

# PLGA microcapsules with novel dimpled surfaces for pulmonary delivery of DNA

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## Abstract

We describe the fabrication of DNA-loaded poly(lactic-co-glycolic acid) (PLGA) microcapsules with novel surface morphologies that will be of use in pulmonary delivery. Our approach was to examine surface morphology and DNA encapsulation efficiency as a function of primary emulsion stability; using two surfactant series based on hydrophile–lipophile balance and hydrophobe molecular weight. Hydrophilic non-ionic surfactants yielded the most stable water-in-dichloromethane emulsions (HLB values >8). These surfactants normally favor convex (o/w) interfacial curvatures and therefore this atypical behavior suggested a relatively high surfactant solvation in the dichloromethane ‘oil’ phase. This was consistent with the large fall in the glass transition temperature for microspheres prepared with Tween 20, which therefore efficiently penetrated the PLGA matrix and acted as a plasticizer. Blends of Pluronic triblock copolymers performed poorly as water-in-dichloromethane emulsifiers, and were therefore used to generate hollow microspheres (‘microcapsules’) with low densities (0.24 g/cm<sup>3</sup>). Although the Pluronic-stabilized emulsions resulted in lower DNA loading (15–28%), microspheres (~8 μm) with novel dimpled surfaces were fabricated. The depth and definition of the dimples was greatest for triblock copolymers with high MW hydrophobe blocks. By cascade impaction, the geometric mean weight diameter of the microcapsules was 3.43 μm, suggesting that they will be of interest as biodegradable pulmonary delivery vehicles.

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**Keywords:** PLGA microcapsules; Non-ionic surfactants; Triblock copolymers; DNA encapsulation; Pulmonary delivery vehicles

## 1. Introduction

There is strong interest in developing PLGA microspheres for delivery of plasmid DNA for DNA-vaccines (Singh et al., 2000), or CpG DNA for stimulation of immune responses (Singh et al., 2001). Plasmid DNA (pDNA) encapsulated, either naked or condensed with polycationic polymers, in microspheres remains transcriptionally active following uptake in cultured macrophages (Benoit et al., 2001). In mice, DNA-vaccines released from microspheres (ca. 5 μm diameter) following their phagocytosis by antigen presenting cells stimulated natural humoral and cellular immune responses (Wang et al., 2004). Moreover, PLGA microspheres are themselves considered vaccine adjuvants by virtue of their controlled release properties (Thomasin et al., 1996; Audran et al., 2003; Zhou et al., 2003). It is apparent that encapsulation efficiencies for naked pDNA are quite low (Barman et al., 2000). This is some-

times overcome by prior DNA complexation with polycations such as protamine sulphate (Dunne et al., 2003). DNA ‘nicking’ (linearization) is clearly to be avoided and loss of supercoiled (to relaxed) isoforms is considered advantageous (Benoit et al., 2001). Optimization of DNA encapsulation has shown that the type and molecular weight of polyester, and surfactant concentration, are important considerations (Capan et al., 1999; Prabha and Labhasetwar, 2004). However, as for protein encapsulation, poly(vinylalcohol) is generally used as the emulsifier (Capan et al., 1999; Bouissou et al., 2004; Prabha and Labhasetwar, 2004). Surprisingly little work has described the stability of the primary emulsion with respect to the surfactant used and the efficiency of DNA encapsulation into microspheres.

Needleless vaccine delivery technologies generally focus on the nasal mucosae (van der Lubben et al., 2003; Yuki and Kiyono, 2003) or bronchus-associated lymphoid tissues (BALT), the latter demonstrated to trigger immunity against influenza virus (Smith et al., 2003). If the technological challenges can be overcome, good bioavailability of macromolecules can be achieved via the lung periphery employing particulate carriers. This has recently been demonstrated by spray-drying

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insulin-loaded chitosan nanoparticles suspended in mannitol solution to create hollow microspheres for pulmonary delivery (Grenha et al., 2005). Therefore, particles targeting the lung periphery and BALT could provide a needleless system able to test a range of vaccine concepts such as DNA-vaccines. If DNA-vaccines are to be delivered to the BALT and lung periphery, the PLGA vehicles would require an aerodynamic diameter above 3–5  $\mu\text{m}$ , preferably with a narrow size distribution. ‘Large porous PLGA particles’, fabricated using emulsion–evaporation techniques, fit this criterion well, even though their size distribution is not strictly controlled (Edwards et al., 1997). The low densities of these particles results in an optimal aerodynamic diameter for a measured diameter of  $>8 \mu\text{m}$ . However, the dispersion and flow of PLGA aerosols could be further improved by minimizing particle–particle contact area. This is suitably demonstrated by albumin aerosols having external corrugations and common puffball spores which have surface protrusions (Geiser et al., 2000; Chew and Chan, 2001). Therefore, an alternative route to achieving PGLA aerosols would be to focus on engineering specific surfaces.

Surface engineering of PLGA microspheres is not currently possible due to a lack of understanding of parameters controlling the evolution of microsphere surface morphology. Work has focused on emulsion parameters (extraction of the oil phase, homogenization, PLGA molecular weight and concentration, etc.) and drying techniques controlling the internal morphology and drug loading–release (Nihant et al., 1994; Yang et al., 2000; Kim and Park, 2004b). For example: (i) lowering the emulsion stability alters the internal pore–morphology from matrix-like to multivesicular (Nihant et al., 1994); (ii) increasing the rate of solvent extraction increases microsphere diameter and size distribution (Yang et al., 2000). In contrast, no mechanism has been proposed for the appearance of differing surface morphologies, although the formation of the surface pores is thought to occur on water extrusion from osmotic imbalance (Yang et al., 2001). Our previous work has shown, empirically, that the type of surfactant used in the primary emulsion influences the microsphere external morphology (Bouissou et al., 2004). Also, a ‘defective skin surface’ has been described as a consequence of low oil-phase volumes (Yang et al., 2001); and tricaprin oil loaded microspheres unexpectedly yielded microspheres with dimpled surfaces (Schaefer and Singh, 2002).

