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A comparative study of a range of polymeric microspheres as potential carriers for the inhalation of proteins

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ABSTRACT

The aim of this study was to compare protein-loaded inhalable microparticles manufactured using a range of biocompatible polymers including hydroxypropyl cellulose (HPC), chitosan, hyaluronic acid, alginate, gelatin, ovalbumin and poly(lactide-co-glycolide) (PLGA). Spray-drying was used to prepare microparticles containing bovine serum albumin labeled with fluorescein isothiocyanate (BSA-FITC). Particles of respirable size and high protein loading were obtained. No evidence of BSA degradation was seen from PAGE analysis. The microparticles were mixed with mannitol as a carrier and powder aerosolization was assessed with a multi-dose dry powder inhaler (DPI) using a multi-stage cascade impactor. The mass median aerodynamic diameter (MMAD) ranged between 2.9 and 4.7 μm. Potential polymer toxicity in the lungs was compared by impinging the particles on Calu-3 monolayers and assessing the cytotoxicity, induction of cytokine release, changes in transepithelial permeability and electrical resistance. No toxic effects were observed with most of the polymers though some evidence of compromised cell monolayer integrity was seen for PLGA and ovalbumin. PLGA and gelatin microparticles caused a significant increase in IL-8 release. Of the polymers studied, PLGA showed the greatest toxicity. Certain polymers showed particular promise for specific protein delivery needs in the lungs, such as HPC to improve flow properties, sodium hyaluronate for controlled release, and chitosan and ovalbumin for systemic delivery.

1. Introduction

Inhalation therapy is a widely used and well-accepted treatment for many lung diseases. As a portal for non-invasive drug delivery, the pulmonary route is attractive. The lungs possess many favorable characteristics including a large absorptive surface area (100 m^2), extensive vasculature, thin alveolar epithelium (0.1–0.2 μm) and short distance of air–blood exchange passage (Patton and Platz, 1992). There has been particular interest in pulmonary delivery of macromolecules for both local and systemic delivery (Scheuch et al., 2006). The most notable being inhaled insulin. Exubera, a dry powder form of insulin was approved in early 2006 but was recently removed from the market (Mack, 2007). Inhalation of proteins/peptides is not without its difficulties. These can include protein denaturation during manufacture, aerosolization and storage, poor powder flowability resulting in inefficient dry powder delivery, rapid clearance from the lungs due to mucociliary clearance, enzymatic degradation and phagocytosis by alveolar macrophages (Boer et al., 2001).

Several approaches have been used to overcome these problems. One of the most versatile strategies is the bioengineering of inhalable particles using biocompatible polymers. Such polymers have been used to improve protein stability and enhance their absorption via the lungs (Sakagami et al., 2001), improve particle aerodynamic properties (Sham et al., 2004), achieve controlled/sustained release (Huang et al., 2002) or escape the natural phagocytic clearance in the lungs (Edwards et al., 1997). Many of these formulations have yet to be commercialized and this delay can be attributed to many factors including: a lack of comparative data on polymer influence on the aerodynamic properties of dry powders for inhalation, the lack of licensed excipients for inhalation and standardized biopharmaceutical testing methods for the pulmonary route, and concerns regarding the safety and clearance of these polymers from the lungs.

This study sought to compare a range of polymers including proteins, polyesters and water-soluble derivatives of proteins and sugars. These included sodium alginate, chitosan, gelatin, hydroxy propyl cellulose (HPC), poly(lactide-co-glycolide) (PLGA), ovalbu-

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min and sodium hyaluronate. Some of these have been investigated as potential carriers for the treatment of diseases such as tuberculosis (Suarez et al., 2001; Bivas-Benita et al., 2004; Zahoor et al., 2005), diabetes (Surendrakumar et al., 2003), asthma (Sakagami et al., 2002) and osteoporosis (Morimoto et al., 2000). Mucoadhesive polymers such as chitosan, sodium hyaluronate and HPC have been found to increase pulmonary absorption by delaying mucociliary clearance (Sakagami et al., 2001). Gelatin, albumin and PLGA have been found suitable for the targeting and controlled delivery of candidates such as salmon calcitonin, tetrandine and insulin to the lungs (Zeng et al., 1995; Edwards et al., 1997; Morimoto et al., 2000). The utility of such polymers as carriers has, therefore, been demonstrated in principle. However, little comparative data on the aerodynamic properties or toxicity and immunogenic potential of these polymers is available to allow formulation scientists to assess their usefulness for particular applications, e.g., local versus systemic delivery.

Inhalable protein-loaded microparticles of the selected polymers were manufactured by spray-drying (Okamoto et al., 2002). A comprehensive comparison of the physico-chemical properties of the microparticles was conducted including—size, morphology, protein loading and stability, protein release and aerodynamic characteristics. The immortalized human airway epithelial cell line, Calu-3 was used to compare the interaction of the polymeric microparticles with the airway epithelium (Grainger et al., 2006). When cultured at an air–liquid interface, they form mucus producing, well differentiated and polarized monolayers with tight junctions that generate significant transepithelial electrical resistance (Foster et al., 2000). A simple, yet reproducible system was developed to impinge the microparticles on to Calu-3 monolayers cultured on Transwell® inserts. Cellular response to particle insult was measured in terms of cell viability (Knebel et al., 2002), cytokine (e.g., IL-8) release (Abe et al., 2000), transepithelial resistance (Blank et al., 2006) and paracellular permeability (Robledo et al., 1999).

