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The effect of polymer coatings on physicochemical properties of spray-dried liposomes for nasal delivery of BSA.

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Abstract: This work describes the development of spray dried polymer coated liposomes composed of soy phosphatidylcholine (SPC) and phospholipid dimyristoyl phosphatidylglycerol (DMPG) coated with alginate, chitosan or trimethyl chitosan (TMC), that are able to penetrate through the nasal mucosa and offer enhanced penetration over uncoated liposomes when delivered as a dry powder. All the liposome formulations, loaded with BSA as model antigen, were spray-dried to obtain powder size and liposome size in a suitable range for nasal delivery. Although coating resulted in some reduction in encapsulation efficiency, levels were still maintained between 60-69% and the structural integrity of the entrapped protein and its release characteristics were maintained. Coating with TMC gave the best product characteristics in terms of entrapment efficiency, glass transition (T_g) and mucoadhesive strength, while penetration of nasal mucosal tissue was very encouraging when these liposomes were administered as dispersions although improved results were observed for the dry powders.

Keywords; nasal, mucosal, liposomes, spray-dried, protein, glass transition, trimethyl chitosan

1. Introduction

Nasal drug delivery has been recognized as a very promising route for drug delivery and antigen delivery in particular since the nasal cavity offers a highly vascularized epithelium, large surface area available for drug absorption, relatively lower rates of enzymatic degradation in comparison with the oral route, as well as the potential for systemic delivery (Pires et al., 2009). However bioavailability may be as low as 10% for some small polar drugs and less than 1% for large molecules (O'Hagan and Illum, 1990) because of the presence of mucus and the mucociliary clearance (MCC) mechanism (Illum, 2003). Moreover degradation in the lumen of the nasal cavity or during the passage across the nasal epithelia barrier can affect negatively the bioavailability of active substances due to the presence of a wide spectrum of metabolic enzymes in nasal tissues (Dimova et al., 2005; Lee and Yamamoto, 1990; Mitra and Krishnamoorthy, 1998).

Liposomes have been extensively investigated for the nasal delivery of proteins; advantages include their protective effect on entrapped protein (Kato et al., 1993; Murramatsu et al., 1999), the ability to cause mucosal membrane disruption (Lee et al., 1991) and the capability of positively charged liposomes to increase residence time at the negatively charged mucosal surface (Law et al., 2001). Unfortunately they are easily removed by MCC. Conversely mucoadhesive polymers can retard the MCC rate but often lack penetration ability offering little improvement in the absorption of hydrophilic and/or large molecules.

Mucoadhesive formulations have been demonstrated to improve the absorption of nasally administered drugs by prolonging the contact duration between drug and nasal mucosa (Gavini et al., 2008, Patil and Sawant, 2009; Ugwoke et al., 2005). Chitosan's positively charged groups can form strong ionic interactions with acidic sialic moieties of mucins, leading to strong mucoadhesion (Bonferoni et al., 2006; Luppi et al., 2010) and can promote the transmucosal absorption of small polar molecules as well as peptide and protein drugs from nasal epithelia (Artursson et al., 1994; Illum et al., 1994), which has been attributed to its capability to open tight junction complexes (Smith et al., 2004) and also to induce redistribution of F-actin (Dodane et al., 1999). However environmental pH has a

marked influence on the permeation capability of chitosan/ chitosan salts (Borchard et al., 1996). Trimethyl chitosan (TMC) and other substituted chitosans have been synthesized (Avadi et al., 2004, 2003; Bayat et al., 2006; Sieval et al., 1998); the degree of quaternization of TMC determines the amount of positive charges available for interaction and thereby influences its drug absorption-enhancing properties and has been shown to improve the paracellular permeability of intestinal epithelia (Thanou et al., 2000). TMC has been investigated for the nasal delivery of proteins and antigens with encouraging results (Amidi et al., 2007, 2006), that have been correlated with the strength of the cationic charge of the polymer (Sadeghi et al., 2008). Among other mucoadhesive polymers, alginates are anionic water-soluble linear polysaccharides that have been widely investigated in mucoadhesive formulations due to their ability to form hydrogen bonds with mucin-type glycoproteins through carboxyl-hydroxyl interactions. Alginates have been investigated for the nasal delivery of actives and it was found that polyanion polymers are more effective bioadhesives than polycation or nonionic polymers (Bernkop-Schnurch 2002; Braccini and Perez, 2001).

Coating liposomes with mucoadhesive polymers can result in increased bioavailability, as previously demonstrated by Jain et al. (2007). Furthermore, formulating liposomes as a powder rather than as a dispersion enables easier application and improved stability over liquids and dried mucoadhesive powders can be more effective than dispersions (Dyer et al., 2002; Gill et al., 1998; Illum et al., 2000; Wang et al., 2006).

Thus, in this study we have aimed to combine the merits of these approaches (liposome formulation and mucoadhesion) to produce spray-dried polymer-coated liposomes for nasal delivery of antigen. Bovine serum albumin (BSA) was used as a model antigen and chitosan, TMC and alginate were evaluated as liposome coating polymers. Uncoated and polymer coated liposome formulations were compared with their corresponding spray-dried liposome powders. The objective of this study is to achieve spray dried BSA-loaded liposomes with high encapsulation efficiency, protection of the protein structure, mucoadhesive properties and penetration across the nasal mucosa.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (LIPOID S 75, 80%), soy phosphatidylcholine (LIPOID S PC, >98%) and phosphatidyl glycerol-Na (DMPG-Na >99%) were obtained from Lipoid (Steinhausen, Switzerland). Cholesterol (PhEur grade), bovine serum albumin (BSA) (Fraction V, lyophilized powder), dithiothreitol (DTT), L-leucine (PhEur grade), trifluoroacetic acid (99%), iron (III) chloride hexahydrate ($\geq 99\%$), alginate (sodium salt from brown algae, medium viscosity), chitosan (medium molecular weight, 75-85% deacetylated, 200-800 cP (1 wt. % in 1% acetic acid), iodomethane ($\geq 99.0\%$), mucin (porcine stomach, Type III, bound sialic acids around 1%), albumin–fluorescein isothiocyanate conjugate (FITC-BSA), sodium iodide ($\geq 99.0\%$), 1-methyl-2-pyrrolidone (99%), sodium hydroxide (PhEur grade), polyvinyl alcohol mounting medium with DABCO[®], ammonium thiocyanate ($\geq 99.0\%$), stearylamine and propidium iodide ($\geq 94.0\%$) were purchased from Sigma-Aldrich (Poole, UK). α , α -trehalose dehydrate (high purity, low endotoxin) was obtained from FERRO Pfanstiehl (Mayfield Heights, USA). Tris (hydroxyl methyl) methylamine AanlaR[®] (99%) was from VWR (Lutterworth, UK). SDS-PAGE loading dye buffer, NuPAGE Novex Bis-Tris (4-12%) minigel, SDS-MOPS running buffer, NuPAGE[®] antioxidant and Simply blue stain were obtained from Invitrogen (Paisley, UK). Tissue-Tek[®] OTC[™] Compound was purchased from Sakura Finetek UK Ltd (Thatcham, UK).

