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Investigation of a pMDI system containing chitosan microspheres and P134a

Robert O. Williams III [a,*], Melisa K. Barron [a], Maria José Alonso [b], Carmen Remuñán-López [b]

[a] *The University of Texas at Austin, College of Pharmacy, Pharmaceutics Division, Austin, TX 78712-1074, USA*
[b] *University of Santiago de Compostela, Faculty of Pharmacy, Department of Pharmacy and Pharmaceutical Technology, Campus Universitario Sur, 15706 Santiago de Compostela, Spain*

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Abstract

Microspheres made of chitosan, a biodegradable polymer, were investigated as a potential carrier for therapeutic proteins, peptides and plasmid DNA for administration to the lung from a pressurized metered dose inhaler (pMDI). Through the use of different cross-linking agents and additives, the physicochemical properties of chitosan microspheres were modified to improve compatibility in a pMDI delivery system. Their density, thermal properties, surface hydrophobicity, surface charge and free amino group content were determined before and after formulation in a pMDI system utilizing P134a. Also, the in vitro delivery characteristics of the pMDI systems were ascertained by cascade impaction. The densities of the non cross-linked and the glutaraldehyde cross-linked chitosan microspheres closely matched that of liquid P134a. An increase in the median particle size and the polydispersity after exposure to P134a was found for all types of chitosan microspheres tested except for those cross-linked with glutaraldehyde. This was due to the presence of water in P134a which hydrated and plasticized the chitosan microspheres causing aggregation during storage of the pMDI formulations. The change in the mass median aerodynamic diameter (MMAD) of the emitted dose of the pMDI systems reflected the influence of water on the particle size distribution of the chitosan microsphere pMDI suspension formulations. The pMDI systems studied produced respirable fractions (%RF) of 18% and multiple determinations of the dose delivery through-the-valve (DDV) of the pMDI systems were consistent. The surface hydrophobicity of the glutaraldehyde cross-linked chitosan microspheres was significantly greater than non cross-linked or tripolyphosphate (TPP) cross-linked chitosan microspheres. The addition of aluminum hydroxide ($Al(OH_3)$) to non cross-linked chitosan microspheres did not significantly influence the surface hydrophobicity. A decrease in the free surface amine content and the zeta potential after exposure to P134a was related to hydration and plasticization by water contained in the pMDI formulations. The non cross-linked and the glutaraldehyde cross-linked chitosan microspheres were found to be potential candidates for carrying biotherapeutic

* Corresponding author. Tel.: +1 512 4714681; fax: +1 512 4717474; e-mail: williro@mail.utexas.edu

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compounds to the lung via a pMDI system due to their compatability with P134a and their physicochemical characteristics.

Keywords: Cross-linking; Chitosan; Dose delivery through-the-valve; Mass median aerodynamic diameter; Microsphere; Pressurized metered dose inhaler; Surface hydrophobicity; Surface charge; Respirable fraction

1. Introduction

Due to the extensive surface area of the lung, and the potential for degradation of drugs administered by the oral route, the pulmonary route has become increasingly popular for the delivery of therapeutic proteins, peptides, plasmid DNA and oligonucleotides (Niven, 1993). Methods of administration to the respiratory tract include intratracheal instillation and inhalation of aqueous or dry powder aerosols (Canonico et al., 1994; Niven et al., 1995; Chan et al., 1997). Nebulizers and dry powder inhalers are currently the most successful lung delivery systems for biological molecules, however the propellant driven aerosol system is an alternative that has recently been proposed (Brown, 1996). Propellant based metered dose inhaler formulations are comprised of drugs that are either solubilized or dispersed in a propellant medium which is liquified under pressure. Suspension pressurized metered dose inhaler (pMDI) formulations may be stabilized by the addition of a surfactant.

Incorporation of a polymeric particulate carrier into a pMDI system may provide additional options for delivery of biotherapeutic compounds to the lung. A polymeric particulate carrier system offers controlled release characteristics as well as protection from chemical degradation of an entrapped molecule (Thanoo et al., 1992). Coadministration of protease inhibitors, absorption enhancers and endosomal releasing agents, which have been shown to enhance the bioavailability of biological compounds in the lung, may be accomplished by adsorbing biotherapeutic molecules to the surface of microspheres while entrapping the adjuvant component within the polymeric matrix (Calis et al., 1995; Hughes et al., 1996; Agerholm et al., 1994; Berthold et al., 1996a; Freeman and Niven, 1996). The biocompatable polymer, chitosan, is a good candidate for use in pulmonary delivery of biotherapeutic compounds. Chitosan is biodegradable, mucoadhesive and enhances the penetration of macromolecules across the intestinal epithelia and nasal mucosa (Lueßen et al., 1996; Illum et al., 1994). Polymeric particles comprised of chitosan have been reported to encapsulate many different compounds and to absorb proteins by passive diffusion (Jameela et al., 1994). Chitosan's polycationic property may facilitate adsorption of biotherapeutic compounds by binding anionic substrates through electrostatic interactions (Imai et al., 1991). Also, a variety of cross-linking agents can be used to modify the surface hydrophobicity of chitosan particle and thus adsorb therapeutic proteins and peptides through hydrophobic interactions. In addition, through cross-linking and the use of additives, the particle density may be modified and the release of an encapsulated agent may be controlled (Bodmeier et al., 1989; Thanoo et al., 1992; Berthold et al., 1996b). Processing techniques for the preparation of chitosan microspheres have been extensively developed since the 1980s. Four main approaches have been proposed: (a) ionotropic gelation with an oppositely charged polyelectrolyte, such as sodium TPP or alginate (b) simple or complex coacervation (c) spray-drying and (d) solvent evaporation. These techniques result in the production of chitosan particles in a wide range of particle size distributions including distributions that are appropriate for deposition in the lung by oral inhalation (Kas, 1997; Lorenzo-Lamosa and Remuñán-López, 1998). In this preformulation study, chitosan microspheres prepared with different cross-linking agents and additives were evaluated for density, susceptibility

to moisture, particle size and free amino group content before and after formulation in the hydrofluoroalkane propellant, P134a. The objective was to determine if chitosan microspheres were compatible with the propellant medium and that the characteristics of pMDI systems containing chitosan microspheres were appropriate for the delivery of biotherapeutic compounds to the peripheral region of the lung. Also the surface characteristics of chitosan microspheres were investigated since the hydrophobicity and charge of solid polymeric surfaces are properties important to adsorption of biotherapeutic molecules and to the interaction of the microspheres with lung tissue.

