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Preparation and characterization of BSA as a model protein loaded chitosan nanoparticles for the development of protein-/peptide-based drug delivery system

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Abstract

Background: The purpose of this study was to develop protein-/peptide-loaded nanoparticle-based delivery system, which can efficiently deliver therapeutic molecules to the lung via pulmonary delivery. The chitosan nanoparticles were prepared by the ionic gelation method, and bovine serum albumin was used as a model protein. These nanoparticles were characterized for size, zeta potential, encapsulation efficiency, cell cytotoxicity, uptake study, release profile and size distribution and uniformity. The chemical interaction of chitosan and protein was studied by XRD and FTIR. The integrity assessment of encapsulated protein into nanoparticle was studied by native and SDS-PAGE gel electrophoresis.

Results: The size and zeta potential of BSA nanoparticles were 193.53 ± 44.97 to 336.36 ± 94.63 and 12.73 ± 0.41 to 18.33 ± 0.96 , respectively, with PDI values of 0.35–0.45. The encapsulation efficiency was in the range of $80.73 \pm 6.37\%$ to $92.34 \pm 1.72\%$. The cumulative release of the BSA from the nanoparticles was $72.56 \pm 6.67\%$ in 2 weeks. The BSA-loaded nanoparticles showed good uptake and no significant cytotoxicity observed into the A549 cell line. In this study, it was also observed that during nanoparticles' synthesis protein structure and integrity is not compromised. The nanoparticles showed controlled and sustained release with initial burst release. In TEM images, it was shown that nanoparticles' distribution is uniform within nanometre range.

Conclusion: From this study, it was concluded that nanoparticles prepared by this method are suitable to deliver protein/peptide into the cells without any degradation of protein during process of nanoparticle fabrication.

Keywords: Nanoparticle, BSA, Chitosan, Ionic gelation and cytotoxicity

Background

The recent advances in the nanotechnology field are revolutionizing the biomedical and pharmaceutical industries with application like targeted drug delivery, nano-medicines, diagnostics and other novel therapies [1]. The nanoparticle-based targeted drug delivery system directs the therapeutics to a specific organ with a controlled release

of drug for prolonged period [2]. The most common nanoparticle-based drug delivery system is polymeric nanoparticles. Chitosan (CS), a natural polysaccharide derived by the deacetylation of chitin, is extensively used as nanoparticle carriers to encapsulate protein, peptides, antibiotics and other therapeutic molecules. The biocompatible and biodegradable features of chitosan make it suitable as a nanoparticle carrier [3]. These carriers are widely used in pharmaceutical applications because of their non-toxicity and easy degradation by the lysozymes without being accumulated into the body [4].

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Chitosan is a cationic polymer, which in the presence of anionic cross-linking agents like sodium tripolyphosphate (TPP) forms a gel structure by the ionotropic gelation method. The amino groups of chitosan have positive charge that can freely interact with the anionic molecules like DNA, protein and phospholipids, leading to their entrapment into the chitosan gel [5]. Chitosan nanoparticles have numerous applications in non-parenteral drug delivery like pulmonary, nasal, mucosal, oral drug delivery, cancer therapy, vaccine delivery as well as drug delivery to brain and gastrointestinal diseases [6, 7].

Protein- or peptide-based chitosan drug delivery system has various advantages such as low immunogenicity, specificity to receptors, easy cross-linking and surface modification [8]. Bovine serum albumin (BSA) is one of the generally and extensively used proteins for nanoparticle formulations. The characteristics of BSA like purity, water solubility, economical and easy accessibility make it a commonly used protein in pharmaceutical industries for drug carrier as well as model protein for nanoparticle formulations [9, 10].

In this study, BSA was used as model protein for the preparation of BSA chitosan nanoparticle by the ionic gelation method. The optimized ratio of chitosan and TPP was used to get fine-tuned efficient particles, which can deliver therapeutic molecules to the lung. These nanoparticles characterized for particle size distribution, zeta potential, encapsulation efficiency and controlled drug release behaviour. The integrity assessment of BSA was characterized by SDS and native PAGE and chemical characteristics by FTIR and XRD analysis. The cell cytotoxicity and cell uptake of BSA nanoparticle formulations were accessed in vitro. The BSA nanoparticle was evaluated as a potential drug delivery system for the controlled release of peptide and protein molecules.

