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Research Article

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Biopolymeric Nanoparticles for Oral Protein Delivery: Design and *In vitro* Evaluation

Nazik Elgindy[1], Kadria Elkhodairy[1], Abdallah Molokhia[2] and Ahmed ElZoghby[1]*

[1]Department of Industrial Pharmacy, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt
[2]European Egyptian Pharmaceutical Industry, PHARCO Corporation, Amreya, Alexandria, Egypt

Abstract

Chitosan (CS) nanoparticles for the oral delivery of the protein, Human Serum Albumin (HSA) were prepared by two techniques (precipitation and ionic gelation) together with two anions (sodium sulfate or tripolyphosphate, TPP). HSA was loaded with CS nanoparticles by adsorption or entrapment loading protocols. The highest HSA association efficiency (93.43%) and loading capacity (58.65%) were obtained using ionic gelation technique with 0.1% w/v TPP as a crosslinker. The particle size of CS-HSA nanoparticles ranged between 100-320 nm with a high specific surface area (703-903 m^2/g) and porosity (1060.99-1350.95 e^{-3}ml/g). Incubation of nanoparticles with lysozyme led to a reduction of 243 nm in particle size within 3 h. CS nanoparticles was redispersible after one month storage. CS/TPP nanoparticles prepared by precipitation/protein entrapment technique slowly released 10.34% HSA over 5 days which is suitable for vaccine or protein delivery while 86.54% of HSA was released from nanoparticles prepared by precipitation/ protein adsorption technique after 8 hr which is suitable for rapid drug release. Using ionic gelation technique, CS/ TPP nanoparticles released 22.47-38.65 % HSA over 5 days at 7:1 to 3:1 CS/TPP mass ratio, respectively. Both techniques retained the structural integrity of HSA after preparation and release processes which was proven via gel electrophoresis.

Keywords: Chitosan nanoparticles; Precipitation; Ionic gelation; Surface area and porosity; Biodegradability

Introduction

Peptides and proteins have become the drugs of choice for the treatment of numerous diseases as a result of their incredible selectivity and their ability to provide effective and potent action [1]. The oral delivery of proteins and peptides HSA become a pressing goal in recent years due to the increased availability of novel therapeutics through the advent of recombinant DNA technology. The main reasons for the low oral bioavailability of biologicals are presystemic enzymatic degradation and poor penetration of the intestinal membrane [2]. The most promising delivery approach is the encapsulation of a protein within biodegradable polymeric nano- or microspheres. Poly (lactide) or poly (lactide-co-glycolide)-based nano and microspheres have been the most studied systems due to the excellent biocompatibility and biodegradability properties of the polymers. However, the main drawback of these systems is the denaturation of some encapsulated proteins due to the manufacturing process conditions [3].

Naturally occurring polymers, especially polysaccharides such as chitosan and alginates, have been extensively researched as carriers for therapeutic protein molecules and as non-viral gene carrying vectors [4,5]. Because of their permeation enhancing effect, enzyme inhibitory capabilities and mucoadhesive properties, chitosan and its derivatives are able to reduce GIT barriers, which makes these polymers important excipients for oral peptide delivery systems [4,5]. Ionic gelation, complex coacervation, emulsion cross-linking and spray-drying are methods commonly used for the preparation of chitosan nanoparticles. Among those methods, ionic gelation and complex coacervation are mild processes occurring in a pure aqueous environment and are ideal for maintaining the in-process stability of proteins and peptides [6,7].

Calvo et al. [8] have developed chitosan/TPP nanoparticles based on ionic gelation technique. Proteins such as bovine serum albumin, tetanus toxoid, diphtheria toxoid and the peptide insulin are examples of macromolecules which have been efficiently associated to these nanoparticles. Protein loading reached values as high as 50% which is the greatest loading capacity reported for nanoparticulate protein carrier. Berthold et al. [9] prepared desolvated chitosan nanoparticles by dropwise addition of sodium sulfate as a precipitating agent into a solution of chitosan and polysorbate 80 under both stirring and ultrasonication. Variation of this technique was later employed for the controlled release of antineoplastic proteoglycans for immunosuppression [10].

In our study, human serum albumin (HSA) was used as a model protein. HSA seems to possess several advantages like being abundant protein in the blood, highly tolerable by the human body, able to carry functional groups which are amenable to surface modifications in addition to passive tumor targeting possibly due to enhanced permeability and retention (EPR) effect [11]. Few researchers had fabricated HSA microspheres using poly (d,l-lactide-co-glycolide), polylactide, poly-dl-lactide-poly(ethylene glycol) and poly (ε-caprolactone)-poly (ethylene glycol) (PECL) copolymers via solvent extraction procedure based on the formation of a w/o/w double emulsion. The highest entrapment efficiency and loading capacity of HSA achieved were 84.45 and 0.86%, respectively [12-14]. The aim of this work is to prepare and characterize CS nanoparticles for the efficient oral delivery of HSA as a model protein drug with a special emphasis on some of the physicochemical properties of chitosan nanoparticles such as surface area, porosity, biodegradability and redispersibilty. The aim was also extended to evaluate the effect of ultrasonication and stirring procedures on the structural integrity of the protein.

***Corresponding author:** ElZoghby, Department of Industrial Pharmacy, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt, Tel: +20 3 3180 658; Fax: +20 3 4873 273; E-mail: aoelzoghby@yahoo.com

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Materials and Methods

Materials

Chitosan, CS (low MW, Brookfield viscosity 20,000 cps, degree of deacetylation 85%), Sodium triphosphate pentabasic (TPP), Glutaraldehyde solution 2.5% v/v in water, Polyethylene sorbitan monooleate (Tween 80), Human Serum Albumin (HSA, 66 kDa, fraction V), Coomassie brilliant blue dye (G-250), Tris-glycine buffer, uranyl acetate and Bradford Reagent were purchased from Sigma-Aldrich (USA). Lysozyme from egg white was purchased from Pacegrove (UK). Sodium metabisulfite (ADWIC, El-Nasr Pharmaceutical Chemicals Co., Egypt). All other chemicals were of analytical grade and used without further purification.

