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Note

A novel method for preparing immune stimulating complexes (ISCOMs) by hydration of freeze-dried lipid matrix

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Abstract

The purpose of this study was to investigate the application of hydration of freeze-dried lipid monophase matrices as a novel technique to produce immune stimulating complexes (ISCOMs) encapsulating lipopeptides as potential sub-unit antigens. Size, polydispersity and morphology of the resulting colloidal particles were measured and characterized by photon correlation spectroscopy and transmission electron microscopy. The homogeneity of ISCOM preparations produced by this method was found to be influenced by the amount of matrix-forming material as well as the ratio of phospholipid:Quil A:cholesterol used for ISCOM preparation. Further, it was observed that more homogeneous ISCOM dispersions were produced if Quil A was included in the hydrating solution compared to incorporating Quil A in the lipid matrix. Entrapment of lipopeptide within ISCOMs was not affected by chain length (C_{12}–C_{16}) or the number of alkyl chains (1–3) and was greater than 80% when loaded at 5% w/w of total lipid. Entrapment efficiency was noted to decrease dramatically on increasing amount of lipopeptide in the ISCOMs from 5% to 10% of total lipid, decreasing to around 40%. All lipopeptide-loaded ISCOMs were observed to aggregate upon storage.

Keywords: Vaccine; ISCOMs; Lipopeptide; Lipid matrix; Lyophilization

1. Introduction

Since reported more than 20 years ago, the use of ISCOMs as adjuvant and delivery system for subunit vaccines has received much attention. It has been shown that ISCOMs can efficiently promote both humoral and cell mediated immune responses against naturally occurring antigens or modified immunogens. As such, ISCOM based vaccines have been approved for veterinary use and are currently undergoing clinical trials for human use [1,2].

ISCOMs are colloidal, cage-like structures comprising of Quil A, phospholipid and cholesterol. Several methods have been described in the literature for the preparation of ISCOMs or ISCOM matrices, and most of these approaches are adaptations of previously reported methods for the preparation of liposomes. In the traditional centrifugation or dialysis methods, a surfactant (e.g., Triton X-100, MEGA-10 or *n*-octylglucoside) is used to solubilize the ISCOM components, and the ISCOMs are formed by surfactant removal during the process of centrifugation or dialysis [3]. The major limitations of these two methods are firstly the tedious or time-consuming nature of the procedure and secondly the possibility of residual surfactant remaining in the preparation following isolation of ISCOMs. Although preparation methods based on ethanol and ether injection have been published as a surfactant-free protocol to form ISCOM matrices [4,5], the processes still require long equilibration time and require removal of all solvent residues. Another surfactant-free procedure based on a modification of Bangham method for the preparation of liposomes has also been reported involving the hydra-

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tion of dried lipid films of phospholipid and cholesterol using an aqueous solution of Quil A [6]. However, it was reported that equilibration times of days or weeks were preferred for this process and hence a freeze-drying and rehydration step was subsequently included in the process to reduce the time required for ISCOM formation [7]. Based on in-house observations, none of the above surfactant-free methods are able to be used to include lipid-modified peptides within ISCOMS as these are typically poorly-soluble in purely organic or aqueous solvents [8]. Lipid modification of peptides is necessary to associate peptide-epitopes with the ISCOM matrix as ISCOMs do not have an entrapped aqueous volume.

Despite the above prior art approaches, ISCOMs prepared by all these methods are in the form of aqueous dispersions, which have associated problem of stability in terms of the potential of hydrolysis and/or possible aggregation leading to reduced shelf-life. A solid-state formulation of ISCOMs able to be reconstituted prior to use could not only overcome such stability issues but also make possible for convenient storage without the requirement for refrigeration. We previously reported on a method of efficiently entrapping lipid-conjugated peptide antigen within liposomes involving hydration of a freeze-dried monophase of phospholipid, antigen and sugar [8]. With consideration of the thermodynamic stability of ISCOMs and similarity of previously reported methods for ISCOM preparation to those used for the preparation of liposomes, we aimed to investigate whether ISCOMs could be prepared using this novel method of hydration of freeze-dried monophase. Further, we aimed to investigate whether lipid-conjugated peptides could be efficiently incorporated into ISCOM matrices using this new method of ISCOM preparation.