Fresh bovine nasal tissue was obtained from a local slaughterhouse and used immediately. All organic solvents were of HPLC grade and all aqueous solutions were prepared with freshly de-ionized water.

2.2. Methods

2.2.1. Preparation of uncoated liposomes

The liposomal dispersions, whose compositions are reported in Table 1, were prepared by dispersing vesicular phospholipid gels (VPGs) using the method developed by Massing et al. (2008). BSA and lipid components were homogenized with the same mass of hydration medium (25 mM Tris HCl containing 15% (w/w) trehalose dihydrate) using a dual asymmetric centrifuge (Speed-Mixer[™] DAC 150 FVZ, Germany). The centrifugation speed of each cycle was maintained at 3,500 rpm for 5 min followed by 2 min rest in an ice bath to avoid heating. The process was performed 15 times. VPGs were converted to liposomal suspensions by dropwise addition of the rest of hydration medium and simultaneous stirring. Then,

liposomal dispersions were continuously stirred for 2 hours in a cold room. The liposome dispersions were either polymer-coated or spray dried directly. Spray drying was performed with a Mini-Spray-dryer (Büchi 190; Flawil, Switzerland). Applied spray parameters were: inlet temperature 100 °C, outlet temperature 68 °C, air-flow 600-650 NI/h, aspirator setting 20 (100%), pump setting 2.5-3 ml/min. A 0.5-mm nozzle was used (Chen et al., 2012). Characterisation for lipid content, size, zeta potential and encapsulation efficiency were performed on fresh samples.

2.2.2. Liposome optimization for spray drying: determination of optimum trehalose concentration

For the preparation of an initial vesicular phospholipid gel (VPG) 25 mM Tris HCl containing either 5, 10 or 15% (w/w) trehalose dihydrate was used as the hydration medium. Subsequently, 5% (w/w) trehalose dihydrate solution was dropwise added into this VPG and then it progressively turned into liposome suspensions. Unentrapped BSA was separated by ultracentrifugation at 90,000 rpm then washing and resuspending the liposome pellets twice (Takeuchi et al., 2005). Finally, the liposome pellets were dispersed in hydration media containing 0.2% (w/w) leucine, as anti-adherent as previously (Chen et al., 2012) and either 5, 10 or 15% (w/w) trehalose, before 4-fold dilution with the same hydration medium prior to spray drying.

2.2.3. Synthesis and characterization of TMC

TMC was synthesized by reductive methylation of the chitosan with methyl iodide in the alkaline condition using the procedure described by Polnok et al. (2004) with some modification. In step 1, 0.5 g of chitosan, 1.2 g of sodium iodide, 2.5 ml of 20% aqueous sodium hydroxide and 20 ml of *N*-methylpyrrolidone were heated in a water bath at 60 °C for 10 min. Subsequently, 3 ml of iodomethane was added to the mixture and the reaction was carried out for 90 min in a reflux condenser. The polymer was precipitated from using ethanol and centrifugation, and then washed twice with diethyl ether. In reaction step 2, the product from the previous step was mixed with 1.2 g of sodium iodide, 2.5 ml of 20% sodium hydroxide and 20 ml of *N*-methylpyrrolidone at 60 °C for 10 min. 3 ml of iodomethane was added to the mixture and the reaction was carried out for 90 min in a reflux condenser. The product was precipitated and washed as described above. The

product from step 2 was dissolved in 100 ml of 10% (w/v) sodium chloride solution to exchange the iodide ion with chloride, and stirred overnight before dialysis and subsequent freeze-drying.

The synthesized polymer was characterized by infrared (IR) and proton nuclear magnetic resonance (^1H -NMR) spectroscopy. The IR spectra of chitosan and TMC were obtained on pellets formed by grinding mixtures of the polymer with KBr before compression, analyses was performed across the range $400\text{ cm}^{-1} - 4000\text{ cm}^{-1}$ using a Fourier Transform Infrared Spectrophotometer model 4100 (FT/IR-4100) (Jasco, Great Dunmow, UK). ^1H -NMR spectra of the TMC samples were obtained with an NMR spectrometer (AVIII-400, Bruker, Switzerland) by hydrating samples of the polymers in D_2O at $80\text{ }^\circ\text{C}$ with suppression of the water peak. The degree of quaternization (DQ) was calculated using data obtained from the ^1H -NMR spectra according to the following Eq. (1).

$$\% \text{DQ} = [(\text{CH}_3)_3] / [\text{H}] \times (100/9) \quad \text{Equation 1}$$

Where $[(\text{CH}_3)_3]$ is the integral of trimethyl amino group at 3.3 ppm. $[\text{H}]$ is the integral of ^1H peaks between 4.7 and 5.7 ppm.

2.2.4. Preparation of polymer-coated liposome powders

Polymer dispersions with concentrations calculated on the basis of the same mole charge ratio were used to coat the liposomes. 0.25% (w/w) alginate and 0.5% (w/w) TMC dispersions were prepared by hydrating them with 25mM Tris HCl buffer containing 10% (w/w) trehalose dihydrate and 0.2% (w/w) L-leucine. 0.25% (w/w) chitosan dispersion was prepared by hydrating with 100 mM acetate buffer (pH 5.0) containing 10% (w/w) trehalose dihydrate and 0.2% (w/w) L-Leucine. Liposomal dispersions in 10% (w/w) trehalose/0.2% (w/w) L-leucine prepared as in 2.2.1 were subjected to ultracentrifugation as in 2.2.2 to remove free BSA, were added dropwise into the desired polymer dispersion at 1:4 (w/w) ratio of liposome dispersion to polymer dispersion. The dispersions were continuously stirred at 900 rpm for 2 h on ice before spray-drying. For the chitosan-coated and TMC-coated liposomes an additional procedure of short period of 2.5 min sonication (10 sec sonication/10 sec rest on ice bath) was applied following the coating procedure in order to reduce the liposomal size as has been described previously (Thongborisute et al., 2006). Spray drying conditions for all liposomes were as described for the uncoated liposomes.

