International Journal of Pharmaceutics 370 (2009) 33–40

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Evaluation of different buffers on plasmid DNA encapsulation into PLGA microparticles

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ARTICLE INFO

Article history:
Received 10 September 2008
Received in revised form 3 November 2008
Accepted 10 November 2008
Available online 18 November 2008

Keywords:
Microparticle
Plasmid DNA
Buffer
PLGA
pH

ABSTRACT

Double emulsion solvent evaporation is a widely used method to prepare poly(DL-lactide-co-glycolide) (PLGA) microparticles encapsulating plasmid DNA. There are inherent problems associated with preparing plasmid DNA in this form, in particular the DNA is liable to degrade during manufacture and the resulting powder has low encapsulation efficiencies. This study compares the use of two buffers, 0.1 M $NaHCO_3$ and 0.07 M Na_2HPO_4 and the effect these have on the encapsulation efficiency and other critical parameters associated with these encapsulated DNA materials. Both buffers preserved the conformation of the original plasmid DNA during the homogenization process, but those made with 0.07 M Na_2HPO_4 had higher encapsulation efficiencies, as well as smaller diameters, compared with those made with 0.1 M $NaHCO_3$ (encapsulation efficiencies of 40.72–45.65%, and mean volume diameters of 2.96–4.45 μm). Buffers with a range of pH from 5 to 12 were investigated, and it was demonstrated that pH 9 was the point at which the highest amount of supercoiled DNA was balanced with the highest encapsulation efficiency. To simulate *in vitro* release, it was shown that microparticles made with 0.07 M Na_2HPO_4 had lower DNA release rates than those made with 0.1 M $NaHCO_3$. These results demonstrate that the use of different buffers can aid in retaining the conformation of plasmid DNA, and can also modulate the amount of DNA encapsulated and the release profiles of microparticles.

1. Introduction

Biodegradable microparticles, such as those made from poly(DL-lactide-co-glycolide) (PLGA), offer many advantages as carriers of plasmid DNA including increased safety in comparison to viral vectors of DNA delivery, protection of DNA from *in vivo* degradation, and the manipulation of release rates of DNA by using different polymers to fabricate the microparticles (Diez and Tros de Ilarduya, 2006; Tinsley-Bown et al., 2000; Wang et al., 1999). Control of release rates of DNA could be advantageous in instances for which the microparticles are used for immunization purposes, for example, to produce a one-shot vaccine or to mimic the initial vaccine/booster effect (Langer et al., 1997; O'Hagan et al., 2006).

Double emulsion ($w_1/o/w_2$) solvent evaporation is one of the most widely used methods of preparing biodegradable microparticles; however, the main problems encountered when encapsulating plasmid DNA using this method is the conversion of the supercoiled form of the plasmid DNA into open-circular/linear form. Conservation of the supercoiled form of plasmid DNA is important as it is generally thought for genetic transfection of foreign DNA into cells (be it prokaryotic or eukaryotic), the supercoiled form of plasmid DNA offers the highest efficiency of transfection (Adami et al., 1998; Hao et al., 2000).

Shear stress has been cited as the main cause of plasmid DNA degradation in methods that use homogenization processes to make the microparticles (Jilek et al., 2004; Kuo, 2003; Lentz et al., 2005). In addition, it has been shown that DNA is pH-sensitive: exposure of plasmid DNA to pH ≤ 3 resulted in rapid loss of transfectivity of HEK293 cells (Walter et al., 1999). Also, large deviances of pH from normal have been shown to contribute to the breakdown of DNA in aqueous solution by way of hydrolysis. Although this process is slow, it can be accelerated if the pH is below 7.5 or above 9.0 (Lentz et al., 2005).

Addition of buffers such as $NaHCO_3$ (Li et al., 2003; Walter et al., 1999) and simple salts such as NaCl (Lengsfeld and Anchordoquy, 2002) have been shown to protect DNA against shear stress and pH-related degradation. In this study, two buffers were selected for their protective effects—0.1 M $NaHCO_3$ (pH 8.3) and 0.07 M Na_2HPO_4 (pH 9.1). These were chosen because they are approved for use in humans. Furthermore, microparticles made with buffers at different pH ranges were also investigated to evaluate the effects on the morphology, encapsulation efficiency, conformation of plasmid DNA and size of the microparticles produced.

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doi:10.1016/j.ijpharm.2008.11.006