Preparation of plain chitosan nanoparticles

Precipitation technique: According to the method reported by Berthold et al. [9] Chitosan (0.25% w/v) was dissolved in an aqueous solution of acetic acid (1% v/v) containing 1% w/v Tween 80. A solution of the precipitating agent (Sodium sulfate 10 or 20 % w/v or 10 % w/v Tripolyphosphate) was added dropwise to chitosan solution during mechanical stirring (4000 rpm) and ultrasonication for 30 min (Julabo sonicator, model USR-3; Julabo Labortechnik, Ceelbach, Germany). The formation of nanoparticles was monitored by turbidity, examined by transmission measurements using the spectrophotometer at 500 nm (Lambda 3B, Perkin Elmer, New York, USA). Percent transmittance (%T) was plotted graphically against the concentration of the precipitant (Figure 1). After the addition of the precipitant, sonication was continued for 15 min. The nanoparticles formed were finally recovered by centrifugation at 17000 rpm for 30 min at 2°C (Sigma laboratory refrigerated centrifuge, model 3K-30, Germany). Then the sediment was resuspended in the original volume of distilled water. These two purification steps were repeated twice before the chitosan nanoparticle suspensions were lyophilized (CRYODOS-50 Freeze-drier, Telstar Cryodos, Spain) [9].

For the preparation of crosslinked chitosan/sulfate nanoparticles, 2 ml of glutaraldehyde solution (25% v/v) was added after nanoparticle preparation and sonication was continued for 30 min. Crosslinking was stopped by the addition of 40 ml sodium metabisulfite solution (12% w/v) and the nanoparticles formed were recovered and purified as previously mentioned.

Ionic-gelation technique: According to the method reported by Calvo et al. [8] Chitosan (0.25% w/v) was dissolved in 1% v/v acetic acid solution at pH 5.5. Sodium tripolyphosphate aqueous solution (0.1% w/v) was then added dropwise under mild magnetic stirring for 30 min (RH basic, Ika labortechnic, Germany). Opalescent suspension was formed spontaneously at room temperature which was further examined as nanoparticles. The formed nanoparticles were recovered, as previously described [8].

Preparation of protein-loaded chitosan nanoparticles

Drug loading was achieved by either incorporating HSA inside CS nanoparticles or by adsorbing HSA after the formation of NPs onto their surface. Entrapment of HSA into the chitosan nanoparticles (Formulae N_3, N_6, N_8, N_9 and N_{10}) was performed by dissolving HSA (0.02 %w/v) in the chitosan solution before the addition of the crosslinking agent then the formed nanoparticles were finally recovered by centrifugation at 17000 rpm at 2°C for 30 min.

The adsorption method was performed by adding 200 µg/ml HSA solution to the preformed chitosan nanoparticle suspension (Formulae N_1, N_2, N_4 and N_5) into glass vials (10 ml each). The vials were shaken for 3 h at 25°C in a thermostatically controlled shaking water bath, model 1083 (M.B.H. & Co., Staufen, Germany). After an incubation period, the suspension was centrifuged (7000 rpm for 10 min at 25°C) to remove the unloaded or aggregated HSA.

The two techniques described above were used to prepare a total of 10 nanoparticle formulations using different experimental variables (Table 1).

Characterization of chitosan nanoparticles

Nanoparticles yield, HSA association efficiency and loading capacity: The nanoparticles yield was calculated by a gravimetric technique. Fixed volumes of nanoparticle suspensions were centrifuged at 17000 rpm at 2°C for 30 min and supernatants were discarded. Sediments were freeze-dried for 24 h. The process yield (P.Y.) was calculated according to Eq. (1):

% P.Y. = (Net wt. of dry NPs obtained/Total wt. of initial solid components used in preparation of this batch) x 100 (1).

The amount of HSA entrapped/adsorbed in the nanoparticles was calculated by the difference between the total amount of HSA added and the free HSA remaining in the aqueous supernatant. The latter amount

Formula	Preparation Technique	Protein loading protocol	Polymer solution (%w/v)	Crosslinker (% w/v)
N_1	Precipitation	Adsorption	CS + 1% Tween 80	20 % Na_2SO_4
N_2	„	Adsorption	CS + 1% Tween 80	20% Na_2SO_4 + 1 ml 25% GA
N_3	„	Entrapment	CS + 1% Tween 80 + (0.02 %) HSA	10 % Na_2SO_4
N_4	„	Adsorption	CS + 1% Tween 80	10 % Na_2SO_4
N_5	„	Adsorption	CS + 1% Tween 80	10 % TPP
N_6	„	Entrapment	CS + 1% Tween 80 + (0.02%) HSA	10 % TPP
N_7	Ionic-gelation	plain	CS	0.1 % TPP (3:1 CS/TPP)
N_8	„	Entrapment	CS + (0.02%) HSA	0.1 % TPP (3:1 CS/TPP)
N_9	„	Entrapment	CS + (0.02%) HSA	0.1% TPP (5:1 CS/TPP)
N_{10}	„	Entrapment	CS + (0.02%) HSA	0.1% TPP (7:1 CS/TPP)

Table 1: Composition of Chitosan Nanoparticle Formulations Prepared by Precipitation and Ionic-Gelation Techniques.