2. Materials and methods

2.1. Materials

Fraction V Bovine Serum Albumin (BSA), BSA-Fluorescein Isothiocyanate (BSA-FITC), low viscosity alginic acid sodium salt from *Macrocystis pyrifera*, low molecular weight chitosan, Gelatin (Type B from bovine skin), and albumin, grade II from chicken egg white were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Low viscosity hydroxy propyl cellulose (HPC-L) was a gift from Nippon Soda Co. Ltd., Japan. Sodium Hyaluronate was sourced from Shandong Freda Biochem Co. Ltd., China. PLGA RG 504H was supplied by Boehringer Ingelheim (Ingelheim, Germany). D-Mannitol (Mannidex®) was a gift from Cerestar, Belgium. All other solvents or chemicals were of HPLC or analytical grade. Calu-3 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cell culture media and supplements were purchased from Gibco BRL (Paisley, Scotland). Tissue culture plastics were from Sarstedt AG & Co. (Germany) and Transwell clear polyester inserts (12 mm diameter, pore size 0.4 μm) from Corning Costar (Corning, NY).

2.2. Spray-drying

BSA-FITC was dissolved in deionized water at 0.01% (w/v) and mixed with aqueous solutions (1%, w/v) of alginic acid sodium salt, gelatin, HPC-L, ovalbumin or sodium hyaluronate. Chitosan was dissolved in 0.1% (v/v) HPLC grade acetic acid. A protein:polymer ratio of 1:100 was used in all cases. In the case of PLGA microspheres, the protein was dissolved in deionized water and emulsified in the organic phase consisting of PLGA dissolved in dichloromethane. Tween 20, at a concentration of 0.1% (w/v), was used as the surfactant. The emulsion and above solutions were spray dried through a 0.5 mm nozzle using a Buchi® 190 spray dryer (Buchi, Flawil, Switzerland) with an inlet temperature between 45 and 140 °C and a feed flow rate of 4–5 ml/min. The recovered particles were stored in a desiccator at 4 °C until further use.

2.3. Determination of size, density and water content

The particle size of the microparticles was determined by laser diffraction (Malvern Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK) following suspension of the microparticles in isopropyl alcohol/ethanol and bath sonication for 1 min. Dry dispersion was employed for particles of HPC-L. Morphological assessment was performed using a Hitachi scanning electron microscope (Model S3500N) after mounting the preparations on studs and sputter-coating with gold (Polaron SC500 Gold Sputter Coater, Quotum technologies, Newhaven, UK). The density (ρ) of the microparticles was determined as the tapped density using a tap density tester (Copley Scientific, Nottingham, UK) (Fiegel et al., 2004). The equilibrium moisture content of the particles was assessed by using a Q-500 thermogravimetric analyzer (TGA) (TA Instruments, New Castle, DE) (Sethuraman and Hickey, 2002).

2.4. Determination of protein loading, stability and distribution

To determine the protein loading, microsphere samples equivalent to approximately 100 μg of protein were dissolved in 10 ml of deionized water and the protein content was analyzed by UV spectrophotometry, at 495 nm using a spectrophotometer (Biochrom, UK). In the case of chitosan microparticles, samples were dissolved in 0.1% acetic acid, precipitated with 0.4 M NaOH and then centrifuged to remove the polymer prior to analysis (Witschi and Mrsny, 1999). PLGA microspheres were disrupted using 2.5%SDS/0.2 M NaOH. The resulting mixture was neutralized to pH 7 by stepwise addition of 1 M HCl (Gupta et al., 1997). To eliminate interference from the reagents used for protein extraction, the samples (from both chitosan and PLGA) were filtered and analyzed for BSA content using reverse-phase HPLC (PerkinElmer, Model Series 200).The system was equipped with a Gemini C_{18} column (5 μm, 250 mm × 4.6 mm, Phenomenex, UK) and a UV detector (PerkinElmer, Model Series 200) using the following conditions: detection wavelength −220 nm; mobile phase A: 0.1% (v/v) trifluoroacetic acid (TFA) in water, mobile phase B: 0.08% (v/v) TFA in acetonitrile; flow rate: 1 ml/min (Kohn et al., 2007). All determinations were performed in triplicate.

The structural integrity of the encapsulated protein was assessed using Polyacrylamide Gel Electrophoresis (PAGE) (Igartua et al., 1998). BSA extracted from the microparticles was compared with native BSA and low molecular weight reference markers (29–205 kDa) (Sigma). Confocal Laser Scanning Microscopy (CLSM) was carried out using an LSM 510 Axio plan 2 upright confocal microscope (Carl Zeiss, Germany) at original magnification ×63 using immersion oil both on the objective and to wet the dry microparticles. A krypton argon laser provided excitation light at 488 nm.

2.5. Determination of aerosolization efficiency

An Anderson Cascade Impactor (Copley Ltd., Nottingham, UK) was used to determine the dispersibility and fine particle fraction (FPF) of each powder/carrier blend through a DPI according to the

manufacturer's instructions. D-Mannitol (Mannidex®, Cerestar, Belgium) was used as the carrier. It was sieved to give particles in the range 60–125 μm. It was then mixed with the microparticles at a ratio of 24:1 (mannitol: microparticles). To achieve uniform distribution, the blend was passed three times through a #125 μm sieve and further mixed in a glass mortar. Blend content uniformity were investigated by analyzing 50.0 ± 0.5 mg samples ($n = 5$) of each blend, according to the method described in Appendix XII H, Test B, Uniformity of Content of the British Pharmacopoeia (2004). Content uniformity for all blends gave a relative standard deviation of less than 5%. Each plate of the impactor was coated with a solution of Tween 80 in acetone (5%, w/v). Acetone was evaporated by placing the plates in an oven at 60 °C for 5 min. About 25 mg of the blend was manually loaded into the DPI and aerosolized by drawing air at a flow rate of 60 l/min for 4 s through the impactor. Ten such doses were discharged into the apparatus. The washings from each stage were assayed for the content of BSA-FITC using HPLC (as described previously). The emitted dose (ED) is the total protein mass exiting the inhaler. Uniformity of emitted dose was determined using Apparatus B (Dosage Unit Sampling Apparatus-DUSA) (USP, 2000). Values were deemed acceptable if they were within 75–125% of the label claim. The recovered dose (RD) was taken as the cumulative mass of protein recovered (inhaler + all stages of the impactor) after each experiment. The fine particle dose (FPD) was calculated as the quantity of protein recovered from Stage 2 to the filter. The fine particle fraction (FPF) is the FPD expressed as a percentage of ED. The experimental mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) of the particles were also calculated (USP, 2000). All experiments were carried out in triplicate.