2. Materials and methods

2.1. Preparation of microparticles

Three hundred mg of PLGA (Lakeshore Biomaterials, USA) (ratio 50:50 DL-lactic acid and glycolic acid, approximately 58.8 kDa, inherent viscosity 0.38–0.48 g/100 ml according to the manufacturer) was dissolved in 5 ml of dichloromethane. An internal aqueous solution containing 200 μg plasmid DNA (200 μl of a 1 mg/ml solution; gWiz Luciferase, Aldevron, USA) and 500 μl 10% (m/v) polyvinyl alcohol (PVA, 13–23 kDa, 87–89% hydrolysed, inherent viscosity of 0.38–0.48 g/100 ml according to manufacturer; Sigma–Aldrich, Poole, UK) dissolved in either distilled water, 0.1 M $NaHCO_3$ or 0.07 M Na_2HPO_4 was emulsified into the polymer/solvent mix using an Ultraturrax T25 homogenizer (Janke & Kunkel IKA Labortechnik, Germany) with a 1 cm diameter probe. The primary emulsion was homogenized for 2 min at a speed of 24,000 rpm. This primary emulsion was then added drop-wise and emulsified into 40 ml of a second aqueous phase containing 2.5% (m/v) PVA (dissolved in either distilled water, 0.1 M $NaHCO_3$ or 0.07 M Na_2HPO_4), with an L4RT homogenizer (Silverson, UK) equipped with a 2.5 cm diameter probe. The secondary emulsion was homogenized for 5 min at a speed of 9500 rpm. After which, a magnetic bar was added into the resulting $w_1/o/w_2$ mixture and this was left to stir at room temperature for about 3–4 h on a magnetic stirrer plate for the solvent to evaporate, the polymer to precipitate and the microparticles to form. After the solvent had evaporated, the microparticles were washed two to three times with water and centrifuged at 15,000 rpm for 15 min before being freeze-dried. All preparative solutions were kept at 4 °C overnight before being homogenized and homogenization of all emulsions was carried out on ice. For particles without DNA ('blank'), 200 ml of Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) was used in the w_1 phase, which was equivalent to the volume of DNA added.

For studies that examined the effect of different pH, buffers were prepared using different ratios of monosodium phosphate (anhydrous) and trisodium phosphate dodecahydrate (BDH, UK) at 0.07 M (so that it is the same molarity as the Na_2HPO_4 solution). The different pH solutions were then used to make up the internal and external PVA solutions. DNA-loaded and blank microparticles were made using the same method as described above.

2.2. Particle characterization

2.2.1. Size

Approximately 2.5 mg of dried particles were re-suspended into 1.0 ml of water. The resulting solution was briefly vortexed (5 s) and sonicated in a bath sonicator (5–10 min). The sample was analysed at an obscuration of 10–20% using a Mastersizer (Malvern Instruments, UK), equipped with a 5 mW Helium-Neon laser (632.8 nm). The mean volume diameter (MVD) (D 4,3), the distributions D[v0.1], D[v0.5] and D[v0.9], and span were recorded.

2.2.2. Zeta potential

Approximately 2.5 mg of dried particles were re-suspended into 1.0 ml of 5% (m/v) D-mannitol (VWR International, UK). The suspension was then sonicated for 10 min before being diluted 1:50 (with 5% (m/v) D-mannitol) for analysis on a Malvern Zetasizer (Malvern, UK). Readings were carried out at count rates were between 2000 and 2500 kcps.

2.2.3. Scanning electron microscopy

Dried particles were mounted onto a stub with double-sided carbon impregnated discs using a fine hair paintbrush. Samples were then sputter-coated with gold using an Emitech K550 Sputter Coater for 2 min at 30 mA. Samples were viewed under a Phillips/FEI XL30 scanning electron microscope.

2.2.4. Extraction of DNA from particles

Ten mg of particles were weighed out into a 1.5 ml centrifuge tube. 200 ml Tris–EDTA buffer was added and the mixture was vortexed briefly. 500 ml chloroform was added and again, the sample was vortexed. The suspension was shaken horizontally in a 37 °C incubator for 90 min. After 90 min, the mixture was centrifuged at 14,000 rpm for 10 min. The aqueous layer (approximately 150 μl or less to avoid contamination from the interface) was taken off and put into a fresh 1.5 ml centrifuge tube. To account for any changes to the plasmid DNA that the extraction process might cause, an equivalent amount of unprocessed DNA was added to 10 mg of blank particles and processed in the same manner.

2.2.5. Analysis of extracted DNA

Plasmid DNA extracted from microparticles were analysed by agarose gel electrophoresis (0.7–0.8% (m/v) of agarose in Tris–borate–EDTA (TBE) buffer (0.089 M Tris base; 0.089 M boric acid; 0.002 M EDTA, final pH 8.3)). The agarose gel was stained with ethidium bromide and viewed under a UV transilluminator. Images were captured using a Syngene GeneGenius Bioimaging system (Syngene, UK), and quantification of the different conformations of plasmid DNA was performed using the software GeneTools v.3.00(h) (Syngene, UK). Percentage of supercoiled DNA was calculated as the ratio of DNA in relation to the other conformations in the sample.

The Picogreen® (Molecular Probes, USA) assay was used to quantify plasmid DNA extracted from microparticles. The analysis was carried out according to the manufacturer's instructions. It was ensured that the samples were diluted to within the working range of the kit (250 pg/ml to 2 μg/ml) and that the same DNA used for encapsulation into the particles was used for the standard. Dilutions were made with Tris–EDTA buffer (pH 8.0). Samples were analysed in black 96-well plates (Fisher Scientific International) and read at excitation 485 nm/emission 535 nm using a Wallac Victor2™ 1420 Multilabel Counter (PerkinElmer Wallac, UK).

2.3. In vitro release of DNA from microparticles

Ten milligrams of particles were accurately weighed out into 1.5 ml centrifuge tubes. 1 ml of phosphate buffered saline (PBS; pH ~7.4) containing 0.02% (m/v) sodium azide was added into each tube. These were placed horizontally into an orbital shaking incubator set at 150 rpm and a temperature of 37 °C. At each time point the samples were centrifuged at 14,000 rpm for 2 min. 1 ml of the supernatant was taken out for analysis. Fresh PBS/sodium azide solution was added to the pellet and the pellet was fully re-suspended before placing back into the incubator. Supernatants were stored at −20 °C if not analysed straight away. Each formulation, was sampled three times at each time point. Samples of microparticles without encapsulated DNA were also incubated under the same conditions to serve as a reference value during analysis. Supernatants were analysed using Picogreen® and agarose gel electrophoresis.