Here we aim to address the issue of DNA encapsulation as a function of primary emulsion (water-in-dichloromethane) stability, and employ the various emulsion systems to investigate alternative routes to engineering microsphere surface morphology. The dichloromethane–water interfacial stability will be characterized for a series of non-ionic surfactant blends guided by their HLB values. Although an empirical value, HLB has been shown to be a predictive variable for the stability of polar/non-polar organic solvent emulsions (Cameron and Sherrington, 1996), and may therefore be of use in respect of the primary emulsions used in microsphere fabrication. The non-ionic surfactant series will be compared to HLB-matched Pluronic<sup>®</sup> PEO–PPO–PEO triblock copolymers (where PEO is poly(ethylene oxide) and PPO is poly(propylene oxide)); in principle imparting a steric contribution to the desorption kinetics

from the interface (Cameron and Sherrington, 1996). A possible experimental route to PLGA particles with novel surfaces would be to use emulsion systems involving self-assembling ‘biocolloids’; as applied to the fabrication of artificial spores from polystyrene (Hemsley and Griffiths, 2000; Hemsley et al., 2003). Although, the polystyrene chains aggregated orders of magnitude slower than does PLGA during solvent extraction, the work inspires the investigation of surfactant penetration into the PLGA matrix upon solvent extraction and surface evolution. We used differential scanning calorimetry to assess the glass transition of the microspheres as an indication of surfactant penetration of the PLGA matrix. The external and internal morphologies of microspheres, and DNA encapsulation efficiency were compared for non-ionic surfactants and triblock copolymers series. The work is of interest to the development of inhalable DNA-vaccines and in a wider context to the development of generic pulmonary drug delivery vehicles.

## 2. Materials and methods

### 2.1. Materials

PLGA (50:50 DL-lactide:glycolide, inherent viscosity 0.88 dl/g) was purchased from Purac Biochem, Netherlands. Poly(vinylalcohol) (PVA) (MW 25 000, 88% hydrolysed), Tween<sup>®</sup> and Span<sup>®</sup> sorbitan surfactants were purchased from Sigma Chemical Company. Water was purified to  $>16 \text{M}\Omega \text{cm}$ . Dichloromethane (DCM) (analytical grade) was obtained Fischer Scientific, UK. Pluronic<sup>®</sup> series triblock copolymers were received as a kind gift from BASF, USA. Plasmid DNA (pGL3-control, Promega, UK) was prepared using a modified method of Sambrook and Russell (2001a); purified to an  $A_{260}:A_{280}$  ratio of 1.8 and analyzed by agarose gel electrophoresis (1% agarose gel, stained with ethidium bromide, following protocols described in Sambrook and Russell, 2001b) for RNA contamination. Hard gelatin capsules and the Monodose dry powder inhaler (DPI) were received as kind gifts from Capsugel, Belgium and MIAT SpA, Italy, respectively.

### 2.2. Preparation of water-in-DCM (w/o) emulsions

Water containing surfactants blends (0.2 to 1%, v/v) were emulsified in DCM using a homogenizer (IKA, T18 basic) at ratios of 1:5 and 1:10 (w:o). Visual inspection at set time points was made to record phase separation (creaming—the first formation of water globules). Stock surfactant blends were made to a specific HLB values as described in Table 1 and diluted appropriately in water before homogenization (stock solutions of Span were dissolved in DCM rather than in water).

### 2.3. Preparation of microspheres

A water-in-oil-in-water (w/o/w) double emulsion–solvent evaporation technique was employed. One hundred microliters of aqueous phase, constituting 300  $\mu\text{g}$  of pGL3-basic plasmid, 1% surfactant or surfactant blend in TE buffer (10 mM Tris, 5 mM EDTA, pH 8.0), was injected into 1 ml DCM containing

Table 1

HLB-matched Pluronic triblock copolymers and Tween/Span sorbitan surfactants series, showing fraction composition of blends where appropriate

Surfactant (Pluronic series)			Surfactant (Tween/Span series)	HLB value
MW PPO: 950–1200	MW PPO: 1800–2400	MW PPO: 3000–3600		
	L81	L121	Span 85	1–2
L31	0.5 L81–0.5 L62	L122	Span 80	4–5
0.9 L31–0.1 L44	0.2 L81–0.8 L62	L92	0.85 Span 80–0.15 Tween 80	6
0.8 L31–0.2 L44	L62	P123	0.65 Span 80–0.35 Tween 80	7–8
0.45 L31–0.55 L44	0.5 L62–0.5 L64	0.5 P123–0.5 P105	0.35 Span 80–0.65 Tween 80 or Tween 85 alone	11–11.5
0.25 L31–0.75 L44	0.25 L62–0.75 L64	0.25 P123–0.75 P105	0.15 Span 80–0.85 Tween 80	13–13.5
0.1 L31–0.9 L44	L64	P105	Tween 80	15
0.85 L44–0.15 L35	P75	0.85 P105–0.15 F108	Tween 20	16.5–17
L35	0.7 P75–0.3 F88	0.6 P105–0.4 F108		19–20
F38	F88	F108		>24

50 mg of PLGA. The mixture was homogenized at 6000 rpm for 1 min (IKA, T18 basic), and transferred immediately into 11 ml of water containing 1% (w/v) PVA, homogenized at 10 000 rpm for 3 min. This w/o/w emulsion was then transferred into a hardening tank containing 100 ml 1% (w/v) PVA in water, stirred at 500 rpm with an overhead impeller (IKA RW11 basic). Stirring was continued for at least 2 h at room temperature to ensure complete evaporation of DCM. Microspheres were harvested by centrifugation (4000–6000  $\times g$ , 3 min), washed three times with distilled water, snap frozen in liquid nitrogen and lyophilized overnight (MicroModulyo, ThermoSavant). For scale-up, 50-fold volume increases were made, also increasing the homogenization speeds to 10 000, 14 000 and 1500 rpm for primary emulsion, secondary emulsion and hardening, respectively. Lyophilized microsphere were kept at 4 °C in a sealed container with silica gel.

#### 2.4. Microsphere size distribution and apparent density

All microspheres were sized prior to freeze-drying by laser diffractometry using Mie scattering theory (Mastersizer 2000, Malvern Instruments, UK), dispersing the microspheres in water until a laser obscuration around 10% was reached. The density of lyophilized microspheres fabricated with Pluronic L92 was calculated from the volume occupied by a known mass of microspheres loaded into a measuring cylinder and tapped 5000 $\times$  (Tap Density Volumeter, Copley Scientific, UK). Volume measurements were repeated for four independent batches of microspheres, subsequently used in the inhaler experiments (below).