Methods

Materials

Chitosan ($\geq 75\%$, deacetylated) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich, Bangalore, India. Bovine serum albumin (BSA) was purchased from HiMedia Laboratories, India. All others chemicals were of analytical grade and obtained from various vendors.

chitosan was prepared in 1% glacial acetic acid by heating at 65 °C on magnetic stirrer followed by filtration through 0.22- μm syringe filter. TPP (1 mg/ml) was prepared into water and filtered through 0.22- μm syringe filter. Twenty microlitres of chitosan solution was taken and kept at magnetic stirring at 700 rpm. Eight microlitres of TPP solution was added drop by drop to the chitosan solution and allowed to stir for 45 min. The nanoparticle suspension was centrifuged at 12,000 rpm for 45 min, and pellet was suspended into distilled water to obtain blank nanoparticles. Different amounts of BSA were taken to prepare 0.5, 1.0, 1.5 and 2.0 mg/ml formulations. BSA was dissolved into chitosan solution, followed by TPP addition to prepare BSA-loaded nanoparticles. The nanoparticles were lyophilized for 24–30 h using 10% trehalose as cryoprotectant. The lyophilized nanoparticles were stored at 4° for further studies.

Characterization of protein-loaded nanoparticles

Size, polydispersity index, and zeta potential

The average particle size, PDI and zeta potential of nanoparticles were analysed using Zetasizer (Nano-ZS90, Malvern, UK). The nanoparticle suspension was diluted with distilled water and sonicated for few minutes. The suspension was then transferred to polystyrene cuvettes and analysed for the size and zeta potential of particles. All the samples were taken in triplicate. The actual size and nanoparticles' uniformity were also determined by transmission electron microscopy (TEM) (Tecnai G20 HRTEM, Thermo Scientific, USA). The sample for TEM was prepared by positioning a drop of nanoparticle suspension on a carbon-coated copper grid. The grid was dried under vacuum at room temperature and examined under TEM at an accelerating voltage of 200 kV.

Encapsulation efficiency

The encapsulation efficiency of nanoparticles was measured using the indirect method. The supernatant after the centrifugation of nanoparticles was collected and stored at 4 °C. The amount of protein present in supernatant was estimated by Lowry's protein estimation method. The absorbance was taken at 600 nm using multimode ELISA plate reader (Biotek Synergy HTX, USA). The amount of the BSA encapsulated into the nanoparticles was calculated by using the following formula:

$$\text{Encapsulation Efficiency \%} = \frac{\text{Total BSA amount} - \text{BSA amount in supernatant}}{\text{Total BSA amount}} \times 100$$

Preparation of nanoparticles

The BSA-loaded chitosan nanoparticles were prepared by the ionic gelation method. One milligram/millilitre

Integrity assessment of encapsulated BSA into nanoparticles

The structural integrity of the protein in the nanoparticles was analysed by native and SDS-polyacrylamide gel

electrophoresis. Ten percentage of resolving gel and 5% stacking gel were cast on the gel plate. Ten milligrams of lyophilized nanoparticles were dissolved into sample buffer and loaded on to the gel. Two milligrams/millilitre concentration of pure BSA was taken as a control. The gel was run at 100 V for 3–4 h. The gel was then stained with Coomassie Brilliant Blue dye for 1 h and then destained till the clear bands were visible.

Cell cytotoxicity studies

The cytotoxicity of the blank and BSA nanoparticles was estimated on the A549 cell line by MTT assay. 1×10^4 cells were seeded into 48-well plates in RPMI media supplemented with 10% FBS. The cells were incubated at 37 °C with 5% CO₂ conditions, until the cells attain at least 60–70% confluence. A dose of 100 µg/ml of blank, BSA nanoparticle and pure BSA treatment were given to the cells for 24 h. In each well, 1X PBS and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reagent were added and incubated for 2–4 h in dark for the emergence of formazan crystals. The formazan crystals were solubilized, and absorbance was taken at 570 and 660 nm by ELISA plate reader.

Cellular uptake study

The uptake study of nanoparticles was evaluated by fluorescence-based absorbance method using multimode ELISA plate reader (Biotek Synergy HTX, USA). For this study, FITC dye-loaded blank nanoparticles were prepared by dissolving 30 µl FITC into TPP and added to chitosan solution. A cell density of 1×10^4 A549 cells was seeded into 24-well plate and incubated at 37 °C with 5% CO₂ in 10% FBS containing RPMI media. A dose-dependent of FITC-loaded nanoparticle, blank nanoparticle and pure FITC dye treatment were given to the cells for 6 h. The cells were lysed using Triton X 100 and NaOH buffer, and the content was transferred to black ELISA plate. The fluorescence was recorded at excitation 485 and emission 535 nm using fluorescence mode of multimode ELISA plate reader.

Release study

1X PBS of pH 7.4 was prepared and autoclaved. In a 50-ml centrifuge tube, 40 ml 1X PBS was taken and 1 ml nanoparticle suspension was added to it. The tube was kept at 37 °C in an orbital shaker incubator with stirring at 80 rpm. One millilitre sample was withdrawn at 24-h intervals followed by the addition of 1 ml fresh PBS. The samples were collected for 15 days and stored at –20. The amount of BSA released into the buffer was estimated by the Lowry protein assay.