2.4. Surface area analysis via gas adsorption

The surface area of select formulations was measured by the Brunaeur–Emmet–Teller (BET) technique using a Beckman Coulter SA3100 Surface Area Analyzer (Beckman Coulter Ltd, High Wycombe). The empty sample holder, stopper and filling rod were first weighed. Then samples (between 0.5 and 1.0 mg) were added carefully into the holder and the whole assembly was weighed again. The samples were outgassed under helium at 30 °C for 300 min and then re-weighed. After the samples were outgassed,

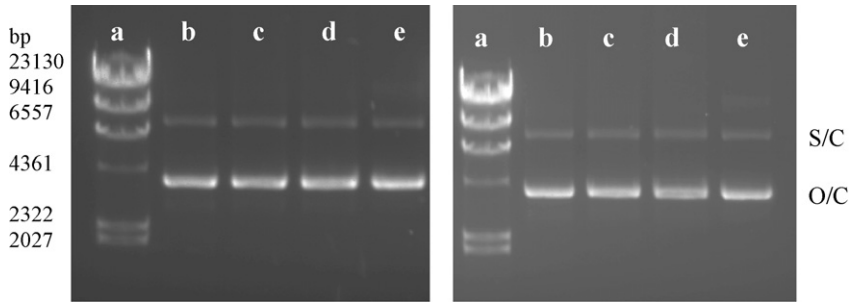

Fig. 1. Agarose gel image of plasmid DNA homogenized at 24,000 rpm for 2 min in buffered or non-buffered solution and with (left) or without (right) 10% (m/v) PVA. (a) DNA/Hind III ladder; (b) in water; (c) in 0.1 M $NaHCO_3$; (d) in 0.07 M Na_2HPO_4; (e) Control DNA. O/C, open circular DNA; S/C, supercoiled DNA.

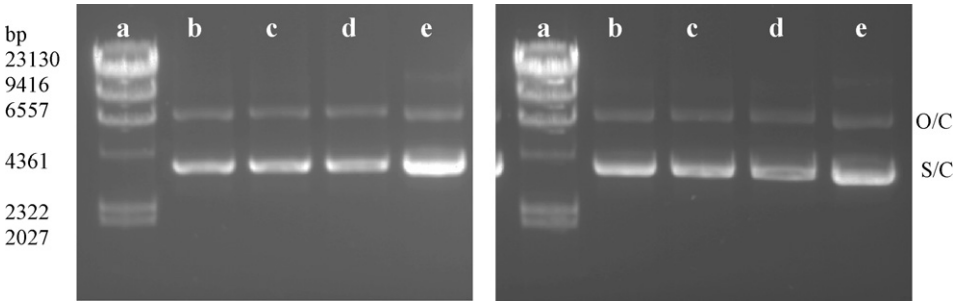

Fig. 2. Agarose gel image of plasmid DNA homogenized at 24,000 rpm for 2 min in presence of dichloromethane. In buffered or non-buffered solution and with (left) and without (right) 10% (m/v) PVA. (a) λ DNA/HindIII ladder; (b) in water; (c) in 0.1 M $NaHCO_3$; (d) in 0.07 M Na_2HPO_4; (e) control DNA. O/C, open circular DNA; S/C, supercoiled DNA.

they were immersed in liquid nitrogen to allow the gas to form a monolayer on the surface of the microparticles. The gas used for adsorption was nitrogen. The surface area was calculated using the SA-VIEW™ Software (Beckman Coulter Ltd, High Wycombe). Each sample was measured twice.

2.5. Statistical analysis

Three batches of microparticles were made for each formulation. All measurements were performed in triplicate and data shown as the mean with standard deviation.

3. Results

3.1. Characteristics of particles made with 0.1 M $NaHCO_3$ or 0.07 M Na_2HPO_4

Initial studies demonstrated that the primary degradation step for plasmid DNA degradation was when the polymer was added to the solution (Figs. 1–3). When the plasmid DNA was homogenized in the presence of polymer with no buffer present the DNA was degraded to its open-circular and linear conformation after the primary emulsification step (Fig. 3, Table 1). This process

Table 1
Particle yield, encapsulation efficiency and zeta potential of particles made with and without buffer. Mean ± S.D. of three readings.

Formulation	Zeta potential (mV)	Encapsulation efficiency (%)	Loading (μg DNA/mg polymer)	Supercoiled content (%)[a]
No buffer (−DNA)	−23.11 ± 2.48	n/a	n/a	n/a
No buffer (+DNA)	−22.90 ± 2.47	32.77 ± 12.96	0.22 ± 0.09	2.81 ± 2.81
$NaHCO_3$ (−DNA)	−23.51 ± 2.31	n/a	n/a	n/a
$NaHCO_3$ (+DNA)	−24.58 ± 1.58	40.72 ± 4.32	0.27 ± 0.03	66.14 ± 3.37
Na_2HPO_4 (−DNA)	−24.52 ± 2.35	n/a	n/a	n/a
Na_2HPO_4 (+DNA)	−23.70 ± 2.16	45.65 ± 5.27	0.30 ± 0.04	61.06 ± 3.83

[a] Unprocessed DNA = 77.01 ± 1.53; $n = 3$.

