Encapsulation and *in vitro* characterization of protein in PLGA-chitosan nanoparticles for efficient drug delivery

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ABSTRACT

Respiratory Syncytial Virus (RSV) is one of the most common causes of severe respiratory tract infections worldwide. An effective RSV vaccine requires the induction of mucosal immunity which is a major hurdle. Development of polymeric nanoparticle delivery systems is one of the strategies to overcome this hurdle. PLGA, a biodegradable copolymer is FDA approved and chitosan, a mucoadhesive polymer, has been suggested to increase the membrane permeability when used intranasally. In this study we encapsulated a model protein, BSA in PLGA-chitosan nanoparticles using emulsion method. SDS-PAGE, FTIR, DSC, zeta potential and cytotoxicity on mouse J774 macrophages were performed to characterize the nanoparticles. The nanoparticles showed encapsulation efficiency > 75 % and low toxicity on J774 macrophage cell line. Our data shows the successful encapsulation and characterization of BSA in PLGA-chitosan nanoparticles. An RSV multivalent protein is currently being evaluated as a vaccine delivery system for RSV.

Keywords: encapsulation, BSA, PLGA, chitosan,

1. INTRODUCTION

Respiratory syncytial virus (RSV) is one of the primary causes for hospitalization for respiratory tract illness especially in young children, immune-compromised and elderly people. A large array of alternative vaccination strategies such as viral antigen, delivery system (live attenuated virus, replicating or non-replicating vectored vaccines, and subunit vaccines), adjuvant and route of administration have been tested against RSV in the last 40 years[1]. Despite these efforts, no effective vaccine has been yet developed. Induction of the mucosal immunity is very important in producing an effective RSV vaccine. But when immunogens are administered via the intranasal route, efficient induction of mucosal immune responses is not achieved. Nanoparticles have been gaining extensive usage in medicine and therapy. The synthesis and nanomedicine formulation of chitosan[2] and PLGA[3] are well reviewed. The objectives of this study were, to encapsulate a model protein BSA with PLGA-chitosan nanoparticles to serve as a protein delivery system. The encapsulated nanoparticles were characterized by physiochemical techniques such as DSC (Differential Scanning Calorimetry), FTIR (Fourier Infrared Spectroscopy) and Zetasizer to analyze the thermal stability, chemical groups, and zeta potential. Cytotoxicity studies were performed to check the safety of the nanoparticles on cells. Recombinant FM2G protein, a multivalent protein vaccine against RSV, has also been subjected to the same studies to evaluate the same as a vaccine delivery system for RSV.

2. MATERIAL AND METHODS

2.1 Preparation of nanoparticles

Double emulsion evaporation method was used to prepare PLGA-chitosan nanoparticles. Briefly, 5% Poly (D, L-lactide-co-glycolide) (PLGA 50:50) was prepared in dichloromethane (DCM). To this mixture was added 500 µg of BSA or rFM2G. The emulsion was sonicated at 22,000 rpm for 15 seconds at 4 ^{o}C. Then 50 ml of 1% PVA was mixed along with this homogenized emulsion and stirred at 400 × g, for 2 h at room temperature. To coat the PLGA with chitosan, 1 mL of chitosan (1%) was added and stirred at 400 × g for 30 min at room temperature. Nanoparticles were harvested using centrifugation (20,000 × g, 10 min, 4 ^{0}C), washed three times with distilled water to remove any residual PVA and lyophilized. Lyophilized nanoparticles were stored at -20 ^{0}C. Nanoparticles prepared with PBS were used as control.

2.2 Protein extraction using an alkaline sodium dodecyl sulphate (SDS) method

About 10 mg of nanoparticles were added to 1 ml of 0.1 N NaOH containing 2% w/v SDS and the solution was shaken overnight at RT. After centrifugation (11269 × g, 5 min), the supernatant was used to estimate the protein content using the BCA assay and analyzed on an SDS-PAGE. All readings were repeated in triplicate. Encapsulation efficiency was calculated using following formula:

$$\frac{\text{Protein encapsulated - Total protein}}{\text{Total protein}} \times 100$$

2.3 Fourier transform infrared spectroscopy

FT-IR spectra were recorded for BSA, and PLGA-chitosan + BSA in attenuated total reflectance (ATR) mode using an IR spectrophotometer (Thermo Fisher Nicolet 380 FT-IR). The spectra were obtained with 64 scan per sample ranging from 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1}.