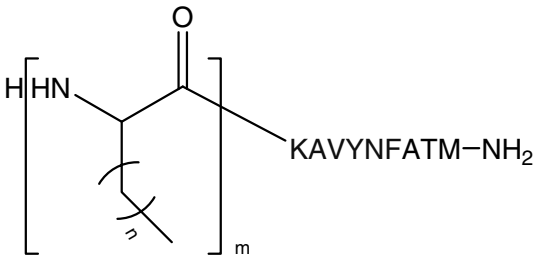
2. Experimental methods

2.1. Materials

Egg phosphatidylcholine (PC) was purchased from Northern Lipids Inc., Vancouver, Canada; Cholesterol (purity $\geqslant$ 95%), sucrose and trehalose were purchased from Sigma–Aldrich Pty., Missouri, USA. Quil A was obtained from Brenntag Biosector, Frederikssund, Denmark. Distilled de-ionized water having a conductivity of less than 0.1 μS (Milli-Q Water system, Millipore, Massachusetts, USA) was used throughout the study. All other chemicals and solvents were of at least analytical grade.

2.2. Synthesis of lipopeptides

Lipopeptides were assembled manually using Boc chemistry on MBHA resin [8]. A range of lipoamino acids (LAA) containing 8, 12, or 16 carbons, respectively, were coupled with the N-terminus of the lymphocytic choriomeningitis virus glycoprotein peptide KAVYNFATM ($LCMV_{33\text{-}41}$). The C12 lipoamino acid was also conjugated in dimeric and trimeric forms (Fig. 1).



n = 13	C_{16}LAA	m = 1	monomeric C_{12}LAA
n = 9	C_{12}LAA	m = 2	dimeric C_{12}LAA
n = 5	C_8LAA	m = 3	trimeric C_{12}LAA

Fig. 1. Chemical structure of synthetic lipopeptides.

2.3. Preparation of ISCOMs by hydration of freeze-dried lipid matrix

ISCOMs were prepared by hydration of a dispersion of lipids in solid sugar matrices. The relative masses of PC, cholesterol, Quil A and sugar investigated are summarized in Table 1. ISCOMs were prepared by two methods: firstly by hydrating the lipid matrix with an aqueous buffer containing Quil A and secondly by hydrating a lipid matrix incorporating Quil A with an aqueous buffer. To obtain the lipid/sugar solid matrix, the required amounts of PC, cholesterol, sucrose (or trehalose) without or with Quil A were weighed and dissolved in a mixture of *tert*-butanol and water (4 mL, v/v 1:1). The resulting monophase solution was snap-frozen in dry ice/acetone, and was freeze-dried overnight (ALPHA 1–2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) at a condenser temperature of −82 °C and pressure of less than 10^{-1} mbar. Four milliliters of phosphate buffer solution (0.01 M, pH 7.4) with or without required amount of Quil A was then added to hydrate the solid matrices and briefly sonicated to facilitate dispersion.

2.4. Characterization of ISCOM dispersions

2.4.1. Photon correlation spectroscopy (PCS)

Size, polydispersity and zeta-potential of ISCOMs were measured using a Zetasizer 3000 (Malvern Instruments, UK) following appropriate dilution in phosphate buffer solution (0.01 M, pH 7.4).

2.4.2. Transmission electron microscopy (TEM)

Samples were placed on carbon-coated, glow discharged copper grids and negatively stained using an aqueous solution of 2% phosphotungstic acid (w/v, pH 5.2) as a contrast agent. Samples were visualised using a JEOL-JEM 1010 electron microscope at an acceleration voltage of 80 kV and typically viewed at a magnification varying from 80,000× to 100,000×. The size of the colloidal structures

Table 1
Mass of PC, Quil A, cholesterol and sucrose used in various formulations and the resulting particle size and polydispersity of samples