Formula	Preparation Technique	Process yield (%w/w)	Loading capacity (%w/w)	Association efficiency (%w/w)	Particle Size (nm)
N_1	Precipitation	34.65±(3.06)	3.6±(0.03)	92.3±(4.25)	320±(4.58)
N_2	„	39.28±(3.59)	3.7±(0.05)	35.1±(1.58)	480±(6.39)
N_3	„	56.12±(2.59)	35.20±(3.49)	64.89±(2.48)	390±(5.20)
N_4	„	27.80±(3.68)	6.7±(0.09)	82.1±(2.33)	150±(3.54)
N_5	„	19.24±(1.49)	5.1±(0.11)	70.1±(3.25)	100±(2.38)
N_6	„	26.93±(3.40)	45.92±(3.77)	72.43±(3.04)	220±(3.92)
N_7	Ionic-gelation	22.67±(3.36)	-	-	161±(2.25)
N_8	„	61.38±(2.57)	58.65±(3.59)	93.43±(4.85)	172±(3.69)
N_9	„	48.35±(3.90)	29.73±(1.22)	84.32±(2.75)	323±(5.19)
N_{10}	„	32.02±(4.75)	23.20±(2.56)	62.50±(3.66)	438±(7.22)

Table 2: Process Yield, HSA Loading Capacity (%LC), Association Efficiency (%AE) and Particle Size of the Prepared Chitosan Nanoparticles (values are mean ± SD, *n* = 3).

was assayed by the Bradford standard protein macro-assay method [15]. 100 µL of each of the protein standard or the unknown sample, 3 mL of the Bradford reagent is added, mixed by gentle vortexing and the absorbance at 595 nm was measured colorimetrically against a reagent blank [15]. The prepared protein standards in PBS (pH 7.4) ranged from 100 to 1000µg/mL of HSA. To each tube containing 100 µL of each of the protein standard or the unknown sample, 3 mL of the Bradford reagent is added, mixed by gentle vortexing and the absorbance at 595 nm was measured colorimetrically against a reagent blank [15].

The protein association efficiency (%AE) and loading capacity (%LC) of the nanoparticles were calculated according to Eqs. (2) and (3):

Association Efficiency (%AE) = (Experimental drug loading / Theoretical drug loading) x 100 (2)

Loading Capacity (%LC) = (The amount of drug entrapped in nanoparticles /total amount of nanoparticles) x 100 (3)

Transmission Electron Microscopy (TEM): The nanoparticle suspensions were diluted 10 folds with distilled water, one drop was deposited on copper grid, dried and stained with 1M uranyl acetate solution. TEM micrographs of nanoparticle samples were obtained with a model JEM-100S, microscope (Joel, Tokyo, Japan) operating at 120 Kv at a magnification of 50,000.

Particle size analysis: The particle size of freshly prepared CS nanoparticle was determined using PCS N5 submicron particle size analyzer (Beckman Coulter, USA) based on the photon correlation spectroscopy (PCS) technique. The particle size measurements were performed in distilled water using a quartz cell in the automatic mode. Each analysis was performed at 25°C with a detection angle of 90°. Measurements on nanoparticle suspension were done triplicate for a single batch of nanoparticles and results were the average of three measurements.

Thermal analysis: Thermograms were obtained using DSC 6 differential scanning calorimeter (Perkin Elmer, USA). Samples (3-4 mg) were directly placed in aluminium pans and heated to 50-200°C at a rate of 10°C /min under a nitrogen atmosphere.

Fourier Transform Infrared Spectroscopy (FTIR)

Samples were finely ground with an infra-red grade of KBr then pressed into pellets and IR spectra were taken in transmission using Spectrum RXI FT-IR spectrometer (Perkin Elmer, USA) over the range of 4000-500 cm^{-1}. The produced charts were examined for possible polymer/drug/crosslinker interaction.

Equilibrium swelling study: Plain chitosan nanoparticles (30 mg) were weighed in an eppendorf tube and incubated with 1 ml PBS (pH 7.4) in a shaking water bath (55 rpm) at 37°C. After 6 h, the samples were centrifuged at 17000 rpm for 15 min and the supernatants were discarded. The wet weight of the nanoparticles was then determined and the percent equilibrium swelling was calculated according to Eq. (4):

Swelling Degree (SD) = $[(W_t - W_0)/ W_0]$ (4)

W_t denotes the weight of swollen NPs at time t (6 h) and W_0 is the initial weight of NPs before swelling. Each swelling experiment was repeated three times and the average value was taken as the swelling degree.

Surface area and porosity: Specific surface area and porosity of chitosan powder and nanoparticles were measured using NOVA 1000 Series surface area analyzer (Quantachrome Corporation, USA). A known weight of nanoparticles was added to a 12 mm bulb sample cell and degassed for a minimum of 3 h. A 5-point nitrogen adsorption isotherm at 77 K was measured and the sample was then analyzed by NOVA Enhanced Data Software via the Brunauer, Emmett and Teller (BET) theory of surface area.

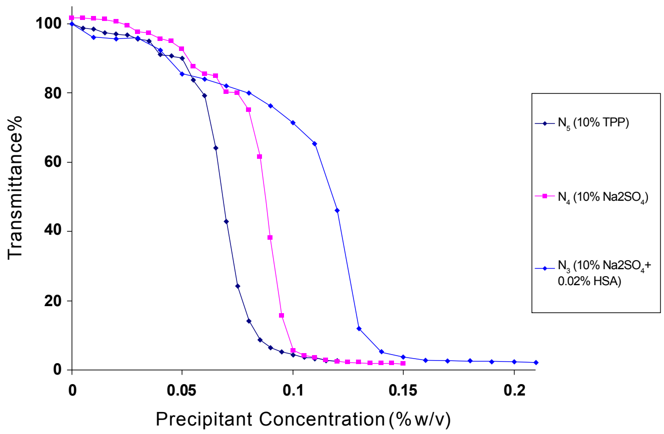

Figure 1: Turbidity monitoring during the precipitation step employed to produce chitosan nanoparticles.