2. Materials and methods

2.1. Materials

Chitosan (Seacure© 123; degree of deacetylation $> 80\%$; M_w 50 kDa; Pronova Laboratories, Drammer, Norway) was used to prepare the microspheres. Fluorescein sodium (Sigma, Madrid, Spain) was entrapped in the chitosan microsphere matrix. The cross-linking agents pentasodium tripolyphosphate (TPP) and glutaraldehyde and the additive, aluminum hydroxide ($Al(OH_3)$) were used as received from Sigma. The propellant, 1,1,1,2-tetrafluoroethane (P134a; DuPont Chemicals, Wilmington, DE) was filtered through a drying filter (Sporlan Valve, Washington, MO) prior to incorporation into pMDI formulations. The analytical reagents, trinitrobenzene sulfonic acid (TNBSA), Rose Bengal dye, sodium tetraborate decahydrate and DL-valine were used as received from Sigma (St. Louis, MO). Purified water was obtained from a Milli-QUV Plus Filtration System (Millipore S.A., Molsheim, France).

2.2. Preparation of chitosan microspheres

Chitosan microspheres were produced by a spray-drying technique (Lorenzo-Lamosa and Remuñán-López, 1998). Polymer solutions (1% w/w; 1000 g) were prepared in 1.5 M acetic acid. Fluorescein sodium with and without a cross-linking agent (glutaraldehyde 25%, TPP 23% by weight of chitosan) or $Al(OH)_3$ (30% by weight of chitosan) were added to the chitosan solution and the mixtures were spray-dried from a 0.3 mm nozzle at a feed rate of 6.6 ml/min (Büchi© Mini Spray Dryer 190, Büchi Laboratoriums-Technik AG, Switzerland). The inlet and outlet temperatures were maintained at 142 ± 3°C and 84 ± 3°C, respectively. The air flow rate through the system was constant at 450 Nl/h. Chitosan microspheres of different compositions were collected, freeze-dried (-30°C, 24 h; Labconco, Kansas City, KA) and stored in a dessicator.

2.3. Chitosan microsphere density

An AccuPyc 1330 (Micromeritics, Norcross, GA) was used to measure the true density (the density of the material itself, exclusive of the voids and intraparticle pores larger than molecular or atomic dimensions in the crystal lattices) of the chitosan microspheres by helium pycnometry. The measurements were conducted in replicates of five.

2.4. Particle size distribution

A SALD-1100 Particle Size Analyzer (Shimadzu, Columbia, MD) was used to determine the particle size distribution of a suspension of chitosan microspheres in 100% anhydrous ethanol by laser light diffraction. The distribution was provided as the cumulative percent of particles undersized by number. The median diameter (M50) was determined at the 50th percentile of particles undersized. The polydispersity was given by a span index which was calculated by $(M90 - M50)/M50$ where M90 is the particle diameter determined at the 90th percentile of particles undersized. The particle size distribution was determined before and after storage in propellant P134a for 3 months at 25°C.

2.5. Scanning electron microscopy

A Jeol JSM 35C electron microscope (Joel, Peabody, MA) was used to obtain the scanning electron microscopy (SEM) photographs of gold-palladium sputter-coated samples of chitosan microspheres. SEM photographs of chitosan

microspheres were taken before and after storage in P134a.

2.6. Susceptibility to hydration

A Photovolt Aquatest 8 (Photovolt, Indianapolis, IN) was used to determine the water content of pMDI formulations of chitosan microspheres in P134a by Karl Fisher titration (Williams et al., 1997). Ten actuations from each chitosan microsphere pMDI formulation were emitted into the test chamber for analysis. The measurements were conducted in triplicate.

A DSC 2920 and Thermal Analyst 2000 software (TA Instruments, New Castle, DE) were used to evaluate the effect of hydration on the chitosan microspheres. A 2.5 mg aliquot of chitosan microspheres was incubated with 10 ml of purified water for 1 h. The hydrated chitosan microsphere samples and the chitosan microspheres recovered after formulating in P134a and storage were compared to the control chitosan microspheres which were not exposed to P134a. Differential scanning calorimetry (DSC) parameters such as glass transition temperature (T_g) and heat capacity (C_p), were controlled at 10°C/min from 20 to 200°C. Hermetically sealed aluminum containers were used and the measurements were conducted in triplicate.

2.7. Preparation of pMDI systems

A Pamasol P2005/P2008 pneumatic crimping and filling laboratory unit (Pamasol Willi Mäder AG, Pfäffikon, Switzerland) was used to prepare pMDI formulations by placing 5.0 or 35.0 mg of chitosan microspheres in epoxy lined aluminum cans (Cebal S.A., Bellegarde, France). The canisters were crimped with 50 μl metering chamber valves (DF10/50; Valois of America, Greenwich, CT) then filled with 10 g of P134a. The chitosan microspheres were readily dispersed in the liquefied P134a propellant. The chitosan microspheres were characterized prior to dispersing in P134a and after storage in P134a at 25°C and 60% RH for 3 months. Chitosan microspheres were recovered from storage in the P134a by puncturing a small hole in the pMDI canisters which were chilled to 4°C and allowing the P134a to evaporate slowly as the pMDI warmed to room temperature.

