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Research paper

Preparation of phytantriol cubosomes by solvent precursor dilution for the delivery of protein vaccines

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ABSTRACT

Different delivery strategies to improve the immunogenicity of peptide/protein-based vaccines are currently under investigation. In this study, the preparation and physicochemical characterisation of cubosomes, a novel lipid-based particulate system currently being explored for vaccine delivery, was investigated. Cubosomes were prepared from a liquid precursor mixture containing phytantriol or glycerylmonooleate (GMO), F127 for particle stabilisation, and a hydrotrope (ethanol or polyethylene glycol (PEG_{200}) or propylene glycol (PG)). Several liquid precursors were prepared, and the effect of varying the concentrations of F127 and the hydrotrope on cubosome formation was investigated. Formulations were prepared by fragmentation for comparison. The model protein ovalbumin (Ova) was also entrapped within selected formulations. Submicron-sized particles (180–300 nm) were formed spontaneously upon dilution of the liquid precursors, circumventing the need for the preformed cubic phase used in traditional fragmentation-based methods. The nanostructure of the phytantriol dispersions was determined to be cubic phase using SAXS whilst GMO dispersions had a reverse hexagonal nanostructure coexisting with cubic phase. The greatest entrapment of Ova was within phytantriol cubosomes prepared from liquid precursors. Release of Ova from the various formulations was sustained; however, release was significantly faster and the extent of release was greater from fragmented dispersions compared to liquid precursor formulations. Taken together, these results suggest that phytantriol cubosomes can be prepared using liquid precursors and that it is a suitable alternative to GMO. Furthermore, the high entrapment and the slow release of Ova *in vitro* highlight the potential of phytantriol cubosomes prepared using liquid precursors as a novel vaccine delivery system.

1. Introduction

New-generation vaccines increasingly utilise highly purified peptides and proteins as the target antigen; however, these are often poorly immunogenic. One of the most promising strategies to overcome the limitations associated with peptide or protein subunit vaccines is to incorporate them into particulate lipid-based carriers [1,2]. Particulate-based systems are unique in that they can be designed to have dimensions which are comparable to pathogens commonly recognised by the immune system, which facilitates recognition and phagocytosis by antigen presenting cells (APCs). Encapsulation within a particulate-based system can also facilitate antigen persistence due to slow release and can offer protection against degradation and/or clearance. This prolongs the circulation time and potential for interaction of the antigen with APCs [3].

Self-assembly of lipids into several well-defined, thermodynamically stable structures such as lamellar, cubic and the hexagonal liquid crystals upon exposure to a polar environment is a well-established phenomenon [4]. Amongst these, the viscous inverse bicontinuous cubic phase (v_2), hereafter referred to as the cubic phase, has attracted considerable attention as a promising pharmaceutical delivery system due to its unique nanostructure. The highly twisted, continuous lipid bilayer and two congruent, non-intersecting water channels provide both hydrophilic and hydrophobic domains and a very large surface area to these systems [5]. This complex structure has been postulated to offer high loading of bioactives and to potentially retard release and protect the encapsulated active against chemical and/or physiological degradation. The ability to formulate such a system into low-viscosity dispersions which retain the internal nanostructure of the parent

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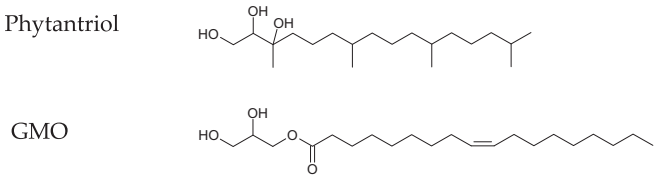

Fig. 1. Chemical structures of phytantriol and glyceryl monooleate (GMO).

non-dispersed system is highly desirable and has been the subject of intense investigation by various research groups [6–12]. These dispersions are termed cubosomes (cubosome is a USPTO registered trademark of GS Development AB Corp., Sweden).

Lipids which are commonly used to prepare cubosomes include emulsifying agents and food additives such as unsaturated mono- and diglycerides, in particular glyceryl monooleate (GMO), mixtures of GMO with other lipids or structural derivatives based on GMO (Fig. 1). Although these lipids are inexpensive and biodegradable, the ester moiety renders them susceptible to hydrolysis. Phytantriol (Fig. 1), a lipid commonly utilised in cosmetic preparations, offers several advantages such as structural stability and higher purity over GMO or GMO-based derivatives [6,13,14].

At present, the most common method of preparation of cubosomes involves the mechanical dispersion of the preformed viscous cubic phase. It is necessary to add stabilisers such as pluronics in these formulations to circumvent aggregation [15]. This method of production is feasible on a small scale and results in cubosomes which have shown stability against aggregation, in some cases for up to a year [15]. However, a major drawback associated with this method of preparation is the prerequisite formation of the cubic phase, which is undesirable for large-scale production. Furthermore, the large amounts of energy required to disperse the cubic phase into submicron-sized cubosomes can limit the incorporation of labile actives, especially peptides and proteins.

Alternative methods for preparing cubosomes to those requiring the mechanical disruption of the performed cubic phase are highly desirable and one such method was described by Spicer et al. [9]. This method is referred to as the liquid precursor or solvent dilution method. It involves the dispersion of a mixture consisting of the liquid crystal–forming lipid, the polymer and a hydrotrope in excess water with minimal energy input to form discrete submicron-sized particles. The hydrotrope is used to dissolve the viscous liquid crystalline phase, and upon dispersion in excess water, the solubility of the liquid crystalline phase is reduced, resulting in the formation of discrete particles by what is presumed to be a nucleation process [9].