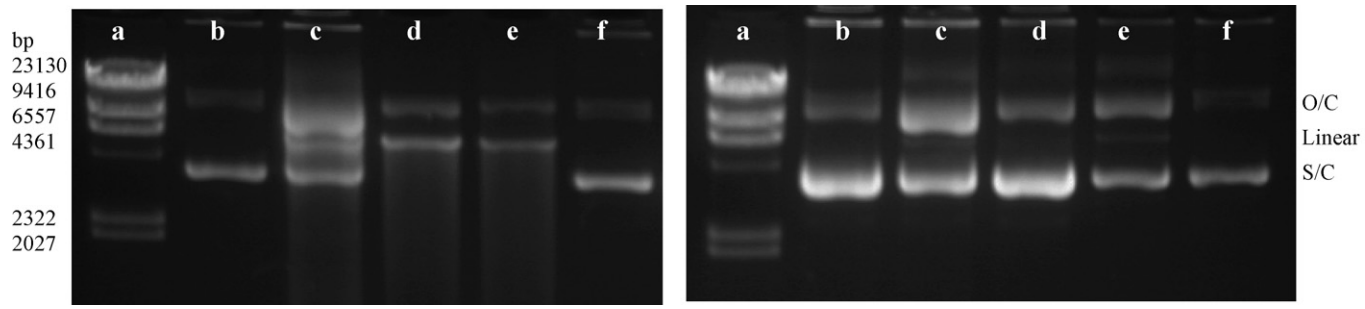

Fig. 3. Agarose gel electrophoresis pictures of plasmid DNA extracted after each stage of the double emulsion solvent evaporation process. Left-hand image shows no buffer. Right-hand image shows with buffer (0.1 M $NaHCO_3$ and 0.07 M Na_2HPO_4 had same results). (a) λ DNA/HindIII ladder; (b) in 10% (m/v) PVA overnight; (c) after primary emulsion; (d) after secondary emulsion; (e) after freeze-drying; (f) untreated DNA. O/C, open circular DNA; S/C, supercoiled DNA.

Table 2
Size distributions of particles made with and without buffer. Mean ± S.D. of three readings.

Formulation	MVD (μm)	D[v10] (μm)	D[v50] (μm)	D[v90] (μm)	Span
No buffer (−DNA)	1.86 ± 0.15	0.73 ± 0.04	1.50 ± 0.14	3.45 ± 0.34	1.81 ± 0.19
No buffer (+DNA)	2.23 ± 0.14	0.79 ± 0.04	1.80 ± 0.16	4.29 ± 0.20	1.96 ± 0.10
$NaHCO_3$ (−DNA)	4.56 ± 0.29	1.78 ± 0.08	4.21 ± 0.34	8.01 ± 0.48	1.48 ± 0.03
$NaHCO_3$ (+DNA)	4.45 ± 0.12	1.78 ± 0.16	4.14 ± 0.13	7.68 ± 0.40	1.42 ± 0.12
Na_2HPO_4 (−DNA)	2.88 ± 0.02	1.32 ± 0.10	2.66 ± 0.07	4.77 ± 0.10	1.30 ± 0.11
Na_2HPO_4 (+DNA)	2.96 ± 0.21	1.31 ± 0.22	2.69 ± 0.28	4.99 ± 0.14	1.41 ± 0.18

Table 3
Encapsulation efficiency and the percentage of supercoiled content of the DNA extracted out from the microparticles made with a higher theoretical loading (0.5% (m/m)). Mean ± S.D. of three readings.

Formulation	Encapsulation efficiency (%)	Loading (μg DNA/mg polymer)	Supercoiled content (%)[a]
No buffer	23.39 ± 1.00	1.17 ± 0.05	0 ± 0.00
0.1 M $NaHCO_3$	61.05 ± 14.42	3.05 ± 0.72	69.67 ± 1.08
0.07 M Na_2HPO_4	28.57 ± 7.22	1.43 ± 0.36	55.42 ± 3.21

[a] Unprocessed DNA = 72.64 ± 2.52; n = 3.

was irreversible, and the electropherisis image showed streaking underneath the bands of DNA, which was indicative of further degradation to the DNA. When the microparticles were prepared in the presence of either 0.1 M $NaHCO_3$ or 0.07 M Na_2HPO_4 the streaking was significantly reduced, which demonstrates that to a large extent the DNA maintained the supercoiled conformation. A more detailed analysis of the conformations of the plasmid DNA from the different formulations demonstrated that the DNA extracted from the microparticles made with 0.1 M $NaHCO_3$ had a slightly higher amount of supercoiled DNA (66.14 ± 3.37%) compared to that of 0.07 Na_2HPO_4 (61.06 ± 3.83%) (Table 1).

All the formulations had similar negative charges, which is probably a reflection of the residual PVA and the phosphate groups of the DNA. The encapsulation efficiencies were notably different between microparticles made without buffer and those made with buffer (Table 1). Although the buffered particles showed similar encapsulation efficiencies, the diameters of microparticles made with 0.07 M Na_2HPO_4 were slightly smaller and with a narrower size distribution compared to the microparticles made with 0.1 M $NaHCO_3$ (Table 2).