2.4 Zeta potential measurement

Approximately, 1-2 mg of lyophilized nanoparticles were suspended in 40 µL of DMSO and 960 µL of distilled water. For measurement of zeta potential of freshly prepared nanoparticles, Zetasizer® (Malvern Instruments, UK) was used which is based on the Photon Correlation Spectroscopy (PCS) techniques. Triplicate samples were analyzed.

2.5 Differential scanning calorimetry

Differential scanning calorimetry (Mettler Toledo Schwerzenbach, USA) was carried out to determine the melting point of the nanoparticles. Samples were previously conditioned at 54% relative humidity and 25°C. The nanoparticles samples (50 mg) contained in aluminum pans were heated from 20 to 120°C at a heating rate of 5°C/min in a nitrogen atmosphere (50 mL/min).

2.6 Cytotoxicity assay

Cytotoxic effects of the BSA encapsulated nanoparticle, rFM2G encapsulated PLGA-chitosan, PLGA and chitosan was performed using the MTT dye reduction assay in J774 cells. The CellTiter96Cell Proliferation Assay kit (Promega, USA) was used to determine the cell viability. Cells were plated in a 96-well plate at a density of 1×10^5 cells/well in 50 µL DMEM complete medium and incubated overnight at 37°C under 5% CO_2. The BSA encapsulated PLGA-chitosan nanoparticles, PLGA, or chitosan were added to the cells in concentrations ranging from 62.5 to 1,000 µg/mL. After incubation for 24, 15 µl of MTT dye solution was added into each well, and the cells were incubated for 4 hours at 37°C under 5% CO_2. To stop the reaction, 100 µl of solubilization solution/stop mix were added to each well and plates incubated in the dark for 2 h at room temperature. Absorbance at 570 nm was measured using an ELISA plate reader (TECAN US Inc., Durham, North Carolina). Cell viability was calculated by extrapolating the optical density reading compared to untreated cells.

3. RESULTS AND DISCUSSION

3.1 Encapsulation efficiency of PLGA-chitosan nanoparticles

Double emulsion evaporation method was used to prepare nanoparticles. Our results indicate a high encapsulation efficiency of 75 % with BSA. The supernatant was analyzed using SDS-PAGE to check the release of the BSA. The gel contained a protein band of ~ 67 kDa that corresponded to the expected size of BSA (Fig. 1.). The encapsulation efficiency of the RSV multivalent protein rFM2G was estimated to be > 90%. Encapsulation of protein has known to increase its stability. We chose PLGA and chitosan because they are one of the most successfully used biodegradable materials for the development of nanomedicines as they undergo hydrolysis in the body to produce biodegradable metabolite monomers.

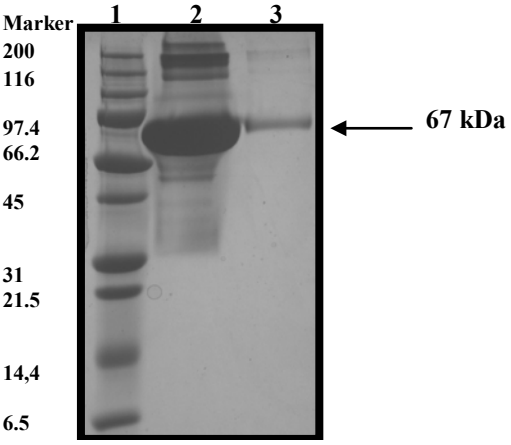

Figure 1: SDS gel showing the release of the BSA protein from PLGA-chitosan nanoparticles. Lane 1: MW marker, Lane 2: BSA alone, Lane 3: BSA released from nanoparticles.