#### 2.5. Plasmid DNA extraction and encapsulation efficiency

A modified method of Barman et al. was adopted (Barman et al., 2000). Approximately 2–5 mg of lyophilized microspheres were accurately weighed and resuspended with 250  $\mu$ l of TE buffer. Two hundred and fifty microliters of chloroform was added to the suspension to solubilize the microspheres. The mixture was rotated end-to-end at room temperature for 60 min. The samples were centrifuged at 12 000  $\times g$  for 5 min and 125–150  $\mu$ l of aqueous supernatant (upper layer) was withdrawn for analysis by absorbance at 260 nm, background corrected against TE buffer. DNA extracted per milligrams of PLGA was

quantified by the Beer–Lambert law (with an absorptivity coefficient of 50  $\mu$ g/ml at  $A_{260\text{nm}} = 1$ ), and analyzed by agarose gel electrophoresis assay (as above).

#### 2.6. In vitro pDNA release

Approximately 10 mg of lyophilized microspheres were accurately weighed and suspended in 1 ml TE buffer with 0.02% (w/v) sodium azide in Eppendorf tubes. The tubes were rotated gently end-to-end at 37 °C for 8 weeks. At predetermined time points (30 min, then 1, 2, 4, 8, 24 and 72 h, then 1, 2, and 4 weeks) Eppendorfs were centrifuged (12 000  $\times g$ , 5 min), the supernatant removed for pDNA quantification (as above), and replaced with fresh buffer. Background readings were obtained using the supernatants from blank microspheres.

#### 2.7. Scanning electron microscopy (SEM)

Approximately 1–2 mg lyophilized microspheres were evenly sprinkled onto a carbon adhesive disk mounted onto an aluminium stub. Samples were coated with a thin layer of gold and imaged on a Jeol JSM-6400 operating at 10 kV, 20 °C and 10<sup>-5</sup> Torr. To image the internal morphology, hydrated microspheres were fractured in situ using a Jeol JSM-6310 cryogenic stage.

#### 2.8. Differential scanning calorimetry (DSC)

The glass transition temperature,  $T_g$ , of a given sample was measured using a differential scanning calorimeter (DSC 822e, Mettler Toledo). Sample analysis was done by accurately weighing approximately 2 mg of microspheres into an aluminium pan which was then hermetically sealed. The reference against which the sample was measured consisted of an empty pin-holed aluminium pan. Both the reference pan and the sample pan were first kept for 5 min at 0 °C to ensure isothermal starting conditions. The pans were then heated at a rate of 10 °C/min from 0 to 85 °C, quench cooled to –20 °C (in order to eliminate any sample history) and heated again to 85 °C at 10 °C/min. The onset  $T_g$  was determined using the bisector method, the  $\Delta C_p$  as the specific heat capacity change over the glass transition, and the enthalpy relaxation as the difference between the specific heat

capacity curve areas over the glass transition before and after quench-cool (Chartoff, 1997).

### 2.9. Cascade impaction

Thirty hard gelatin capsules (type 3) were each loaded with  $30 \pm 2$  mg of dried PLGA microcapsules. A multi-stage liquid impinger (MSLI) (Copley Scientific, UK) was assembled with 20 ml of TE buffer dispensed into stages 2–4, with 15 ml into stage 1 and a glass fiber filter (Gelman Sciences) placed on stage 5 (Department of Health, 2002). The pump (Model HCP4, Copley Scientific) was connected to the MSLI outlet and the pressure was adjusted to 2–4 kPa (Critical Flow Controller Model TPK, Copley Scientific) with the inhaler mouthpiece attached to the induction port, giving an equivalent flow rate of  $60 \pm 5$  l/min (Flowmeter Model DFM, Copley Scientific). (Providing effective cut-off diameters at impaction stages 1, 2, 3, 4 and 5 of 13, 6.8, 3.1, 1.7 and  $<1.7$   $\mu\text{m}$ , respectively.) With the capsule pierced, 4 l of air was drawn through the inhaler and MSLI over 4 s. This was repeated for the subsequent 29 capsules. The glass filter was soaked in 20 ml buffer to extract the DNA, the induction port, inhaler and capsule washings were collected, and the MSLI was agitated rigorously. All solutions were kept unstirred for 24 h in their respective compartments, after which a 1 ml sample from each stage, filter, induction port, inhaler and capsules, was removed, centrifuged at  $12\,000 \times g$  for 5 min, and the pDNA concentration quantified as above. Calculation of geometric mean weight diameter ( $d_g$ ) and the geometric standard deviation ( $\sigma_g$ ) was made from the log-probability plot for cumulative % frequency undersize versus particle size, as described by Martin (1993a).

## 3. Results

### 3.1. Surfactant stabilization of water-in-DCM mixtures is atypical of HLB predictions

We investigated water:DCM ratios of 1:5 and 1:10 for the primary (w/o) emulsion since these are commonly encountered in microsphere preparation. For both ratios, emulsions stabilized with 0.2–1.0% non-ionic sorbitan surfactants (Tween/Span<sup>®</sup>) were relatively stable for blends with HLB values  $\geq 6$  (Figs. 1 and 2). For low surfactant concentrations of 0.05% (w/v), the optimal HLB was  $\geq 11$ . Below these respective HLB values, phase separation was very rapid, occurring almost instantaneously. For low DCM fractions (1:5, w/o), emulsion stability was favored for surfactant blends with an HLB value between 8 and 13 (Fig. 1), whereas hydrophilic surfactants with an HLB  $>16$  appeared to favor high oil fractions (1:10, w/o) (Fig. 2). Blends of surfactants were also seen to yield more stable emulsions compared to HLB-matched single surfactant systems. Most notably, water-in-DCM emulsions were comparatively less stable for addition of Triton 85 compared to the HLB-equivalent Span 80/Tween 80 (0.35/0.65) blend. Tween 20 (HLB 16.5–17) and Span 80 (HLB 4–5) were selected for microsphere preparation in order to observe the effect of good and poor primary emulsion stability, respectively.

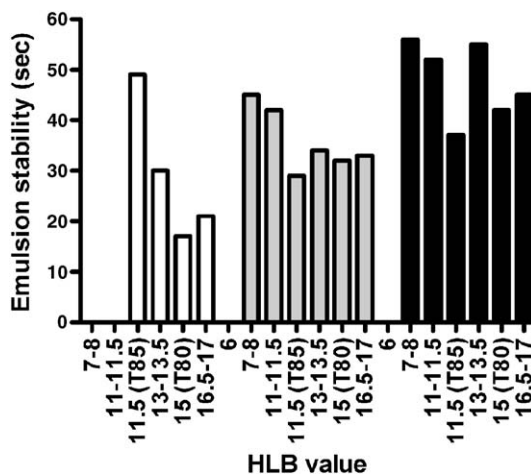


Fig. 1. Stability of water-in-DCM (1:5) emulsions, measured as the time till the first appearance of droplet coalescence. Clear, grey and black bars represent surfactant concentrations of 0.05, 0.2, 1.0% (v/v), respectively. The HLB values shown are calculated for blends of Tween and Span, unless shown as T85 (Tween 85) and T80 (Tween 80).