2.6. In vitro protein release

A Franz diffusion Cell fitted with a 0.45 μm pore cellulose acetate membrane filter was used to monitor *in vitro* protein release (Leitner et al., 2004). The receptor compartment contained pH 7.4 phosphate buffer maintained at 37 °C ± 1 °C. Microparticles (~10 mg) were deposited on the membrane at the air–liquid interface established by the filter. At selected times, 250 μl of the buffer was sampled from the receptor compartment and the volume replaced. The quantity of BSA-FITC released was determined using UV spectrophotometry as mentioned before. The *in vitro* drug release data was fitted using the Korsmeyer–Peppas (KP) equation, $M_t/M_\infty = Kt^n$, where M_t and M_∞ corresponds to the amount of drug released at time t and ∞, respectively, k is the kinetic constant, t is the release time and n is the diffusional component for drug release.

2.7. Cell culture

Calu-3 cells, an adenocarcinoma cell line derived from a 25-year-old Caucasian male were purchased from the American Type Culture Collection (Rockville, MD, USA) at passage 14 and used between passages 20 and 50 (Grainger et al., 2006).

2.8. Cell viability

Calu-3 cells were plated at a density of 3×10^4/well in a 96-well format at 37 °C in 5% CO_2. After 24 h of culture, a suspension containing 2 mg of empty (unloaded) microparticles per ml of growth media was added to each well and incubated for either 4 or 6 h at 37 °C and 5% CO_2. Cellular viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (Gupta and Gupta, 2004).

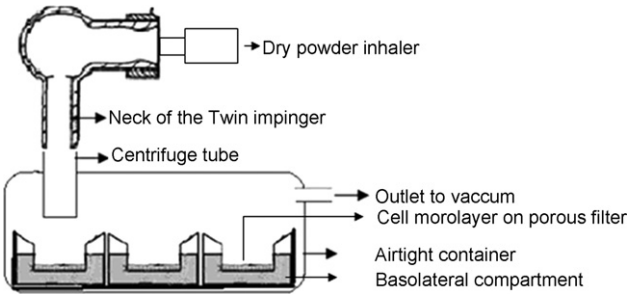

Fig. 1. Apparatus used for particle impingement on cell monolayers.

2.9. Microparticle application on Calu-3 monolayers

Calu-3 cells were seeded on to Transwell clear polyester inserts at a seeding density of 0.5×10^6 cells/cm^2. They were grown at 37 °C in a 90% humidified incubator with 5% CO_2. For cells cultured under LLI (liquid–liquid interphase) conditions, 500 μl of media was added to the apical chamber and 1500 μl to the basolateral chamber. For cells cultured at an ALI (air–liquid interface) condition, the apical culture medium was removed 1 day after seeding and the basolateral medium was reduced to 650 μl. For both culture conditions, the media was replaced once daily. Blank microparticles were impinged on the monolayers on the 12th day after seeding. In the case of LLI monolayers, the apical media was aspirated and the basolateral volume was reduced to 650 μl before impingement. ALI grown monolayers were used as such. For the application of microparticles, the multi-well plate with the monolayers was placed in an airtight container as shown in Fig. 1. 1 mg of the microparticles was weighed into the DPI. The vacuum pump was adjusted to deliver a constant suction. Filter inserts containing the monolayers were each placed in the first well under the tube. Microparticles were aerosolized onto the monolayers for 3 s. The inserts were then returned to their respective wells.

To obtain data on the reproducibility of particle deposition, preweighed Transwell® inserts containing no cells ($n = 5$) were slightly wetted with water and treated as described above for each polymer. After impingement, the inserts were left to dry and reweighed to determine the quantity deposited.

2.9.1. Fluorescein-sodium transport across monolayers

After particles were aerosolized onto the cell monolayers, the filter inserts were placed into new wells containing 1500 μl of bicarbonated Krebs–Ringer (KRB) solution in the basolateral compartment. 500 μl of a 50 μM sodium fluorescein (flu-Na) solution in KRB was added to the apical compartment of each well. 100 μl samples were taken at predetermined intervals up to 4 h from the basolateral compartment and replaced with an equal amount of fresh buffer. The fluorescence of flu-Na was measured in 96-well plates using a fluorescence plate reader (Wallac Victor, PerkinElmer, Cambridge, United Kingdom) at excitation and emission wavelengths of 488 and 530 nm, respectively. The apparent permeability coefficient values were calculated using the following equation:

$$P_{app} = \frac{1}{AC_0}\frac{dQ}{dt}$$

where Q, cumulative amount of Na-flu permeated across the cell monolayers over time t; A, surface area of transwells (1.12 cm^2); C_0, initial Na-flu concentration in the apical chamber.

2.9.2. Bioelectric measurements

Transepithelial electrical resistance (TEER) was measured with an EVOM® Epithelial Voltohmmeter device equipped with "chopstick electrodes" (World Precision Instruments, Sarasota, FL, USA).