2.8. Fluorescein sodium content

A Hitachi F-2000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) was used to determine the amount of fluorescein sodium per milligram of chitosan microsphere. A 5.0 mg aliquot of chitosan microspheres was dispersed in 5.0 ml of 0.1 M borax buffer, pH = 8, and mixed for 60 h. The samples were filtered to remove the chitosan microspheres and the filtrate was analyzed for fluorescence emission at 513 nm. Standardization of fluorescein sodium in 0.1 M borax buffer produced a correlation coefficient of 1.0. Measurement of each sample of chitosan microsphere was conducted in triplicate.

2.9. Aerodynamic particle size distribution

An 8-stage cascade impactor (Andersen I ACFM Non-Viable 8-Stage Cascade Impactor with a USP Induction Port, Mark II, Graseby-Andersen, Smyrna, GA) was used to characterize the aerosol dose emitted from the chitosan microsphere pMDI formulations in terms of the mass median aerodynamic diameter (MMAD) and percent respirable fraction (%RF) which is the fraction by weight of the emitted aerosol that is less than 4.7 μm. Sixty actuations were collected for each cascade impaction measurement. Glass fiber filter paper (Graseby-Andersen, Smyrna, GA) was used as the collection substrate. 0.1 M borax buffer, pH = 8, was used to solubilize fluorescein sodium contained in the chitosan microspheres from the glass filter substrate. The mass of fluorescein sodium deposited onto each stage and in the induction port and actuator was evaluated by fluorescence spectrophotometry.

2.9.1. Dose delivery through-the-valve (DDV)

After priming by actuating three times into a waste container, three actuations from each pMDI formulation were collected in a dosage unit sampling tube (26.6 × 37.7 × 103.2 mm; 50 ml volume, Jade, Huntingdon Valley, PA). Subsequently, 15 ml of 0.1 M borax buffer was added to the dosage unit and the amount of drug emitted per actuation was

determined by fluorescence spectrophotometry. The measurement was conducted in triplicate.

2.9.2. Microsphere surface characteristics

A procedure used to determine the hydrophobicity of polystyrene microspheres was modified and applied to the chitosan microspheres prepared in this study (Müller et al., 1997). The surface hydrophobicity of chitosan microspheres was quantitated by determining the extent of adsorption of the hydrophobic dye, Rose Bengal. Increasing weights of chitosan microspheres (increasing surface area) were dispersed in a buffered solution of Rose Bengal (0.1 M Borax, pH = 8). After 2 h, the samples were filtered to remove the chitosan microspheres and the filtrate was collected for analysis. The amount of Rose Bengal adsorbed by the chitosan microspheres was quantitated by determining the difference in the initial amount and the amount remaining in solution by comparison to a standard curve of Rose Bengal obtained by UV absorbance at 548 nm ($r^2 = 0.9996$). The slopes of the straight lines obtained from a plot of Rose Bengal partition quotient (the ratio of the amount adsorbed and the initial amount) versus microsphere surface area were used to measure the degree of surface hydrophobicity; the greater the slope, the greater the relative hydrophobicity. Repeated analysis ($n = 3$) produced consistent slopes.

A Brookhaven ZetaPlus Zeta Potential and Particle Size Analyzer (Brookhaven Instruments, Holtsville, NY) was used to determine the zeta potential of dilute aqueous dispersions of glutaraldehyde cross-linked chitosan microspheres at pH 5.2, 7.0 and 9.0 prior to and following storage in P134a. The measurement was conducted in triplicate.

A procedure used to quantitate the free surface amino group content of bovine serum albumin microspheres was modified and used in this study (Andry et al., 1996). Since the TNBSA-amine complex forms in a 1:1 mole ratio, the free surface amino group content was determined by measuring the depletion of TNBSA in a dispersion of chitosan microspheres in 100% anhydrous ethanol. An excess of TNBSA reagent was used and the reaction was completed after 18 h. The samples were filtered to remove the chitosan microspheres and the filtrate was collected for analysis. The remaining, unbound TNBSA in the filtrate was determined by UV absorbance at 340 nm and compared to a standard prepared with DL-valine ($r^2 = 0.9854$). Analysis of four replicate samples was conducted.

2.9.3. Statistical analysis

The data were compared using one-way ANOVA where the results were judged to be significant based upon the 95% probability values ($p < 0.05$).

3. Results and discussion

3.1. Particle density

Formulation and stabilization of a suspension of chitosan microspheres carrying a drug in a liquefied non-polar propellant medium involves basic principles utilized in aqueous suspension systems. The velocity of sedimentation is expressed by Stokes' law: $v = 2r^2(\rho_s - \rho_o)g/9\eta_o$ where v is the sedimentation velocity; r is the radius of the dispersed particles; ρ_s is the density of the dispersed phase; ρ_o is the density of the dispersion medium; g is the gravitational force constant; and η_o is the intrinsic viscosity of the dispersion medium. The difference in the density of the dispersed phase, ρ_s, and the density of the dispersion medium, ρ_o, should be minimized to reduce the rate of sedimentation and produce an optimum suspension formulation (Martin, 1993). In a pMDI the dispersion medium is the liquified propellant. The hydrofluoroalkane propellant, P134a, used in this study has a liquid density of 1.21 g/ml at 25°C. The true density of chitosan microspheres was modified by using different cross-linking agents and by the addition of $Al(OH)_3$. The resulting true densities of the chitosan microspheres prepared in this study are listed in Table 1. The density of the non cross-linked chitosan microspheres was 1.48 g/ml. Cross-linking with glutaraldehyde produced a density of 1.42 g/ml which was not significantly different from the density of the non cross-linked chitosan microspheres ($p < 0.05$). However, cross-linking with TPP increased the density of the chitosan microspheres to 1.62 g/ml. Also, the

Table 1
Particle size and density of chitosan microspheres prior to and after storage in P134a for 3 months at 25°C and 60% RH

Chitosan microsphere description	True density (g/ml)	Particle size (μm)			
		Before storage		After storage	
		M50	Span index	M50	Span index
Non cross-linked	1.48 (0.009)[a]	3.8	1.3	1.3	2.1
TPP cross-linked	1.62 (0.005)	5.8	0.86	2.4	1.3
Glutaraldehyde cross-linked	1.42 (0.002)	3.4	1.8	4.0	1.5
$Al(OH)_3$ additive	1.54 (0.003)	3.7	1.4	9.0	2.7

[a] Standard deviations are given in parenthesis.

addition of $Al(OH)_3$ significantly increased the density of the chitosan microspheres to 1.54 g/ml. According to their density, both the non cross-linked and the glutaraldehyde cross-linked chitosan microspheres were the most suitable for suspending in P134a.