The Pluronic series of triblock copolymer surfactants were grouped by increasing molecular weight of the hydrophobe (PPO) (Table 1). For each group, a series of blends with HLB values equivalent to the Tween/Span series could therefore be generated. However, in contrast to the relatively hydrophilic Tween/Span blends, the Pluronic copolymers proved to be very poor emulsifiers of water-in-DCM mixtures; phase separation occurring within seconds (data not shown). Increasing the concentration of Pluronic in the emulsion did not improve emulsion stability (as was similarly observed for increasing concentrations of Tween/Span blends with HLB values  $<6$ ). Therefore, for water-in-DCM mixtures containing Pluronic surfactants, neither the HLB nor the fraction hydrophobe were useful predictors of emulsion stability. For this reason, Pluronic copolymers chosen

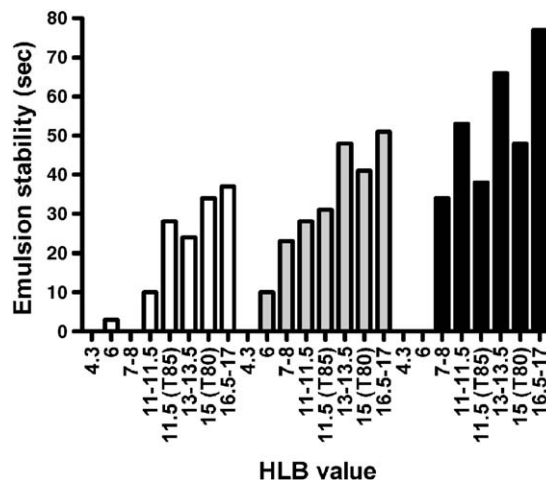


Fig. 2. Stability of water-in-DCM (1:10) emulsions, measured as the time till the first appearance of droplet coalescence. Clear, grey and black bars represent surfactant concentrations of 0.05, 0.2, 1.0% (v/v), respectively. The HLB values shown are calculated for blends of Tween and Span, unless shown as T85 (Tween 85) and T80 (Tween 80).

Table 2

Encapsulation efficiencies and size parameters for microspheres fabricated with surfactants indicated added to the primary emulsion

Surfactant type and fraction in blend	HLB	Median diameter ( $\mu\text{m}$ )	Span	Encapsulation efficiency (%)
PVA	–	13.4	1.39	55.5
Tween 20	16.5–17	14.3	1.32	67.7
Span 80	4.3	10.6	1.28	47.2
L31 <sup>a</sup>	4–5	11.4	1.66	15.0
0.25 L31–0.75 L44	13–13.5	10.5	1.80	26.6
0.25 L62–0.75 L64	13–13.5	12.7	1.80	16.1
0.2 L81–0.8 L62	6	13.7	1.85	28.3
L92	6	12.4	1.68	17.2

<sup>a</sup> Refer to Table 1 for hydrophobe (PPO) MW of the Pluronic surfactants; span =  $(d(0.1) - d(0.9))/d(0.5)$ , where  $d(0.1)$ ,  $d(0.5)$  and  $d(0.9)$  represent the measured diameters at the 10%, 50% (median) and 90% cumulative volumes, respectively. All sample measurements were monomodal and normally distributed in profile.

for microsphere preparation could not be selected on the basis of emulsion stability. However, since the Tween and Span surfactants selected represented high and low HLB values, Pluronic surfactants and blends were selected with similar HLB values (Table 2). PVA was used as a reference given its common use in the literature.

### 3.2. Loading and release of pDNA is dependent on the surfactant used in the primary emulsion

For each surfactant system, pDNA encapsulation efficiency was greatest for the more stable primary emulsion systems (PVA was used as the emulsifier in the secondary emulsion for all systems) (Table 2). The range of encapsulation efficiencies was comparable to previous data for pDNA-loaded microspheres (Walter et al., 1999). Comparison between surfactant type showed that Pluronic-stabilized emulsions performed more poorly than emulsions containing Span 80, despite the latter also demonstrating poor emulsifying activity. Analysis of the pDNA extracted from the microspheres was performed using agarose gel electrophoresis. The two main isoforms of the pDNA, supercoiled and relaxed (open circle), can be clearly observed in Fig. 3. Linear pDNA, which is sometimes seen to arise through homogenization (Benoit et al., 2001), was not observed (and was therefore at most a minor component). This suggested that

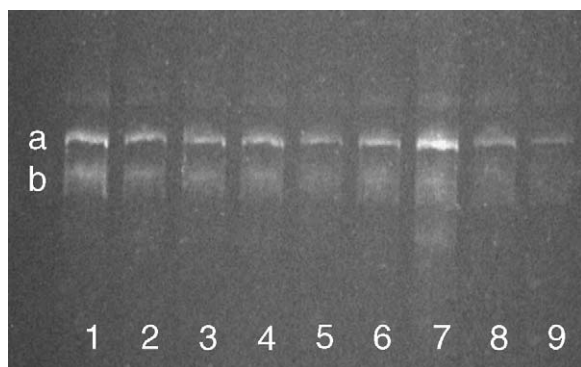


Fig. 3. Analysis of pDNA released from microspheres by agarose gel electrophoresis (lanes 2–8). The major isoforms of pDNA can be seen as relaxed (a) and supercoiled (b). Lane 1 shows pDNA purified from *E. coli*. The surfactants used in the primary emulsion of microsphere fabrication are: 2, PVA; 3, Pluronic L31; 4, Pluronic 0.25 L31–0.75 L44; 5, Pluronic L92; 6, Pluronic 0.25 L62–0.75 L64; 7, Pluronic 0.2 L81–0.8 L62; 8, Span 80; 9, Tween 20.

nicking of the pDNA was not problematic during the emulsion protocols employed.