FTIR analysis

The FTIR spectra of the chitosan, BSA drug and BSA nanoparticle were recorded on PerkinElmer spectrum 2 (USA) by the KBr method. The samples were scanned in the wavelength range of 4000 to 400 cm⁻¹.

XRD analysis

The X-ray pattern of BSA and BSA-loaded nanoparticles was obtained by Rigaku Miniflex 600 desktop X-ray diffraction system (Tokyo, Japan). The samples were scanned over the diffraction angle of 5°–50° 2θ with CuKα radiation at wavelength 1.55 Å.

Statistical analysis

The data were recorded in triplicate and expressed as mean ± standard deviation. The value of $p < 0.05$ was regarded as statistically significant.

Results

Size, zeta potential and polydispersity index

The particle size, zeta potential and polydispersity index of the various formulations are listed in Table 1. The size of the various sample was in the range of 193.53 ± 44.97 nm to 333.36 ± 94.63 nm with zeta potential from 12.73 ± 0.419 to 18.33 ± 0.96 mV (Fig. 1). The blank nanoparticles showed slightly less particle size and zeta potential as compared to BSA-loaded particles. The different formulations of BSA nanoparticles showed a proportional relationship between the amount and the size of the samples. However, the difference was not very significant as the particle size and zeta potential showed a slight increase in their values with increase in amount of BSA into the formulations. The BSA-loaded formulations showed an insignificant increase in the zeta potential with the increasing concentration of BSA. This increment in surface charge is due to the interaction of BSA protein with chitosan and TPP ionic gel system [11]. The polydispersity index (PDI) of the blank and loaded nanoparticles was 0.356 to 0.459. The low PDI values show that the particles are uniform, monodisperse and stable.

Table 1 Size, zeta potential and PDI of blank and different concentrations of BSA-loaded (NP01, NP02, NP03 and NP04 represent 0.5, 1, 1.5 and 2 mg of BSA, respectively) nanoparticles

Sample ID	Particle size (nm)	Zeta potential (mV)	PDI
Blank NP	193.53 ± 44.9	12.73 ± 0.41	0.356
NP01.5	234.2 ± 50.27	14.46 ± 1.65	0.441
NP02	292.03 ± 62.62	17.37 ± 0.82	0.459
NP03	333.36 ± 94.63	15.78 ± 2.72	0.401
NP04	257.3 ± 47.51	18.33 ± 0.96	0.459

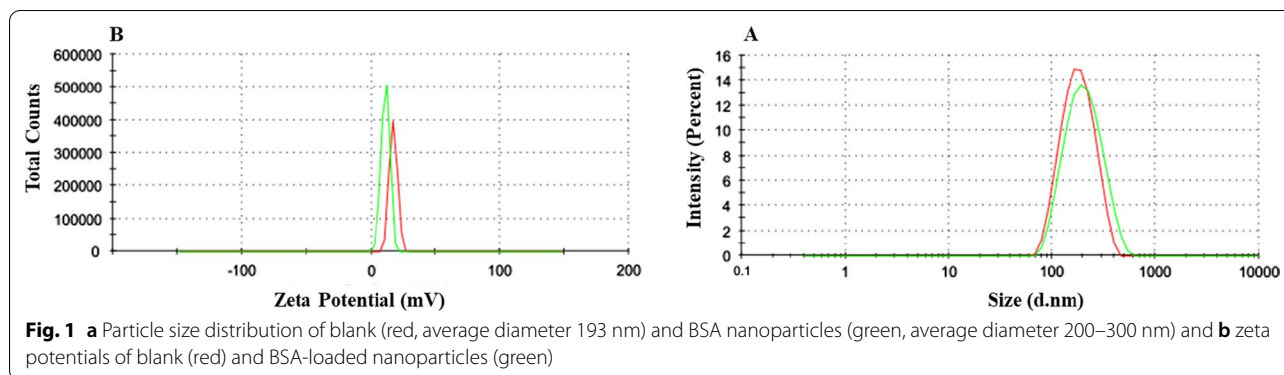


Fig. 1 a Particle size distribution of blank (red, average diameter 193 nm) and BSA nanoparticles (green, average diameter 200–300 nm) and b zeta potentials of blank (red) and BSA-loaded nanoparticles (green)