The aim of this study was to prepare and characterise the physicochemical properties of cubosomes prepared with phytantriol using the liquid precursor method. The effect of hydrotrope type (ethanol, polyethylene glycol (PEG_{200}) and propylene glycol (PG)), hydrotrope concentration and the lipid-to-stabiliser (Pluronic F127) ratio on cubosome formation was investigated. Optimised formulations were investigated for their potential to incorporate and retain ovalbumin (Ova), a model hydrophilic protein routinely utilised in vaccine research. GMO-based systems were studied as a comparator to phytantriol systems.

2. Experimental section

2.1. Materials

Phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecanetriol) was purchased from A & E Connock (Hampshire, England) and glyceryl monooleate (GMO) (RYLO MG 20 PHARMA), a distilled monoglyceride (min. 95%) with a high GMO content (90%), was a gift from Danisco (Brabrand, Denmark). Both lipids were used as received. RYLO MG 20 has a similar composition to other GMO-based products published elsewhere in the literature with its phase behaviour shown to be representative of GMO [16,17]. Pluronic F127 (PEO98PPO67PEO98), with an average molecular weight of 12,500, was purchased from BASF (Ludwigshafen, Germany). Ethanol, polyethylene glycol 200 (PEG_{200}) and propylene glycol (1,2-propanediol or PG) were purchased from BDH Chemicals Ltd. (Poole, UK). Chicken egg albumin, ovalbumin (Ova) (grade V, Sigma, St. Louis, Missouri), was conjugated to fluorescein isothiocyanate (FITC) (Isomer I, Sigma) as described previously [18]. Triton® X-100 (octophenol-polyethylene glycol ether) and phosphate-buffered saline (PBS) sachets (pH 7.4, 0.01 M) were all purchased from Sigma–Aldrich Pty. Ltd. (New Zealand). Chloroform (99–99.4% purity) was purchased from Merck, Darmstadt, Germany. All water was ion exchanged, distilled and passed though a Milli-Q water purification system (Millipore, Bedford MA, USA).

2.2. Preparation of phytantriol- and GMO-based dispersions by fragmentation

Phytantriol or GMO was heated to 45 °C, and approximately 300 mg was weighed into 5-mL vials. Water (25% w/w to lipid) was gently layered on the surface of the lipids, and the vials were sealed. Samples were incubated at ambient temperature for a minimum of 3 days to allow for the formation and equilibration of the cubic phase. The matrices were then mixed with a solution of F127 (9:1, lipid: F127) to form a coarse dispersion. This dispersion was subsequently homogenised using an Ultra-Turrax (IKA Labortechnik, Germany) for 40 min at 16,000 min^{-1}.

To prepare cubosomes containing the model protein FITC-Ova, phytantriol or GMO was heated to 45 °C and a concentrated solution of FITC-Ova (1 mg/10 μL) at 10% w/w to the lipid was added and mixed using a magnetic stirrer (250–300 rpm) until the samples were visually homogeneous. The lipid and protein mix was then weighed into 5-mL vials (approximately 303 mg; 300 mg lipid and 3 mg FITC-Ova) [6]. Samples were then processed as described previously. The concentration of lipid in the resulting dispersions was typically 2% w/w.

2.3. Preparation of phytantriol- and GMO-based dispersions using liquid precursors

2.3.1. Optimisation of formulation

Varying amounts of phytantriol and F127 were dissolved completely in approximately 1 g of ethanol by vortexing for 5 min. The ethanol was subsequently evaporated on a rotary evaporator under a stream of N_2 (outlet pressure 80 kPa) followed by a further 20 min of evaporation under vacuum to remove any remaining traces of ethanol. This mixture was then dissolved in ethanol, PEG_{200} or PG at the required concentration. When PG and PEG_{200} were used, the mixture had to be heated (40 °C in a water bath) to facilitate the mixing process. To form the dispersions, a 100-μL aliquot of the precursor formulation was dispersed in 2 mL of Milli-Q water by hand shaking and vortexing for 5–10 min.

2.3.2. Liquid precursor-based dispersions containing FITC-Ova

Phytantriol or GMO (100 mg), F127 (15 mg) and PG (70% w/w) were dissolved completely in chloroform (~10 mL), replacing ethanol as in the optimisation studies described earlier. Chloroform was used as both lipids and F127 had greater solubility in this solvent and it evaporated significantly faster when compared to ethanol. The chloroform in this modified mixture was subsequently evaporated under a stream of N_2. Then, 10 μL of water or FITC-Ova solution (1 mg/10 μL water) was added to the lipid mixture.

The liquid precursors were dispersed with 5 mL of Milli-Q water by vortexing for 5–10 min.

2.4. Physicochemical characterisation of dispersions

2.4.1. Particle size distribution and zeta potential

Particle size distribution (*Z*-average), polydispersity (PDI) and zeta potential (based on the Smulochowski model) [19] of the dispersions were determined using photon correlation spectroscopy (PCS) (Malvern Zetasizer 3000, Malvern, UK). Measurements were performed at 25 °C, and the results presented are the mean of three successive measurements of 100 s of at least three independent samples. Samples were diluted with water to adjust the signal level. The stability of the diluted formulations at 30 days post-preparation stored at ambient temperature was also investigated.

2.4.2. Cryo-field emission scanning electron microscopy (Cryo-FESEM)

Selected formulations were loaded into brass rivets and plunge frozen in liquid propane or nitrogen. The samples were then transferred into the cryo chamber (Gatan, Alto 2500, UK) of the microscope (JEOL, JSM-6700F, Japan), which was held at −140 °C. The samples were then sublimed at −90 °C for 2–5 min to remove any surface frost and then coated with platinum for 2 min. The coated samples were then viewed at −140 °C at an acceleration voltage of 3 kB and a working distance of 6 mm.