2.2.5. Lipid content, size and zeta potential analysis

Lipid content, expressed as phospholipid concentrations, was assessed by first extracting the lipids according to the method of Bligh and Dyer (1959) and then performing the Stewart assay (Stewart, 1980). Liposome size and Zeta potential were determined with a ZetaSizer 3000HS (Malvern Instruments, Malvern, UK) at a temperature of 25 ± 0.1 °C. Uncoated-, alginate- and TMC- coated liposomes were dispersed into 2.5mM TrisHCl buffer of pH 7.2 while chitosan-coated liposomes were dispersed into 2.5 mM acetate buffer of pH 5.0. For size analysis, 4 replicate measurements were performed. Zeta potential was measured 10 times; values are reported as mean $\pm$ standard deviation.

2.2.6. Encapsulation efficiency of BSA

The encapsulation efficiency (EE%) of BSA entrapped in the liposome and polymer-coated liposomal dispersions was calculated based on the ratio of encapsulated to total BSA concentration. Entrapped BSA was calculated by subjecting dispersions to ultracentrifugation at 90,000 rpm (Beckman Coulter Optima TLX-120 Ultracentrifuge, High Wycombe, UK) for one hour at 4°C and then determining the concentration of free BSA in the supernatant by using HPLC. Total BSA content was determined by dilution then membrane disruption using isopropanol followed by HPLC analysis as below.

2.2.7. HPLC method for analysis of BSA

Reverse phase HPLC method was used for quantification of BSA with some modification of the conditions by Umrethia et al. (2010). HPLC was carried out using a Waters system (Waters 1525 Binary HPLC Pump, Waters IN-Line Degasser AF, Waters 2487 Dual λ Absorbance Detector, Waters 717 plus Autosampler, Waters, Milford, USA). Jupiter C-5 column (300 Å, 250 X 4.6 mm 5 μ m, Phenomenex, Torrance, USA) was used at 30°C and the detector was set to 220 nm. The mobile phase consisted of a gradient of 0.2% TFA in acetonitrile (A) and 0.2% TFA in water (B). The gradient schedule was: (a) 0-20 min, A/B=5:95 (v/v) $\rightarrow$ A/B=65:35 (v/v), flow rate of 1 ml/min; (b) 20-23 min, A/B=65:35 (v/v) $\rightarrow$ 100% (v/v) A, flow rate of 1 ml/min; (c) 23-24.5 min, 100% (v/v) A, flow rate of 1 ml/min $\rightarrow$ 1.5 ml/min;

(d) 24.5-30.5 min, 100% (v/v) A, flow rate of 1.5 ml/min; (e) 30.5-31 min, 100% (v/v) A, flow rate of 1.5 ml/min → 1 ml/min; (f) 31-34 min, 100% (v/v) → A/B=5:95 (v/v), flow rate of 1 ml/min; (g) 34-44 min, A/B=5:95 (v/v), flow rate of 1 ml/min.

2.2.8. Effect of Processing on BSA integrity and secondary structure

The integrity of BSA in the liposomes following processing was analysed using SDS-PAGE. The dispersions were diluted until a concentration of ~ 50 µg/ml of BSA was obtained. A 13 µl aliquot was mixed with 5 µl of SDS-PAGE loading dye buffer and 2 µl of dithiothreitol and heated at 70 °C for 10 min. Subsequently, 10 µl aliquots were loaded onto a NuPAGE Novex Bis-Tris (4-12%) minigel, run at 200 V under reducing conditions using SDS-MOPS running buffer. When the dye front reached the bottom of gel, electrophoresis was stopped and the gel was carefully removed from the cassette, washed and stained for one hour using Simply Blue stain (Invitrogen Corporation, Paisley, UK) and destained overnight using water.

The secondary structure of BSA in the liposomes following processing was investigated using circular dichroism (CD). The control solution of BSA containing 30 µg /ml was made by dissolving BSA powder into 25 mM Tris HCl, pH=7.2. Spray-dried uncoated/polymer-coated liposomes were rehydrated with water, followed by ultracentrifugation (90,000 rpm) for 30 minutes. Afterwards, supernatants containing untrapped BSA were collected and subsequently subjected to centrifugation using Amicon Ultra-0.5mL centrifugal filter (molecular weight cut-off of 100KDa, Millipore, Watford, UK). The filtrate was withdrawn and then concentrated using Amicon Ultra-0.5mL centrifugal filter (molecular weight cut-off of 10KDa, Millipore, as before). The BSA concentration was determined using the HPLC method (as before) then adjusted to 30 µg/ml for CD measurement. Since the interference of TMC and chitosan could not be completely excluded from CD analysis, we adopted an alternative approach to indirectly calculate the maintenance of secondary structure of BSA in chitosan- and TMC-coated liposome following spray drying and rehydration. Data for simulated chitosan- and TMC-coated liposomes encapsulating BSA were obtained from samples produced by following the same preparative procedures of these two polymer-coated vesicles but without the inclusion of the polymer excipients. Baseline correction for solvent was made prior to CD analysis of all samples. Spectra were acquired using a Jasco J815 spectropolarimeter (JASCO, Great Dunmow, UK) and using the following settings: cell

path length 1 cm, band width 1.0 nm, wavelength range 260–190 nm far UV, scanning rate 20 nm/min, time constant 4 s, wavelength step 0.2 nm. CD spectra for all samples were recorded in an average of 3 scans. Machine units Θ_{obs} (millidegrees) were converted to $\Delta\epsilon$ for secondary structure prediction using Equation 2 (Janda and Casadevall, 2010):

$$\Delta\epsilon = (\Theta_{\text{obs}} \times \text{MRW}) / (10 \times c \times l \times 3298) \quad \text{Equation 2}$$

Where MRW is the mean residual weight of the BSA (molecular weight/number of amino acids in sequence), c is the BSA concentration in mg/ml and l is the path length in cm. The extents of secondary structures of BSA (e.g. α -helix & β -strand) were determined from the delta epsilon ($\Delta\epsilon$) using K2D3 software (Louis-Jeune et al 2012).