3.2 FT-IR analysis

FT-IR was used to identify variations in chemical functional groups in the nanoparticle. FT-IR for BSA provides information about its secondary protein structure (Fig. 2.). Amide I linkage is seen at 1625 cm-1. In the other spectra of PLGA–chitosan +BSA (red); the peaks around 2920 cm^{-1} is due to NH^{3+} vibrations, which is present in chitosan. The peak around 1800 cm^{-1} -2000 cm^{-1} is due to asymmetric vibrations of C=O that are present in PLGA. A distinct separated peak around 1600 cm-1 is due to amide I vibration (1600-1700 cm-1) of the protein. The FTIR shows successful encapsulation of BSA in PLGA-chitosan nanoparticles.

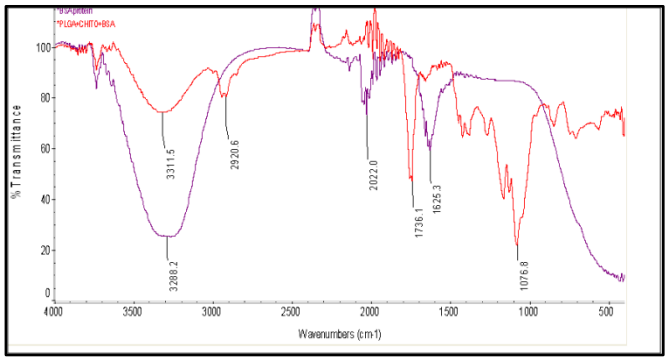

Figure 2: FTIR analysis of PLGA-chitosan nanoparticles. Purple: BSA protein alone, Red: PLGA-chitosan + BSA.

3.3 Physiochemical characterization of nanoparticles

The Zeta potential charge distributions for PLGA-chitosan and BSA encapsulated PLGA-chitosan were determined from an average of three readings. Our results indicate that the charge of PLGA-chitosan without BSA was approximately -16mV (Fig. 3A.). However, for PLGA-chitosan + BSA the charge was approximately -28.9 mV (Fig. 3B.). Zeta potential of the BSA-loaded PLGA-chitosan nanoparticles can greatly influence their stability in suspension through electrostatic repulsion between the particles. The effect of BSA loading increased the zeta potential of the particles. The particles in a colloidal suspension or emulsion usually carry an electrical charge. If the repulsion between approaching particles is large enough they will bounce away from one another and that will keep the particles in a state of dispersion. If the repulsive force is not strong enough, the particles will come together and may stick in a permanent doublet. If the electric charge alone can keep the system in a disperse state then the zeta potential above 25 mV (positive or negative) is considered to be optimum.

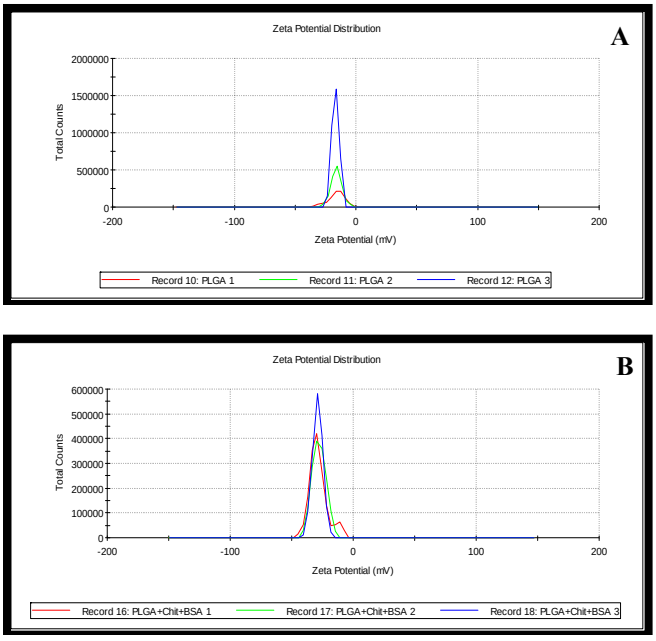

Figure 3: Zeta potential analysis of PLGA-chitosan with and without BSA. Approximately 1 mg of nanoparticle was suspended in deionized water. The zeta potential was measured using zetasizer.