2.4.3. Small-angle X-ray scattering (SAXS)

Measurements were performed on a Bruker Nanostar SAXS camera with pin-hole collimation for point focus geometry. The instrument source was a copper rotating anode (0.3 mm filament) operating at 45 kV and 110 mA, fitted with a cross-coupled Göbel mirror, resulting in Cu Kα radiation wavelength of 1.54 Å. The SAXS camera was fitted with a Hi-star 2D detector (effective pixel size 100 μm). The sample to detector distance was chosen as 650 mm, which provided a *q*-range of 0.008–0.32 $Å^{-1}$. Samples were loaded into a custom-made stainless steel gel holder with Kapton windows and temperature was controlled by a Peltier-cooled sample stage within ±0.1 °C. The samples were equilibrated at 25 °C for 30 min prior to the collection of scattering pattern for 30 min. Optics and sample chamber were under vacuum to minimise air scatter. Scattering files were background subtracted and normalised to sample transmission and then integrated using Bruker AXS software v4.1.18. The diffraction patterns obtained were converted to plots of intensity versus *q*-value, which enabled the identification of peak positions, and their correlation with Miller indices, to identify the different liquid crystalline structures and space groups for the dominant internal nanostructure of the sample [4].

2.4.4. Entrapment of FITC-Ova in phytantriol- and GMO-based dispersions

Aliquots (200 μL) of the dispersions were centrifuged for 30 min at 14,000 rpm to separate the entrapped protein fraction from the unentrapped. An aliquot of the supernatant was diluted with 5% w/v Triton® X-100 (PBS, pH 7.4) to lyse any lipid fragments and was analysed by spectrofluorimetry at $\lambda_{excitation}$ = 490 nm and $\lambda_{emission}$ = 520 nm. Absorbance values obtained were analysed against standard curves prepared on each day of the analysis from solutions of FITC-Ova kept under same conditions as the formulations. FITC-Ova entrapment was then determined by calculating the difference between the total FITC-Ova added and the free fraction of FITC-Ova in the supernatant.

2.4.5. In vitro release of FITC-Ova from phytantriol- and GMO-based dispersions

To investigate the release of FITC-Ova from the formulations upon dilution, 200-μL aliquots of the various dispersions were added to vials containing 1 mL of PBS (pH 7.4, and 37 °C). The release study was conducted in a 37 °C water bath with a horizontal shaker (60 strokes/min). At fixed time intervals, a vial was removed and three to four 200-μL aliquots were taken and centrifuged at 14,000 rpm for 30 min. The supernatants were removed, diluted with 5% w/v Triton® X-100 and analysed by spectrofluorimetry as in the entrapment protocol described earlier.

2.5. Statistical analysis

Standard curves were constructed and assessed using regression analysis. A one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons was used to assess statistical significance where required. All statistical analysis was performed using Minitab® Statistical Software Release 12.1 (Minitab Inc., Pennsylvania, USA).

3. Results

3.1. Particle size distribution and zeta potential of liquid precursor–based formulations

3.1.1. Effect of solvent type and concentration

The resulting average particle size of freshly prepared dispersions was between 150 nm and 300 nm (Fig. 2). There was a trend towards a decrease in particle size with increasing solvent concentration although this was not statistically significant ($p > 0.05$). The PDIs (Fig. 2) ranged from 0.15 to 0.3 and was lowest for formulations containing the higher concentrations of solvents. However, this trend appeared to be independent of solvent type ($p > 0.05$).

Particles were smallest when freshly prepared; however, upon storage for a month, small aggregates were visible. Analysis of the samples after the aggregates were redispersed by vortexing resulted in size and PDI values comparable to those of freshly prepared samples (Fig. 2) ($p > 0.05$).

The zeta potential of all freshly prepared formulations was negative (Table 1). Ethanol-containing formulations had the most negative zeta potential of around −45 mV, whilst PEG_{200} and PG containing formulations had zeta potentials of around −35 mV. However, the differences were not statistically significant ($p > 0.05$).

3.1.2. Effect of varying stabiliser (Pluronic F127) concentration

The size of freshly prepared dispersions ranged from 200 to 300 nm (Fig. 3). There was no statistical difference in particle size with a decrease in the lipid-to-polymer ratio up to the 5:1 ratio. At a ratio of 4:1, a statistically significant decrease in the size of the dispersions prepared from liquid precursors was only observed for formulations prepared using ethanol as a hydrotrope ($p < 0.05$). Again, particles were smallest when freshly prepared, with small agglomerates visible upon 1 month of storage that were redispersible with vortexing. When reanalysed, there were no significant changes in particle size ($p > 0.05$). The PDIs of the freshly prepared and stored dispersions ranged from 0.15 to 0.3 with no statistically significant ($p > 0.05$) changes observed as a function of the amount of polymer used. The zeta potential of all freshly prepared dispersions was highly negative at around −35 mV (Table 1).

3.1.3. Effect of preparation method, lipid type and presence of FITC-Ova

Dispersions prepared using modified liquid precursors of phytantriol and GMO were characterised and compared to dispersions prepared by fragmentation (Table 2). Blank dispersions of GMO prepared by the fragmentation method were significantly smaller

