ionic gelation. Calcium alginate was used as a reinforcing agent. Size of the prepared nanoparticles ranged from 116 to 615 nm with PDI lower than 0.3. TEM Analysis demonstrated that nanoparticles were not spherical. At higher sodium alginate concentration smaller mean particle size was obtained. High concentration of sodium alginate solution resulted in lower entrapment efficiency. Higher degree of deacetylated chitosan lowered particle size and improved the entrapment efficiency. This study showed that reinforcement of chitosan-TPP nanoparticles with calcium alginate significantly lowered the burst release and consequently the BSA release rate. Increased theoretical loading of BSA and degree of deacetylation of chitosan led to increased burst release and release rate.

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