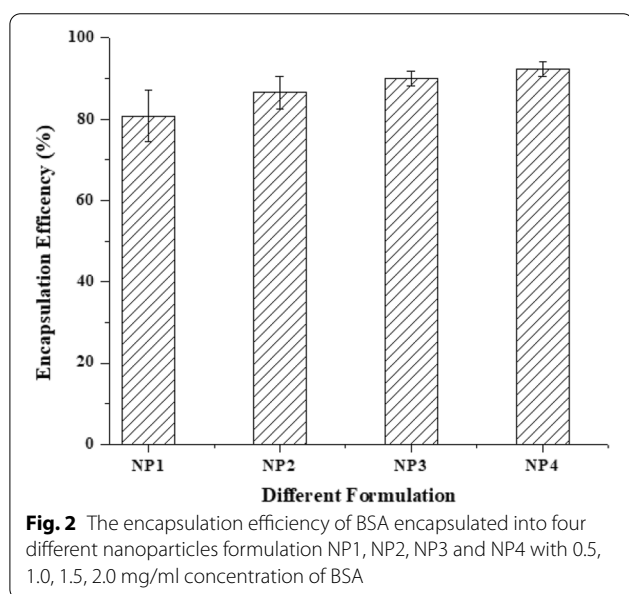


Fig. 2 The encapsulation efficiency of BSA encapsulated into four different nanoparticles formulation NP1, NP2, NP3 and NP4 with 0.5, 1.0, 1.5, 2.0 mg/ml concentration of BSA

Encapsulation efficiency

The amount of BSA entrapped into the nanoparticles was estimated by encapsulation efficiency. The nanoparticles showed an increase in encapsulation efficiency with the increment of BSA amount in different formulations. The percentage of encapsulation for 0.5 mg/ml concentration was found to be $80.73 \pm 6.37\%$ that increased to $92.34 \pm 1.72\%$ in 2 mg/ml BSA formulation (Fig. 2). One milligram/millilitre concentration of chitosan and TPP was used throughout the study, and this concentration was proved efficient for successful cross-linking and encapsulating the BSA. The increasing concentration of BSA does not have any adverse effect on the ionic gelation between chitosan and TPP [12].

Integrity assessment

The SDS-PAGE and native PAGE electrophoresis study show the intact and stable bands of BSA that were encapsulated into the nanoparticles (Fig. 3a, b). All the