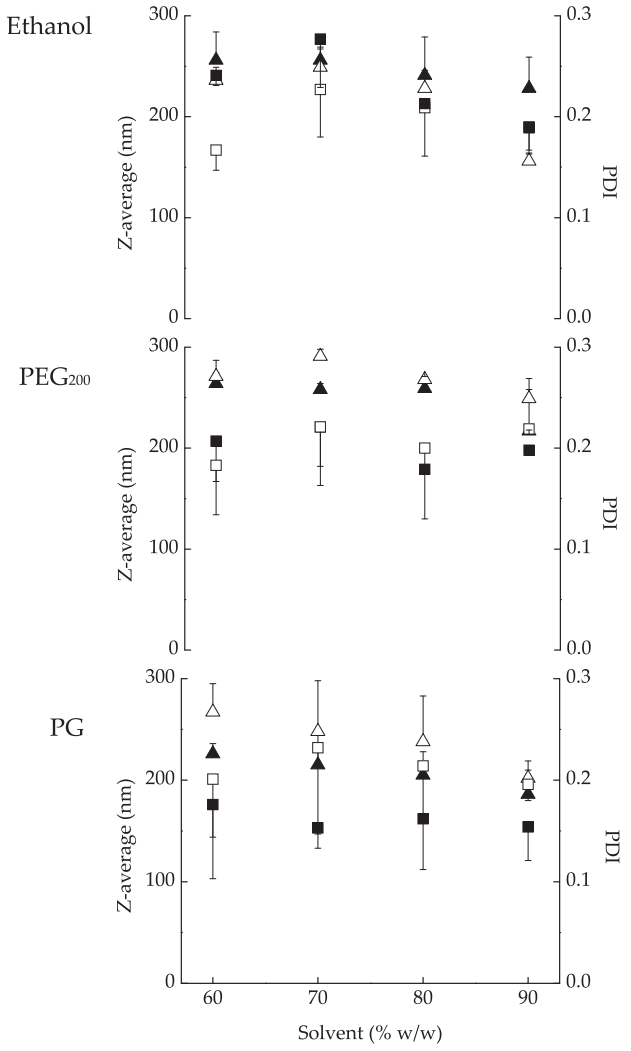

Fig. 2. Effect of solvent type and concentration on particle size (nm) (triangles) and polydispersity index (PDI) (squares) of freshly prepared dispersions (closed symbols) and dispersions stored at ambient temperature for 30 days (open symbols). Liquid precursors were prepared with 200 mg phytantriol and 30 mg F127 with varying solvent type (ethanol, PEG_{200} or PG) and solvent concentration (60–90% w/w). Data presented are the mean ± SD of three independent experiments.

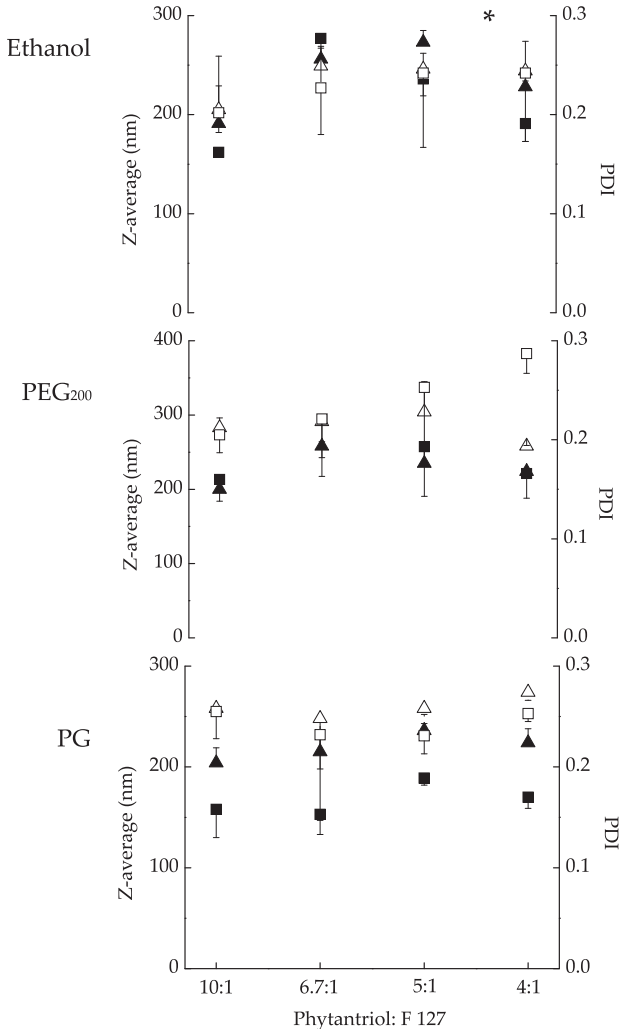

Fig. 3. Effect of stabiliser (F127) concentration on particle size (nm) (triangles) and polydispersity index (PDI) (squares) of freshly prepared dispersions (closed symbols) and dispersions stored at ambient temperature for 30 days (open symbols). Liquid precursors were prepared with 200 mg phytantriol and 70% w/w of either ethanol, PEG_{200} or PG with varying ratios of F127 to the lipid. Data presented are the mean ± SD of three independent experiments.

Table 1
Zeta potential (mV) of liquid precursor-based formulations prepared using different solvent types (ethanol, PEG_{200} or PG) and varying solvent and stabiliser concentration. The liquid precursors were prepared using 200 mg phytantriol and 30 mg F127 with varying solvent concentrations or with 70% w/w solvent whilst varying the F127-to-lipid ratio. Data presented are the mean ± SD of three independent experiments.

Parameters	Ethanol	PEG_{200}	PG
Solvent (%w/w)			
60	−44.8 ± 3.0	−30.0 ± 2.1	−33.9 ± 4.1
70	−39.7 ± 8.0	−30.1 ± 4.5	−36.4 ± 3.3
80	−39.1 ± 5.9	−36.5 ± 2.4	−38.0 ± 4.6
90	−39.3 ± 3.2	−23.9 ± 4.3	−33.9 ± 5.9
Phytantriol:F127			
10:1	−35.4 ± 5.6	−33.0 ± 3.7	−32.8 ± 6.0
6.7:1	−39.7 ± 8.0	−30.1 ± 4.5	−36.4 ± 3.3
5:1	−37.1 ± 2.1	−34.6 ± 1.5	−34.8 ± 3.2
4:1	−36.1 ± 3.3	−33.5 ± 1.7	−34.0 ± 4.8

Table 2
Particle size (nm), polydispersity index (PDI) and zeta potential (mV) of blank dispersions (unshaded) and dispersions containing FITC-Ova (shaded) prepared from modified liquid precursors (LP) (70% w/w PG) or by fragmentation (Frag) of the preformed cubic phase. Data presented are the mean ± SD of three independent experiments of freshly prepared samples characterised on the day of formulation.