3.2. Particle size distribution

As indicated by Stokes' law, the particle size of a pMDI suspension formulation influences the dispersion and sedimentation properties of the suspension. The particle size of a pMDI suspension formulation will also influence the deposition of the emitted dose from a pMDI in the respiratory tract because the aerodynamic particle size distribution of the emitted dose from a pMDI is predominately determined by the particle size distribution of the suspended particles in the liquefied non-polar propellant medium (Byron, 1987). In a healthy state with normal breathing patterns, aerosol particles which range between 5 and 10 μm are preferentially deposited in the central airways. Aerosol particles which are less than 3 μm are deposited in the peripheral airways (Heyder et al., 1986). The chitosan microspheres were produced by a spray drying technique which was optimized to yield a particle size distribution that is appropriate for deposition in the peripheral region of the lung via a pMDI system (Lorenzo-Lamosa and Remuñán-López, 1998). The particle size distribution of the chitosan microspheres described in Table 1 shows that the M50 of the non cross-linked and glutaraldehyde cross-linked chitosan microspheres was 3.8 and 3.4 μm, respectively. The particle size of the non cross-linked chitosan microspheres was not significantly influenced by the addition of $Al(OH)_3$ ($p > 0.05$). Chitosan microspheres prepared with TPP as the cross-linking agent were significantly larger in median diameter (5.8 μm) but the distribution was less polydisperse as indicated by a span index of 0.86 ($p < 0.05$). As shown in Table 1, the particle size distribution of the non cross-linked and the TPP cross-linked chitosan microspheres after formulation and storage in P134a for 3 months at 25°C was increased about 4-fold, compared to the original diameter, to 13 and 24 μm, respectively. The particle size distribution of the chitosan microspheres prepared with glutaraldehyde as the cross-linking agent was not significantly affected by exposure to P134a ($p < 0.05$). SEM photographs of the chitosan microspheres after storage in P134a are shown in Fig. 1. It was observed that after storage, the non cross-linked microspheres were slightly fused together. No change in the physical appearance of the glutaraldehyde cross-linked chitosan microspheres was found from the SEM photographs. Fusion of the non cross-linked chitosan microspheres may be responsible for their increase in particle size distribution during storage in P134a for 3 months at 25°C and 60% RH.

3.3. Susceptibility to hydration

The presence of water is inherent to P134a even when an in-line desiccating filter is used during

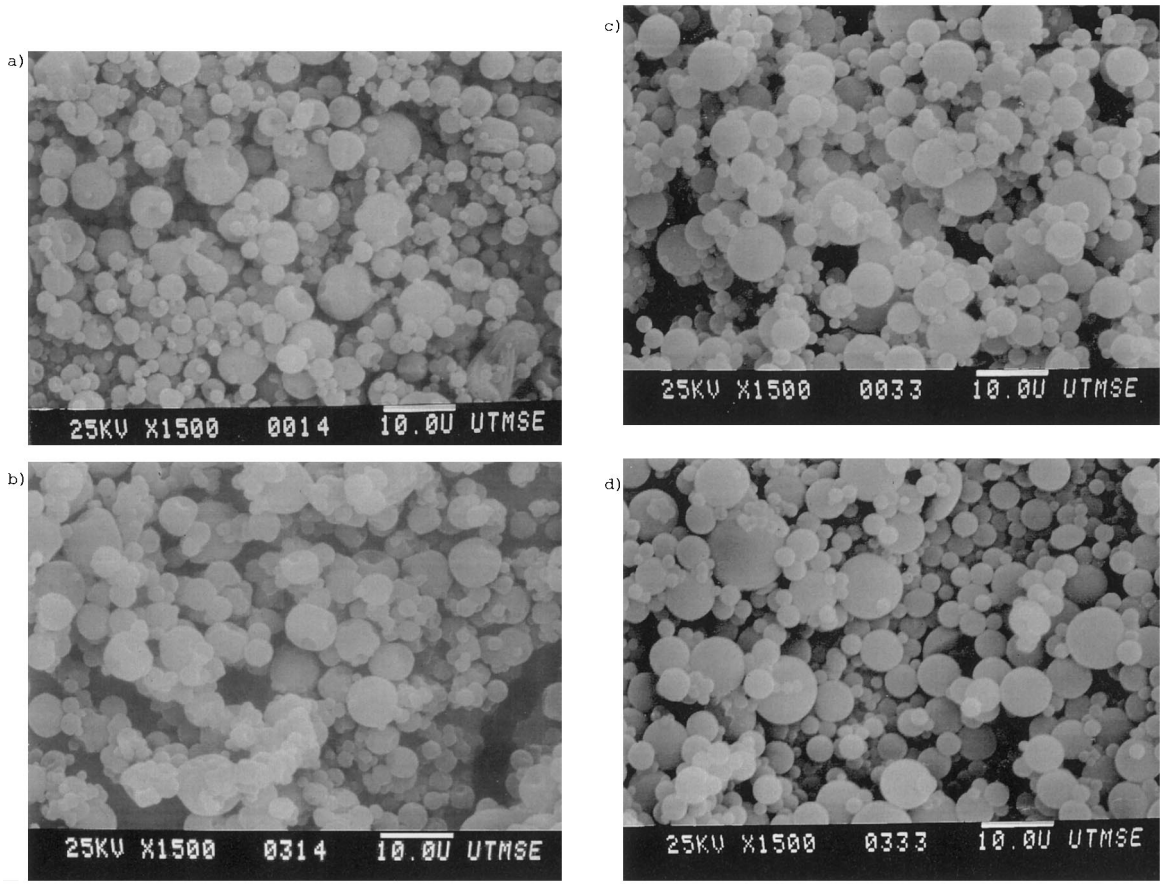

Fig. 1. SEM photographs of non cross-linked chitosan microspheres prior to (a) and after (b) storage in P134a for 3 months at 25°C, 60% RH and glutaraldehyde cross-linked microspheres prior to (c) and after (d) storage in P134a for 3 months at 25°C, 60% RH.