2.2.9. Mucoadhesive strength of liposomal powders

Mucoadhesive strength was determined using a microtensiometer (KRÜSS, Hamburg, Germany) based on the method of Dalpiaz et al., 2008 and Albertini et al. (2009), using bovine nasal mucosa. Briefly, spray-dried powders were compressed into a mini-tablet (weight of 40 mg, diameter 0.5 mm) by a single punch press (type Korsch, Korsch, Maschinenfabrik No 1.0038.86, Berlin, Germany), attached to the top plate of the instrument and hydrated with 2% mucin (in 0.9% NaCl, pH 7.0) solution. The turbinates were fully exposed by a longitudinal incision through the nose and then stored on cold Krebs's Bicarbonate Ringer's (KBR) buffer during transportation to our laboratory. The respiratory mucosa was carefully removed from the underlying bone cutting with scalpel and gently pulling the mucosa off with forceps, washed with KBR solution and cut into small pieces of adequate size. The tissue samples were fixed to the bottom stage of the apparatus with cyanoacrylate adhesive and then hydrated with 2% mucin solution. The excessive mucin fluid was removed from both nasal mucosa and mini-tablet with tissue paper. The bottom plate was moved up until minimum contact between nasal mucosa and sample was attained. After one minute rest, the top plate was moved up until the tablet completely detached from mucosa. The force (p) applied to separation of nasal mucosa and mini-tablet was recorded on the display (dyne/cm). This specific force (p) was then transformed into tensile force (T_F , dyne) using the following equation:

$$T_F = p \times 2\pi r \quad \text{Equation 3}$$

where r is the mini-tablet radius. The detachment force (D_F , dyne/cm²) was then calculated: dividing the tensile force by the area of the exposed face of the mini-tablet: $D_F = T_F / \pi r^2$. The results are reported as mean values $\pm$ S.D. (mN/cm²) ($n=8$)

2.2.10. Comparative BSA Release from liposomes

The dialysis membrane method was used to compare BSA release from the different formulations. Spray-dried powders were dispersed into de-ionized water and then loaded into a dialysis membrane (Spectra/Por CE. Molecular weight cut-off of 300 KDa, Spectrum Laboratories P.V., Breda, Netherlands). The dialysis membrane bag was placed in a vial containing 50 ml of phosphate buffered saline (pH 7.2) in a shaking incubator (37 ± 0.5 °C, 60 rpm, 25 mm throw; Unitron, Infors HT, Switzerland). At predetermined time intervals, 2 ml aliquots were withdrawn from the release medium and replaced with conditioned phosphate buffered saline. BSA concentrations were determined using the HPLC method as described above.

2.2.11. Physicochemical characterisation of spray dried liposome powders

Modulated temperature differential scanning calorimetry (MTDSC) analysis was performed on the dried powders using a TA Q100 Differential Scanning Calorimeter (TA Instruments, New Castle, PA, USA), which had been calibrated for temperature, enthalpy and heat capacity. Products were sealed into hermetic aluminum pans and after equilibration at 0 °C, were heated at 2 °C /min to 250 °C with a modulation of ± 0.4 °C/40 s. Glass transition (T_g) values were recorded as onset values from the reversing heat flow signal. Thermogravimetric analysis was performed to measure the water content of the powders using a TGA (Q500, TA instruments, as before), which had been calibrated for mass and temperature. Samples were heated from 20 to 250 °C at a scan rate of 10 °C/min in an inert nitrogen atmosphere, 3 replicate measurements for each sample were performed.

Morphological examination was performed using SEM; powders were coated in gold with a sputter coater and their surface morphology was observed using a scanning electron microscope (JEOL 6500F field emission scanning electron microscope; JEOL, Tokyo, Japan).

Powder particle size was measured using a Laser Light Diffraction Analyzer (HELOS/BR, Sympatec, Clausthal-Zellerfeld, Germany). Approximately 5 mg of powder was suspended in chloroform in a 50 ml glass cuvette and stirred with a magnetic bar at 1000 rpm. A short

period of sonication (60 s) using an 8.5 mm diameter ultrasound tip at a power of 60 W (CUVETTE, Sympatec Clausthal-Zellerfeld, Germany;) was applied before sizing (Minne et al., 2008). A R4 lens was used allowing measurements in the range 0.45-875 μm . 4 replicate measurements were made for each sample.

2.2.12. Ex-vivo penetration study across bovine nasal mucosa

Freshly prepared bovine nasal mucosa was mounted in a Franz-type permeation cell with a diffusional area of 1.5 cm^2 . To maintain the freshness of the nasal mucosa, the aforementioned process was completed as soon as possible. Material was obtained from freshly slaughtered animals and the experiments were completed within 4 hours of collection (Wadell et al 1999 demonstrated that nasal mucosa remained viable for up to 8 h after removal). Fresh bovine nasal mucosa obtained from a local slaughter-house was carefully peeled off using forceps, washed with Krebs' Bicarbonate Ringer's solution and cut into small pieces of adequate size. At time zero, sample (either powder or reconstituted liposome dispersion) was loaded into the donor compartment. Powders were carefully spread over the entire surface of the tissue. The receiver compartment filled with Krebs bicarbonate ringer buffer (adjusted to pH 7.4) was maintained at 37 $^{\circ}\text{C}$ by means of a surrounding jacket and constant stirring. Unencapsulated FITC-BSA was used as a control, the amount used was calculated by measuring the total content of FITC-BSA within the respective spray-dried uncoated- and polymer-coated liposome formulations using HPLC, and then adjusting the mass of formulation delivered so that each contained the same mass of FITC-BSA. After 1 h, the formulations were removed from the tissue, which was rinsed twice with further buffer.

For the microscopic analysis, slices perpendicular to the mucosa surface, 10 μm in thickness, were cut from the nasal tissue, embedded in the Optimal Cutting Temperature compound, by using a cryostat (Leica CM1900, Wetzlar, Germany) at -20 $^{\circ}\text{C}$. Cell nuclei were stained with propidium iodide solution and slides were mounted using polyvinyl alcohol mounting medium with DABCO[®] and a cover glass. The slides were observed using confocal laser scanning microscope (CLSM) (BIORAD- μ Radiance Confocal Scanning System, attached to an Olympus BX-60 microscope (Southend-On-Sea, UK) using λ_{ex} of 485 nm and λ_{em} of 515 nm for the visualization of FITC and λ_{ex} of 530 nm and λ_{em} of 625 nm for the visualization of propidium iodide.