	Z-average (nm)	PDI	Zeta potential (mV)
Frag			
Phytantriol	235 ± 9	0.23 ± 0.04	−39.0 ± 5.9
	179 ± 4	0.11 ± 0.01	−27.2 ± 2.6
GMO	175 ± 8	0.12 ± 0.04	−19.7 ± 1.1
	159 ± 8	0.12 ± 0.01	−21.3 ± 1.0
LP			
Phytantriol	256 ± 16	0.206 ± 0.031	−23.9 ± 1.7
	247 ± 10	0.274 ± 0.052	−34.1 ± 1.2
GMO	269 ± 40	0.394 ± 0.068	−18.6 ± 6.0
	233 ± 15	0.252 ± 0.018	−30.2 ± 1.4

in size and showed a narrower size distribution (lower PDI) when compared to phytantriol-based dispersions ($p < 0.05$). This difference was not apparent in formulations made by the liquid precursor method.

No significant differences in particle size or PDI were observed for liquid precursor–based dispersions prepared with or without

FITC-Ova ($p > 0.05$) and containing either lipid. In contrast, entrapment of FITC-Ova within phytantriol cubosomes prepared by fragmentation lead to a significant decrease in particle size and PDI ($p < 0.05$). Interestingly for similar GMO-based formulations, no differences were observed when compared to their blank counterparts ($p > 0.05$).

The zeta potentials for all formulations were negative, where phytantriol-based samples were more negative than dispersions prepared with GMO ($p < 0.05$). Furthermore, with the exception of phytantriol dispersions made by the fragmentation method, addition of model protein lead to dispersions which were more negative than their blank counterparts ($p < 0.05$) and is likely due to the negative charge on Ova in solution [20].

3.2. Morphology of dispersions observed under cryo-FESEM

The morphology of selected liquid precursor-based formulations was investigated by cryo-FESEM (Fig. 4). Panel A shows a representative micrograph of the resulting dispersion prepared from liquid precursors containing 60% w/w ethanol. The particles are generally spherically shaped, around 250–300 nm in size, with a rough nodular surface as described previously for cubosomes prepared by fragmentation [7]. The micrographs in Panels B and C appear more spherical with a smoother surface. The long filaments and ridges evident in the micrographs are believed to be artefacts caused by freezing of the solvents PEG_{200} and PG as they are virtually absent from the ethanol system.

3.3. Phase determination by SAXS

Fig. 5 shows the plot of intensity versus the scattering vector, q, obtained from formulations prepared using phytantriol (Fig. 5A) and GMO (Fig. 5B). Phytantriol-based dispersions (Blank and FITC-Ova loaded) prepared by fragmentation displayed three peaks, where the first two were strong whilst the third peak was relatively weak. These peaks were observed with relative positions at ratios $\sqrt{2} : \sqrt{3} : \sqrt{4}$ and are in accordance with the bicontinuous cubic-phase structure with Pn3m space group, indicating cubosomes with a D-type cubic nanostructure [4]. There was a small increase in the lattice parameter observed for dispersions of phytantriol with incorporation of FITC-Ova, from 67.8 Å to 71.7 Å (Table 3).

Phytantriol-based dispersions (Blank and FITC-Ova loaded) prepared from modified liquid precursors again showed strong diffraction peaks with relative positions at ratios $\sqrt{2} : \sqrt{3}$ and a weaker peak at $\sqrt{4}$, again indicating the successful formation of cubosomes with a D-type cubic nanostructure.

Blank and FITC-Ova-loaded GMO dispersions prepared by fragmentation had identical diffraction patterns as shown in Fig. 5B. Generally, three peaks were observed with relative positions at $\sqrt{1} : \sqrt{3} : \sqrt{4}$, where only the first peak observed was strong, whilst the other two were weak. Peaks with this reciprocal spacing ratio indicate dispersions with an inverse hexagonal (H_2) microstructure, or the formation of hexosomes. In addition to these peaks, shoulders either side of the main peak were also evident (~0.11 and 0.13 $Å^{-1}$) in both GMO samples, which index to spacing ratios of $\sqrt{2}$ and $\sqrt{3}$, again indicating coexistence of the Pn3m cubic-phase structure. The lattice parameters were essentially identical for the two phases between the two samples, the cubic phase giving lattice parameter of 84.5 and 83.3 Å for blank and FITC-Ova-loaded GMO dispersions, respectively, whilst the lattice parameter for the H_2 phase was 61.4 Å in both cases (Table 3).

Blank formulations of GMO prepared using the liquid precursor method displayed the reflections indicative of H_2 phase $\sqrt{1} : \sqrt{3} : \sqrt{4}$ with a lattice parameter of 54.3 Å (Table 3). Dispersions of GMO containing FITC-Ova gave weaker reflections, but showed peaks due to the Pn3m cubic phase (lattice parameter = 85.6 Å), and in this case a weaker peak corresponding to the H_2 phase. Although the H_2 phase cannot be unambiguously assigned on the basis of the one peak, the lattice parameter derived from the position of this peak alone is 61.4 Å, identical to that found for the fragmented sample which was indexed from three peaks, providing full confidence in this assignment (Table 3).