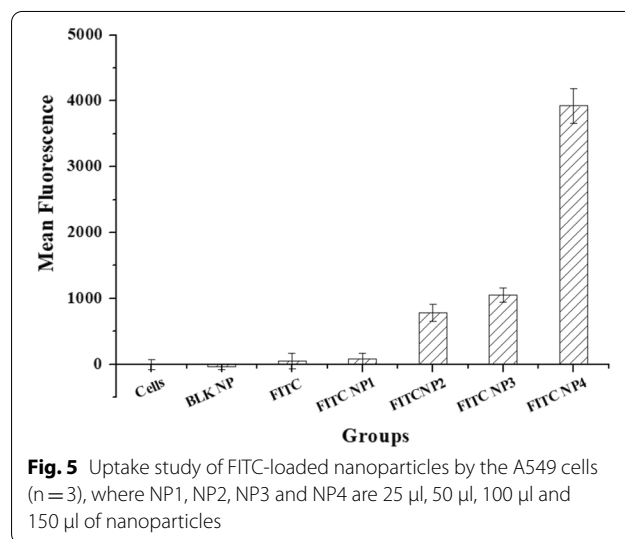
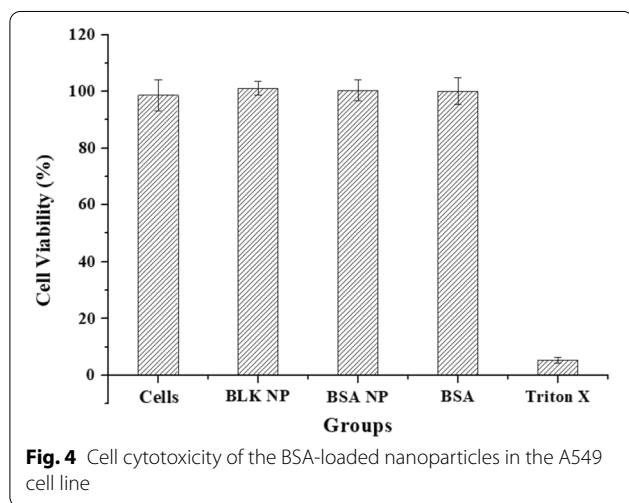
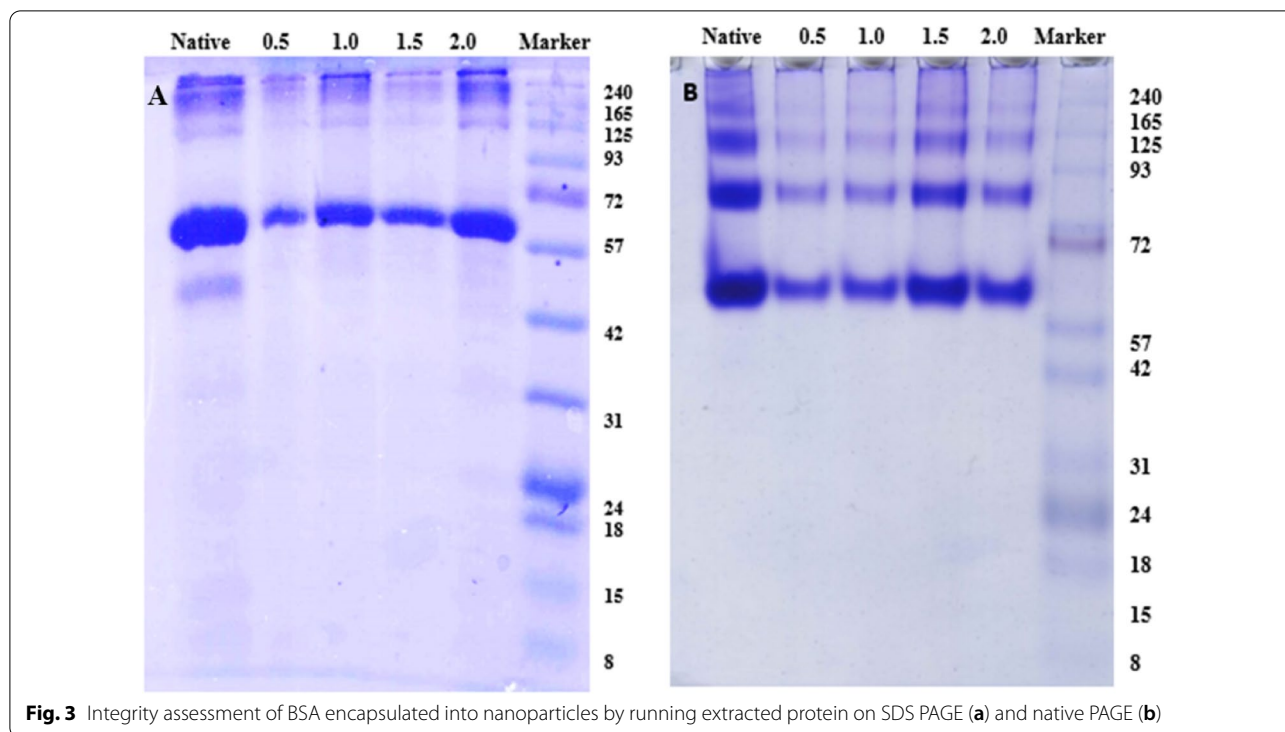
formulations of BSA nanoparticles were loaded on the gel with pure BSA as control. The gel image exhibits the similar movement of BSA particles as well as the control BSA drug of the molecular weight of 66.5 kDa. The bands do not show any alteration or deterioration of the structure of BSA protein. The study confirms that the BSA loaded into the nanoparticle had maintained its structural integrity.

Cell cytotoxicity by MTT

The cytotoxicity effect of nanoparticles was performed by MTT assay. MTT is a colorimetric assay that measures the cell metabolic activity based on the reduction of the tetrazolium salt into insoluble formazan crystals by mitochondrial dehydrogenase enzyme of viable cells [13]. The amount of formazan crystals produced is equivalent to the number of viable cells [14]. The formazan crystals are solubilized in dimethyl sulphoxide (DMSO), and viable cells are measured by taking absorbance at 570 nm and 660 nm as reference wavelength. The blank and BSA nanoparticles show the same viability as the cell control ($99.99 \pm 16.97\%$), whereas BSA drug showed significant increase in cell viability ($108.36 \pm 25.69\%$) (Fig. 4). The MTT assay confirms that the nanoparticles do not have any toxic effect on the cells.