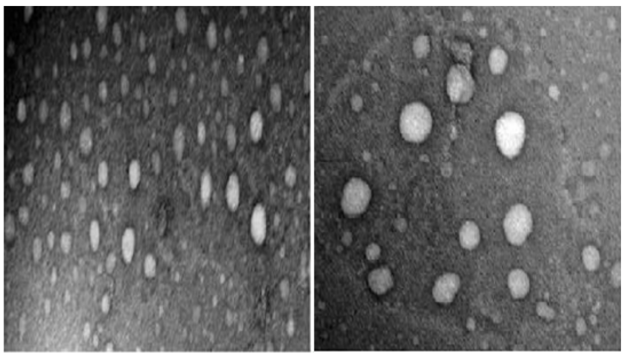

Figure 2: Transmission electron micrographs (X 50,000) of some selected chitosan nanoparticles prepared by (a) Precipitation technique (to the left side) (b) Ionic-gelation technique (to the right side) [The lower photos are magnified 3 folds].

Biodegradability of nanoparticles in lysozyme: The stability of fresh plain nanoparticles (Formula N_7) was monitored following their incubation with a 2 mg/ml solution of lysozyme in PBS pH 7.4 at 37°C under mild horizontal shaking for 3 h. At appropriate time intervals (5, 30, 60, 120 and 180 min), the mean particle size was analyzed using the submicron particle size analyzer.

Redispersibility of nanoparticles (reconstitution test): An aliquot of CS nanoparticle suspension (Formula N_8) was freeze-dried and stored at room temperature. After one month, 10 mg of lyophilized nanoparticles was resuspended into 10 ml of distilled water and the suspension was vortexed for 5 sec. Reconstituted sample was then evaluated for any change in their particle size.

***In-vitro* HSA release from chitosan nanoparticles:** A known quantity of protein-loaded nanoparticle suspension (40 ml) was centrifuged at 17000 rpm for 30 min and the supernatant was discarded. The collected nanoparticles were resuspended in 20 ml PBS (pH 7.4) with controlled agitation (100 rpm) at 37°C in a shaking water bath. At predetermined time intervals, 2 ml samples were centrifuged and replaced by an equal volume of prewarmed PBS. The amount of HSA released at various time intervals in 1 ml of the supernatant was determined using the Bradford protein micro-assay method [15]. The prepared protein standards in PBS (pH 7.4) ranged from 1-10 µg/mL of HSA. To each tube containing 1 mL of each protein standard or the unknown sample is added, 1 mL of the Bradford reagent is added and mixed by gentle vortexing [15]. All measurements were performed in triplicate.

SDS-Polyacrylamide Gel Electrophoresis (PAGE): The structural integrity of the HSA extracted from nanoparticles and after *in-vitro* release process was analyzed by SDS-PAGE, Minigel slab cell (Biometra, USA) to evaluate the effect of the fabrication technique and release processes on the protein integrity. For the detection of HSA, 17 µl of each sample was loaded on 5% upper stacking gel and was separated with 10% lower resolving gel in Tris-glycine electrophoretic buffer (pH 8.6). Polyacrylamide gels were run for approximately 2 h at 90V. After migration, the gel was stained with Coomassie brilliant blue (G-250) to reveal the protein. Each experiment was repeated twice.

Results and Discussion

Optimization of conditions for fabricating CS nanoparticles

The ability to control and modulate the properties of chitosan nanoparticles, in particular the particle size, is essential in determining not only the preparation method feasibility but also the reproducibility of the *in vivo* performance of the nanoparticles. Chitosan nanoparticles in our study were prepared by two different techniques; precipitation and ionic gelation (Table 1).

The extent of precipitation was controlled by the concentration of the precipitating agent and monitored by a turbidity measurement. The transmission in relation to the added amount of sodium sulfate or tripolyphosphate is shown in Figure 1. Initially the addition of the precipitant led to a slow decrease in % T, then transmittance fell down sharply till attaining a minimum value after which no significant change in transmittance was recorded. The optimum amount of sulfate added was determined from the graph at the point after which no significant change in % T was observed. On the other hand, upon using TPP as a precipitant, the optimum amount of TPP added was corresponding to about 50% T. Further increase in the amount of precipitant added beyond this optimum concentration was found to increase the nanoparticle size in case of sulfate whereas particle agglomeration occurred in case of TPP. These findings were in agreement with the findings of both Berthold et al. [9] and Jain et al. [16].

It can be seen that less amount of TPP was required for the formation of chitosan nanoparticles than that required of Na_2SO_4. This can be explained on the basis of charge density where TPP carries five negative charges while the sulfate carries only two charges. On the other hand, when HSA was added to chitosan solution, a higher amount of the precipitant was required for formation of nanoparticles.

Preliminary experiments were done to determine the formation zone of nanoparticles using the ionic gelation technique. Starting with a clear chitosan solution, stepwise addition of tripolyphosphate led to formation of the nanoparticles which was indicated by a very light turbidity compared to nanoparticles prepared by the precipitation technique. The formation of nanoparticles was confirmed by particle size analysis using a submicron particle size analyzer. It was found that when chitosan/TPP mass ratio was in the range of 7:1-3:1, nanoparticles of different sizes could be obtained. These findings were in agreement with the work done by Wu et al. [17] who noted that three different zones were identified during chitosan NP formation; clear solution, opalescent suspension and aggregates.

Characterization of the fabricated nanoparticles

Process yield, protein association efficiency (%AE) and loading capacity (%LC) of nanoparticles: As shown in Table 2, the comparison of the yield values of plain and the corresponding protein-loaded nanoparticles indicated that the entrapment or adsorption of protein on the nanoparticles led to a significantly higher production yield. Similar results were obtained by Grenha et al. [18] who found that the entrapment of insulin into CS/TPP NPs increased the production yield of the nanoparticles. Increasing the sodium sulfate concentration used in nanoparticle preparation from 10 to 20 % w/v increased the production yield from 27.8 to 34.65 % w/w (Formulae N_4 and N_1, respectively). The incorporation of increasing amounts of TPP with respect to CS led to a significant increase in the process yield of loaded nanoparticles prepared by ionic gelation technique. The maximum yield (61.38 % w/w) was achieved for the 3:1 CS/TPP mass ratio which is the optimum condition for nanoparticles formation.