As these were preliminary experiments, the loading of plasmid DNA was kept low (0.67 μg DNA/mg polymer); however, when higher theoretical loadings were used (5 μg DNA/mg polymer) the actual loading of DNA in the microparticles were able to be increased (Table 3). There were differences in the encapsulation efficiencies and the supercoiled content of the microparticles made with higher theoretical loading, and from the results it looks like those made with 0.1 M $NaHCO_3$ had better overall properties. It is unclear at present without doing further experiments why these differences occurred.

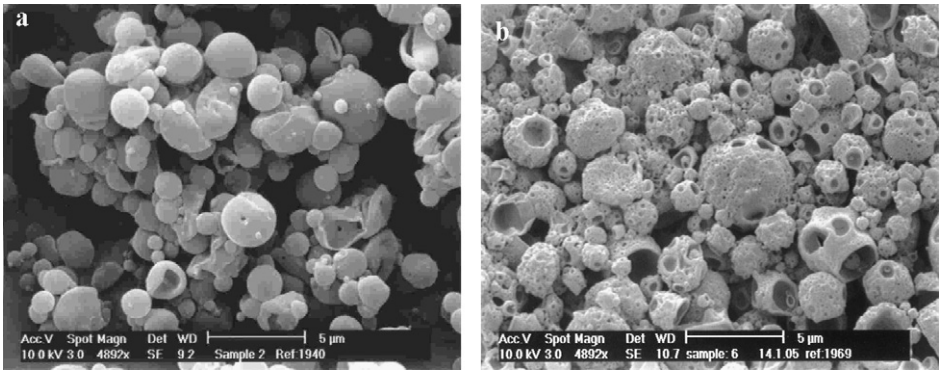

Fig. 4. Scanning electron microscopy images of microparticles: (a) without buffer and (b) with buffer (both types of buffer showed similar morphologies). Note the porous nature of the buffered microparticles. DNA-loaded and blank particles showed no difference in morphology.

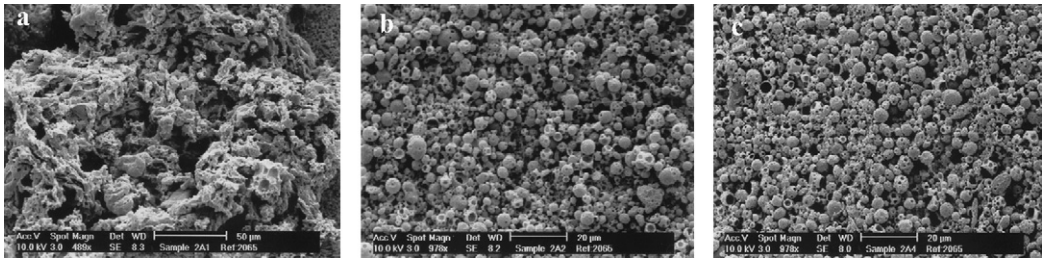

Fig. 5. SEM images of the morphology of microparticles made with 0.1 M $NaHCO_3$ as buffer at different stages of $w_1/o/w_2$ double emulsion solvent evaporation process. Samples were taken out after each step and air dried onto 0.22 μM filter paper before being sputter coated and examined under SEM: (a) after primary emulsion; (b) after secondary emulsion; (c) after secondary emulsion and after 4 h evaporation. Microparticles made without buffer or with 0.07 M Na_2HPO_4 had similar results in that the morphologies of the particles were formed after the secondary emulsion step but before the washing step.

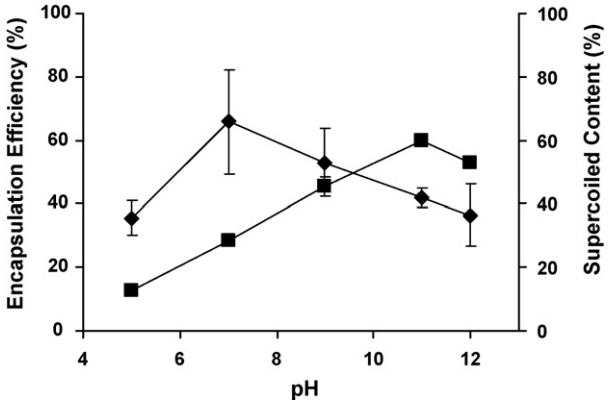

Fig. 6. Graphical representation of % encapsulation efficiency (♦) and % supercoiled content (■) vs. pH.

The morphologies of the microparticles produced with and without buffer in the aqueous phase also demonstrated marked differences (Fig. 4). The microparticles produced in presence of buffer had a porous structure and there was also an influence on the diameters of the particles. For instance, the D[v50] of microparticles encapsulating DNA made without buffer was 1.80 ± 0.16 μm, whereas those made with 0.1 M $NaHCO_3$ and 0.07 M Na_2HPO_4 were 4.14 ± 0.13 and 2.69 ± 0.28 μm, respectively. Indeed, the added porosity of the microparticles made with buffer also increased the overall surface area of these microparticles as demonstrated by results from BET (Table 4).

A further study was carried out where the emulsions were taken out at different stages of the manufacturing process to observe when the porosity occurred. Morphologies of all particles were formed after the secondary emulsion and during the evaporation process (Fig. 5). It was hypothesized that the salt from the buffer particles would dissolve out during the washing process due to the concentration gradient and leave the porous structures behind, but it has been demonstrated that this is not the case since the morphologies are formed before the washing step (Fig. 5).

Table 4
Surface area analysis of microparticles encapsulating plasmid DNA. Mean ± S.D. of three readings.

