Formulation Optimization for the Nanoparticles-in-Microsphere Hybrid Oral Delivery Systems Using Factorial Design

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ABSTRACT

The objective of the present study was to optimize the processing parameters for nanoparticles-in-microsphere oral delivery system (NiMOS) and to mathematically relate the process parameters with the selected attribute (particle size) of the hybrid system. Fluorescein Isothiocyanate (FITC) labeled gelatin nanoparticles were encapsulated in poly(epsilon caprolactone) (PCL) micropshere and this system was optimized for particle size using a full 3^3 factorial design. Particles with size less than 10 micrometer were processed based on the results obtained from factorial design. These particles can be used for the oral delivery of macromolecules such as peptide, proteins, and other therapeutics.

Keywords: nanoparticle, microsphere, factorial design, response surface plot, oral delivery

INTRODUCTION

Advancements made in biotechnology and molecular engineering has afforded the use of polymeric systems as oral delivery devices for therapeutic/antigenic proteins and other macromolecules [1-3]. Despite the progress of knowledge in this field, present limitations of nanoparticles as transmucosal macromolecular delivery systems include their instability in contact with the gastrointestinal fluids and their limited interaction and transport across mucosal barriers [4]. Several researchers have suggested the use of microspheres prepared using biodegradable polymers to enhance the gastrointestinal stability of the encapsulated proteins and peptides. In order to enhance the targetability of the microspheres to the intestinal mucosae, designing of the microspheres below the size of 10 μm has been reported [5–9].

In this study, we have developed the formulation of gelatin nanoparticles encapsulated in PCL microspheres as a potential delivery system which can be used for intestinal mucosal delivery of therapeutic or antigenic proteins. The encapsulation of nanoparticles in microspheres was conceived with the intention of making these nanoparticles more stable when in contact with physiological fluids. The idea behind this concept was that the microsphere shell would hinder protein/enzyme adsorption, thereby avoiding the harsh environment to which the particles are exposed until they reach the absorbing epithelium.

Factorial design based on response surface method was adopted to optimize particle size of NiMOS encapsulating, a model system, FITC labeled gelatin nanoparticles. A 3^3 full factorial design was employed to evaluate the combined effect of the selected variables on the size (in microns) of the prepared NiMOS.

EXPERIMENTAL METHODS

Preparation of FITC-labeled gelatin nanoparticles

For the preparation of FITC-labeled gelatin, gelatin was dissolved in borate buffer (pH 8.5) at 37 ^{o}C. In a separate beaker FITC was dissolved in borate buffer (pH 8.5). The above solutions were mixed and incubated for 3 h at room temperature. The mixture was then extensively dialyzed against distilled water to remove any free FITC molecules.

Nanoparticles of FITC-labeled gelatin were prepared by desolvation and controlled precipitation as previously described [10].

Preparation of NiMOS

NiMOS were prepared using the “double emulsion like” technique. Briefly, FITC-labeled gelatin nanoparticles were suspended in distilled water and homogenized with PCL in dichloromethane using a Silverson lab mixture (Model L4RT-A, Silverson Lab Machines, Bucks, England) until formation of a stable emulsion like system. The resulting system was homogenized with poly(vinyl alcohol) solution in distilled deionized water. This dispersion was then magnetically stirred at room temperature to allow the dichloromethane to evaporate and harden the microspheres. The formed microspheres were collected by centrifugation, washed with distilled deionized water to remove PVA and lyophilized.

Particle size analysis

Freshly prepared suspension of FITC-labeled gelatin nanoparticles was analyzed for particle size and size distribution by light scattering method using 90Plus particle size analyzer, Brookhaven Corp. (Holtsville, NY). Particle size analysis was carried out at a scattering angle of 90^{o} and a temperature of 25^{o}C. Similarly, freshly prepared NiMOS were characterized for particle size and size distribution using

Multisizer™ 3, Beckman Coulter (Fullerton, CA). The instrument measures the change in the electrical resistance produced by non-conductive particle in the electrolyte solution. NiMOS were diluted with water and the diluted suspension was added to the glass beaker containing the standard electrolyte solution until a suitable concentration was obtained for particle size measurement. All measurements of particle size determination for NiMOS were carried out at room temperature.