3.4 DSC analysis

The thermogram of BSA encapsulated in PLGA-chitosan was around 100 ^{0}C (Fig. 4.). These results indicate that BSA encapsulated in PLGA-chitosan is stable at very high temperatures. Nanoparticle complex stability can be considered as one of the most important parameters for efficient protein delivery. The complex must keep its structure and shape and not dissociate before entering the cells. DSC is one of the most general methods to find out the physiochemical interaction between protein and polymer in a formulation. It is well known that protein alone is not stable at high temperature. PLGA-chitosan encapsulation resulted in increasing the thermostability. The degradation of PLGA occurs in two steps: first, chain cleavage as indicated by a decreasing molecular weight; second, polymer erosion as indicated by a loss of matrix weight[5]. Degradation of chitosan occurs in four stages which involves loss of water attributed to positive-charged amino groups. Deacetylation and dehydrations occurs in the second and third stages. The last stage, degradation, is mainly due to chain scission[6].The DSC profile demonstrates that PLGA-chitosan in which BSA was encapsulated shows the existence of unique molecular interactions in these nanoparticles.

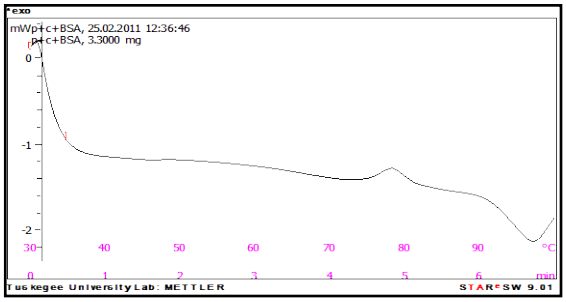

Figure 4: Thermal stability analysis of BSA encapsulated PLGA-chitosan using DSC. The nanoparticle was heated at a rate of 10°C/min from 20°C to 120°C under nitrogen. Endothermic peak is ~100 °C.

3.5 Cytotoxicity studies of PLGA-chitosan nanoparticles with protein BSA

The cytotoxicity of PLGA, chitosan, BSA encapsulated in PLGA-chitosan nanoparticles and rFM2G encapsulated PLGA-chitosan were evaluated in J774 mouse macrophage cell lines by the MTT assay. Fig. 5 shows the percent viability of J774 cells exposed to PLGA with concentrations ranging from 1,000 µg/mL to 62.5 µg/ml for 24 h. Approximately 20% of the cells died at 1,000 µg/mL concentration of PLGA at 24 h. Cytotoxicity of chitosan was also tested with concentrations ranging from 62.5 µg/ml to 500 µg/ml for 24 h. As seen in Fig. 6 cell death at 24 h was minimal (10%). Likewise, BSA encapsulated in PLGA-chitosan was tested for cytotoxicity in J774 cells for 24 h. At 24 h, the cell viability was > 80% (Fig. 7). For the rFM2G encapsulated PLGA-chitosan after 24 h the cell viability was > 90% (Fig. 8). Research has been done on metal, carbon and semiconductor based nanoparticles. In general, cells can survive short-term exposure to low concentrations (< 10 µg/mL) of nanoparticles[4]. However at high doses cytotoxic effects have been found to emerge in a dose- and time-dependent manner for all of the nanoparticles tested.

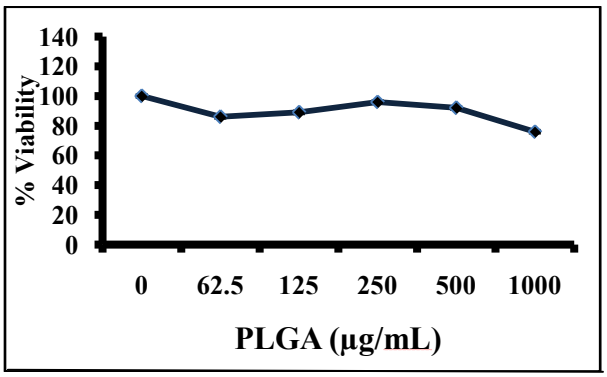

Figure 5: Cytotoxicity effect of PLGA (50:50) in mouse J774 macrophages after incubation for 24 hours. MTT assay was done to measure the viability of cells based on the activity of mitochondrial enzymes in living cells that reduce MTT to formazan. Data represent the mean ± SD of triplicate samples.