Sample	Mass (g)[a]	BET surface area (m^2/g)	
		Reading 1	Reading 2
No buffer	0.81844	6.705	6.825
0.1 M $NaHCO_3$	0.72456	12.814	12.976
0.07 M Na_2HPO_4	0.75351	13.019	13.051

[a] Mass of microparticles after outgassing.

Table 5
Size distributions of particles made with different pH buffers. Mean ± S.D. of three readings.

pH	MVD (μm)	D[v0.1] (μm)	D[v0.5] (μm)	D[v0.9] (μm)	Span
5	2.06 ± 0.29	0.70 ± 0.03	1.38 ± 0.13	3.89 ± 0.22	2.32 ± 0.08
7	2.68 ± 0.15	0.94 ± 0.02	2.10 ± 0.04	5.31 ± 0.59	2.08 ± 0.25
9	2.48 ± 0.19	0.89 ± 0.11	1.96 ± 0.17	4.65 ± 0.15	1.93 ± 0.15
11	3.58 ± 0.12	1.59 ± 0.21	3.21 ± 0.16	6.05 ± 0.01	1.39 ± 0.14
12	5.18 ± 0.09	2.57 ± 0.09	4.78 ± 0.09	8.34 ± 0.07	1.21 ± 0.03

3.2. Effect of different pH on plasmid DNA and particle parameters

To elucidate the effect of pH on the parameters of the microparticles, a range of phosphate buffered solutions were made at 0.07 M concentrations and at pH ranging from 5 to 12. There was a correlation between the pH and amount of DNA encapsulated and also between the pH and supercoiled content, as shown in Fig. 6. For the pH ranges 7–12, there is a linear decrease in the encapsulation efficiency. But, for the supercoiled content, there is a linear increase between the pH ranges of 5 and 11. It is proposed that pH 9 is the best compromise for combined encapsulation efficiency and degree of supercoiled content and this also happens to be the pH of the buffer solution 0.07 M Na_2HPO_4.

The mean microparticle size, as measured by laser diffraction, was about 2 μm for formulations prepared at low pH, and for pH above 9 there seemed to be an increase in microparticle size (Table 5). This trend was confirmed by SEM and the images also showed that at higher pHs there was a change in porosity (Fig. 7). At pH 5, the particles were smooth and spherical, for pH 7 and 9 there was a degree of porosity but also intense aggregation of the microparticles, which was decreased at pH 11 and 12 (Fig. 7).

3.3. Release of DNA from microparticles

A burst release of DNA was observed within 24 h of the study, which then proceeded at a steady rate (Fig. 8). Formulations made with 0.1 M $NaHCO_3$ demonstrated the most rapid release rate, and also released the greatest amount of DNA within the time stud-

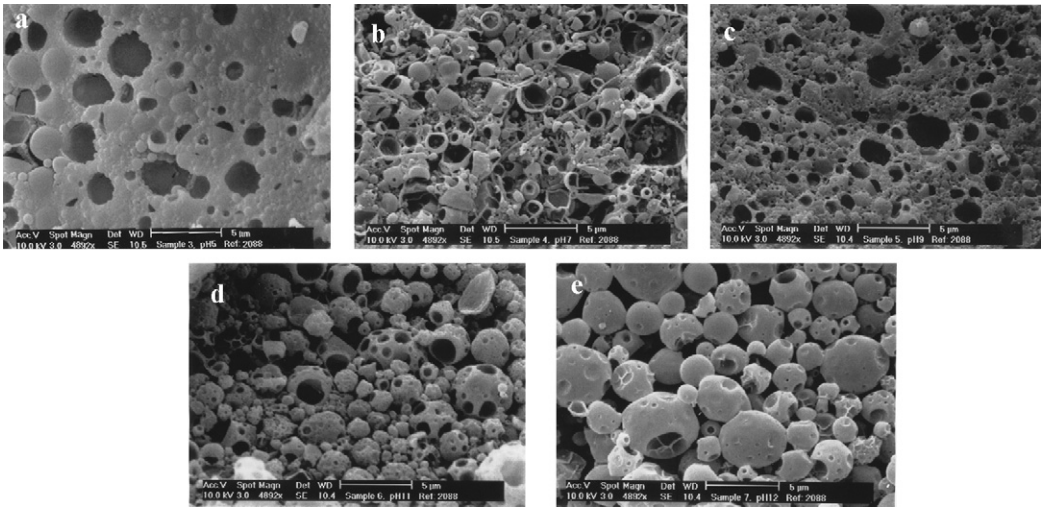

Fig. 7. SEM pictures of microparticles made with different pH: (a) pH 5, (b) pH 7, (c) pH 9, (d) pH 11 and (e) pH 12.