Scanning electron microscopy (SEM)

Freeze-dried FITC-labeled gelatin nanoparticles and NiMOS were mounted separately on an aluminum sample mount and sputter coated with gold-palladium to minimize surface charging. The sputter coated samples were then observed for surface morphology under Hitachi S-4800 field emission scanning electron microscope (Pleasanton, CA).

Fluorescence microscopy studies

NiMOS prepared using FITC-labeled gelatin nanoparticles were centrifuged, freeze-dried, and re-dispersed in distilled deionized water to form a suspension. The suspension was then mounted on a glass slide and enclosed with a cover-slip. The particles were observed for fluorescence resulting from entrapment of FITC-labeled gelatin nanoparticles. Bright field and fluorescence microscopy images were acquired using Nikon TE-2000U scanning fluorescence confocal microscope (Melville, NY) and digital images were processed using Adobe Photoshop and Image-J software.

Data analysis

The effect of process parameters on the particle size of NiMOS was measured and expressed in micrometers (μm). Statistical comparisons were made with ANOVA. The level of significance was taken as $p < 0.05$.

RESULTS AND DISCUSSIONS

NiMOS encapsulating FITC labeled gelatin nanoparticles were formulated using "double emulsion like" technique. The formulation was optimized for particle size using a 3^3 full factorial design. FITC, used as a fluorescent maker, was covalently attached to gelatin in an alkaline buffer. The desolvation of gelatin with organic solvent is a commonly used method for the preparation of protein nanoparticles. The mean particle size of the precipitated nanoparticles, as measured with the particle size analyzer, was 100 nm (80–300 nm) with a narrow size distribution and was verified by SEM as seen in Figure 1.

Preliminary batches of NiMOS using double emulsion like technique were prepared to obtain discrete microspheres.

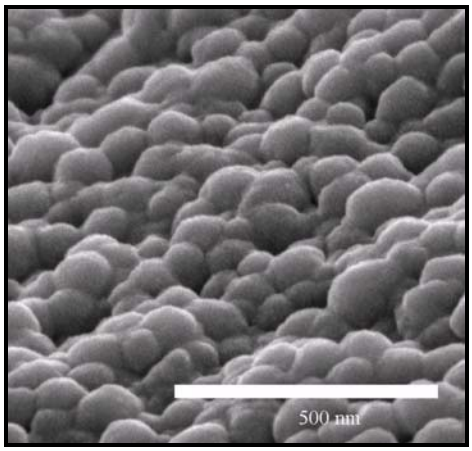

Figure 1. Scanning electron micrograph of fluorescein isothiocyanate-labeled gelatin nanoparticles.

At high polymer concentration, relatively large microspheres were produced; whereas, at high stirring speeds, increase in the amount of internal phase and low polymer concentration, relatively smaller microspheres were obtained. Three variables affecting the particle size of NiMOS were (1) concentration of PCL in the organic phase (X_1), (2) amount of nanoparticles forming the internal phase (X_2) and (3) speed of homogenization for formation of double emulsion (X_3). Using these three variables, 27 experiments were performed based on a 3^3 factorial design. Other independent process parameters such as volume of external phase and time of homogenization were kept constant during processing of NiMOS. All batches of NiMOS prepared with the proposed experimental design layout yielded free flowing microspheres.

The attribute or the response selected for observation based on the factorial design was particle size in micrometers of NiMOS. Particle size measurements for NiMOS were done using Multisizer™ 3 and the results corroborated well with the micrographs of NiMOS obtained from SEM analysis. Figure 2 shows the SEM image of representative NiMOS. Based on the results obtained from Coulter analysis, a quadratic statistical model, presented below, was generated by regression analysis in order to evaluate the response.

$$Y = 11.789 + 4.804X_1 - 0.648X_2 - 4.646X_3 + 0.562X_1^2 + 2.482X_2^2 + 1.543X_3^2 - 0.870X_1X_2 + 0.955X_2X_3 - 2.701X_1X_3 - 0.431X_1X_2X_3 \quad (1)$$

The particle size values for the 27 batches show a wide variation in response i.e., the response ranges from a minimum of 6.8 μm to a maximum of 31.6 μm. This data clearly indicates that the particle size value is strongly dependent on the selected variables. The close resemblance between the observed and the predicted response indicates the validity of the generated model.