Table 1
Size, density and water content of polymeric microparticles (mean ± S.D., $n = 3$)

Polymer	Geometric diameter (d) (μm)	Density (ρ) (g/cm^3)	d_{aer} (calculated) (μm)	Water content (%)
Chitosan	5.49 ± 0.32	0.28 ± 0.02	2.91	5.03 ± 0.17
Alginate	3.23 ± 0.25	0.22 ± 0.02	1.52	6.73 ± 0.09
PLGA	5.5 ± 0.41	0.11 ± 0.02	1.82	0.49 ± 0.01
Gelatin	2.88 ± 0.2	0.18 ± 0.02	1.22	4.84 ± 0.07
HPC-L	4.61 ± 0.16	0.09 ± 0.04	1.38	1.8 ± 0.08
Ovalbumin	4.29 ± 0.38	0.11 ± 0.05	1.42	10.47 ± 0.76
Sodium hyaluronate	3.61 ± 0.35	0.12 ± 0.02	1.25	3.22 ± 0.05

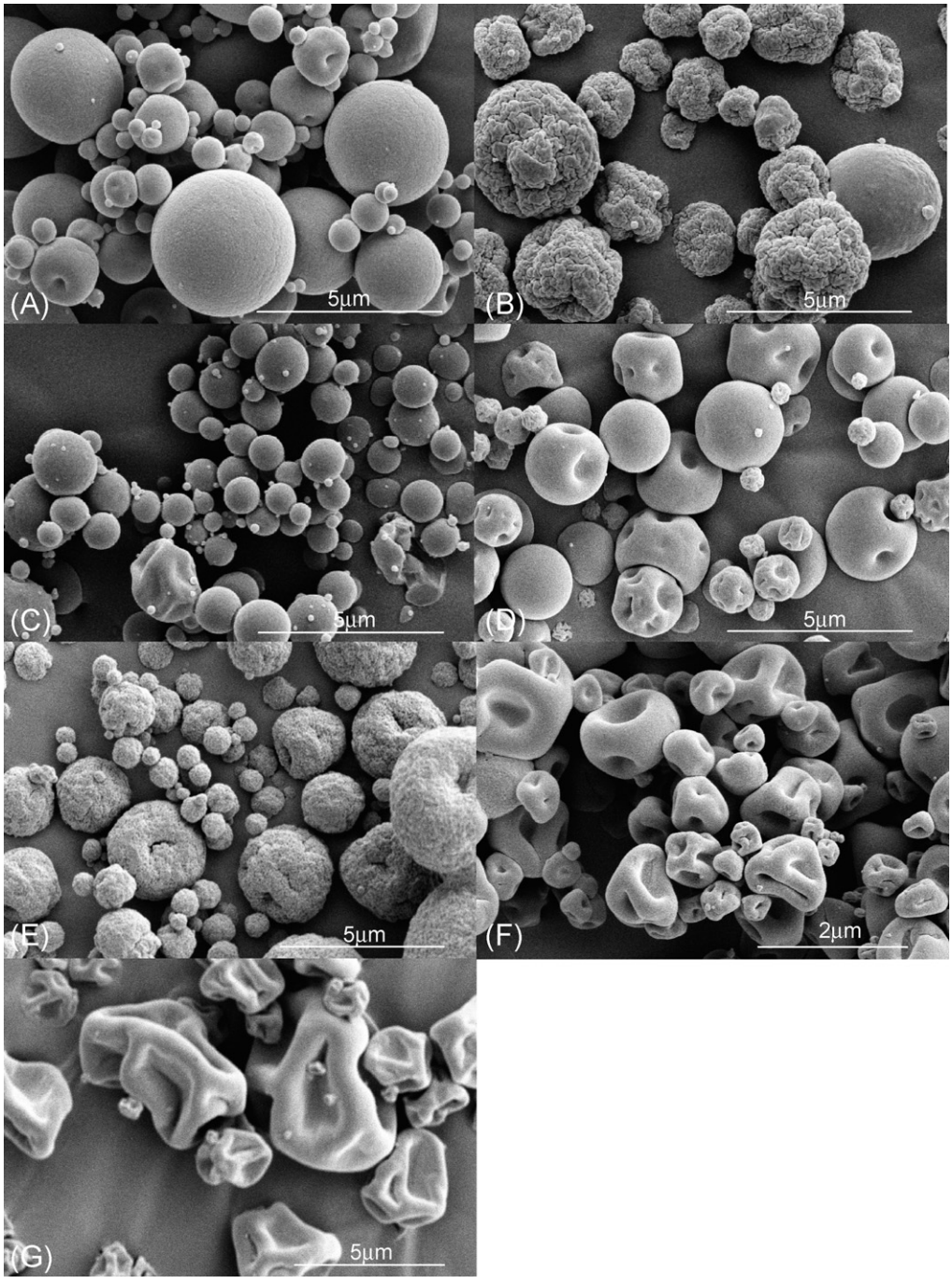

Fig. 2. Scanning electron micrographs of BSA loaded microparticles: alginate (A); chitosan (B); PLGA (C); gelatin (D); sodium hyaluronate (E); ovalbumin (F); HPC (G).