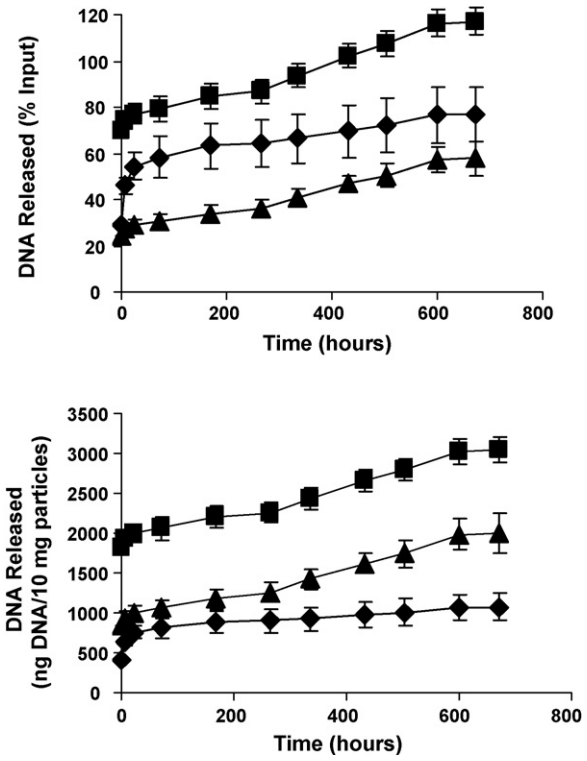

Fig. 8. Cumulative percentage and total amount of DNA released from each formulation over a period of 28 days. No buffer (♦); 0.1 M $NaHCO_3$ (■); and 0.07 M Na_2HPO_4 (▲). Mean ± S.D.; $n = 3$.

ied. By contrast, particles made with 0.07 M Na_2HPO_4 showed the lowest release rate (only 57.81 ± 7.70% after 28 days), but with an intermediate amount of DNA released, owing to its higher initial encapsulation efficiency.

4. Discussion

For the studies that investigated the effect of homogenization on the plasmid DNA conformation, it was shown that plasmid DNA was not affected by shear stress or presence of organic solvent. It was not until the addition of polymer that there was a change, and this is in agreement with previous studies (Walter et al., 1999). Although, in this case, homogenization did not degrade plasmid DNA that was in its original solution (i.e. without $NaHCO_3$ or Na_2HPO_4), which is in contrast to previous findings. This may be due to Walter et al. using sonication, which has a higher energy output into the system than homogenization (Walter et al., 1999). Degradation of the DNA by sonication was prevented in the presence of $NaHCO_3$, but it did not prevent degradation during spray-drying. In a later study, the authors are unclear whether degradation was caused directly by the spray-drying process, or whether the DNA degraded during sonication in the presence of polymer (Jilek et al., 2004).

A similar study was carried out with 0.9% NaCl, 0.1 M $NaHCO_3$ (pH 8.0) and PBS (pH 8.0) as buffers to protect DNA during primary emulsification (Li et al., 2003). It also confirmed the protective effect of salt on the conformation of the plasmid DNA, with 0.1 M $NaHCO_3$ having the greatest protective effect. The presence of polymer during primary emulsification also led to more degradation of the plasmid DNA (Li et al., 2003).

It is uncertain why the addition of polymer at the emulsification step should cause the DNA to change conformation, but there are a number of possible mechanisms. The addition of polymer could result in an increase in the viscosity of the solution would increase the shear stress on the DNA and therefore cause strand breakage. Alternatively, there maybe complex interactions occurring between the organic and aqueous phases causing entanglement effects between the polymer, PVA and DNA chains (Boury et al., 1995; Lee et al., 1999). PVA is used in a wide range of industrial, commercial, medical and food applications (DeMerlis and Schoneker, 2003), and in $w_1/o/w_2$ double emulsions it is well known for its properties as a stabilizer of the emulsion formed (Jeffery et al., 1991, 1993; Prabha and Labhasetwar, 2004). It has been proposed that the hydrophobic vinyl acetate part of partially hydrolysed PVA serves as anchor polymer at the oil interface, and once it is entangled with the polymer, it is much more difficult to remove from the surface (Boury et al., 1995; Lee et al., 1999). These entanglement effects between the polymer and PVA could trap the plasmid DNA and therefore cause conformational changes to the DNA due to shear stress.

There are many other factors which might affect DNA condensation including electrostatic forces, hydrodynamic interactions, base composition and length of the DNA and the ionic strength of the solution in which the DNA is present and these have been discussed previously in the literature (Bloomfield, 1996; Lyubchenko and Shlyakhtenko, 1997; Schlick et al., 1994).

Previous literature has shown that the structure of supercoiled DNA changes easily depending upon the ionic strength of the solution (Lyubchenko and Shlyakhtenko, 1997). Computational models on the effect of different NaCl concentrations demonstrated that Na^+ ions shield the phosphates on the DNA backbone, which can lead to coils moving into closer proximity and therefore, to a reduction in diameter. If there are not enough ions present, then the coils are more open in structure (Schlick et al., 1994). It is likely that the protective effect of 0.1 M $NaHCO_3$ and 0.07 M Na_2HPO_4 buffers is to reduce the diameter of the plasmid DNA so that it is less likely to be damaged during homogenization (Li et al., 2003; Walter et al., 1999). In this study, it seemed that microparticles made with 0.07 M Na_2HPO_4 offered more advantageous characteristics compared with those made with 0.1 M $NaHCO_3$, in terms of a narrower diameter distribution and higher encapsulation efficiency. Additional benefits may include the porosity of the microparticles formed, which might make them useful for pulmonary delivery (porous particles being less dense), and thus facilitate different deposition kinetics that could be advantageous for delivery into the deep lung (Bosquillon et al., 2001).