A response surface plot generated using the quadratic equation is presented in Figure 3 to observe the responses in

particle size of NiMOS obtained from changing independent variables.

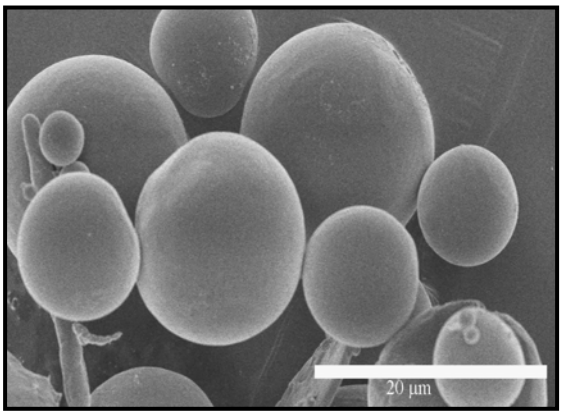

Figure 2. Representative scanning electron micrograph of NiMOS.

The variable X_3 was kept at a constant high value (9000 rpm) since it was observed that a high stirring speed was required for formulating NiMOS of relatively smaller size.

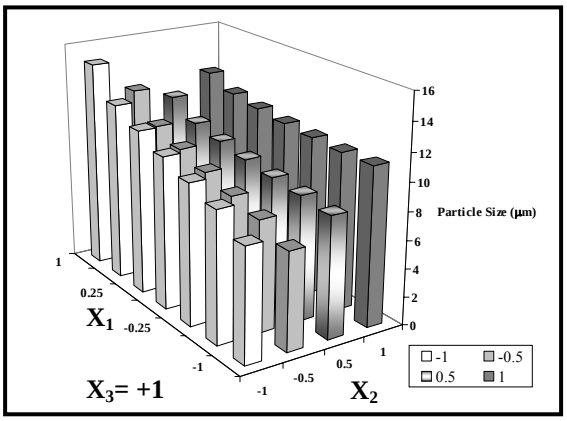

Figure 3. Response surface plot generated using the statistical model obtained from multiple regression analysis. This plot shows the effect of the selected parameters on the particle size of NiMOS keeping X_3 at a high level.

A large particle size obtained with high polymer concentration shows that as the polymer concentration is increased in a fixed organic phase it favors the formation of large particles. Similar result for PCL microparticles has been reported by other researchers [11, 12]. The speed of homogenization obviously had a negative effect on the particle size of NiMOS, with high speed of homogenization leading to the formation of the smaller particles. These results are in agreement with the general theory of microsphere formation. The amount of gelatin nanoparticles (X_2) as internal phase, however, had a negative effect on the particle size of NiMOS.

NiMOS were further observed under fluorescence microscope to check for the entrapment of FITC labeled gelatin nanoparticles. Confocal Z stacking images of NiMOS were obtained showing the presence of FITC labeled gelatin nanoparticles within the microsphere (data not shown). Fluorescent image along with the Z stacking images indicates that it is possible to entrap FITC-labeled gelatin nanoparticles in the PCL microspheres. Figure 4 shows bright field (a) and fluorescence (b) of NiMOS.

We were successful in formulating NiMOS less than 10 µm in size. Based on the two dimensional contour plot presented in Figure 5 processing parameters were chosen to formulate NiMOS less than 10 µm in size (X_1=3% w/v of polymer, X_2=31 mg of nanoparticles as internal phase and X_3=9000 rpm as speed of homogenization).