Protein loading in chitosan nanoparticle system was achieved by one of two methods either adsorption or entrapment. CS/SO_4 NPs incubated with 200 µg/ml HSA solution showed high association efficiencies of 92.3 and 82.1 % for formulae N_1 and N_4, respectively (Table 2). The values of protein association efficiency (%AE) and loading capacity (%LC) of chitosan nanoparticles prepared by precipitation or ionic-gelation in which HSA was entrapped are presented in Table 2. It was noticed that both parameters were higher using TPP than sodium sulfate which may be due to higher TPP charge density. Formula N_{10} showed the highest %AE of 93.43 and %LC of 58.65 which are higher than those previously reported for HSA entrapment.

The effect of CS/TPP mass ratio on protein encapsulation was studied at a mass ratio of 3:1, 5:1 and 7:1 with a fixed chitosan concentration of 0.25 % w/v and HSA concentration of 0.02 % w/v. Results presented in Table 2 showed that HSA association efficiency decreased from 93.43 to 62.5% when CS/TPP mass ratios increased from 3:1 to 7:1. This reinforces the suggestion that a lower CS/TPP mass ratio favors protein encapsulation during the formation of the CS–HSA nanoparticles. The high TPP mass ratio may cause a rise in solution pH, with a consequential effect on increased overall negative surface charge carried by the protein molecules enhancing electrostatic interactions between CS and HSA molecules [19].

Transmission Electron Microscopy (TEM): TEM photos (Figure 2) confirmed the formation of spherical and regular nanoparticles with solid dense structure mostly in the nanosize range.

Particle size analysis: Particle size is one of the most significant determinants in mucosal and epithelial tissue uptake of nanoparticles and in the intracellular trafficking of the particles [20]. Plain CS/TPP NPs prepared by ionic gelation displayed a particle size of 161 nm compared to 100, 150 and 320 nm for plain CS NPs prepared by precipitation using 10% TPP, 10% and 20% w/v Na_2SO_4, respectively (Table 2).

The effect of CS/TPP mass ratio on the particle size of HSA-loaded CS NPs prepared by ionic gelation was studied for formulae N_8-N_{10}. Table 1 revealed that nanoparticle size decreases with decreasing the CS/TPP mass ratio with the smallest (N_8, 172 nm) being obtained for the lowest CS/TPP ratio (3:1). This provides a simple processing window for manipulating and optimizing the nanoparticle size for intended applications. These results were in accordance with the work done by Grenha et al. [18].

Thermal analysis: The endothermal dehydration of the chitosan was shifted to 64°C in plain CS/TPP NPs. On the other hand, the DSC thermogram of plain CS/SO_4 NPs showed two additional endothermic peaks at about 236° and 275°C. These findings strongly support that an ionic interaction between chitosan and TPP or Na_2SO_4 had occurred [21] (Figure 3, Table 4). Upon encapsulation of protein in the nanoparticles, the peaks of protein disappeared possibly because of the relatively low amounts of protein relative to the polymer or may be due to ionic interactions occurring between the hydrophilic polymer and protein in the nanoparticles [22].

Fourier Transform Infrared Spectroscopy (FTIR): The spectrum of plain CS NPs prepared with TPP showed that the amino group absorption is shifted from 1659 to 1642 cm^{-1}, indicating creation of ionic interaction with TPP (Figure 4). These interactions reduced CS solubility and are responsible for CS separation from the solution in the form of nanoparticles. Chitosan hydroxyl groups remains almost at the same position in the formed nanoparticles [23].

In the CS/SO_4 NPs, a shift from 3434 to 3354 cm^{-1} is shown with the peak at 3354 cm^{-1} becomes wider, this indicates that hydrogen bonding is enhanced. Similar observations were reported by Borges et al. [21] who reported that the sulfate ions interact with the primary amino groups of chitosan, resulting in the formation of crosslinked CS NPs.

Equilibrium swelling study: The CS/TPP NPs (Formula N_7) prepared by ionic gelation were able to imbibe more aqueous medium after 6 h of hydration in PBS than CS/SO_4 NPs (Formula N_4) prepared by precipitation. Formula N_7 had an equilibrium swelling degree of 8.7±0.03 as compared to 6.4±0.05 for formula N_4. The more porous CS/TPP NPs showed a higher swelling due to uptake of aqueous medium by a capillary action.

Surface area and porosity: BET theory aims to explain the physical adsorption of gas molecules on a solid surface and serves as the basis for an important analysis technique for the measurement of the specific surface area of a material. The BET method is based on adsorption of gas, usually nitrogen, on a solid surface, the amount of gas adsorbed at a given pressure allows to determine the surface area. The total pore volume is derived from the amount of vapor adsorbed at a relative pressure close to unity by assuming that the pores are then filled with liquid adsorbate [24].

The addition of TPP to chitosan led to the formation of plain CS NPs (Formula N_7) by ionic-gelation with a very high specific surface area and porosity compared to chitosan powder. Entrapment of HSA within those CS NPs was found to reduce both parameters significantly as the protein molecules may occupy the pores within CS/TPP matrix (Formula N_8) (Table 3).

On the other hand, CS/SO_4 NPs prepared by precipitation (Formula N_4) showed a lower specific surface area and porosity compared to CS/TPP NPs. Similarly, HSA adsorption onto those nanoparticles decreased the specific surface area and porosity (Formula N_3). The higher specific surface area and porosity of CS/TPP NPs compared to CS/SO_4 NPs may be due to the mild nature of ionic-gelation process which allows the formation of more porous matrix compared to the more dense structure of CS/SO_4 NPs created by the precipitation process.