The *in vitro* release profiles showed that the majority of the encapsulated pDNA was released within the first 1–2 days (Fig. 4). Subsequent to this initial release phase a second, slower release phase was observed to occur over the following month. These release profiles are typically reported and suggestive of pDNA encapsulated within the PLGA matrix of the polyester particles, consistent with the emulsion–evaporation protocol (Capan et al., 1999; Walter et al., 1999; Zhou et al., 2003; Wang et al., 2004). The relative amounts of pDNA released during the secondary release phase were highly variable, with respect to the amount of pDNA released during the initial “burst” release. For Pluronic surfactants with low MW PPO groups in particular (L31 and L44), the extent of pDNA released over this period appeared to be considerable. Similar secondary release profiles have been described and, as discussed below, may be representative of pDNA degradation over this period (Walter et al., 1999).

### 3.3. Pluronic surfactants cause microsphere surface dimpling

The external morphologies of the pDNA-loaded microspheres fabricated using the selected surfactants (Table 2) in

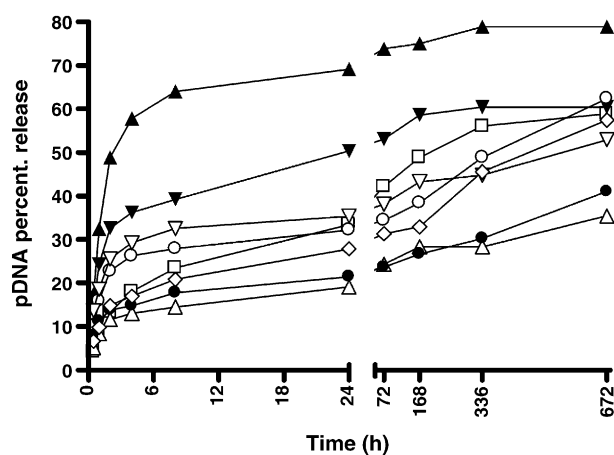


Fig. 4. *In vitro* release profiles of pDNA from microspheres prepared with the following surfactants in the primary emulsion: (□) PVA; (▲) Tween 80; (▼) Span 80; (◇) Pluronic L31; (○) Pluronic 0.25 L31–0.75 L44; (△) Pluronic L92; (▽) Pluronic 0.25 L62–0.75 L64; (●) Pluronic 0.2 L81–0.8 L62.

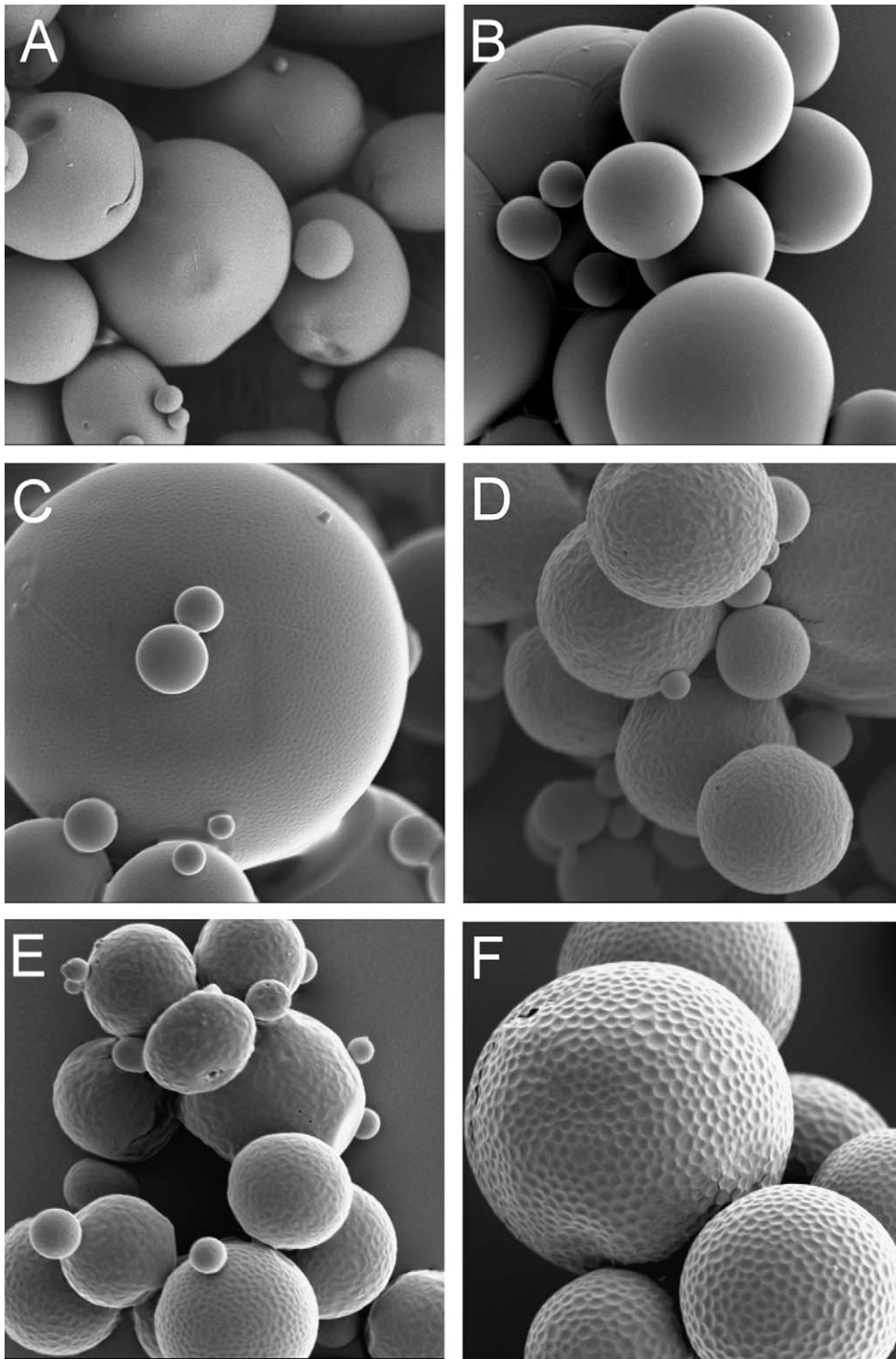


Fig. 5. . Surfaces morphologies of the microspheres fabricated using PVA in the secondary emulsion and the following surfactants in the primary emulsion: (A) PVA; (B) Tween 20; (C) Span 80; (D) Pluronic 0.25 L31/0.75 L44; (E) Pluronic 0.2 L81/0.8 L62; (F) Pluronic L92 (magnification 3500 $\times$ ).