Uptake study

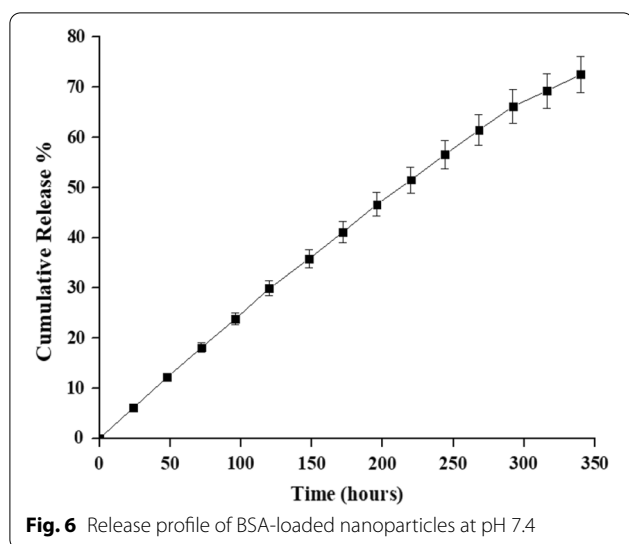
The in vitro cellular uptake was performed on the A549 cell line by using FITC-loaded nanoparticles. The fluorescence activity of FITC dye loaded into the particles was quantified by measuring fluorescent at excitation 485 and emission 535 nm in fluorescence mode of ELISA plate reader. The dose-dependent treatment of FITC nanoparticles was given to the cells with blank nanoparticles (without dye) and FITC dye as control. As shown in Fig. 5 with the increase in the FITC nanoparticle dose, there was increase in fluorescence by the cells. The lowest dose of 25 $\mu\text{g/ml}$ showed less fluorescence by the cells that increased drastically in the highest dose of 150 $\mu\text{g/ml}$.



ml treatment suggesting the cell uptake is concentration-dependent [15]. Apart from nanoparticle concentration, particle size, surface charge, incubation time and temperature play an important role in the cell uptake of the nanoparticles [16]. As the average particle size of nanoparticle is in range of 200–300 nm, the particles can easily enter into the cells [17]. The uptake study showed that the nanoparticles have successfully taken up by the A549 cells.

Release profile study

The in vitro release profile of BSA nanoparticles in PBS at pH 7.4 was studied over a period of 15 days. The BSA nanoparticle shows the initial release of $6.10 \pm 0.55\%$ in 24 h and a cumulative release of $72.56 \pm 6.67\%$ in 2 weeks (Fig. 6). The particles showed slow and constant release with more or less similar rate in 12 days. However, after that the release rate from the particles was decreased slowly. The exponential pattern displayed by the release



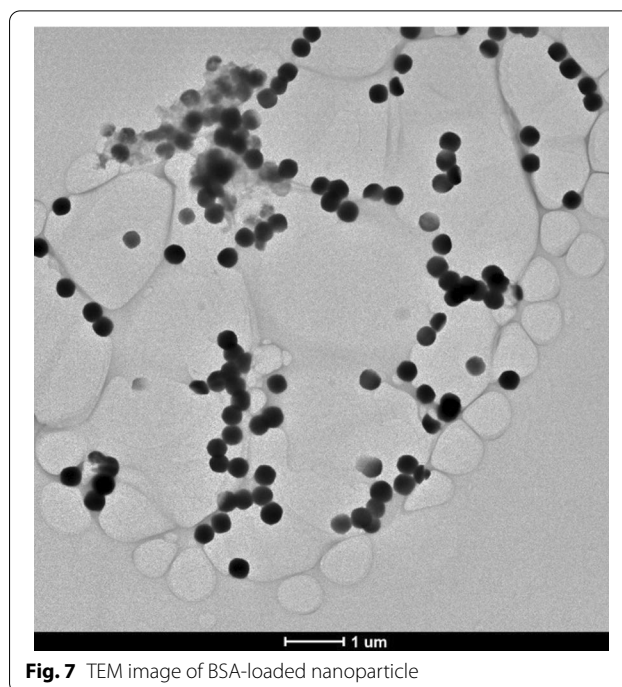
profile of BSA nanoparticles indicates that the system is suitable for sustained release of therapeutics [18]. Amount of BSA and chitosan as well as the degree of deacetylation of chitosan plays an important role in the release rate. The higher deacetylation of chitosan has a higher number of ammonium group that forms ionic interactions with TPP, resulting in the formation of dense particles. This results into the lower permeability of nanoparticle surface and decrease in release rate [19]. The larger the amount of BSA-encapsulated leads to higher diffusion rate due to the formation of concentration gradient between the chitosan and buffer matrix [20]. Though the particles showed 80% encapsulation, the release rate was slow that is because of the chitosan used in the study is of low concentration (1 mg/ml) and high degree of deacetylation.