Biodegradability of nanoparticles in lysozyme: The nanoparticle

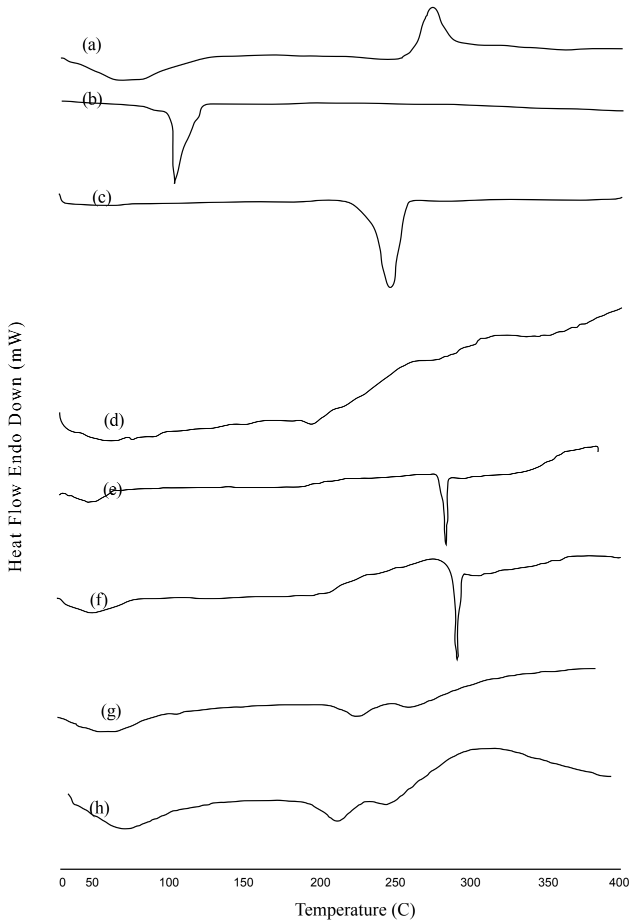

Figure 3: DSC thermograms of chitosan (a), TPP (b), Na_2SO_4 (c), HSA (d), plain CS/TPP NPs (N_7) (e), HSA-loaded CS/TPP NPs (N_8) (f), Plain CS/Na_2SO_4 NPs (N_4) (g) HSA-loaded CS/Na_2SO_4 NPs (N_3) (h).

Formula	Preparation Technique	Specific Surface Area (m^2/g)	Total Pore Volume (e^{-3}ml/g)	Average Pore Radius (Angstrom)
Chitosan	-	1669.75 ± (23.32)	2437.67± (21.53)	29.20± (2.75)
HSA	-	1136.91± (14.58)	1631.05± (12.07)	28.70± (1.65)
N_4(plain)	Precipitation	3008.96± (29.44)	2430.45± (14.75)	28.71± (3.04)
N_3(loaded)	„	703.53± (10.34)	1060.99± (11.88)	29.10± (2.65)
N_7(plain)	Ionic-gelation	3227.18± (25.87)	4730.62± (22.60)	29.32± (1.84)
N_8(loaded)	„	903.02± (21.09)	1350.95± (17.33)	29.23± (2.37)

Table 3: Specific Surface Area and Porosity of Selected Plain and HSA-Loaded Chitosan Nanoparticles (values are mean ± SD, *n* = 3).

Formula	**Peak (°C)**	**Height (mW)**	**Area (mJ)**	**ΔH (J/g)**
chitosan	62.56	8.49	83.86	53.46
TPP	117.72	12.85	193.75	84.35
Na_2SO_4	243.54	12.37	198.33	99.16
HSA	50.24	N.A.	N.A.	N.A.
plain CS/TPP NPs (N_7)	275.05	11.08	209.22	64.93
HSA-loaded CS/TPP NPs (N_8)	286.45	15.86	217.12	94.84
Plain CS/Na2SO4 NPs (N_4)	230.38	8.12	120.21	57.71
HSA-loaded CS/Na2SO4 NPs (N_3)	220.10	9.56	135.46	64.33

ΔH: Enthalpy of fusion of lyophilized dispersion.
N.A.: Not applicable due to very broad peak.

Table 4: Thermotropic Parameters of polymer, HSA crosslinkers, plain and protein-loaded nanoparticles with regard to Endothermic Peak of Phase Transition.

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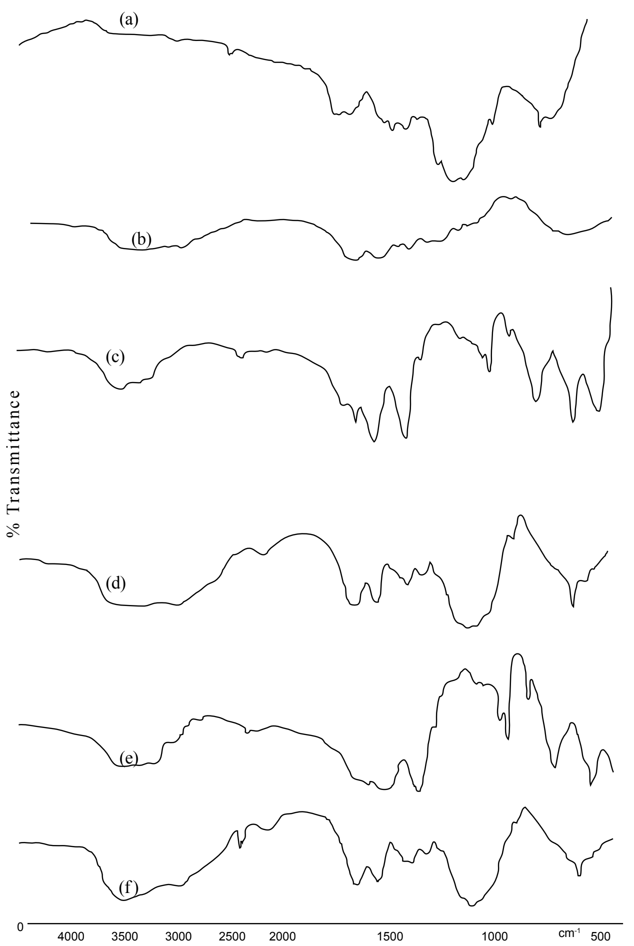

Figure 4: FT-IR transmission spectra of chitosan (a), HSA (b), plain CS/TPP NPs, N_7 (c), HSA-loaded CS/TPP NPs, N_8 (d), plain CS/SO_4 NPs, N_4 (e), and HSA-loaded CS/Na_2SO_4 NPs, N_3 (f).