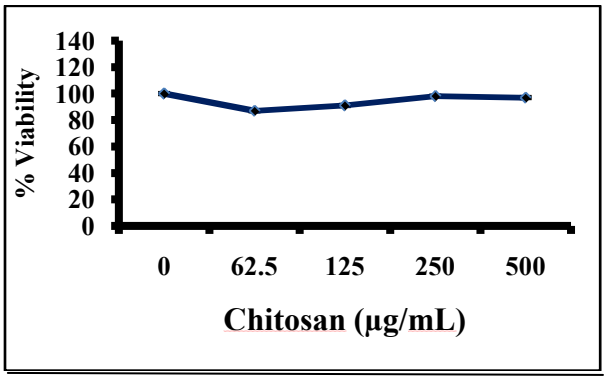

Figure 6: Cytotoxicity effect of chitosan in mouse J774 macrophages after incubation for 24 hours. MTT assay was done as described in Figure 5. Data represent the mean ± SD of triplicate samples.

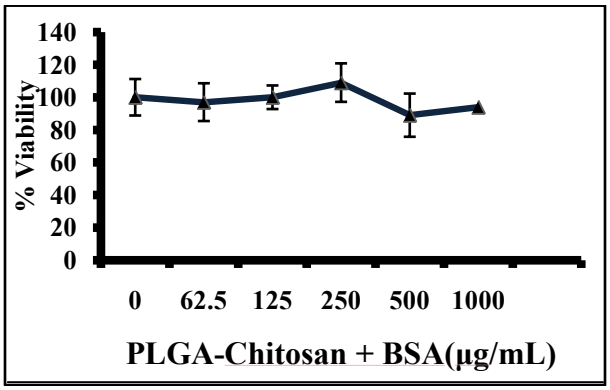

Figure 7: Cytotoxicity effect of BSA encapsulated in combined PLGA-chitosan in mouse J774 macrophages after incubation for 24 hours. MTT assay was conducted as indicated in Figure 5. Data represent the mean ± SD of triplicate samples.

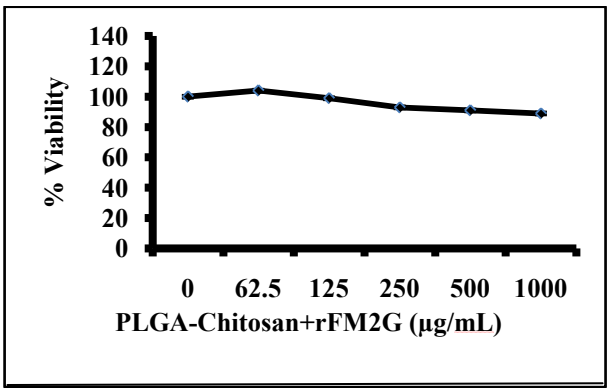

Figure 8: Cytotoxicity effect of rFM2G encapsulated in combined PLGA-chitosan in mouse J774 macrophages. Macrophages were incubated with various concentrations of PLGA-chitosan-encapsulated rFM2G for 24 hours. MTT assay was done to measure the viability of cells as described in Figure 5. Data represent the mean ± SD of samples run in triplicates.

In summary, the findings from these studies clearly indicate the successful encapsulation of the model protein BSA in combined PLGA-chitosan nanoparticles. Physiochemical characterization studies confirmed BSA encapsulation in these nanoparticles. PLGA-chitosan nanoparticles overall are safe for *in vivo* studies as they exhibited little or no toxicity to mouse macrophages. Moreover, of utmost interest is that we have similarly encapsulated a multivalent RSV protein in PLGA-chitosan nanoparticles and characterized the encapsulated protein to serve as a vaccine delivery system for RSV.

ACKNOWLEDGMENT

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