# Pulmonary Delivery of Proteins Using Nanocomposite Microcarriers

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*Received 7 May 2015; revised 15 July 2015; accepted 9 September 2015*

*Published online 27 October 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24681*

**ABSTRACT:** In this study, Taguchi design was used to determine optimal parameters for the preparation of bovine serum albumin (BSA)-loaded nanoparticles (NPs) using a biodegradable polymer poly(glycerol adipate-co-ω-pentadecalactone) (PGA-co-PDL). NPs were prepared, using BSA as a model protein, by the double emulsion evaporation process followed by spray-drying from leucine to form nanocomposite microparticles (NCMPs). The effect of various parameters on NP size and BSA loading were investigated and dendritic cell (DC) uptake and toxicity. NCMPs were examined for their morphology, yield, aerosolisation, *in vitro* release behaviour and BSA structure. NP size was mainly affected by the polymer mass used and a small particle size ≤500 nm was achieved. High BSA (43.67 ± 2.3 μg/mg) loading was influenced by BSA concentration. The spray-drying process produced NCMPs (50% yield) with a porous corrugated surface, aerodynamic diameter 1.46 ± 141 μm, fine particle dose 45.0 ± 4.7 μg and fine particle fraction 78.57 ± 0.1%, and a cumulative BSA release of 38.77 ± 3.0% after 48 h. The primary and secondary structures were maintained as shown by sodium dodecyl sulphate poly (acrylamide) gel electrophoresis and circular dichroism. Effective uptake of NPs was seen in DCs with >85% cell viability at 5 mg/mL concentration after 4 h. These results indicate the optimal process parameters for the preparation of protein-loaded PGA-co-PDL NCMPs suitable for inhalation. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:4386–4398, 2015
**Keywords:** nanoparticles; biodegradable polymers; polymeric drug delivery systems; particle size; pulmonary delivery; protein delivery and formulation; spray-drying

## INTRODUCTION

Pharmaceutical research has recently focussed on developing delivery systems for macromolecules such as proteins, peptides and antigens as they become the preferred therapeutics because of their greater selectivity, lower disruption of normal biological processes and reduced clinical development time with a shorter United States Food and Drug Administration approval period.[1] However, to reach a therapeutic level most of these macromolecules must be administered repeatedly in an invasive manner.[2] The pulmonary delivery of macromolecules is a viable alternative because of the attractive physiological properties of the lungs; the pulmonary epithelium is more permeable and lower enzymatic activity than the gut. In addition, it has a large surface area that is highly vascularised with thin epithelium in the alveolar lung tissue.[3] In addition, the pulmonary epithelium has many immunological properties.[2] Most organisms causing respiratory infections attack the host via the mucosal membrane. Consequently, the non-invasive pulmonary delivery of antigens can provide protection to mucosal membranes at the site of infection and potentially provides a first-line defense against invading microorganism.[4] The extensive dendritic cell (DC) networks that line the respiratory epithelium are considered an ideal target to initiate a strong immune response.[2] Moreover, pulmonary delivery reduces the risk of cross contamination due to the reuse of needles and syringes, and eliminates needle stick injuries, in both patients and medical personnel.[4]

Nanoparticles (NPs) are a useful delivery system for pulmonary macromolecules because of their potential for targetted drug delivery, sustained release and reduced dosing frequency, hence improving patient compliance and convenience.[5] They provide an additional advantage for antigen delivery systems, with studies suggesting NPs of about 500 nm or less were optimal for DCs uptake.[6,7] A most common method for the encapsulation of water-soluble drugs such as protein, peptides and antigens in biodegradable polymer-based NPs is the water-oil-water (w/o/w) double-emulsion solvent

**Abbreviations used:** BCA, bicinchoninic acid; BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; CD, circular dichroism; DMSO, dimethyl sulfoxide; DCM, dichloromethane; DCs, dendritic cells; DL, drug loading; DPIs, dry powder inhalers; EAP, external aqueous phase; FITC, fluorescein isothiocyanate; FPD, fine particle dose; FPF%, fine particle fraction percentage; HDL, high drug loading; IAP, internal aqueous phase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MEM, minimum essential medium; MMAD, mass median aerodynamic diameter; NCMPs, nanocomposite microparticles; NGI, next-generation impactor; NPs, nanoparticles; OP, organic phase; PBS, phosphate buffer saline; PGA-co-PDL, poly(glycerol adipate-co-ω-pentadecalactone); PLGA, poly(lactic-co-glycolic acid); PVA, poly(vinyl alcohol); SDS-PAGE, sodium dodecyl sulphate poly(acrylamide) gel electrophoresis; S/N, single-to-noise ratio; SPS, smaller particle size; SEM, scanning electron microscopy; TGA, thermogravimetric analysis; w/o/w, water-in-oil-in-water.

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Journal of Pharmaceutical Sciences, Vol. 104, 4386–4398 (2015)

evaporation method.[2] Biodegradable poly(glycerol adipate-co-ω-pentadecalactone, PGA-co-PDL) has previously been investigated as a novel delivery system of small molecule, for example, dexamethasone phosphate,[8] model drugs, for example, ibuprofen[9] and sodium fluorescein,[10] and macromolecules, for example, DNase I,[11] via dry powder inhalation (DPIs). However, particles smaller than 1 μm show poor lung deposition and are likely to be exhaled because of their low inertia, whereas particles larger than 5 μm cannot pass the oropharynx effectively.[12] Hence, to achieve particle deposition in the respirable region of the lung, the particles should be in the size range 1–5 μm. NPs can be formulated into microparticle carriers with an aerodynamic diameter between 1 and 5 μm to form nanocomposite microparticles (NCMPs) as DPIs by spray-drying.[13–16]