2.2.13. Statistical analysis

Student's *t*-test and the one-way analysis of variance (ANOVA) with Student-Newman-Keuls all pairwise multiple comparisons were performed to compare two or multiple groups, respectively. Analyses were run using the SigmaPlot 12 software and differences were considered to be significant if *p* values were in the range 0.01 to 0.05; very significant if between 0.001 and 0.01 and extremely significant if <0.001 .

3. Results and discussion

3.1. Liposomal formulation selection and optimization

Table 1 shows the effect of different lipid formulations on size and BSA encapsulation efficiency of the freshly prepared suspensions. Formulations of SPC and cholesterol with a 9:2 mole ratio and of SPC:DMPG:cholesterol with a 9:1:2 mole ratio displayed smaller sizes, <100 nm, than those comprised of SPC: stearylamine: cholesterol (9:1:2 mole ratio). The negative charge associated with the SPC and cholesterol liposomes was attributed to the presence of small percentage of other phospholipids such as phosphatidylserine and phosphatidylinositol that have a negative charge, which has been observed previously (Garg et al., 2007 and Mosca et al. 2011). Although inclusion of stearylamine allowed nearly complete BSA entrapment that was attributed to electrostatic interaction between the positively charged components and the negative charge of the BSA at physiological pH ($PI \cong 4.7$, Lee et al., 2002), the liposome size markedly increased and phase separation occurred during overnight storage. This was attributed to the very low zeta potential allowing aggregation and fusion (Table 1). Therefore the formulation including negatively charged DMPG was selected for polymer coating and subsequent spray drying.

It has previously been shown that liposomes can be damaged during drying processes and that the addition of disaccharide (protectant) and L-leucine (as an anti-adherent) can prevent powder aggregation (Chen et al., 2012). Therefore the concentration of trehalose in the dispersions was optimized to achieve stable liposomes. Table 2 shows that inclusion of higher concentrations of trehalose offered the best protection to liposomes according to size change and encapsulation efficiency of BSA. It is evident that a combination of 10% (w/w) trehalose/ 0.2% (w/w) leucine in the hydration medium was capable of protecting liposome against spray-drying damage and this protective effect did not appear to markedly increase

with the increasing concentration of trehalose. Therefore the formulation containing 10% trehalose and 0.2% leucine was selected. Although some levels of BSA leakage were found (Table 2), the entrapped efficiency was higher than previous studies (Dhoot and Wheatley, 2003).

3.2. Characterization of synthesized trimethyl chitosan (TMC)

Supplementary Figure 1 shows the chemical characteristics of the synthesized TMC compared with chitosan. The IR spectra of chitosan and TMC show a new deformation vibration of C-H (δ_{C-H} (CH₃)) at 1486 cm⁻¹ in the TMC IR spectrum, indicating that the primary amino groups of chitosan had been substituted by methyl groups, and the successful trimethylation of chitosan. The ¹H-NMR analysis reported in Supplementary Figure 2 revealed that the degree of quaternization of the polymer was 50%. This value is close to the value of 48% found in the literature by Hamman et al. (2003) to give the best effect on TEER and transport of hydrophilic macromolecules across Caco-2 monolayer cells; higher degrees of quaternization (55% and 59%) did not improve these properties, which they attributed to steric hindrance by the additional methyl groups and increased flexibility of the molecules, a similar effect was observed by Boonyo et al. (2007).

3.3. Characterization of spray-dried liposomes

The liposome formulation SPC:DMPG:cholesterol (9:1:2 mole ratio) containing 10% w/w of trehalose as protectant and 0.2% w/w of leucine as anti-adherent was coated using 0.25% w/w of alginate and chitosan solutions and 0.5% w/w of TMC solution. The characteristics of spray-dried uncoated/coated liposomes are reported in Table 3 and compared with their properties after reconstitution. After coating liposomes with alginate a small but significant increase in particle size and polydispersity was seen. This was much smaller than the increase observed for chitosan- and TMC-coated liposomes. This difference was attributed to the difference in charge between the polymers. Chitosan and TMC offer the possibility of increased electrostatic interaction with the negatively charged liposome surface, which caused aggregation of the liposomes. A short period of 2.5 min sonication was therefore applied to reduce their size. However after reconstitution, the liposome size increased again, this together with the increase in liposome size and reduction in BSA encapsulation efficiency after spray drying and rehydration indicate that the introduction of polymer into

the formulation hampered the protective effect of trehalose on the integrity of the liposomes. It has been suggested that interaction between protectants such as trehalose and the polar head groups of the phospholipids at the liposome surface is necessary for a protective effect because of a physical spacing effect that prevents the vesicles from coming into contact with each other (Strauss et al., 1986) and hydrogen bonding between the protectant and lipid head groups (Crowe et al., 1994). Both mechanisms are likely to contribute. Polymer coating can disrupt the trehalose liposome interaction but does not itself offer a specific protective effect thus allowing the liposomes to aggregate/fuse during drying and rehydration (Takeuchi et al 1998); or even for the polymers to become inserted into the liposomal lipid bilayers during rehydration, changing the membrane fluidity and leading to the leakage of entrapped solutes (Dhoot and Wheatley, 2003). As alginate did not cause size increase to the same extent as the other polymers, it is suggested that chitosan and TMC lead to vesicular aggregation through electrostatic interaction and disruption of the interaction between trehalose and the head groups of the phospholipids that is necessary for stabilization.

All of the formulations exhibited excellent encapsulation efficiency before spray drying and the combination of trehalose and leucine in the hydration medium was capable of protecting the uncoated liposomes during spray-drying (Table 3). But following spray-drying and rehydration the encapsulation efficiency of the polymer-coating liposomes dropped substantially; the ability to retain BSA decreased in the following order: TMC > chitosan > alginate. Again this was attributed to the polymers interference with the interaction between trehalose and the phospholipids; the better encapsulation efficiency of the positively charged polymers was attributed to their better electrostatic interaction with the negatively charged protein. The period of sonication required for the chitosan and TMC-coated liposomes did not adversely affect the encapsulation efficiency.

3.4. BSA preservation in the liposomes

SDS-PAGE analysis (supplementary Figure 3) showed that the structural integrity of BSA loaded into uncoated and polymer-coated liposomes followed by spray-drying and rehydration was preserved. SDS-PAGE analysis reflects changes in the primary structure of protein only, while CD offers additional information about secondary structure. Figure 1

shows the CD spectra in which two negative bands are visible in the far-UV region at 208 nm and 222 nm. These are characteristic of the α -helical structures of protein (Liu et al., 2004).