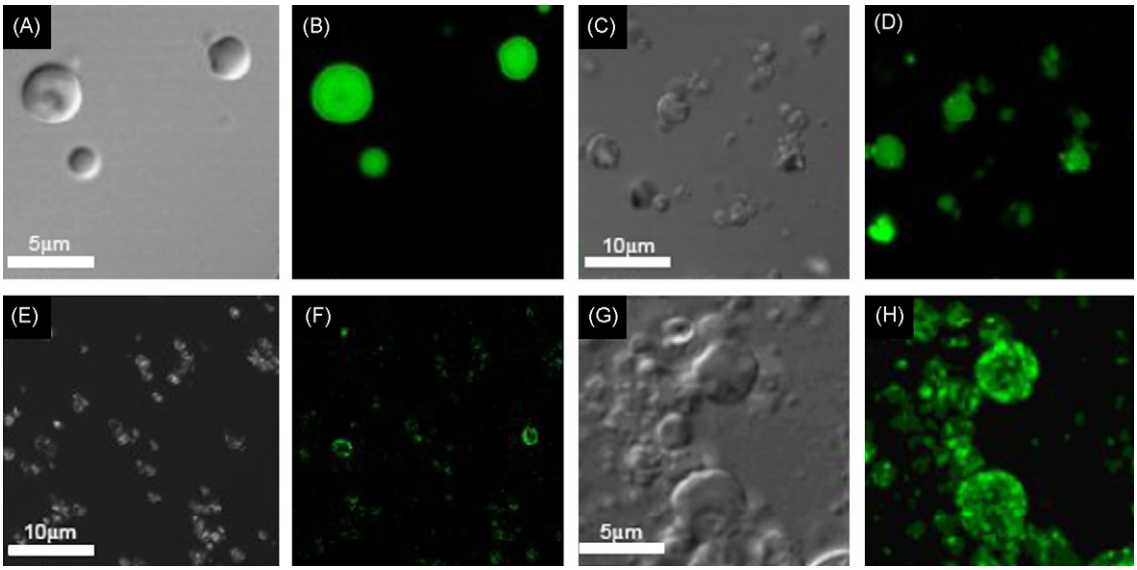

Fig. 3. Confocal laser scanning microscopy images of microparticles containing BSA-FITC (green), differential interference contrast image (grey); (A and B) alginate; (C and D) chitosan; (E and F) PLGA; (G and H) gelatin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Monolayers grown under LLI conditions were used directly. Monolayers grown under ALI conditions had 750 and 1500 μl of prewarmed media added to the apical and basolateral sides, respectively, and were allowed to equilibrate for 10 min in an incubator before the TEER was measured.

2.9.3. Cytokine release

Basolateral media collected after 4 h exposure to the microparticles were analyzed for IL-8 levels using ELISA MAX™ Kit (Biolegend, Inc., San Diego, CA, USA). The assay detection limit was 30 pg/ml. Collected media was diluted with the sample diluent and analyzed following manufacturers' protocols mentioned in the kit.

2.10. Statistical analysis

Results are expressed as mean ± S.D. One-way ANOVA was used to test for differences between treatments. In cases where the differences in the mean values were significant, *post hoc* pair wise comparisons were done using Neumann–Keuls multiple comparison tests. A p-value <0.05 was taken as significant.

3. Results

3.1. Size, density and water content

The particle size of the microspheres obtained after spray-drying was dependent on the concentration (polymer and protein) of the spray solution and the spray flow rate (amount of compressed air needed to spray the solution). These parameters were optimized to obtain particles in the respirable size range. Table 1 gives the density and geometric diameter in μm of the microparticles. The density was least in the case of HPC-L microspheres (0.09 ± 0.02 g/cm^3) and highest in the case of chitosan microspheres (0.28 ± 0.02 g/cm^3).

The theoretical aerodynamic diameter of individual particles, d_{aer}, is related to the geometric diameter d (assuming particle sphericity) by the following expression:

$$D_{aer} = d \times \sqrt{\frac{\rho}{\rho_1}}$$

where ρ is the particle density and ρ_1 is 1 g/cm^3 (Hinds, 1999). The d_{aer} was calculated for each (Table 1) and found to be in the range 1–3 μm.

The scanning electron micrographs (Fig. 2) show that the particles were smooth and spherical in the case of PLGA and alginate. Chitosan and sodium hyaluronate particles were spheroidal and corrugated. Ovalbumin and gelatin particles were toroidal and smooth. Particles made of HPC were crumpled. The water content (WC) from TGA analysis was least in the case of PLGA particles (0.49 ± 0.01%, w/w) and highest in the case of ovalbumin particles (10.47 ± 0.76%, w/w) (Table 1).

3.2. Protein loading, stability and distribution

The protein loading was determined as outlined in Section 2.4 and compared with the theoretical loading to calculate protein encapsulation efficiency (%EE). %EE was highest in the case of alginate microspheres (96.9 ± 0.55%) and least in the case of microspheres manufactured from chitosan (59.2 ± 0.78%). %EE for the other polymers was 62.6 ± 1.2% (PLGA), 95.8 ± 1.2% (gelatin), 88.9 ± 1.3% (HPC), 96.7 ± 1.4% (ovalbumin), and 89.2 ± 1.2% (sodium hyaluronate). The effect of spray-drying on the structural integrity of BSA was investigated using SDS-PAGE. No degraded protein fragments or covalent aggregates could be detected in all the formulations tested (data not shown).

CLSM can be used as a non-destructive visualization technique for microparticles containing a fluorescently labeled protein (Lamprecht et al., 2000). A homogeneous distribution of BSA-FITC can be seen within the loaded polymeric particles except in the case of gelatin and PLGA (Fig. 3). In the batches of PLGA and chitosan, empty microspheres were seen which may explain their relatively low %EE compared to the other polymers.

3.3. Aerosolization efficiency

Microparticles blended with mannitol were aerosolized at 60 l/min through a DPI using an Andersen cascade impactor. The deposition data is shown in Table 2. The recovered dose (RD) ranged between 83 and 95% of the loaded dose. The fine particle fraction (FPF) was highest in the case of HPC-L particles (26.1%) and

Table 2
Deposition in an anderson cascade impactor after aerosolization of mannitol blended microparticles from a DPI at 60 l/min (mean ± S.D., $n = 3$)

Polymer	FPF (%)[a]	MMAD[b]	GSD[c]
Chitosan	15.4 ± 5.1	3.9 ± 0.2	2.06 ± 0.1
Alginate	20.7 ± 6.1	4.1 ± 0.3	1.88 ± 0.07
PLGA	13.9 ± 4.3	2.9 ± 0.2	2.05 ± 0.1
Gelatin	18.9 ± 4.8	4.7 ± 0.4	1.92 ± 0.12
HPC-L	26.1 ± 3.9	3.3 ± 0.6	1.91 ± 0.05
Ovalbumin	11.9 ± 4.3	4.2 ± 0.3	1.85 ± 0.1
Sodium hyaluronate	19.9 ± 7.2	4.6 ± 0.2	1.89 ± 0.2