Surface morphology of nanoparticles

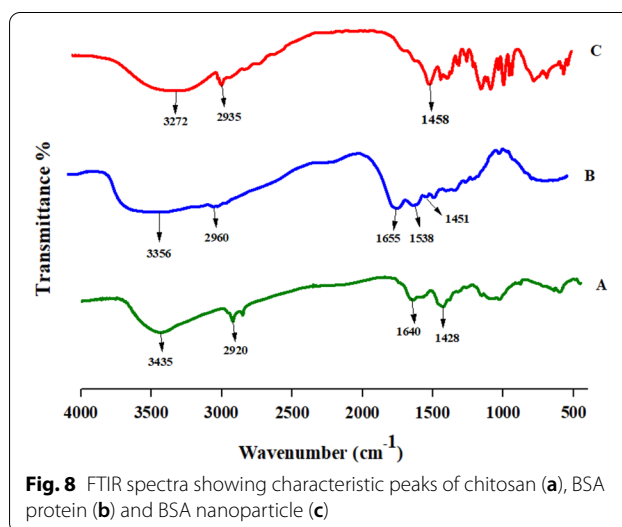
The image obtained by TEM showed the spherical, smooth morphology and almost uniform size distribution of BSA nanoparticles (Fig. 7). The size of the particle was found to be approximately 200–300 nm. The image shows that the particles are monodispersed; some of them are linked together and are forming chain-like structure without losing their individual shape, whereas few particles have tended to form aggregate and have taken a distorted shape.

FTIR analysis

The FTIR spectra of chitosan, BSA protein and BSA nanoparticle are shown in Fig. 8. The IR spectra of chitosan show –NH and –OH group stretching at 3435 cm^{-1} and –CH stretch at 2920 cm^{-1} and the C=O stretching of amide I and II bands at 1639 cm^{-1} and 1428 cm^{-1} ,



respectively (Fig. 8a) [20, 21]. The broad band at 3356 cm^{-1} in BSA protein spectra shows the –NH stretch and –CH stretching vibration at 2960 cm^{-1} with peaks at 1655 cm^{-1} and 1538 cm^{-1} responding to C=O stretching and –NH bending of amide I and II band, which are characteristic peaks for protein structure (Fig. 8b) [22, 23]. The BSA nanoparticle shows a band at 3272 cm^{-1} , which seems to be shifted from 3356 cm^{-1} peak in BSA. The shifting is indicative of the –NH stretch and increased –OH bonding due to overlapping of –OH bonds of



chitosan as well as BSA protein [20, 24]. The peak at 2959 cm^{-1} and 1461 cm^{-1} represents the $-\text{CH}$ stretch and $-\text{CH}$ bending, respectively. The enhanced $-\text{OH}$ bonding as well as the disappearance of bands between 1530 and 1660 cm^{-1} in nanoparticle suggests the interaction between the chitosan and BSA protein (Fig. 8c).

XRD analysis

The XRD pattern of BSA protein and BSA nanoparticle is shown in Fig. 9. The broad peak of BSA protein exhibits its amorphous nature (Fig. 9a). The XRD pattern of BSA nanoparticle shows the characteristic peaks at 2θ values of 9.66° , 14.6° , 18.7° , 20.4° , 21.1° , 23.4° , 29.4° and 33.5° (Fig. 9b). The percentage of crystallinity shown by the BSA nanoparticles is 65.91%.

Discussion

Nowadays nanoparticles are widely used for therapeutics and diagnostics in pharmaceutical and biomedical applications [1]. The distinct features of nanoparticles like large surface area, small size, easy solubility, prolonged stability and high bioavailability and biocompatibility make them an efficient drug delivery carrier [25, 26]. Nanoparticle-based targeted delivery system provides site-specific delivery of bioactive compounds with better efficiency and penetration at the targeted tissues [10].

The proteins and peptides are widely used as a therapeutic agent as they are natural, biocompatible, less toxic and are more efficient and potent than the chemical drugs [27]. Though the proteins and peptides have great potential as a therapeutics, they also possess some limitations like immunogenicity, agglutination and poor stability and passage into the biological system [28, 29]. To overcome these limitations, proteins and peptides can be

encapsulated into the polymeric nanoparticles without affecting their efficacy.

Chitosan nanoparticles are common polymer-based drug delivery carriers that are actively used in pharmaceutical and therapeutic applications [30]. It is a natural polymer which is biodegradable, less toxic and is biocompatible with a range of active compounds like peptide, protein, DNA, antibiotics and vaccines [3, 20]. The chitosan nanoparticles are prepared by various methods like emulsification, precipitation and ionotropic gelation method. The gelation method employs mild preparation conditions like low shear forces and agitation and avoids harsh organic solvent and heat application [31]. The drug delivery of proteins and peptides is quite challenging as these are prone to enzymatic degradation. The chitosan delivery system can easily deliver the proteins at the specific site due to its mucoadhesive properties and solubility in aqueous solution at different pHs [32]. Moreover, the chitosan shows the controllable and tunable chemical modifications such as polymerization, methylation and alkylation. The modifications can facilitate the attachment of different functional groups to reform the cationic or anionic properties of the drug delivery system as per the biomedical application requirements [33]. The gelation method used in this work is proved to be effective for the encapsulation of BSA.