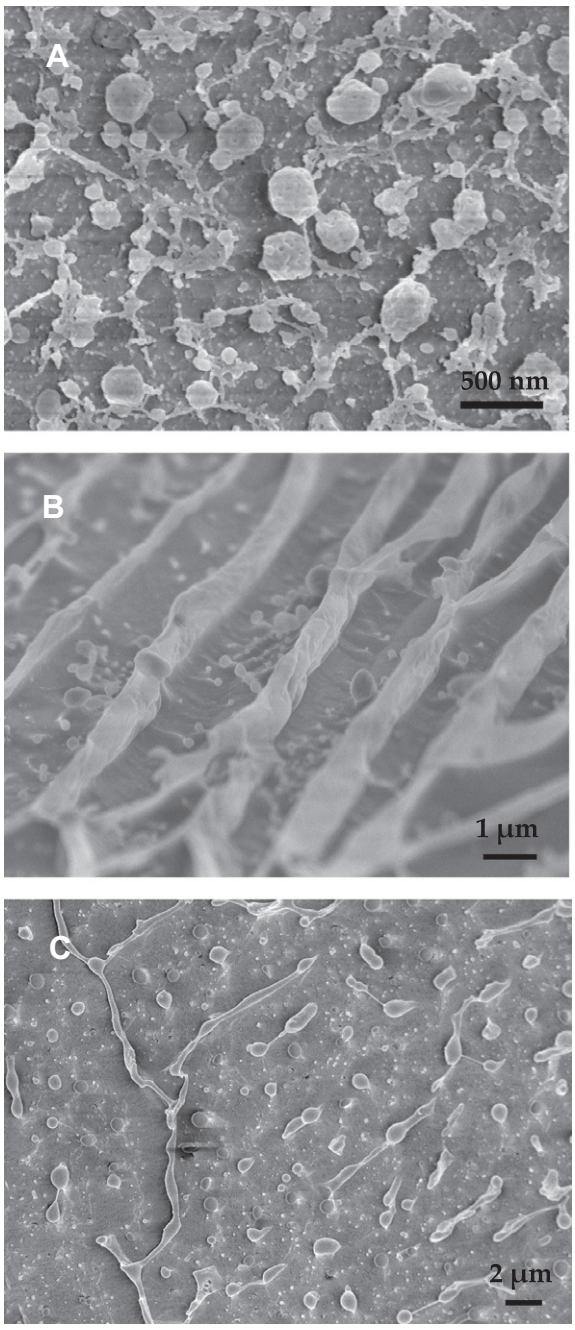

Fig. 4. Cryo-FESEM micrographs of dispersions prepared from liquid precursors containing 200 mg phytantriol, 30 mg F127 (6.7:1, phytantriol:F127) and 60% w/w ethanol (A) or 70% w/w PEG_{200} (B) or 70% w/w PG (C). The long filaments and ridges evident in Panels B and C are believed to be artefacts caused by freezing of the solvents PEG_{200} and PG.

3.4. Entrapment of FITC-Ova

Entrapment of FITC-Ova in formulations prepared with phytantriol and GMO using either method was analysed within 1 h

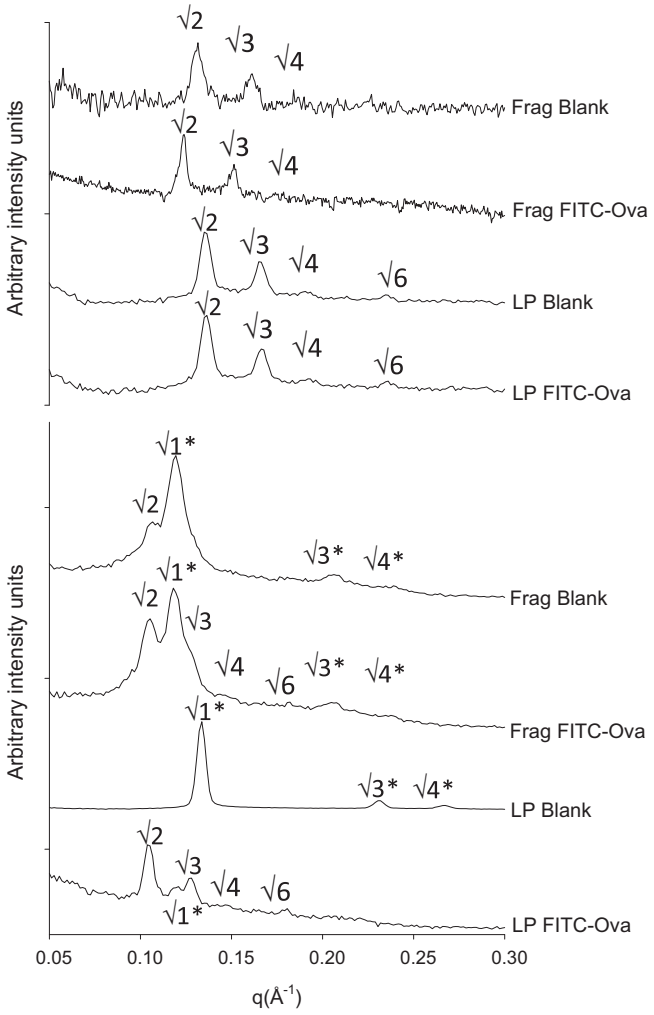

Fig. 5. Intensity vs. q plots obtained from SAXS measurements of formulations prepared with phytantriol (A) and GMO (B). Formulations were prepared by fragmentation (Frag) of the preformed cubic phase or by dispersion of PG-based liquid precursors (LP). Formulations were prepared without model protein (Blank) and containing 1 mg per 100 mg of FITC-Ova (FITC-Ova). Peaks with the reciprocal spacing ratios observed for each formulation are indicated. H_2 peaks are marked with an asterix.

Table 3
Phase structure and lattice parameters obtained from SAXS measurements of blank dispersions (unshaded) and dispersions containing FITC-Ova (shaded) prepared from modified liquid precursors (LP) (70% w/w PG) or by fragmentation (Frag) of the preformed cubic phase.