the primary emulsions are shown in Fig. 5. SEM micrographs were obtained for at least three different batches of microspheres to ensure that the morphologies produced were consistent. The micrographs show that the type of surfactant added to the primary emulsion dominated the resultant surface morphology. In this case, the sorbitan surfactants (Tween 20 and

Span 80) produced similar, smooth surfaces, also seen for PVA. In contrast, the Pluronic triblock copolymers revealed an interesting potential to generate dimpled surfaces. For low MW PPO copolymers (Pluronics L31 and L44) the microsphere surface appeared irregular. At intermediate PPO MWs (Pluronics L62 and L64), shallow, dimpled surfaces morphologies

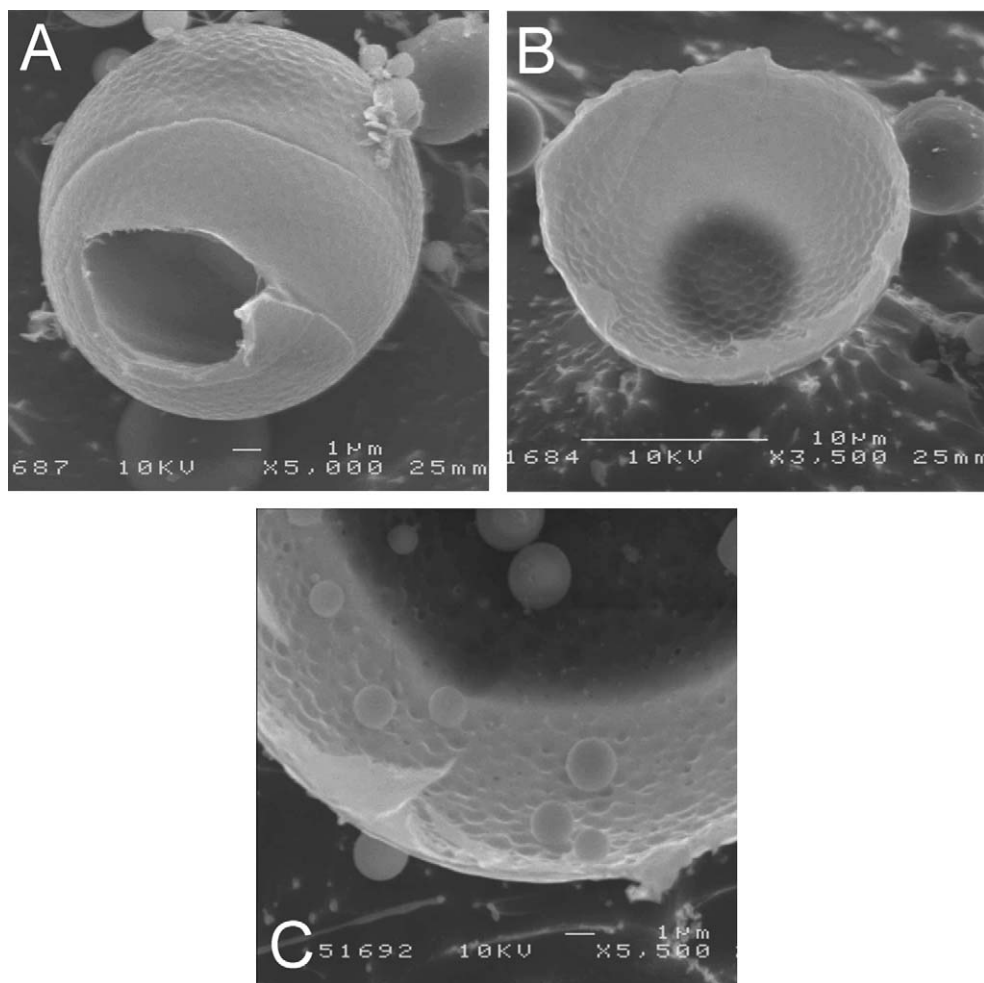


Fig. 6. (A–C) SEM micrographs prepared using freeze-fracture techniques showing the internal morphologies of microspheres fabricated with Pluronic L92 added to the primary emulsion. The microspheres are clearly hollow ('microcapsules') and show the same dimpled morphologies on the internal wall as the surface.

were observed, though more pronounced than for the Pluronic L31/L44 blend. At high PPO MWs (Pluronic L92) the dimples on the microspheres' surfaces were clearly visible. Although the formation of these dimpled surfaces may depend on the inherent instability of the Pluronic-stabilized emulsions, this does not follow given the smooth surfaces seen with Span 80. However, the increased depth and definition of the dimples may have been related to the increasing MW of the PPO block.

Freeze-fracture cryo-SEM imaging clearly showed that the dimpled microspheres prepared with Pluronic L92 were entirely hollow, and may be better described as microcapsules (Fig. 6). Comparison of the scale bar in Fig. 6C against the capsule wall suggests a wall thickness of around 100–300 nm. This morphology is consistent with earlier work demonstrating that unstable primary emulsions in w/o/w systems lead to capsular microspheres due to coalescence of the internal water droplets (Nihant et al., 1994; Schugens et al., 1994). A similar dimpled surface was observed for the internal wall of the microcapsule. However, despite the fragile appearance of the microcapsules, they did not fracture readily. For this reason freeze-fracture techniques were needed to image the internal morphology.

#### 3.4. Tween and Pluronic surfactants cause plasticization of PLGA

The DSC method included a quench-cool step to delete any thermal history. This was done in order to negate any influence acquired during lyophilization, such as aging and changes in water content which are known to induce plasticity. Since the heating rate influences the transition around the  $T_g$  and the values presented here are not absolute but useful for purposes of comparison. For blank microspheres containing no surfactant the measured  $T_g$  corresponded with the manufacturers data (ca. 45 °C) and the difference in the  $T_g$  before and after quench-cool was small (Table 3). Addition of PVA as the emulsifier appeared to have little affect on the  $T_g$  of the microspheres following lyophilization. However, this was due to the large enthalpy of relaxation, seen as the overheating (or overshoot) curve superposed on the glass transition in Fig. 7. Following quench-cool the  $T_g$  was significantly (>2 °C difference) lower than for blank microspheres containing no surfactant. Therefore, PVA interacted with the PLGA polymer network in a manner consistent with that of a plasticizing agent (Passerini and Craig, 2001). The plasticizing affect of PVA was small when com-

Table 3

Thermal properties of blank PLGA microspheres synthesized using the various surfactants as indicated in the primary emulsion, with PVA in the secondary emulsion