Spray-drying is a process where the formulation is presented as a feed solution, suspension or emulsion and converted into fine droplets, followed by exposure to rapid hot air-stream resulting in dry respirable-sized powders. In addition to the composition of the feed formulation, several operational parameters greatly affect the quality and quantity of the final formulation such as inlet temperature, air flow, aspiration capacity and feed rate.[17–20] Biocompatible excipients (carbohydrates, amino acids and lipids) are typically added to the formulation feed to afford dry powders with bulk and to promote the production of a desirable aerodynamic particle size which upon inhalation allows rapid release of NPs in the lung fluid.[10,13,17,21–23] In addition, the excipients can protect the NPs and encapsulated agents against the extreme spray-drying process conditions such as high temperatures and shear forces.[24] Delivering these microparticle carriers as DPIs via the pulmonary route offer many advantages such as not requiring trained medical personnel, eliminates cold-chain requirements and offers increased physical and chemical stability of macromolecules in comparison with liquid formulations.[4]

The Taguchi design is a useful method for studying a large number of parameters and interactions as it has the ability to optimise many parameters simultaneously and extract quantitative data from only a few experiments compared with the traditional factorial design.[25,26] For example, a single replicate of four parameters and three level experiments would require 81 runs for a full factorial analysis. However, using the Taguchi method will require only nine runs. The Taguchi approach has previously been used in the improvement of dosage forms.[25] Moreover, the Taguchi design concentrates on product robustness against uncontrollable (noise) factors. It employs a signal-to-noise (S/N) ratio to quantify variations. These ratios are meant to be used as measures of the effect of noise (uncontrollable) factors on performance characteristics. S/N ratios take into account both amount of variability in response data and closeness of average response to the target (1). In Taguchi design, S/N ratio can be defined as the measure of the deviation of the response from the desired value. So, 'signal' presents the mean value and 'noise' presents the SD. It means that lower variability in the process is ensured through maximising the S/N ratio (2). The variability of a characteristic is due to the noise factor such as environmental factors. Thus, optimising process parameters by the Taguchi design leads to bringing the average quality near to the target value, and also to simultaneously decrease the variation in quality (3). The experimental condition having the maximum S/N ratio is considered the optimum condition, as the variability of characteristics is in inverse proportion to the S/N ratio (4).

In this study, we have formulated PGA-co-PDL NPs encapsulating bovine serum albumin (BSA), a model protein, using Taguchi design to optimise NPs size and drug loading (DL) for effective uptake by DCs. The PGA-co-PDL NPs were then incorporated into L-leucine microparticle carriers via spray-drying using condition optimised using Taguchi design to produce NCMPs carriers suitable for pulmonary delivery via DPIs, maintaining BSA structure and activity.

## MATERIALS AND METHODS

### Materials

Poly(glycerol adipate-co-ω-pentadecalactone) (MW of 16.7 kDa) polymer was synthesised and characterised in our laboratory as previously described by Thompson et al.[27] BSA was obtained from (MW 67 kDa) Avenchem, UK. Poly(vinyl alcohol) (PVA; MW of 13–23 kDa, 87%–89% hydrolysed) was obtained from Clariant GmbH, Frankfurt, Germany. Dichloromethane (DCM) was purchased from BDH, Laboratory Supplies, Poole, UK. QuantiPro bicinchoninic acid (BCA) protein assay kit, L-leucine, phosphate buffer saline tablet (PBS; pH 7.4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium with L-glutamine and sodium hydrogen carbonate ($NaHCO_3$), tween 80, albumin tagged with fluorescein isothiocyanate (FITC-BSA) were purchased from Sigma–Aldrich, Gillingham, UK. Minimum essential medium (MEM) alpha-nucleosides were obtained from Gibco by Life Technologies, Paisley, UK. 75 $cm^2$/tissue culture flask (vented cap), 96-well flat bottom plates, acetone, antibiotic/antimyotic solution (100×), dimethyl sulfoxide (DMSO) and paraformaldehyde were purchased from Fisher Scientific, Loughborough,UK. CVS10D omniPAGE vertical gel electrophoresis system, proto-Gel stacking buffer, protein molecular weight markers in the range 10–220 kDa, protein loading buffer blue (2×) [0.5 M Tris–HCl (pH 6.8), 4.4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and bromphenol blue in distilled/deionised water], Tris–glycine–sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (10×) containing 0.25 M Tris base, 1.92 M glycine and 1% (w/v) SDS were purchased from Geneflow Limited, Lichfield, UK. 4-Nitrophenyl acetate esterase substrate (NPAES) was purchased from Sigma–Aldrich, Gillingham, UK. Heat inactivated foetal calf serum (FCS) was purchased from Biosera, Uckfield, UK. Adenocarcinomic human alveolar basal epithelial cell line, A 549 (CCL-185TM) and immature DCs; monocyte, mouse, JAWS II (CRL-11904™) were purchased from American Type Culture Collection (ATCC), Teddington, UK; 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) and Wheat Germ Agglutinin Texas RedR-X conjugate (WGA TR) were purchased from Invitrogen, Ltd., Paisley, UK.

### Experimental Design and NP Preparation

Taguchi design L36 orthogonal array was constructed through Minitab 16 Statistical Software® (Minitab Inc., Pennsylvania, USA). It was composed of eight variables set at two levels or three levels (Table 1). This design was used to identify the important parameters that would influence the NP size and BSA loading. A high signal-to-noise (S/N) ratio indicated the optimum conditions. The signal factor (S) was the outcome, that is, particle size or BSA loading and noise factors (N) included room temperature, humidity, experience of researcher and so on.