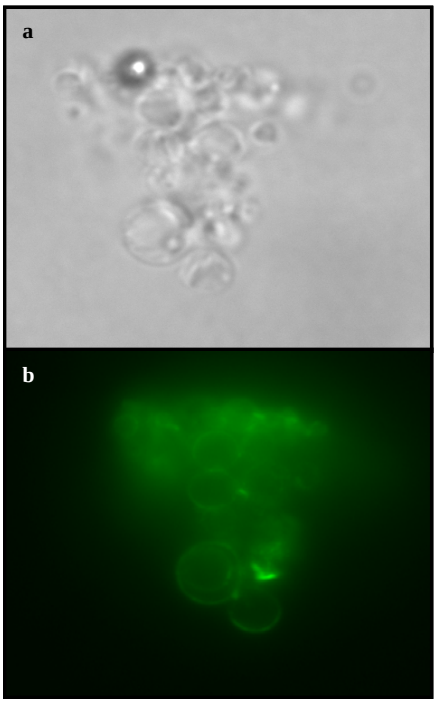

Figure 4. Bright field (a) and fluorescent (b) images of NiMOS showing entrapment of fluorescein isothiocyanate-labeled gelatin nanoparticles in poly(ε-caprolactone) microspheres.

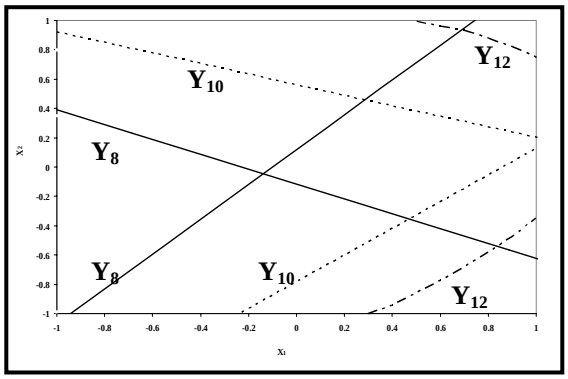

Figure 5. Two dimensional contour plot for Y_8, Y_{10} and Y_{12}.

A particle size of 8.0 μm was obtained for the check point based on the calculations using the model and the experimentally determined particle size value for the check point was found to be 9.5 μm. Figure 6 shows the SEM micrograph of the selected check point. These NiMOS could be used for delivery of therapeutic agents including a biological agent or immunogen for uptake by the M-cells of the Peyer's patches of the small intestine to generate mucosal immunity.

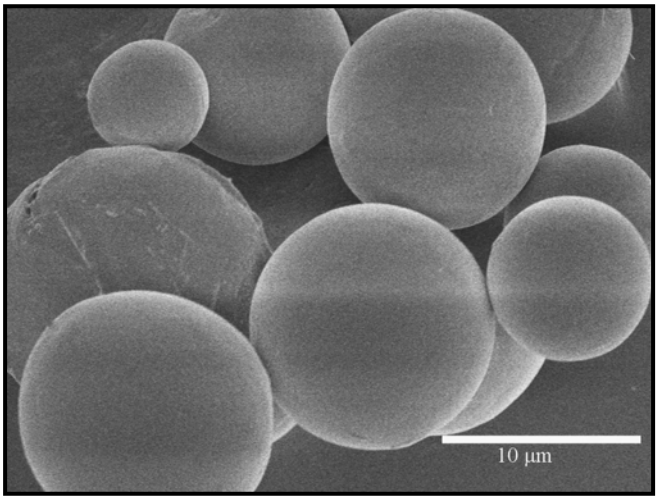

Figure 6. Scanning electron micrograph of NiMOS with less than 10 μm particle diameter.

The uptake of microparticles below the size of 10μm by the Peyer's patches of the small intestine has been reported by many researchers [13–15, 16, 17]. Further more, it has also been reported that the uptake of microparticles by the Peyer's patches increases with decreasing particle size [16].

CONCLUSION

We were successfully able to formulate NiMOS of less than 10 μm in diameter by double emulsion like technique using a 3^3 full factorial design. The multiple regression analysis of the obtained results led to a statistical model that describes adequately the influence of the selected variables at different levels on the responses under study in the present work. NiMOS of size less than 10 μm could be used for oral mucosal delivery of proteins, peptides and small drug molecules.

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