Surfactant used in primary emulsion	Glass transition temperature, $T_g$ (onset) ( $^{\circ}\text{C}$ )	Specific heat capacity, $\Delta c_p$ (mW/g)	Enthalpy of relaxation, $\Delta H$ (mJ)
No surfactant	44.86	40.24	5.1
No surfactant <sup>a</sup>	43.42	40.12	
PVA	44.51	45.46	15.96
PVA <sup>a</sup>	38.82	46.36	
Pluronic L92	41.55	36.76	6.48
Pluronic L92 <sup>a</sup>	35.68	38.68	
Tween 20	37.59	46.00	12.13
Tween 20 <sup>a</sup>	29.67	46.51	

<sup>a</sup> Parameters for microspheres following quench-cool;  $T_g$ ,  $\Delta c_p$  and  $\Delta H$  were calculated as described in the methodology.

pared against the fall in the  $T_g$  brought about by the use of Pluronic L92 and Tween 20 as surfactants, both before and after quench-cool (Table 3). Tween 20 in particular appeared to be a strong plasticizing agent, reducing the  $T_g$  well below body temperature.

It was interesting to note that the microspheres aged rapidly – during overnight lyophilization, given the obvious overheating peak (Fig. 7). This was particularly true for microspheres prepared with PVA and Tween 20 in the primary emulsion. Following quench-cool the overheating peak was greatly attenuated as would be predicted by the removal of thermal history for the sample. Typically, as seen here, aging was accompanied by an increase in the  $T_g$ . These data clearly demonstrated that mechanical changes occurred to the microspheres on inclusion of surfactants during emulsification, involving rearrangement of the PLGA chains. However, there did not appear to be a relationship between plasticization of PLGA and surface morphology.

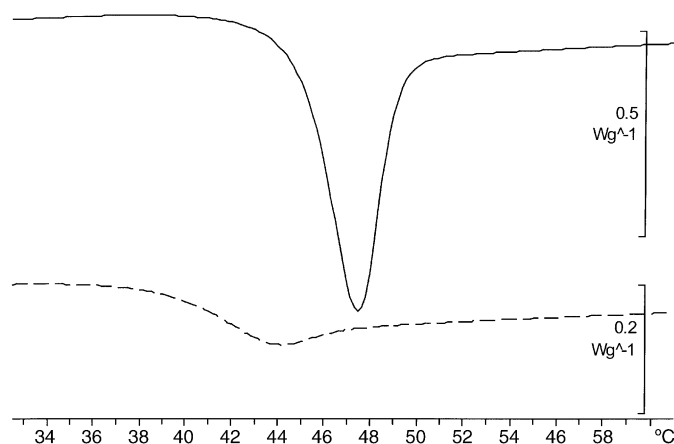


Fig. 7. Representative specific heat capacity curves for microspheres fabricated with PVA immediately following lyophilization in the region of the glass transition. Solid and dashed curves represent the heating measurement before and after quench-cool, respectively. Each curve has a corresponding scale and endothermic peaks point downwards.

### 3.5. PLGA microcapsules fabricated with Pluronic L92 have good aerosol properties

The PLGA microcapsules fabricated with Pluronic L92 had low apparent densities of  $0.24 \text{ g/cm}^3$  (Table 4); lower still than the densities of the large porous particles (Edwards et al., 1997). During scaled-up fabrication, the median diameter of the microcapsules measured by laser diffractometry was seen to be slightly reduced compared to small-scale fabrication ( $7.85 \mu\text{m}$  versus  $12.4 \mu\text{m}$ ). This may have been due to the higher homogenization speeds used. The calculated aerodynamic diameter ( $d_a$ ) for the microcapsules was  $3.81 \mu\text{m}$ , which could therefore be predicted to be deposited in the lung periphery. The flow and dispersion of the microcapsules was further investigated by cascade impaction using an MSLI. The determined geometric mean weight diameter ( $d_g$ ) was  $3.43 \mu\text{m}$ . The log-probability plot reflected the normal size distribution determined by laser diffractometry, the estimated geometric standard deviation ( $\sigma_g$ ) being 1.46 (Table 4). The  $d_g$  therefore supported the prediction that the microcapsules would be useful pulmonary delivery vehicles. The measured span and corresponding  $\sigma_g$  of the microcapsules was reasonable and typically observed for emulsion fabrication methods (Schiavone et al., 2002).

## 4. Discussion

Surprisingly, the sorbitan surfactants with a more hydrophilic nature (e.g. Tween 20) best stabilized the concave interfacial w/o curvature (Winsor type II emulsions (Binks, 1998)). It follows that these surfactants had significant interaction or solvation within the DCM ‘oil’ phase with respect to the aqueous phase. This is in contrast with their behavior predicted by their high HLB values ( $>10$ ) which imply a preferential stabilization of (non-volatile) oil-in-water emulsions, where the greater degree of solvation in the water phase leads to convex interfacial curvatures (Binks, 1998). The behavior of emulsions containing volatile solvent in place of oil can be atypical, although the HLB values remain in certain cases (as seen here with the Tween-Span blends) to be predictive (Cameron and Sherrington, 1996). Given that the degree of interaction with the DCM phase is high, this should be reflected in the penetration of surfactant within the nascent PLGA matrix upon extraction of DCM during secondary emulsion.

Unsurprisingly, emulsion stability improved as sorbitan surfactant concentrations increased, with little or no change observed for surfactants with HLB values  $<6$ . The critical micelle concentration (CMC) of Tween/Span surfactants is low, around  $\sim 0.005\%$  (v/v) in TE buffer (Helenius et al., 1979). Therefore, stabilization of the water-in-DCM emulsions requires surfactant concentrations at least 40-fold above the CMC. Similarly, the improved stabilization afforded by blends of surfactants versus single surfactants (Figs. 1 and 2) is commonly encountered and employed in the calculation of the required HLB of a system (Martin, 1993b). Since the Pluronic triblock copolymers were disappointingly poor water-in-DCM emulsifiers, the solvation of the PPO group into the DCM phase was probably minimal. As for Tween 20, the extent of PPO solvation within



Table 4  
Physical parameters for the PLGA microcapsules fabricated with Pluronic L92 in a scaled-up fabrication process

Median diameter ( $d$ ) ( $\mu\text{m}$ )	Span	Apparent density ( $\rho$ ) ( $\text{g}/\text{cm}^3$ )	Calculated aerodynamic diameter ( $d_a$ ) ( $\mu\text{m}$ )	Geometric mean weight diameter ( $d_g$ ) ( $\mu\text{m}$ )	Geometric standard deviation ( $\sigma_g$ )
$7.85 \pm 0.16$	$1.53 \pm 0.05$	$0.24 \pm 0.04$	$3.81 \pm 0.21$	$3.43 \pm 0.06$	$1.46 \pm 0.04$

Data are expressed as the mean  $\pm$  standard deviation for four independent batches.

the oil phase during primary emulsification may ultimately be reflected by interaction the PLGA chains. We therefore used DSC to characterize surfactant-induced mechanical changes to the microspheres.