[a] FPF is the fine particle fraction.
[b] MMAD is the mass median aerodynamic diameter.
[c] GSD is the geometric standard deviation.

lowest with ovalbumin particles (11.9%). The other polymers produced FPF values between 14 and 21%. The MMAD values were larger than the theoretical d_{aer} (calculated in Section 3.1) and ranged between 2.9 and 4.7 μm possibly due to particle aggregation. Emitted dose uniformity for all formulations as tested using DUSA were in the acceptable range (80.9–91%). The values were 82.4 ± 7.5% (chitosan), 84.4 ± 6.1% (alginate), 85.5 ± 8.7% (PLGA), 80.9 ± 2.3% (gelatin), 91.1 ± 4.5% (HPC), 81.3 ± 3.2% (ovalbumin), and 80.2 ± 2.6% (sodium hyaluronate).

3.4. In vitro protein release

To determine the effect of polymers on protein release rates, studies were carried out as described in Section 2.6. Fig. 4 shows the cumulative BSA-FITC release profiles from the microparticles. At the end of 3 h, near complete release was seen with particles made of HPC and gelatin. Release from PLGA particles was negligible (~0.2%). For all other polymers, the release ranged between 17 and 43% after 3 h. Application of the Peppas model to determine release kinetics resulted in coefficient of determination (R^2) values between 0.93 and 0.99 for all polymers except PLGA. The diffusion component (n) ranged between 0.39 and 0.43 for alginate, chitosan, gelatin, ovalbumin and sodium hyaluronate. The value of n was 0.65 for HPC.

3.5. Polymer toxicity

3.5.1. MTT assay

For the MTT assay, a particle dose of 2 mg/ml was used based on the theoretical concentration of dry powder that would deposit in the central and intermediate lung after inhalation of a standard 40 mg dose (Newman and Busse, 2002; Widdicombe, 2002; Newhouse et al., 2003). Table 3 shows that after 4 or 6 h exposure to the microparticles, no significant difference ($p > 0.05$) in viability was observed between the cells treated with microparticles and the untreated control cells.

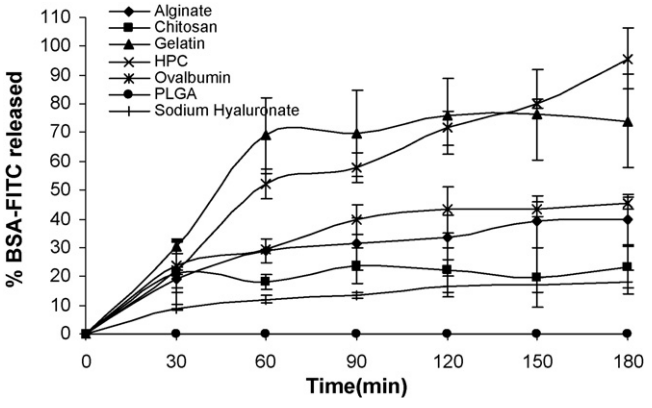

Fig. 4. Protein release profiles from spray-dried microparticles carried out using Franz diffusion cell (mean ± S.D., $n = 3$).

3.5.2. Bioelectric measurements

TEER measurements were used in order to assess the effect of the polymers on the tight junctional integrity of Calu-3 cell monolayers. From deposition reproducibility studies with blank filters, it was found that 576 ± 128 μg of microparticles was delivered on each insert (area of insert: 1.1 cm^2) using the apparatus shown in Fig. 1. The exposure dose used is approximately 250 times greater than what would be expected based on the ICRP66 model (ICRP, 1994). Prior to particle impingement, cells grown under LLI conditions exhibited a mean TEER value of 1870 ± 48 Ω cm^2 and those grown under ALI conditions exhibited a mean value of 774 ± 16 Ω cm^2. On removal of apical media from the LLI monolayers for particle impingement, a sharp drop in electrical resistance was observed which did not recover during the course of the study. Four hours post-treatment, a further drop in TEER was observed for monolayers treated with chitosan, gelatin, sodium hyaluronate and ovalbumin. Three days later, the TEER values for the treated cells had recovered to values between 450 and 650 Ω cm^2 for all polymers except PLGA and ovalbumin. A TEER value ≥450 Ω cm^2 is indicative of a tight Calu-3 monolayer (Sanjar and Matthews, 2001). In contrast, monolayers grown under ALI conditions showed no significant drop in TEER value after treatment except in the case of PLGA and ovalbumin microparticles (Fig. 5). After 3 days, however, TEER values returned to initial levels for all treated cells except those impinged with PLGA.

3.5.3. Transport study with sodium fluorescein

The transport of a paracellular marker, sodium fluorescein across Calu-3 cell monolayers, cultured under ALI and LLI conditions, was studied in the presence of impinged microparticles. Flux and P_{app} were calculated from a plot of the cumulative amount of Na-Flu transported as a function of time. Only PLGA and ovalbumin caused a significant increase in Na-Flu transport ($p < 0.05$) (Table 3). An increase in Na-Flu transport was also seen with chitosan for cells cultured under ALI conditions, although this was not significant.

3.5.4. Cytokine production

Upon stimulation by immunogenic airborne particulates, airway epithelial cells release cytokines such as Interleukin-8 (IL-8) which attracts and activates inflammatory cells in the airways and contributes to the pathogenesis of airway disorders. After 4 h of apical exposure to the polymeric microparticles, polarized Calu-3 cells secreted IL-8 at levels from 240 to 2100 pg/ml consistent with previous studies (Witschi and Mrsny, 1999). As shown in Table 3, IL-8 production was significantly increased ($p < 0.05$) from the control (air impinged) monolayers in the case of gelatin and PLGA.