filling liquid P134a into sealed pMDI canisters. It has been demonstrated in our laboratory that the water content of P134a contributed the largest amount of water by weight in a model pMDI suspension formulation containing ethanol and micronized drug. The presence of minute amounts of water influence suspension stability and drug solubility and may have negative effects on the emitted dose of a pMDI (Williams et al., 1997). Therefore, the water content of the chitosan microsphere pMDI formulations was measured and is shown in Fig. 2. After storage for 3 months at 25°C and 60% RH, the water content of the pMDI formulations was approximately 30 μg/actuation or 400 ppm. It is known that chitosan films swell due to hydration in aqueous solutions. The water uptake and swelling capacity is related to the extent of cross-linking and the type of cross-linking agent where non cross-linked chitosan microspheres take up more water and swell to a greater extent than glutaraldehyde cross-linked chitosan microspheres. Changes in mechanical properties of polymer films are correlated to plasticization by the addition of small molecular weight compounds. Lower puncture strength and higher percent elongation values were found by exposure of chitosan films to water indicating that the water acts as a plasticizer (Remuñán-López and Bodmeier, 1997). Since plasticization causes polymeric materials to become soft and rubbery with a decreased internal viscosity and increased molecular mobility, the hydrated chi-

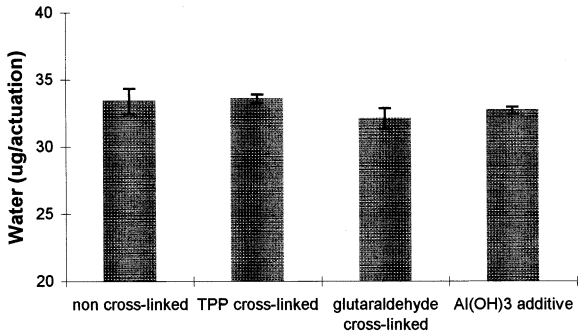

Fig. 2. The water content of chitosan microsphere pMDI formulations after storage in P134a for 3 months at 25°C, 60% RH.

tosan microspheres may fuse together much like film coating of surfaces with colloidal polymer dispersions (Bodmeier et al., 1994). In thermal calorimetry studies, the addition of a plasticizer to a polymer was shown to decrease the glass transition temperature (T_g) and increase the heat capacity (C_p) of the polymer (Hancock and Zografi, 1994). In the present study, the T_g was determined in order to investigate if the chitosan microspheres were hydrated and thus plasticized by the water contained in the P134a during storage. The thermal properties of chitosan microspheres, chitosan microspheres hydrated in purified water and chitosan microspheres recovered after formulation and storage in P134a are shown in Table 2. The thermographs show a significant decrease in the T_g of the non cross-linked chitosan microspheres after hydration in purified water from 68.9 to 50.0°C ($p < 0.05$). However the T_g of the glutaraldehyde cross-linked microspheres was increased from 48.4 to 68.3°C ($p < 0.05$). The C_p is the heat required to raise the temperature of 1 mole of a substance by 1 degree, and is also an indication of hydration of a polymer (Danjo et al., 1995). The C_p of both types of chitosan microspheres were significantly increased after hydration in purified water. The C_p of the non cross-linked chitosan microspheres was calculated using the Thermal Analyst 2000 software and was found to increase from 298.3 to 2570 J/g. The C_p of the glutaraldehyde cross-linked chitosan microspheres increased from 330.8 to 2011 J/g. Although both types of microspheres were hydrated after the addition of purified water, the thermal data indicated that water was a more effective plasticizer for the non cross-linked microspheres than for the glutaraldehyde cross-linked microspheres due to the decrease in T_g. Also in Table 2, the thermal properties of the chitosan microspheres hydrated in purified water were shown to be similar to the thermal properties of the chitosan microspheres recovered after storage in P134a for 3 months at 25°C and 60% RH. The T_g of the non cross-linked chitosan microspheres decreased from 68.9 to 48.9°C ($p < 0.05$). Although not statistically significant, the T_g of the glutaraldehyde cross-linked chitosan microspheres was increased from 48.4 to 51.1°C ($p > 0.05$). The C_p of both types of chitosan microspheres was found to also be influenced by exposure to P134a at 25°C for 3 months. The C_p of non cross-linked chitosan microspheres recovered after storage in P134a was increased from 298.3 to 341.3 J/g. The C_p of glutaraldehyde cross-linked chitosan microspheres recovered after exposure to P134a was decreased from 330.8 to 238.2 J/g. The decrease in T_g and increase in C_p indicated that hydration, subsequent swelling and plasticization of the non cross-linked chitosan microspheres by water

Table 2
Thermal properties of chitosan microspheres before dispersion and storage in P134a, hydrated in water and after dispersion and storage in P134a for 3 months at 25°C and 60% RH

DCS parameters	Non cross-linked chitosan microspheres			Glutaraldehyde cross-linked chitosan microspheres		
	Before storage	Hydrated	After storage	Before storage	Hydrated	After storage
T_g (°C)	68.9 (1.39)[a]	50.0 (1.08)	48.9 (0.580)	48.4 (1.45)	68.3 (1.33)	51.1 (1.52)
C_p (J/g)	298.3 (6.940)	2570 (14.50)	341.3 (2.669)	330.8 (4.306)	2011 (18.33)	238.2 (6.023)

[a] Standard deviations are given in parenthesis.