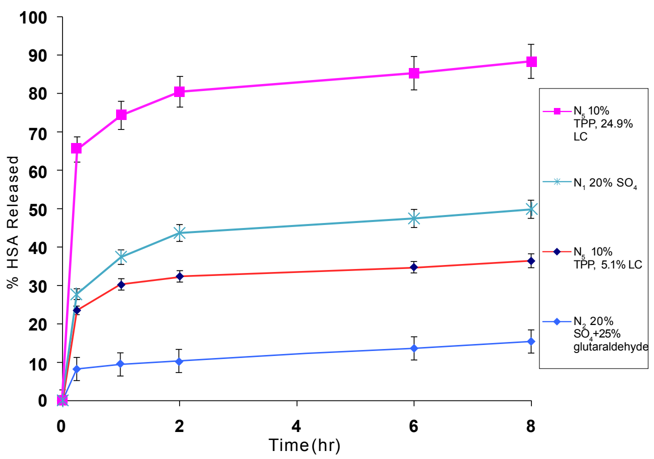

Figure 5: The *in vitro* release of HSA from CS nanoparticles prepared by precipitation technique (adsorption method) in PBS (pH 7.4).

size (Formula N_7) was found to decrease by increasing the incubation period with the enzyme. The tested formulation showed a decrease in the nanoparticle size immediately after the initial contact with the enzyme followed by a gradual decrease. The incubation of fresh nanoparticles with lysozyme led to a total reduction of 243±6.36 nm (55.5 %) in the particle size within 3 hr. These results were predictable, considering that lysozyme can attack chitosan and hydrolyze the glycoside bonds between the acetylglucosamine units [18].

Redispersibility of nanoparticles: For a long-term storage of nanoparticles, aqueous solutions of the nanoparticles are essentially required to be lyophilized as solid products and to be reconstituted immediately before use. As the nanoparticles were prepared with a tremendous increase in surface area and a very high surface activity, aggregation and particle fusion are reported to occur after a long period of storage [25]. The lyophilized CS NPs (Formula N_8), stored at room temperature for one month, were found to be easily reconstituted by simple hand-agitation. However, it was observed that the average nanoparticle size was increased slightly with respect to the initial values (from 172±3.69 to 194.7±5.93 nm), probably because of some particle aggregation. Storage stability at room temperature revealed no significant changes in the particle size of the HSA-loaded CS NPs.

In-vitro **HSA release from chitosan nanoparticles:** CS-HSA NP formulations were tested for *in-vitro* release in PBS (pH 7.4) at 37°C (Figures 5 and 6). The release profiles of HSA from 4 CS NP formulations prepared by the precipitation technique using TPP and sodium sulfate as precipitating agents are shown in Figure 5. In these formulations the model protein HSA was associated to the prepared plain CS NPs by the adsorption (incubation) method. All formulae showed biphasic release profiles with a rapid burst effect.

The CS/TPP NPs (Formula N_5) with two different protein loadings (5.1 and 24.9 % LC) were tested. Calvo et al. [8] observed that the percentage release of BSA from CS NPs was greater for those formulations containing a higher protein loading. Our finding is consistent with their observation where the higher the drug loading, the faster its release from the nanoparticles. Although 86.62±1.45 % of HSA was released after 1 h when loaded with 24.9% LC, only 33.89±0.62 % of the total protein was released at 5.1 % LC. This observation suggests that the remainder of the drug was trapped within the matrix where most of the incorporated protein would be released by degradation or by erosion of the polymer matrix providing a sustained release effect. The release of HSA from uncrosslinked CS/SO_4 NP formulation (N1) was compared with its release from CS/SO_4 NP formulations (N2) chemically crosslinked with glutaraldehyde. After 8 h, the uncrosslinked nanoparticles showed a faster release of protein (50.04±2.23 %) than the crosslinked formulations (10.23±0.47 %) [10].

The *in-vitro* release behavior of HSA from CS NPs prepared by precipitation/protein entrapment technique was illustrated in Figure 6. CS/TPP NPs (Formula N6) showed a faster release of protein compared with CS/SO_4 NPs (Formula N3). The interesting observation was that a consistent low portion of HSA (10.34±0.66 % and 5.35±0.18 % of loaded HSA for CS/TPP and CS/SO_4 NPs, respectively) was released over the 5 days period. The majority of release occurred in the first 1 and 8 h for CS/SO_4 and CS/TPP NPs, respectively. The initial burst release may arise from the desorption of those loosely attached HSA from the surface of the polymeric matrix. Though dissociation appears to be the principle mechanism, other factors, such as diffusion of physically entrapped HSA, may also have a role in the release process [26].

The small size of the nanoparticles is also a major factor, which influences the release rate. These nanoparticles have a large surface area due to their small size. Therefore a significant portion of HSA will be at

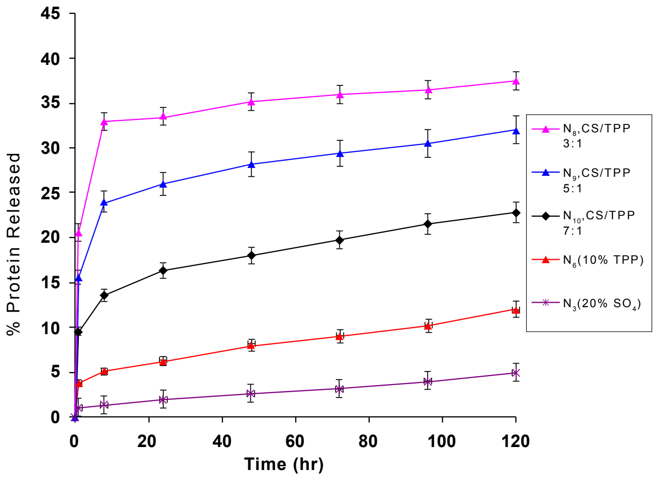

Figure 6: The *in vitro* release of HSA loaded by entrapment method from CS nanoparticles prepared by ionic gelation (N_8, N_9 and N_{10}) or precipitation (N_3 and N_6) techniques in PBS (pH 7.4).