Not many researchers have used buffers/salts in the w_1 and w_2 phases, which makes it more difficult to interpret and compare results directly. In previous studies that have used 0.1 M $NaHCO_3$ (Walter et al., 2001), the morphology of the particles shown in images were not porous, although this is probably due to the different methodology used to make the particles (a single w/o emulsion, which was then spray-dried); and the different polymers used (a range of PLGA Resomer from Boehringer, Ingelheim, Germany). In another study where an o/w emulsion was used to entrap quinidine sulphate, the addition of various inorganic salts (NaCl, KSCN, Na_2SO_4, NaBr, NaSCN and $NaClO_4$) led to different effects on the loading efficiency, with some salts increasing loading efficiency and others reducing it. This was explained by the salts acting in two ways, either by depressing the drug aqueous solubility or by increasing the organic solvent solubility in the aqueous phase (Al-Maaieh and Flanagan, 2001). The salts used for the present study may also act in a similar way and this could help to explain the morphologies obtained. If the presence of salt increases the solubility of dichloromethane, then the evaporation rate maybe faster thus producing more pores.

The pH dependence results obtained in this report are in agreement with a previous study where a range of 6–9 demonstrated an increase in plasmid DNA stability with pH 9 offering the best protection (Evans et al., 2000). There appears to be a trend whereby

higher salt contents cause an increase in the total DNA encapsulation efficiency and an increase in the protection of the supercoiled DNA. This maybe linked to the pH dependence and is likely to be due to the compactness of the DNA structure.

Various studies have highlighted the effect of using different polymers (e.g. different ratios of lactide to glycolic acid and molecular weights) to control the release rate of DNA (Diez and Tros de Ilarduya, 2006; Tinsley-Bown et al., 2000; Wang et al., 1999). However, it is unclear how or what release profile would be ideal for DNA vaccines. The release profile of these microparticles is in agreement with previous studies that have encapsulated model plasmid DNA into PLGA 50:50 micro/nanoparticles using the double emulsion evaporation method (Cohen et al., 2000; Diez and Tros de Ilarduya, 2006; Mohamed and van der Walle, 2006), i.e. a rapid initial release, followed by a more prolonged release of lower quantities of DNA. The actual mechanics of how substances are released and how PLGA degrades in a microparticulate form are still a matter of debate. There have many studies that have analysed or modelled release rates of proteins or other entities from microparticles (O'Hagan et al., 1994; Cleland et al., 1997; Capan et al., 1999; Yang and Alexandridis, 2000; Wang et al., 2002); and the degradation profiles of PLGA polymer (Reed and Gilding, 1981; O'Hagan et al., 1994; Gopferich, 1996; Shive and Anderson, 1997) and therefore, details will not be discussed here. It was also not the intention of this study to analyse how polymer degradation and release profiles were related, and so, this aspect will not be discussed further. However, it is generally thought that the burst release is related to the rapid release of DNA that is located at the surface of the microparticles and therefore be more readily available to diffuse out (O'Hagan et al., 1994; Cleland et al., 1997; Capan et al., 1999); and the slower release is according to the gradual erosion of the polymeric microparticle, which will also depend on the degree of porosity and the size of the pores (Herrmann and Bodmeier, 1995; Ehtezazi and Washington, 2000; Lemaire et al., 2003; Klose et al., 2006). In addition, as mentioned above, it is possible that the entanglement effects of the PVA, polymer and DNA could also affect the release rates. The results obtained here do not present a clear relationship between degree of porosity and release rate, especially for those made with 0.07 M Na_2HPO_4, which had the lowest rate in contrast to its surface area. It is not possible to make a conclusion regarding the correlation between surface properties and dissolution rates without further experiments.

Others have argued that release profiles obtained *in vitro* do not take into account enough factors that would be useful to predict an immune response *in vivo* (Balenga, 2006) and so improved strategies for analysing release profiles of drugs from microparticles are needed. A number of researchers have previously looked at this, including the use of a continuous sampling system (Wang et al., 2002), and the use of more suitable buffers (Blanco-Príetoa et al., 1999; Jiang et al., 2002) for a more accurate *in vitro* and *in vivo* correlation.

The exact level of encapsulated DNA required for effective vaccination has not been studied in detail, but it would seem logical to have as high a loading of DNA into microparticles as possible. In this article, low loading was achieved (for example, 0.33 ± 0.04 mg DNA/μg polymer for microparticles made with 0.07 M Na_2HPO_4). This can be improved by increasing the initial concentration of DNA in the formulation, however there is a need for other approaches such as through condensing the DNA with polymer having a positive charge, for example, chitosan (Borchard, 2001) and poly-L-lysine (Gebrekidan et al., 2000), or by changing the parameters of formulation (for example, the volume of the aqueous phases or the polymer concentration). Parameters of the formulation can be explored efficiently with the use of experimental design as demonstrated by O'Hara and Hickey (2000), Hedberg et al. (2004), and Lopez-Hurtado (2005).

5. Conclusions

Preservation of the supercoiled conformation of plasmid DNA during double emulsion solvent evaporation is paramount to ensure that the highest transfectability potential of the DNA is maintained. This work has shown that this can be achieved quite simply by adding a buffer to the aqueous phase of the formulation; however, this also affects the encapsulation efficiency and release profile of the microparticles. This method could be used in conjunction with other techniques such as using different ratios of co-polymers to adjust release profiles and encapsulation efficiencies of microparticles intended for delivering plasmid DNA into mammalian cells. It would be useful to conduct *in vivo* studies of these formulations to assess their impact on immune responses, especially as it is still unclear what release profiles and what loading of DNA is ideal for effective vaccination.

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