Figure 1a shows that the CD spectrum of BSA standard solution almost overlapped with those of uncoated and 0.25% alginate-coated liposomes, suggesting that the secondary structure of BSA was preserved throughout the coating, drying and rehydration processes. The α -helix content of the BSA standard solution was 62.1%, in agreement with the literature (Di Sabatino et al., 2012). The negative control heat treatment at 80 °C for 1h indicates the change in spectrum caused by denaturation (46.3% α -helix content). The α -helix content values of BSA loaded into uncoated and 0.25% alginate-coated liposomes (59.8% and 62%, respectively) were very close to the reference solution (62.1%)

There was no observed effect of environmental pH on BSA structure (Figure 1b), with α -helix contents determined to be 63.1% in both neutral and acidic pH using the K2D3 software. Furthermore, BSA in simulated TMC- and chitosan-coated liposomes displayed similar spectra to the standard solution with α -helix contents determined to be 62.1% for both coatings. It is suggested that the secondary conformation of BSA in TMC- and chitosan-coated liposomes was maintained through spray drying and rehydration. These results indicate the structural integrity of the entrapped BSA in all of the liposomes investigated.

3.5. Characterization of spray-dried liposome powders

Spray-dried uncoated and polymer-coated liposome powders displayed sphere-like microparticles with wrinkled surfaces (Figure 2). The size distribution of all dried powders were similar with median particle size by volume in the range of 3-4 μ m, as reported in Table 4, indicating that polymer coating had no significant effect on either water content or spray drying properties of the formulations. Additionally, similar acceptably low levels of water content were found for all liposomal powders although the TMC powders exhibited significantly higher Tg values ($p < 0.05$) than the other formulations, indicating that the inclusion of TMC may also offer a small benefit in terms of thermal stability of the powders. As there were no significant differences in either the particle size or the water content of this formulation compared with the others it is hypothesized that this effect is caused by interaction of the polymer with the trehalose, which is the main contributor to the Tg of the powder. This effect has not previously been noted for the interaction of TMC with trehalose and may be important in development of TMC containing formulations that require spray or

freeze-drying.

Figure 3 shows the *in vitro release* profile of the samples. The dialysis membrane method is used to discriminate between the release profiles of the different liposomal formulations not to mimic *in vivo* release (Tiwari et al., 2009); the main barrier to release is the dialysis membrane as evidenced by the release profile for BSA alone. 0.25% alginate-coated and 0.25% chitosan-coated liposomes exhibited a slightly higher release rate than uncoated and TMC-coated liposomes, although there were no significant differences between any of the coatings nor between coated or uncoated liposomal formulations (Figure 3). The sustained release properties were as expected for liposomal formulations and are preferable to the typical burst release profile that is often displayed by smaller particles, which may lead to protein degradation in the hostile environment of the mucus. Conversely slower release from liposomes protects protein from degradation while it is carried through the mucus.

As regards the mucoadhesion of spray-dried uncoated and polymer-coated liposome powders, chitosan and TMC-coated liposomes exhibited a significantly higher mucoadhesive strength than alginate-coated or uncoated liposomes on the basis of the equivalent mole charge ratio (Figure 4), which was ascribed to the positive charge of chitosan and TMC that can interact with the sialic groups on mucin.

The confocal laser scanning microscopy (CLSM) microphotographs obtained from the *ex vivo* penetration study show that pure FITC-BSA freely diffused into nasal mucosal tissues after 1h penetration (Figure 5a). The uncoated-liposomes (Figure 5 b and c) administered as either powders or reconstituted suspensions, displayed a similar penetration pattern as the FITC-BSA solution. The polymer-coated liposomes exhibited varying ability to penetrate the nasal mucosa. For the alginate-coated liposomes (Figure 5 d and e), reconstituted suspensions gave an inferior penetration compared to the reconstituted uncoated-liposome suspensions, whilst this effect could be improved by administering as the spray-dried powder. Therefore alginate liposomes performed similarly to the uncoated liposomes when administered both as powder and as liposomal dispersion, showing no advantage as a powder. Chitosan-coated liposomes (Figure 5 f and g) exhibited the poorest penetrating ability that was attributed to their increased liposome size after rehydration (Table 3) (Takeuchi et al., 2005) and the reduced solubility of chitosan at a higher pH (Borchard et al., 1996). TMC-coated liposome dispersions (Figure 5 h) offered no improvement in

penetration capability compared with the other formulations presumably due to their increased size, but when the same TMC-coated liposomes were administered as powders (Figure 5 i) they performed better, possibly because the powders draw water from the nasal mucosa consequently resulting in transient dehydration of epithelial membrane and opening of tight junctions (Callens and Remon, 2000; Dyer et al., 2002; Fernandez-Urrusuno et al., 1999; Illum et al., 2000; Wang et al., 2006). There have been some contradictory effects related to the effect of liposome size reported in the literature (Cao et al., 2009; Jain et al., 2007; Takeuchi et al., 2005). The results obtained in this study suggest that liposome size is of more importance than liposome charge as long as the charge is sufficient to maintain repulsion between the liposome surfaces and prevent aggregation. The benefit of larger liposomes therefore may lie in the improved encapsulation efficiency and thus higher effective concentration of protein at the mucosal membrane. This then acts as a passive driving force for membrane permeation as has been postulated by di Cagno et al. (2013).

Alginate-coated liposomes retained the smallest size after coating and rehydration but chitosan- and TMC-coated liposome powders displayed higher mucoadhesive strength and encapsulation efficiency. All of the spray-dried powders displayed sphere-like morphology with some roughness and similar size regardless of the presence and type of polymers. Among the polymer-coated liposomes, those coated with TMC retained the highest BSA encapsulation ($p < 0.05$). The structure of BSA within the uncoated and polymer-coated liposomes was preserved throughout the preparative process and the polymer coating did not affect the *in vitro* release rate of the liposomal BSA. A reduction in penetrating ability across bovine nasal mucosa was observed in the reconstituted chitosan- and TMC-coated liposome suspensions, although the TMC-coated liposomes showed a marked improvement when administered as a powder. Taking all of the results together it can be seen that while incorporation into liposomes offered only marginal improvement over FITC-BSA alone, the possibility for improved physicochemical properties e.g. high Tg, encapsulation efficiency and increased mucoadhesion for the TMC-coated liposomes may offer benefits for nasally administered vaccine material especially if administered in a dry powder format.