Formulation	Phase structure and lattice parameter (Å)	
	Pn3m	H_2
Frag		
Phytantriol	67.8	
	71.7	
GMO	84.5	61.4
	83.3	61.4
LP		
Phytantriol	65.8	
	65.1	
GMO		54.3
	85.6	61.4

of preparation. The entrapment of FITC-Ova within particles, prepared by fragmentation using either phytantriol (33.2 ± 3.4%) or GMO (46.8 ± 10.7%), was comparable ($p > 0.05$). The entrapment

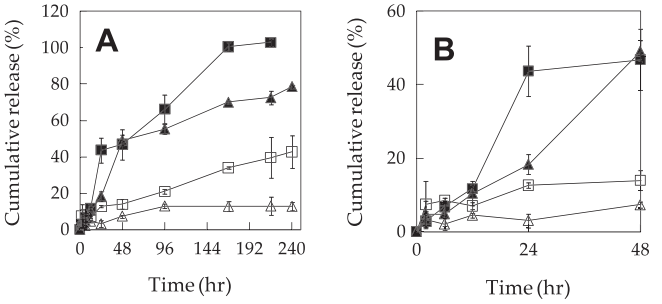

Fig. 6. Cumulative release (%) of entrapped FITC-Ova from formulations prepared either by fragmentation of the preformed cubic phase (closed symbols) or by dispersion of PG-based liquid precursors (open symbols) over 240 h at 37 °C (PBS, pH 7.4) (A). Release from the various formulations for the first 48 h of the release study (B). Formulations contained 1 mg FITC-Ova per 100 mg of phytantriol (triangles) or GMO (squares). Data presented are the mean ± SD of three independent experiments.

of FITC-Ova within phytantriol-based formulations (73.2 ± 2.0%) prepared by dispersion of liquid precursors was significantly greater ($p < 0.05$) than that of liquid precursor formulations prepared using GMO (53.0 ± 0.5%). Furthermore, significantly more FITC-Ova ($p < 0.05$) was entrapped within phytantriol formulations prepared using liquid precursors when compared to formulations prepared by fragmentation of the preformed cubic phase.

3.5. In vitro release kinetics of FITC-Ova

In vitro release kinetics of entrapped FITC-Ova from phytantriol and GMO-based dispersions prepared using both methods was examined over time (Fig. 6A). For dispersions prepared by fragmentation, release of FITC-Ova was comparable from either phytantriol or GMO-based formulations for the first 48 h (Fig. 6B) where around 50% of the entrapped FITC-Ova was released. However, after 96 h, significantly greater release was observed from GMO-based dispersions ($p < 0.05$) with almost all entrapped FITC-Ova released within 168 hr, whilst only around 70% of the entrapped FITC-Ova was released from phytantriol-based dispersions.

Release of FITC-Ova occurred to a lesser extent from formulations prepared by dispersion of liquid precursors when compared to formulations prepared by fragmentation ($p < 0.05$). The kinetics of FITC-Ova release was comparable from liquid precursor-based dispersions prepared using either lipid for the first 12 h of the study. Thereafter, release was significantly greater from GMO-based dispersions ($p < 0.05$), with a constant rate of release over the study period of 240 h. In contrast, release of FITC-Ova from phytantriol-based cubosomes reached a plateau after 96 h with only 13% of the entrapped protein released over the release study period.

4. Discussion

Several approaches are currently under investigation to improve the efficacy of subunit vaccines. However, it is evident from a review of the literature that there is still a pressing need for the development and formulation of novel vaccine delivery systems that are capable of stimulating strong immune responses towards a range of subunit vaccines [1,2,21]. Cubosomes are appealing candidates, and their application as carriers of subunit peptides and proteins has not been fully explored.

Cubosomes are often prepared by fragmentation of the preformed cubic phase using high-energy methods resulting in the production of relatively uniform-sized dispersions [12,22]. How-

ever, the limitations associated with this approach include difficulties in large-scale processing and the risk of disrupting the cubic liquid crystalline structure due to input of excessive energy into the system. Furthermore, the risk of physical degradation of the entrapped bioactive renders this method of production less than ideal. Hence, some research has been conducted into alternative methods for the preparation of cubosomes [9,23,24]. Spicer et al. recently described a simple process where liquid precursor mixtures of GMO–ethanol, when diluted with aqueous F127 solution, lead to the formation of cubosomes with minimal input of energy [9]. This approach is extremely attractive as it circumvents the need for high-energy processes and the formulation of the pre-equilibrated cubic phase.

Spicer et al. compared the particle size distribution of GMO-based dispersions prepared by dilution of liquid precursors with those prepared using high-energy methods [9]. They showed that the dilution-based process resulted in dispersions with smaller particle size and distribution. The observed results were attributed to the inclusion of ethanol in the formulation, where the ethanol increased the solubility of GMO and thereby significantly reducing its operative viscosity.

Here, we report the first preparation of phytantriol cubosomes using liquid precursors which precipitate to form submicron-sized particles upon dilution in solvent. Formulations of phytantriol and GMO were also prepared using fragmentation, and the physicochemical properties of the formulations prepared using the two methods are discussed. Furthermore, the work presented also highlight some of the advantages of using phytantriol as the liquid crystal–forming lipid over GMO.

