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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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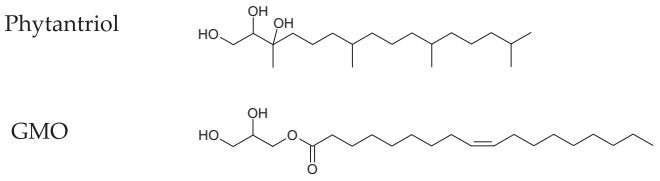

Fig. 1. Chemical structures of phytantriol and glyceryl monooleate (GMO).

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.