4. Conclusions

For nasally administered products good penetration, is of little use if the formulations are not able to remain in contact with the mucosal surface for a long enough time to enable penetration to occur, therefore mucoadhesion is a key characteristic of a nasally administered formulation where local mucosal delivery is required. Additionally administration of powders rather than liquids enables better storage stability with easier handling and should offer improved stability of the therapeutic payload; all powders exhibited good Tg and water content values. We therefore conclude that although polymer coating resulted in some drug loss and increase in liposomal size, the use of an optimized protectant formulation ensured that the protein was stabilized during manufacture and rehydration. The increased mucoadhesive properties and ability to penetrate the nasal mucosa afforded by the TMC coating outweigh this loss. *In vivo* studies would be required to determine the importance of mucoadhesion and mucosal penetration in tandem.

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Figure captions

Figure 1 (A) CD spectra of BSA standard solution, BSA in uncoated- and 0.25% alginate-coated, as well as BSA with heat treatment at 80 °C for 1 h; (B) CD spectra BSA standard in Tris HCl buffer, pH=7.2, BSA standard in acetate buffer, pH=5, BSA in simulated chitosan (CHI)-coated and simulated trimethyl chitosan (TMC)-coated liposomes.

Figure 2. SEM images of spray-dried powders of (A) uncoated liposomes; (B) Alginate-coated liposomes; (C) Chitosan-coated liposomes; (D) TMC-coated liposomes.

Figure. 3. Release profiles of BSA from reconstituted uncoated- and polymer-coated liposome suspensions (0.25% alginate-coated liposome, 0.25% chitosan-coated liposome and 0.5% trimethyl chitosan-coated liposome) through the dialysis membrane over 48 h. Error bars represent standard deviation of three measurements (n=3).

Figure. 4. Comparison of mucoadhesive strength of spray-dried uncoated and polymer-coated liposome powders. Each bar represents mean $\pm$ SD of eight measurements (n=8). * P<0.05 (One Way ANOVA analysis with Student-Newman-Keuls all pairwise multiple comparisons) as compared to uncoated liposome powders, and + p<0.05 as compared to 0.25% alginate-coated liposome powders.

Figure. 5. CCLSM Images showing longitudinal sections of bovine nasal mucosal tissue following 1 h penetration of a) FITC-BSA solution b) uncoated liposome reconstituted suspension c) uncoated liposome powder d) alginate coated liposome reconstituted suspension e) alginate coated liposome powder f) chitosan liposome reconstituted suspension g) chitosan liposome powder h) TMC-coated liposome reconstituted suspension i) TMC-coated liposome powder Each figure shows overlay of propidium iodide (red) and FITC-BSA (green) stain (left) and FITC-BSA stain only (right)

Table 1. Characterization of freshly prepared liposomal suspensions in 25 mM Tris HCl buffer consisting of differently charged lipids. Values represent mean $\pm$ S.D of three batches and n denotes replicate measurements of each batch. Statistical significance of differences between groups ($P < 0.05$) was determined by one-way ANOVA analysis with Student-Newman-Keuls all pairwise multiple comparisons.

Formulation (mole ratio)	Size (nm) & PI (n=3)	Zeta potential (mV) (n=10)	Total BSA content (mg/ml) (n=2)	EE (%) & entrapped BSA (mg/ml) (n=1)
SPC:CHOL=9:2	74.6 $\pm$ 19.0 [*] (0.56 $\pm$ 0.14 [*])	-21.2 $\pm$ 3.6	2.07 $\pm$ 0.07	69.5 $\pm$ 5.0 [*] (1.43 $\pm$ 0.06 [*])
SPC:DMPG:CHOL=9:1:2	88.5 $\pm$ 4.1 [*] (0.65 $\pm$ 0.05 [*])	-42.9 $\pm$ 5.5	1.94 $\pm$ 0.05	72.9 $\pm$ 2.9 [*] (1.41 $\pm$ 0.02 [*])
SPC:SA:CHOL=9:1:2	474.7 $\pm$ 206.0 (0.90 $\pm$ 0.18)	2.0 $\pm$ 14.6	2.03 $\pm$ 0.17	100.0 $\pm$ 0.2 (2.03 $\pm$ 0.17)

* denotes $P < 0.05$ compared to liposome formulations comprised of SPC:SA:CHOL.

Table 2. Characterization of liposomes in the presence of 0.2% leucine and varying concentrations of trehalose prior to spray-drying and after rehydration. Numbers in parentheses are values after rehydration. Each value represents the mean $\pm$ SD of three batches and n denotes the number of replicates per batch.

	5% trehalose	10% trehalose	15% trehalose
Size (nm) (n=3)	229.7 $\pm$ 10.6 [*] (285.3 $\pm$ 25.8)	210.0 $\pm$ 14.1 (218.4 $\pm$ 7.2)	227.5 $\pm$ 10.3 (233.5 $\pm$ 9.2)
PI (n=3)	0.06 $\pm$ 0.03 [*] (0.56 $\pm$ 0.20)	0.06 $\pm$ 0.03 (0.13 $\pm$ 0.06)	0.08 $\pm$ 0.05 (0.11 $\pm$ 0.09)
Total BSA Content (μ g/ml) (n=2)	242.0 $\pm$ 23.3 (247.0 $\pm$ 31.6)	230.2 $\pm$ 24.9 (233.6 $\pm$ 19.1)	187.1 $\pm$ 22.1 (209.9 $\pm$ 9.0)
EE (%) (n=1)	98.0 $\pm$ 0.4 (60.7 $\pm$ 4.2)	98.4 $\pm$ 1.3 (85.6 $\pm$ 2.4) ⁺	96.7 $\pm$ 3.4 (88.7 $\pm$ 1.6) ⁺
SPC content (mg/ml) (n=2)	8.13 $\pm$ 0.37 (8.68 $\pm$ 0.49)	8.29 $\pm$ 0.33 (8.66 $\pm$ 0.52)	10.35 $\pm$ 0.89 (10.92 $\pm$ 0.53)
Loading efficiency (μ g BSA/mg lipid) (n=2)	29.2 $\pm$ 2.3 (17.2 $\pm$ 1.4)	27.3 $\pm$ 2.0 (23.1 $\pm$ 1.6)	17.7 $\pm$ 0.8 (17.1 $\pm$ 0.6)

* P<0.05 (t-test) in comparison of liposomes prior to spray-drying and after rehydration.