Table 3
The aerodynamic particle size distribution and DDV of chitosan microsphere pMDI suspension formulations

pMDI formulation description	MMAD (μm)[a]	GSD[b]	RF (%)[c]	DDV (μg/actuation)[d]
Non cross-linked	5.08 (0.36)[e]	5.15	18.5 (2.41)	5.90 (0.216)
Glutaraldehyde cross-linked	2.46 (0.40)	3.89	18.1 (1.33)	0.848 (0.002)

[a] Mass median aerodynamic diameter.
[b] Geometric standard deviation.
[c] Respirable fraction.
[d] Dose delivery through-the-valve.
[e] Standard deviations are given in parenthesis.

present in the P134a, was responsible for their increased particle size distribution found after storage. Since the magnitude of change in the thermal properties of a polymer is dependent upon the amount and the effectiveness of the plasticizer, the T_g and C_p determined for the glutaraldehyde cross-linked microspheres after storage in P134a may indicate that water is an antiplasticizer for this type of polymeric matrix (Guo, 1993). Hence, the particle size distribution of the glutaraldehyde cross-linked microspheres was not significantly changed after formulating and storage in P134a because they were not as susceptible to swelling due to hydration and plasticization by water.

3.4. Aerodynamic particle size distribution and DDV

Chitosan microsphere suspension pMDI formulations were prepared with non cross-linked and glutaraldehyde cross-linked microspheres because of their optimal densities and particle size distributions. After storage for 3 months at 25°C and 60% RH, the aerodynamic particle size distribution produced by the pMDI systems were determined and are described in Table 3. The MMAD of the glutaraldehyde cross-linked chitosan microsphere pMDI system was 2.46 mm. An MMAD ≤ 3 μm is optimum for deposition in the peripheral region of the lung (Heyder et al., 1986). Even though the M50 of the non cross-linked chitosan microspheres recovered after formulation and storage in P134a was found to be 13 μm, the MMAD of the aerosol emitted from the pMDI was found to be 5.08 μm. This was because the measurement of the aerodynamic particle size distribution determined by cascade impaction which is an in vitro model of the oropharynx, bronchial and alveolar regions of the respiratory tract, excludes particles which are greater than 10 μm. In cascade impaction those particles are impacted in the induction port of the apparatus which corresponds to the oropharynx region in vivo and are not deposited in the lung. Table 3 also shows that the aerodynamic particle size distributions of both pMDI formulations were polydisperse as indicated by the magnitude of their geometric standard deviations (GSD) greater than 1.22 (Moren et al., 1993). Since the particle size distributions of the suspended chitosan microspheres were polydisperse it is not surprising that the aerodynamic particle size distributions of the emitted aerosols were polydisperse. Interestingly, the %RF, which is the portion of the emitted aerosol having a particle size less than 4.7 μm, however, was not affected by the difference in MMAD of the pMDI formulations. The %RF of the non cross-linked and the glutaraldehyde cross-linked pMDI formulations was 18.5 and 18.1%, respectively. The %RF of the chitosan microsphere pMDI suspension formulations fall within the range of %RFs produced by model suspension pMDI systems (Williams et al., 1997). The DDV of the chitosan microsphere pMDI formulations is also shown in Table 3. The DDV is a measure of the concentration of drug which is emitted per actuation from the valve of the pMDI. Multiple determinations produced low standard deviations of the DDV for both the non cross-linked and glutaraldehyde cross-linked chitosan microsphere pMDI formulations. By preparing the chitosan microsphere

pMDI formulations in clear glass vials it was observed that the chitosan microspheres were well dispersed in the propellant medium and were easily resuspended after a long period of sedimentation. The highly consistent DDVs produced may be a result of their favorable dispersion and sedimentation characteristics in P134a. The higher DDV for the non cross-linked chitosan microsphere pMDI was due to their increased encapsulation efficiency compared to the glutaraldehyde cross-linked chitosan microspheres. 27.73 μg of fluorescein and 3.517 μg of fluorescein was found to be encapsulated per mg of non cross-linked and glutaraldehyde cross-linked chitosan microspheres, respectively. Therefore, the %RF found for the glutaraldehyde cross-linked chitosan microsphere pMDI would correspond to a much lower dose of an encapsulated or surface adsorbed drug and would require inhalation of a higher mass of loaded microspheres to be equivalent to the non cross-linked chitosan microsphere pMDI formulation.

3.5. Particle surface characteristics

Dispersion and suspension characteristics as well as surface interactions of biotherapeutic compounds and tissue components, are influenced by the surface properties of polymeric microspheres (Müller et al., 1997). Hydrophobic interactions and electrostatic interactions are the primary mechanisms of adsorption of therapeutic proteins and nucleic acids to solid surfaces (Mientus and Knippel, 1995). Adsorption of biological molecules to the surface of chitosan particles may be utilized to overcome the low loading efficiencies and to avoid irreversible denaturation and low biological activity resulting from encapsulation and cross-linking processes (Calis et al., 1995). Chitosan particles have been shown to adsorb drugs such as prednisolone sodium phosphate, trypsin and lipoproteins through electrostatic interactions (Berthold et al., 1996a; Shi et al., 1996). The role of hydrophobic interactions was represented when lipophilic steroids were found to adsorb to chitosan microspheres in lower amounts than their hydrophilic derivatives (Berthold et al., 1996a). Since electrostatic interaction is the primary mechanism for the adsorption of anionic substances to the positively charged chitosan surface (Imai et al., 1991) it may be the primary mode of interaction with polyanionic substances such as plasmid DNA and oligonucleotides (Roth and Lenhoff, 1993). Although the structure of proteins and peptides vary, they usually carry a positive or neutral charge which would repel the positively charged chitosan particle surface. Also, the negative charge of a protein or peptide is usually internalized and inaccessible for ionic bonding (Lee and Kim, 1974). Therefore adsorption of therapeutic proteins and peptides to polymeric surfaces may be primarily attained through hydrophobic interactions (van de Steeg et al., 1992). Since the surface hydrophobicity and surface charge of chitosan microspheres may be manipulated through the use of different cross-linking agents, the surface characteristics of chitosan microspheres may be tailored to facilitate the predominate mode of interaction between the drug molecule and the microsphere surface. In addition, dispersion and sedimentation characteristics of chitosan microsphere suspensions in non-polar propellant media may be optimized by modifying their surface hydrophobicity and surface charge.