As previously described (Capan et al., 1999), poor emulsion stability leads to lower pDNA encapsulation efficiencies. Presumably, coalescence of water-droplets results in mixing of the dispersed and continuous aqueous phases and escape of pDNA. It was interesting that Span 80, despite being a poor emulsifier retained relatively high pDNA-loading efficiencies with respect to the Pluronic surfactants. The mechanism for this is unclear but clearly related to surfactant interfacial behavior. Although the Pluronic surfactants yielded low pDNA-loading efficiencies, this does not necessarily imply that such microspheres will be ineffective DNA-vaccines (Wang et al., 2004). Further work will be required to determine what threshold is required to bring about a clinically useful cell transfection-gene expression response, or CpG-stimulated immune response.

Encapsulated pDNA was released over two phases: an initial burst release followed by a secondary phase which was more variable in nature (Fig. 4). For encapsulation of compounds into PLGA microspheres in general, this secondary phase is thought to represent microsphere morphological affects and/or bulk erosion of the matrix (von Burkersroda et al., 2002; Kim and Park, 2004a). For microspheres prepared with Pluronic L31 and L44 blends, the secondary phase was significant. However, this does not imply morphological changes or more efficient incorporation of pDNA into the PLGA matrix. This is because the pDNA released during the secondary release is thought to become degraded on account of acidic PLGA breakdown products within the microsphere; this gives rise to artificially high release profiles over this period when DNA concentration is measured by UV absorption (Walter et al., 1999). The same reason is likely to be responsible for the small discrepancies observed between pDNA encapsulation efficiency and percentage pDNA release.

The micrographs acquired for microspheres prepared with the selected Tween/Span and Pluronic surfactants (Fig. 5) did not suggest a relationship between HLB and surface morphology, as suggested by a previous study (Bouissou et al., 2004). However, this may be because in that study different surfactant types were used and the protein encapsulated had intrinsic emulsifying activity (cf. the absence of emulsifying activity for pDNA). This study does suggest that surface morphology can be dependent on surfactant type, specifically, the appearance of surface dimples with the use of Pluronic surfactants. More pronounced surface dimples appeared to be related to high MW PPO blocks; this may be due surface desorption rates (Cameron and Sherrington, 1996) but further work is required to determine the underlying

mechanism. The rate of DCM extraction–evaporation and PLGA concentration was not altered between the batches fabricated and therefore was unlikely to have influenced surface morphology in this case (Yang et al., 2001). Surface dimpling may improve particle dispersion via reduction in particle–particle contact energies (cf. smooth surfaces), this would need to be further tested using atomic force microscopy (Young et al., 2002). To the best of our knowledge, similar control of surface morphology with the particular aim of fabricating effective aerosols is only described for bovine serum albumin (BSA) powders (Chew and Chan, 2001; Chew et al., 2005). These BSA particles showed corrugations that were more pronounced than the dimples observed here and their protrusion required measurement of the spherical envelope (Chew and Chan, 2001). However, recent fabrication of BSA particles progressing from smooth to highly corrugated surfaces has shown that only modest corrugations are required to considerably increase aerosol performance (Chew et al., 2005).

Tween 20 was shown to have a strong plasticization effect on the microspheres, which may therefore experience collapse upon warming in vivo above their  $T_g$  (Royall et al., 2001). The penetration of Tween 20 with the PLGA matrix is consistent with the good emulsifying activity observed. In addition, the surfactants may be acting as plasticizing agents by facilitating the adsorption of water within the matrix (Passerini and Craig, 2001). Residual water could also remain in the microspheres if the lyophilization process did not remove strongly adsorbed water. Unfortunately, the end point of the secondary drying phase could not be determined with the lyophilizer used. The microspheres aged during the lyophilization, seen as an overheating peak superposed on the glass transition. The aging of vitrified glassy polymers is commonly encountered on account of the immobilized polymer chains in non-equilibrium conformations and is accompanied by an increase in the  $T_g$  (Chartoff, 1997). While the DSC data clearly demonstrated mechanical changes to the microspheres by inclusion of surfactants, there did not appear to be a relationship between plasticization of PLGA and surface morphology.

While the median diameter of PLGA microcapsules prepared with Pluronic L92 was  $7.85 \mu\text{m}$ , their hollow nature and concomitant reduction in apparent density may be of particular use to pulmonary delivery. Similar strategies have been adopted for pulmonary deposition using hollow lipidic particles (Bot et al., 2000) and large porous particles (Dunbar et al., 2002). For such particles with densities ( $\rho$ )  $< 1 \text{ g}/\text{cm}^3$ , although their median diameter ( $d$ ) may be  $> 5 \mu\text{m}$  (as for the dimpled microspheres), their aerodynamic diameter ( $d_a$ ) is small given that  $d_a = d \times \sqrt{\rho}$ . For the DNA-loaded microcapsules, the determined geometric mean weight diameter ( $d_g$ ) by cascade impaction was  $3.43 \mu\text{m}$ ,

consistent with the calculated  $d_a$  (Table 4). Therefore, these microcapsules show good potential as inhalable particles for pulmonary delivery. To increase the fraction of microspheres deposited in the deep lung, a smaller span (and corresponding  $\sigma_g$ ) would be required. Microspheres with uniform size distributions have been described for membrane emulsification (Ma et al., 1999) and microchannel-mixing techniques (Freitas et al., 2003). While the latter is less suitable for microspheres  $<5 \mu\text{m}$  and scale-up the former technique shows promise (Liu et al., 2005) and we are currently pursuing this route.

In summary, an understanding of water-in-DCM stability for various surfactants and HLB values can improve pDNA-loading into PLGA microspheres and generate novel surface morphologies. The hollow nature of microspheres prepared with Pluronic L92, coupled with their surface dimpling, will be of interest to the development of generic pulmonary delivery vehicles.

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