+ P<0.05 (One Way ANOVA analysis with Student-Newman-Keuls all pairwise multiple comparisons) compared with 5% trehalose+0.2% leucine.

Table 3. Comparison of properties of polymer-coated liposomes prior to spray-drying and after reconstitution. Each value represents mean $\pm$ SD of three batches and n denotes the number of replicates per batch.

	Uncoated liposome		0.25% ALG-coated liposome		0.25% CHI-coated liposome			0.5% TMC-coated liposome		
	Before spray-drying	After reconstitution	Before spray-drying	After reconstitution	Before spray-drying		After reconstitution	Before spray-drying		After reconstitution
					2hr coating	2.5 min sonication		2hr coating	2.5 min sonication	
Size (nm) (n=3)	210.0 $\pm$ 14.1	218.4 $\pm$ 7.2	268.1 $\pm$ 12.3	303.3 $\pm$ 10.0 ^{*♣}	902. $\pm$ 185.2 ⁺	354.3 $\pm$ 15.4	996.4 $\pm$ 68.1 ^{*♣}	2264.2 $\pm$ 281.4 ^{+♯}	316.0 $\pm$ 9.7	848.7 $\pm$ 203.0 ^{+♣}
PI (n=3)	0.06 $\pm$ 0.03	0.13 $\pm$ 0.06 [*]	0.42 $\pm$ 0.11	0.45 $\pm$ 0.06 [♣]	0.95 $\pm$ 0.14 ⁺	0.32 $\pm$ 0.09	1.00 $\pm$ 0.00 ^{+♣}	1.00 $\pm$ 0.00 ⁺	0.10 $\pm$ 0.07	1.00 $\pm$ 0.00 ^{+♣}
Zeta potential (mV) (n=10)	-49.7 $\pm$ 3.3	-54.6 $\pm$ 2.6 [*]	-54.2 $\pm$ 1.8	-60.3 $\pm$ 1.2 [*]	42.8 $\pm$ 3.3	42.7 $\pm$ 1.0	44.0 $\pm$ 1.8 ⁺	36.1 $\pm$ 2.6 [#]	35.0 $\pm$ 1.1	32.7 $\pm$ 1.4 ⁺
Total BSA (μ g/ml) (n=2)	230.2 $\pm$ 24.9	233.6 $\pm$ 19.1	257.3 $\pm$ 12.1	247.4 $\pm$ 7.2	239.5 $\pm$ 30.2	241.6 $\pm$ 27.5	244.5 $\pm$ 26.9	278.1 $\pm$ 22.5	262.4 $\pm$ 16.6	271.3 $\pm$ 12.3
EE (%) (n=1)	98.4 $\pm$ 1.3	85.6 $\pm$ 2.4 [*]	99.2 $\pm$ 0.0	61.3 $\pm$ 4.8 ^{*♣}	97.8 $\pm$ 0.5 ^{+♯}	89.2 $\pm$ 0.9	60.7 $\pm$ 1.0 ^{+♣}	97.5 $\pm$ 1.0 ^{+♯}	87.6 $\pm$ 2.6	69.2 $\pm$ 3.2 ^{+♣}
SPC content (mg/ml) (n=2)	8.29 $\pm$ 0.33	8.66 $\pm$ 0.51	7.93 $\pm$ 0.53	8.57 $\pm$ 0.31	8.82 $\pm$ 0.66	8.74 $\pm$ 0.74	9.12 $\pm$ 0.56	8.78 $\pm$ 0.67	8.64 $\pm$ 0.63	9.29 $\pm$ 0.79
Loading efficiency (μ g BSA/mg lipid) (n=2)	27.3 $\pm$ 2.0	23.1 $\pm$ 1.6 [*]	32.3 $\pm$ 3.6	17.7 $\pm$ 1.7 [*]	26.8 $\pm$ 5.2 [#]	24.8 $\pm$ 3.8	16.4 $\pm$ 2.7 ⁺	31.0 $\pm$ 3.9 [#]	26.7 $\pm$ 2.6	20.3 $\pm$ 2.0 ⁺

* P<0.05 (t-test) comparison of formulation prior to spray-drying and after rehydration.

+ P<0.05 (One Way ANOVA analysis with Student-Newman-Keuls all pairwise multiple comparisons) as compared to the corresponding formulations after 2.5-min sonication.

P<0.05 (One Way ANOVA analysis with Student-Newman-Keuls all pairwise multiple comparisons) as compared to their reconstituted liposomes.

♣ P<0.05 (One Way ANOVA analysis with Student-Newman-Keuls all pairwise multiple comparisons) as compared to reconstituted uncoated liposomes.

Table 4. Size distribution, water content and Tg values of spray-dried uncoated and polymer-coated liposome powders. Each value represents mean $\pm$ SD of three batches and n denotes replicate measurements of each batch.

	Particle size distribution				Water content (%) (n=3)	Tg ($^{\circ}$ C)(n=3)
	d[v, 10] μ m	d[v, 50] μ m	d[v, 90] μ m	SPAN (n=4)		
Uncoated liposome	1.18 $\pm$ 0.01	3.89 $\pm$ 0.05	8.68 $\pm$ 0.18	1.93 $\pm$ 0.02	3.2 $\pm$ 0.2	60.4 $\pm$ 0.9
ALG- liposome	1.18 $\pm$ 0.01	3.86 $\pm$ 0.02	8.58 $\pm$ 0.09	1.92 $\pm$ 0.01	3.2 $\pm$ 0.2	60.1 $\pm$ 0.9
CHI- liposome	1.17 $\pm$ 0.01	3.84 $\pm$ 0.03	8.52 $\pm$ 0.11	1.91 $\pm$ 0.01	3.5 $\pm$ 0.2	58.6 $\pm$ 1.7
TMC- liposome	1.18 $\pm$ 0.01	3.92 $\pm$ 0.04	8.82 $\pm$ 0.14	1.95 $\pm$ 0.01	3.3 $\pm$ 0.3	64.3 $\pm$ 2.3

d[v, X]: particle distribution at X% of the volume distribution.

SPAN: width of the volume distribution relative to the median diameter (d[v, 90]-d[v, 10])/ d[v, 50].

Graphical abstract