3.6. Surface charge

The surface charge of the glutaraldehyde cross-linked chitosan microspheres, which is related to the number of free surface amino groups, is presented in Fig. 3. The zeta potential is a measure of charge per unit surface area and was found to increase as the pH of the aqueous suspension medium was decreased. At lower pH values a greater fraction of amino groups on the microsphere surface was protonated to form alkylaminium ions which carry a positive charge in aqueous solution (Solomons, 1988). The surface charge of the glutaraldehyde cross-linked microspheres was shown to decrease after formulation and storage in P134a at 25°C for 3 months. However the decrease in zeta potential was probably due to the increase in surface area of the glutaraldehyde cross-linked chitosan microspheres after exposure to P134a for 3 months at 25°C.

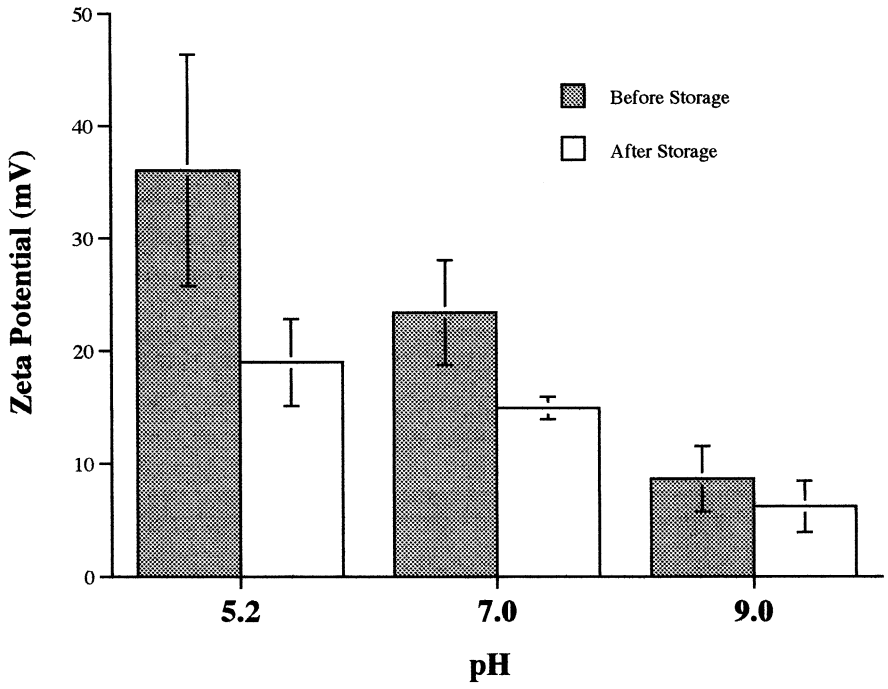

Fig. 3. The zeta potential of glutaraldehyde cross-linked chitosan microspheres at various pH before and after storage in P134a for 3 months at 25°C, 60% RH.

The data presented in Table 1 indicate that the median particle diameter of the glutaraldehyde cross-linked microspheres increased from 3.4 to 4.0 μm during storage in P134a. This increase in particle diameter corresponds to a 15% increase in surface area calculated by the equation for the surface area of a sphere ($A = 4\pi r^2$) and may account for the slight decrease in the zeta potential of the glutaraldehyde cross-linked chitosan microspheres recovered after formulation and storage in P134a. The charged surface of the chitosan microspheres may be beneficial for the stability and dosing reproducibility of the pMDI formulations. By inducing a repulsive particle-to-particle ionic interaction, the positive surface potential of chitosan microspheres may stabilize the pMDI suspension (Martin, 1993). The repulsive particle-to-particle ionic interaction may be partly responsible for the well dispersed characteristic of the pMDI formulations and enabled the chitosan microspheres to be easily resuspended after a long period of sedimentation.

3.7. Degree of cross-linking

The extent of cross-linking determines the number of free amino groups which are responsible for the positively charged chitosan microsphere surface. Minimizing the extent of cross-linking will increase the positive potential of the chitosan surface and enhance electrostatic interactions with anionic adsorbates. Cross-linking may also stabilize the suspension of chitosan microspheres by reducing the effect of hydration and thereby decrease the swelling capacity so that the particle size is maintained (Remuñán-López and Bodmeier, 1997). The extent of cross-linking of chitosan microspheres was evaluated by determining the total number of free surface amino groups. Table 4 describes the quantification of the free surface amino groups prior to and after exposure to P134a for 3 months at 25°C and 60% RH. The non cross-linked chitosan microspheres were found to have about 20% more free surface amine groups than the TPP or glutaraldehyde cross-linked microspheres. The amount of free surface

amine for the TPP cross-linked and glutaraldehyde cross-linked microspheres was 2.410 and 2.597 μmol, respectively. Therefore, cross-linking with either TPP or glutaraldehyde was equally effective under the processing conditions employed to prepare the chitosan microspheres. A decreasing trend in the free surface amino group content of the chitosan microspheres was found after storage in P134a for 3 months at 25°C and 60% RH. However, it is well documented that chitosan matrices absorb moisture and the amount of absorbed water is quantitated by gravimetric techniques (Remuñán-López and Bodmeier, 1997). Since 5.0 mg aliquots of chitosan microspheres were analyzed prior to storage and after storage, the mass of chitosan microspheres after storage would be diluted due to the absorption of water contained in the propellant. The decrease in the number of free surface amino groups was probably due to an increase in mass of the chitosan microspheres caused by hydration during storage in P134a. Therefore the chemical composition of the microsphere surface was not affected by exposure to P134a for 3 months at 25°C.