BSA is a common protein that is frequently used in biochemical and pharmaceutical applications. It is stable, economical and free from cross reactions with other proteins. These features make it ideal for carrier molecule or as a model protein for initial stage of pharmaceutical observations [10]. The BSA chitosan nanoparticle used in this study was easy to formulate and optimize by controlling various parameters like concentration of polymer and gelation agent, pH, quantity of BSA, magnetic stirring speed and time and centrifugation speed. One milligram/millilitre concentration of chitosan and TPP solvent in 5:2 ratios was optimized for BSA encapsulation. Encapsulating increasing amount of BSA into different formulations does not show any adverse effect on the gelation between chitosan and TPP. However, the increase in amount of BSA showed insignificant to slight increase in the size, zeta potential, PDI and EE in formulations.

The native and SDS-PAGE confirmed that the integrity of the protein structure encapsulated into the nanoparticles is not compromised. The nanoparticle system is stable, and the encapsulated protein is fully functional and intact. The FTIR study confirms the successful interaction of chitosan and TPP. The protein nanoparticles are shown to have immunogenic activity on the cells. However, the cell uptake and cell cytotoxicity activity

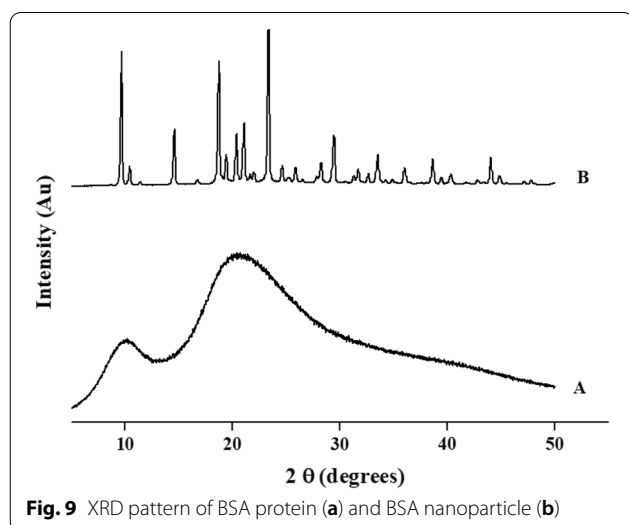


Fig. 9 XRD pattern of BSA protein (a) and BSA nanoparticle (b)

observed on A549 cells showed that the nanoparticle system is non-toxic and is successfully taken up by the cells. This proved that this nanoparticle system is potentially fit to be used for targeted delivery of protein.

The surface morphology study showed that the nanoparticles are regular in shape with uniform size distribution. The *in vitro* release of BSA from nanoparticles showed very low initial burst with prolonged and slow release over a 2-week period. The release of BSA from the nanoparticles is facilitated by diffusion, erosion or degradation of polymer matrix. The type of process involved and its speed determine the initial burst and sustained release amount of the BSA. Initial burst release is one of the prominent limitations of this nanoparticle system that can be improved by the further modifications [3]. The purpose of the study was to formulate and optimize a nanoparticle-based system for the efficient delivery of peptide and protein at specific site using BSA as a model protein. The system can be used for encapsulating different target proteins for the administration by nasal, subcutaneous and inhalation routes [7, 34–36].

Conclusion

The BSA nanoparticles were successfully prepared by ionic gelation of chitosan and TPP as a drug delivery system for controlled release. The amount of BSA has slight-to-insignificant effect on the particle size, surface charge and encapsulation efficiency of the nanoparticles. The encapsulation efficiency was higher in the formulations containing increased amount of BSA. The FTIR study confirmed the successful interaction of chitosan and BSA, whereas the integrity assessment proved that the structure of BSA protein was intact in the nanoparticle matrix. The release profile showed a sustained and controlled drug release. The *in vitro* studies showed that the BSA chitosan nanoparticles are easily taken up by the cells and do not have cytotoxicity affects. These results showed that the BSA nanoparticles are suitable drug delivery systems for controlled release of proteins and peptides. Further *in vivo* studies are required to confirm the application of this drug delivery system. To conclude, the nanoparticle formulation can be used as a drug delivery system for encapsulating various peptides, proteins and drugs with slight modifications for the administration via subcutaneous, nasal or inhalation route.

Abbreviations

BSA: Bovine serum albumin; FTIR: Fourier transform infrared spectroscopy; XRD: X-ray diffraction; SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PDI: Polydispersity index; CS: Chitosan; TPP: Sodium tripolyphosphate; EE: Encapsulation efficiency; NP: Nanoparticle; MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; FITC: Fluorescein

isothiocyanate; PBS: Phosphate buffer saline; TEM: Transmission electron microscopy.

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Authors' contributions

PY performed the experiments and compile data for manuscript preparation and ABY conceived the idea design experiments, helped in data analysis, supervised the project and gave the final shape to the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

All data and materials are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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