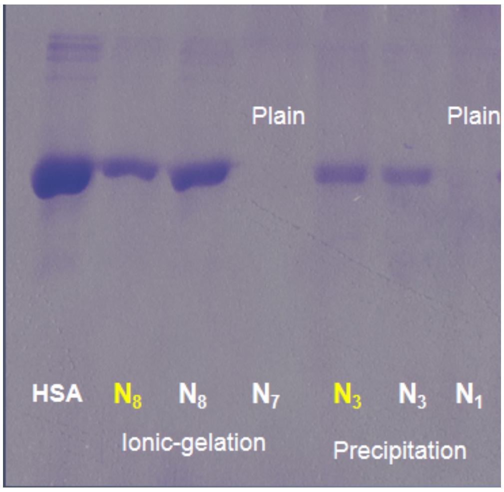

Figure 7: SDS-PAGE results of different HSA samples.
Lane 1: HSA standard (MW 66 kDa);
Lane 2: HSA entrapped into CS/TPP NPs prepared by ionic-gelation (N_8);
Lane 3: HSA released from CS/TPP NPs prepared by ionic-gelation (N_8);
Lane 4: Plain CS/TPP NPs prepared by ionic-gelation (N_7);
Lane 5: HSA entrapped into CS/SO_4 NPs prepared by precipitation (N_3);
Lane 6: HSA released from CS/SO_4 NPs prepared by precipitation (N_3);
Lane 7: Plain CS/SO_4 NPs prepared by precipitation (N_1).

or near the particle surface and can be readily released. Furthermore, the diffusion distances encountered in the particles are small which allows the release medium to diffuse in readily and to exchange with HSA. However, due to the large molecular size of HSA, it is expected to diffuse out slowly even when it becomes dissociated [26].

The release rate of protein from CS NPs is found to be highly affected by the protein loading procedure, namely adsorption (Figure 5) and entrapment (Figure 6). The protein-loaded CS/TPP NPs (Formula N_5) prepared by precipitation/adsorption technique released 86.54 % of protein after 8 h whereas only 10.34 % of protein HSA been released from CS/TPP NPs (Formula N_6) with a higher protein loading but prepared by precipitation/entrapment technique. Similarly protein loaded CS/SO_4 NPs (Formula N_1) by precipitation/adsorption technique was released at a much faster rate than from CS/SO_4 NPs (Formula N_3) prepared by the precipitation/entrapment technique. Thus, a slow protein release over an extended period of time was obtained from NPs in which protein was loaded by the entrapment technique while a fast release was obtained from NPs in which protein was loaded by the adsorption technique.

The effect of CS/TPP mass ratio on HSA release from CS/TPP NPs prepared by ionic gelation technique was studied at the mass ratios of 3:1, 5:1 and 7:1. Results presented in Figure 6 showed that when CS/TPP mass ratio decreased from 7:1 to 3:1, total HSA release after 5 days was increased from 22.47±2.93 to 38.65±4.05 %. The nanoparticles prepared with a lower CS/TPP mass ratio had a greater overall release, reflecting a higher protein encapsulation at lower CS/TPP mass ratio. This was in agreement with Gan and Wang [19].

SDS-Polyacrylamide Gel Electrophoresis (PAGE): Figure 7 showed SDS-polyacrylamide gel electrophoresis of some selected CS NPs formulations prepared by the two techniques. The electrophoretic analysis of the entrapped and released HSA showed identical bands for the native HSA. There were no additional bands to indicate the presence of molecular weight aggregates or fragments greater or less than 66 kDa (M.W. of HSA). These data suggest that the structural integrity of HSA was not significantly affected by the entrapment or the release procedures. Therefore, it is assumed that no chemical polymerization, non-covalent aggregation or substantial degradation of HSA occurred during these processes.

The encapsulation process of HSA into CS/TPP NPs prepared by ionic gelation did not affect the structural integrity of HSA. With this mild method the protein was not exposed to potentially harsh conditions, such as the contact with organic solvents, mechanical agitation or sonication. This was in agreement with the work done by Amidi et al. [27]. Ultrasonication and mechanical agitation employed in the precipitation technique were expected to dramatically affect the protein integrity. Nevertheless, no degradation was observed in case of HSA entrapped or released from CS/SO_4 NPs prepared by the precipitation technique. This may be attributed to the stabilizing effect of CS NPs by entrapping the protein within its matrix providing some sort of a physical protection.

Conclusions

A biodegradable nanoparticle system solely made of the hydrophilic polymer, chitosan (CS), for the oral delivery of a model protein drug, HSA was developed. Precipitation and ionic-gelation techniques were successfully used for the preparation of nanoparticles. The physicochemical characterization of these nanoparticles revealed that they have a homogenous and adjustable size with a great capacity for association of proteins. The prepared nanoparticles exhibited a high specific surface area and porosity and were biodegradable in presence of lysozyme solution. A slow protein release over an extended period of time was obtained from NPs where the protein was loaded by entrapment method which is suitable for delivery of vaccines and protein drugs used for chronic diseases. On the other hand, a fast release was obtained from NPs where the protein was loaded by adsorption method which is suitable for delivery of protein drugs therapeutically used for acute cases. Retention of both the nanoparticle integrity following freeze-drying and reconstitusion and the structural integrity of the associated protein following preparation and release processes were proved.

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