Table 3
Toxicity and immunogenicity of microparticles in Calu-3 cells: P_{app} of fluorescein-sodium, cell viability and cytokine release

Treatment	P_{app} ($\times 10^{-7}$ cm/s) of fluorescein-sodium		% Cell viability[a]		IL-8 (pg/ml)[a]
	ALI	LLI	Exposure		
			4 h	6 h	
Air impinged	1.19 ± 0.12	0.85 ± 0.02	NA	NA	274 ± 33
HPC-L	1.38 ± 0.22	1.28 ± 0.27	101 ± 3	89 ± 5	382 ± 64
Alginate	1.61 ± 0.23	1.71 ± 0.28	91 ± 18	82 ± 12	352 ± 87
Chitosan	2.29 ± 0.02	1.28 ± 0.17	97 ± 2	95 ± 2	467 ± 108
Gelatin	1.61 ± 0.19	1.71 ± 0.09	83 ± 12	94 ± 15	1964 ± 219[b]
Sodium hyaluronate	1.66 ± 0.74	0.93 ± 0.13	85 ± 4	78 ± 7	417 ± 83
Ovalbumin	11.9 ± 2.89[b]	20.72 ± 1.88[b]	129 ± 23	113 ± 2	468 ± 51
PLGA	9.52 ± 3.29[b]	6.91 ± 1.15[b]	98 ± 2	97 ± 1	538 ± 108[b]

[a] Mean ± S.D. ($n = 4$).
[b] Significantly different compared to control ($p < 0.05$).

4. Discussion

Spray-drying produced microspheres with a protein encapsulation efficiency between 59 and 97%. The microspheres had a calculated aerodynamic diameter (d_{aer}) in the range 1–3 μm. Particles with a d_{aer} between 0.02 and 0.05 μm and 2–5 μm are capable of alveolar deposition (Hanes et al., 2003).

A homogenous protein distribution within all the polymeric particles except gelatin and PLGA (as seen by CLSM) is expected since the feed to the spray dryer is a neat solution of the protein and polymer dissolved in purified water. The punctate and non-uniform distribution of BSA within the gelatin microspheres produced from a neat solution could indicate a protein–protein interaction. In the case of PLGA particles where a w/o emulsion was used, BSA could be expected to accumulate at the water–solvent interface because of its surfactant properties. The fluorescent corona seen in this case is similar to those previously reported (Lamprecht et al., 2000).

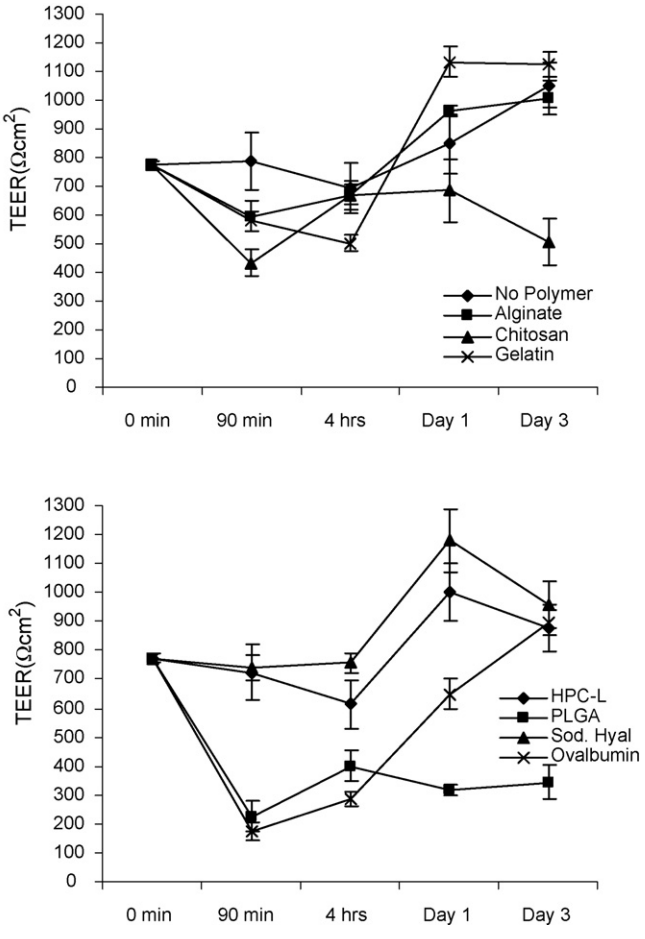

Fig. 5. Time course of change in TEER values in Calu-3 cell monolayers after exposure to polymeric microparticles under ALI conditions (mean ± S.D., $n = 4$).

When aerosolized alone, the forces generated within the DPI were insufficient to entrain the microparticles as a result of poor flow and particle aggregation. Entrainment was aided by the addition of a coarse carrier, mannitol (Chan et al., 1997). In addition to powder entrainment, one of the key factors that determines FPF is the ability of the inhaler to generate forces sufficient to deagglomerate the microparticles from the carrier. The FPF values for the polymeric microparticles in the present study were 12–26%. The degree to which the microparticles are released from mannitol during aerosolization is influenced by factors such as the size of the microparticles, their shape, surface roughness and moisture content. In the case of irregularly shaped particles or those with a rough surface, the area of contact is reduced. These particles are thought to experience reduced adhesive forces (Maugis, 1996). The SEMs indicated a polymer dependent difference in the shape and surface properties of the microparticles manufactured in this study. The collapsed, raisin-like shape of HPC particles coupled with a low residual moisture content could have facilitated deagglomeration resulting in a relatively high FPF (Table 2). The low FPF seen with ovalbumin particles could be due to particle aggregation as a result of high residual moisture. Incomplete powder deaggregation in the DPI could have also resulted in the experimental MMADs of the microparticles being 2–3 times larger than the theoretical aerodynamic diameter, d_{aer} (Bosquillon et al., 2001) as calculated in Table 1.