3.8. Surface hydrophobicity

The choice of cross-linking agent and the extent of cross-linking may influence the surface charge of chitosan microspheres as well as the surface hydrophobicity. The surface hydrophobicity may be optimized to facilitate adsorption of biological molecules through hydrophobic interactions to influence uptake and absorption of the polymeric particles by lung tissue (Müller et al., 1997). The use of a hydrophobic cross-linking agent such as glutaraldehyde may produce a more hydrophobic surface than a hydrophilic cross-linking agent such as TPP. The relative surface hydrophobicity of the chitosan microspheres is shown in Fig. 4. It was found that the type of cross-linking agent employed affected the surface hydrophobicity of the chitosan microspheres. As indicated by an increase in the slope of the Rose Bengal dye partition quotient versus the microsphere surface area, cross-linking with TPP increased the hydrophobicity of the microsphere surface compared to non cross-linked chitosan microspheres. The slopes of the linear plots of non cross-linked and TPP cross-linked chitosan microspheres was 0.028 and 0.040 ml/m^2, respectively. A greater increase in surface hydrophobicity was found by cross-linking with glutaraldehyde which produced an increase in the corresponding slopes of the linear plots from 0.028 to 0.157 ml/m^2. The addition of $Al(OH)_3$ to the non cross-linked chitosan microsphere matrix produced a slope of 0.024 ml/m^2 and therefore was not an influence on the surface hydrophobicity.

Table 4
The surface free amine content of chitosan microspheres prior to and after storage in P134a for 3 months at 25°C and 60% RH

Chitosan microsphere description	Amine (μmol)	
	Before storage	After storage
Non cross-linked	3.009 (0.3104)[a]	2.458 (0.4631)
TPP cross-linked	2.410 (0.1669)	2.436 (0.3067)
Glutaraldehyde cross-linked	2.597 (0.1549)	2.355 (0.1203)
$Al(OH)_3$ additive	2.901 (0.2490)	2.545 (0.2373)

[a] Standard deviations are given in parenthesis.

4. Conclusions

The results of this preformulation study indicated that the density of chitosan microspheres was influenced by the degree of cross-linking, the type of cross-linking agent and the addition of $Al(OH)_3$. The density of the non cross-linked and the glutaraldehyde cross-linked chitosan microspheres was most similar to that of P134a. Water present in P134a influenced the median particle size of the suspension pMDI of non cross-linked chitosan microspheres but did not affect the suspension characteristics of the glutaraldehyde cross-linked chitosan microsphere pMDI. Changes in the median particle size of

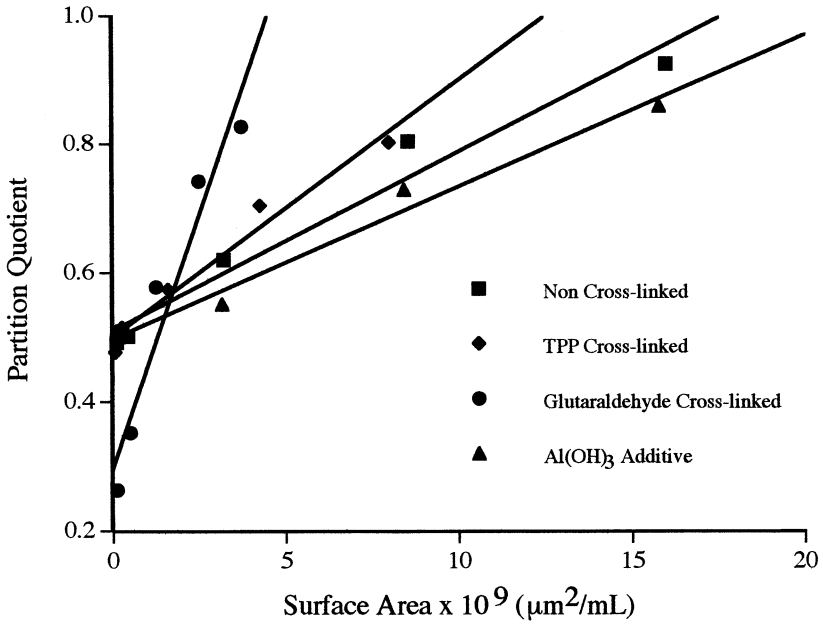

Fig. 4. The relative surface hydrophobicity of chitosan microspheres.

the chitosan microsphere pMDI suspensions resulted in changes in the aerodynamic particle size distribution of the dose emitted from the pMDI. Dosing from chitosan microsphere pMDI formulations was reproducible and the %RF was acceptable. However due to differences in the loading capacities, a much higher dose of the glutaraldehyde cross-linked chitosan microspheres would be required to administer an equivalent dose of an encapsulated biotherapeutic compound compared to the non cross-linked chitosan microsphere pMDI formulation. The surface hydrophobicity of chitosan microspheres was influenced by the degree of cross-linking and by the type of cross-linking agent. The addition of $Al(OH)_3$ did not significantly influence the surface hydrophobicity. The surface charge and the free surface amine content was not influenced by exposure to P134a. Due to the biocompatibility and the increased loading capacity of the non cross-linked microspheres and the compatibility of the glutaraldehyde cross-linked microspheres with P134a, both types of chitosan microspheres appear to be potential candidates for carrying biotherapeutic compounds to the lung via a pMDI system.

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