GMO-based formulations prepared by fragmentation resulted in particles which were smaller and more homogenous when compared to similar phytantriol dispersions. Differences in the degree of interaction and the principal mode of stabilisation of GMO- and phytantriol-based dispersions by F127 may explain these results, where the PPO portion of F127 has a lesser affinity for the phytantriol bilayer resulting in simple adsorption of F127 to the particle surface when compared to GMO [25]. Interestingly, these differences were not significant when the formulations were prepared using liquid precursors. This may be due to the presence of hydrotropes which may also be involved in transiently stabilising the particles during the dilution process. Spicer et al. proposed that cubosomes prepared by dilution of liquid precursors have a more homogenous distribution of the stabilising polymer (e.g. F127) on their surface during the presumed nucleation process when compared to cubosomes prepared by mechanical dispersion [9]. Therefore, the lack of differences observed between the various liquid precursor-based formulations may also be explained by the homogenous distribution of the F127 on the particle surface. The sizes of the liquid precursor-based formulations prepared using both lipids were between 200 and 300 nm and consistent with those reported by Spicer *et al.* for GMO-based cubosomes [9].

The differences observed in the surface charge between phytantriol and GMO formulations may be due to differences in the interaction of F127 with the lipid. It is likely that a greater proportion of F127 may be located at the interface in the case of phytantriol and may influence interactions with the hydroxyl ions present in the surrounding aqueous environment explaining the differences observed in the surface charge (Table 2).

Surface activity of proteins such as albumin/Ova has been described in the literature, where they were shown to adsorb to the air or lipid–water interface [26–28]. Competitive adsorption kinetics may be responsible for the decrease in size and zeta potential observed for FITC-Ova containing phytantriol formulations prepared by fragmentation. The weak interaction of F127 with phytantriol may allow Ova to displace the F127 to a certain extent from the particle and itself interact with the bilayer leading to a reduction in particle size. Furthermore, lower coverage of the particle surface with F127 may result in reduced adsorption of hydroxyl ions again resulting in the reduction in the observed zeta potential.

The internal nanostructure of the particles prepared by solvent dilution (data not shown for ethanol- and PEG_{200}-based dispersions) largely reflected that of the bulk phases and particles prepared by fragmentation. Phytantriol dispersions appear to be very stable under the influence of hydrophilic additives [25] and in this study retained the internal Pn3m cubic nanostructure with both preparation methods and in the presence of FITC-Ova. The GMO dispersions displayed the H_2 mesophase (indicating formation of hexosomes), either alone or coexisting with Pn3m cubic-phase nanostructure of dispersed particles. SAXS was performed on samples 2 weeks after preparation, and the small fraction of cubic phase present may indicate an initial dominance of this phase with gradual conversion over time to the H_2 phase. The conversion of GMO systems from the Pn3m to the H_2 phase is most likely a consequence of hydrolysis of GMO to oleic acid as previously reported [6,16,29]. The finding of the Pn3m phase is significant as GMO dispersions in F127 are well known to undergo a transition to the Im3m cubic phase [25,30,31]. This indicates that the preparation method and/or degradation of the GMO prior to analysis counteract the change in curvature induced by the F127. Taken together, the SAXS results indicate the lability of GMO to degradation and support the choice of phytantriol as a more appropriate candidate lipid for the preparation of cubosome particles for deployment as pharmaceutical carriers.

Entrapment of FITC-Ova within dispersions was remarkably high. Incorporation of the FITC-Ova into either a low-water lipid precursor mixture prior to fragmentation, or the solvent precursor prior to dilution, enables the FITC-Ova to be incorporated and retained largely within the aqueous domains of the particles rather than be equally distributed throughout the aqueous phase as is the case when preparing liposomes by the lipid film method. Some surface adsorption is also likely indicated by burst release kinetics (Fig. 6) and modulation of particle charge in the presence of FITC-Ova (Table 2).

Retarded release of FITC-Ova from non-dispersed systems of phytantriol [6] and GMO [6,32] has been reported. Release of FITC-Ova from the particles over time after an initial burst was slow. This is to some degree surprising as small-molecule drugs are known to release virtually immediately from cubosomes by virtue of the high surface area-to-volume ratio of submicron particles [5,23,33,34]. However, FITC-Ova, being a relatively large (~45,000 Da)[35] and somewhat surface active protein [26–28], appears to be retained in the matrix for a period of days, slowly leaking from the matrix into the surrounding bulk fluid. The significance of the generally faster release from the GMO particles compared to phytantriol is not immediately apparent; however, the lattice dimension of the Pn3m cubic phase observed for GMO is higher than that for phytantriol, which may in part explain the difference. However, SAXS indicated a large proportion of the system was in the H_2 phase which is known to provide substantially slower release than the cubic-phase structures [36]. The issue of release behaviour of large proteins and other biological entities from liquid crystalline particles has not been appropriately addressed in the literature, and the slow-release behaviour reported here for FITC-Ova has significant ramifications for their use as pharmaceutical carriers generally and has stimulated us to investigate this issue further in subsequent studies.

The slower release from the solvent-based systems compared to the fragmented systems may be related to the 'closed' nature of the *in vitro* release studies. The medium, although it is a sink condition for FITC-Ova, will retain the hydrotrope after dilution, albeit at a relatively low concentration. It is possible that the residual solvent

increases the propensity for FITC-Ova to interact with lipid interfaces, driving more association with the particles relative to the bulk liquid. The differences in release between the fragment and solvent-based particles should be observed with caution and is anticipated to be insignificant in an 'open' *in vivo* scenario in which solvent would be expected to diffuse away from the site of administration.

Further work is required to fully understand the effects of the hydrotropes on phase behaviour of the selected lipids. This information may be required for large-scale production of cubosomes, optimising their loading capacity and to assist in making informed decisions with regard to addition of guest molecules within the formulation.

In conclusion, cubosomes were successfully prepared from liquid precursors and the effect of incorporating the model protein Ova within cubosomes was investigated. The high entrapment of the model protein and the *in vitro* release kinetics suggest that phytantriol-based cubosomes prepared via dilution of liquid precursors have the potential to act as a novel sustained-release antigen delivery systems. Current work is in progress and has shown these cubosomes to generate significantly greater antigen-specific immune responses in *in vivo* models when compared to liposomes [Rizwan et al., manuscript in preparation].

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