The amount of liquid available for the dissolution of a therapeutic aerosol in the lungs is very small (Patton, 1996). For protein release studies, open membrane systems have been employed previously as a representative technique (Witschi and Mrsny, 1999). A novel approach using Franz diffusion cells was used in this study (Franz, 1975). These cells are not suited for prolonged drug release studies because of the evaporation of release media from the lower compartment. This is a limitation of the device. No pharmacopeial methods are presently available to understand drug release from a formulation following its deposition in the lungs. The diffusion coefficient (n) values with the KP equation were less than ≤0.43 for alginate, chitosan, gelatin, ovalbumin and sodium hyaluronate particles indicating protein release by Fickian diffusion through water-filled pores in the polymer network (Peppas, 1985). Matrix swelling/erosion effects were not evident possibly due to the small volume of release media that seeps into the upper chamber. Polymer relaxation effects were seen, however, with HPC particles. The n value in this case was 0.65 indicating anomalous (non-Fickian) release kinetics. Drug release rates in the case of Fickian diffu-

sion is governed by factors such as polymer hydrophilicity, pore size of the polymer network and overall net charge carried by the protein/polymer. One or more of the above factors could have contributed to the differences in protein release rate observed between the polymers. The rapid release observed with gelatin could be due to the uncoiling of its triple helical structure when exposed to body temperatures. In the case of HPC, both diffusion and polymer erosion combined to give a high release rate. Sodium hyaluronate displayed the lowest release rate (excluding PLGA). This could indicate that it underwent hydration more rapidly than the other polymers. Hydration produces a gelatinous mass, the thickness of which then determines the diffusional path length for the protein. The longer the path length, the slower would be the rate of release (Sujja-areevath et al., 1998; Surendrakumar et al., 2003). Negligible protein release from PLGA particles could be attributed to its strongly hydrophobic nature resulting in poor wetting. Indeed the $t_{1/2}$ for hydrolysis of PLGA in the lungs is expected to be in the order of several months (Dunne et al., 2000). Chitosan and sodium hyaluronate appear best suited for sustained release of proteins/peptides in the lungs. Their mucoadhesive properties could also be exploited to reduce particle clearance by ciliary movement. Another important factor in sustained release to the lungs using microparticles is alveolar macrophage avoidance. Some strategies to reduce particle phagocytosis include coating with phospholipids, precoating with BSA and nonproteinaceous macromolecules (Cryan, 2005), and control of the particle size and density (Edwards et al., 1997).

The polymers considered in the present study have all received the G.R.A.S (Generally Regarded As Safe) status from the US FDA. However, the toxicity of a substance varies greatly with the route of administration and excipients considered safe for ingestion may not be safe for inhalation. Upon inhalation and release of the drug payload, the polymers and their degradation products must be nontoxic and non-immunogenic. It is believed that exposure of lung epithelial cells to foreign particulate matter can cause disruption of the epithelial tight junctions and formation of intercellular channels for the passage of molecules from airway lumen to blood. The MTT assay indicated no major cytotoxicity up to 6 h after exposure of the cells with the polymeric microparticles. Exposure of submerged cell monolayers to suspended particles is a traditional approach to study particle-cell interactions and their effect on monolayer integrity. To better mimic the *in vivo* situation of an air–liquid interface, a novel method was devised in this study to impinge dry microparticles directly on to Calu-3 cells grown on porous supports.

In spite of the extremely high particle exposure dose, after 72 h cytotoxicity was observed only with ovalbumin and PLGA (under LCC conditions) and only with PLGA (under ALI conditions). Consistent with the TEER observations, P_{app} values for sodium fluorescein were highest in the case of PLGA and ovalbumin (Table 3). Alteration of the barrier function could occur by a number of mechanisms including tyrosine phosphorylation or dislocation of junctional proteins (Atkinson and Rao, 2001).

A decrease in epithelial integrity on treatment with ovalbumin has previously been reported (Evans et al., 2002). In the present study, treatment of ALI cultured Calu-3 cells with ovalbumin microparticles caused only a transient disruption of the barrier properties and TEER recovered to pre-impingement values after 24 h. The increase in P_{app} for sodium fluorescein in the presence of chitosan (though not significant) for cells under ALI culture is consistent with previous studies showing the epithelial permeability enhancing effects of chitosan (Smith et al., 2004). The transient opening of epithelial tight junctions in the case of ovalbumin and chitosan could be exploited for absorption enhancement in systemic protein delivery via the lungs.

Production of the inflammatory cytokine, IL-8 was significantly increased ($p < 0.05$) in the case of PLGA and gelatin microparticles which is indicative of their immunogenicity. The highest secretion (1964 ± 219 pg/ml) was observed in the case of gelatin. This could be due to endotoxin contamination in the raw material used (Witschi and Mrsny, 1999).

5. Conclusion

While the concept of controlled and targeted delivery is well established for oral and parenteral use, its application for pulmonary drug delivery is a new area lacking fundamental data. Commercial formulations based on these technologies have yet to make it to market and this can be attributed in some part to the lack of comparable data of potential excipients, concerns regarding the safety and clearance of these polymeric carriers from the lungs and the dearth of standardized biopharmaceutical testing methods for the pulmonary route. In this paper, a comprehensive comparison of a range of protein-loaded polymeric particles for inhalation was conducted. The effects of the polymers on the delivery and release of proteins in the lungs and their biocompatibility was modelled using innovative methods. From our studies certain polymers would appear suited to meet different respiratory delivery needs, e.g., high delivery efficiency (HPC), low toxicity and controlled release (sodium hyaluronate and chitosan), and improved systemic delivery (ovalbumin and chitosan). Interestingly, one of the most commonly studied excipients for controlled release in the lungs, PLGA, had the greatest toxicity of the polymers studied.

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