Formulation	PC (mg)	Quil A (mg)	Cholesterol (mg)	Sugar (mg)	Mean size[b] (nm)	Zeta potential (mV)	Polydispersity
1	8	8	4	200 (S)	59 ± 10	−22.9 ± 0.7	0.27 ± 0.08
				200 (T)	56 ± 12	−27.6 ± 0.5	0.20 ± 0.08
2	10	6	4	200 (S)	108 ± 9	–	0.29 ± 0.06
				200 (T)	105 ± 13	–	0.31 ± 0.07
3[a]	8	8	4	200 (S)	83 ± 11	–	0.32 ± 0.10
				200 (T)	81 ± 10	–	0.31 ± 0.12
4[a]	10	6	4	200 (S)	123 ± 18	–	0.36 ± 0.09
				200 (T)	121 ± 16	–	0.35 ± 0.15

S and T represent sucrose and trehalose, respectively.
[a] Represent samples prepared incorporating Quil A into the freeze-dried matrix.
[b] Represent intensity mean ± SD ($n = 3$) as measured by PCS.

was measured using AnalySIS® software (Soft Imaging Systems, Megaview III, Munster, Germany) to confirm the results of PCS.

2.5. Incorporation of lipopeptides within ISCOMs

For the incorporation of lipopeptides within ISCOMs, lipopeptides together with PC (8 mg), cholesterol (4 mg) and sucrose (200 mg) were dissolved in the mixture of *tert*-butanol and water. Following snap-freezing in dry ice/acetone, the system was freeze-dried overnight. The resulting solid matrices were hydrated with 4 mL Quil A in phosphate buffer solution (2 mg/mL) with a brief period of sonication. The ISCOM dispersion was subsequently extruded through 400 nm polycarbonate membranes (Lipex Extruder™, Northern Lipids, Vancouver, Canada) to remove non-entrapped lipopeptide. The amount of lipopeptide incorporated into ISCOM matrices was estimated by measuring the concentration of lipopeptide in the ISCOM extrudate as previously described and validated using liquid chromatography coupled to electrospray mass spectroscopy (LC–MS) [8].

3. Results and discussions

The mass ratio of each lipid component in the formulations, namely phosphatidylcholine (PC), Quil A and cholesterol, was selected based on the pseudo-ternary diagram reported by Demana et al. [9]. According to the phase diagram, ISCOM matrices were found as the predominant colloidal structures in systems containing 40–50% PC, 30–40% Quil A and 10–30% cholesterol upon hydration. Therefore, freeze-dried lipid matrices were initially prepared with a lipid mass ratio of 8:8:4 (w/w, PC:Quil A:cholesterol) and a total lipid mass of 20 mg. Initially, the effect of varying the amounts of sucrose as the water soluble matrix-forming agents was investigated in the range from 100 to 400 mg. It was revealed that the sucrose/lipid ratio in the formulation plays a key role in determining the size and type of structures produced upon hydration of the freeze-dried matrix with Quil A buffer solution. For formulations containing low sucrose/lipid ratio of 5:1 (100 mg sucrose), liposomes were found as the predominant colloidal structures upon hydration. This was confirmed by photon correlation spectroscopy in which the typical size of the particles was between 150 and 200 nm, which is consistent with the size of liposomes produced by this method [8]. The formation of liposomes rather than ISCOMs may signify that the lipids are not adequately dispersed in the sugar matrix, forming large lipid "mini-domains" into which the Quil A present in the hydrating solution is not able to sufficiently penetrate before the lipids hydrate to form bilayer structures. At a higher sugar/lipid ratio of 10:1 (200 mg sucrose), ISCOMs were found as the predominant colloidal structures and relatively homogeneous preparations could be produced. The size of the particles obtained was in the range of 50–60 nm as measured by photon correlation spectroscopy. TEM (Fig. 2A) confirmed the size as well as showing the morphology of the colloids to be that of hollow cage-like structures, typical of the morphology of ISCOM matrices. The surface charge of these colloidal particles was approximately −20 mV in phosphate buffer, which is also again characteristic of ISCOMs as a result of glucuronic acid present in the saponin.

Upon further increasing the sugar/lipid ratio to greater than 15:1 (300 or 400 mg sucrose), the occurrence of ISCOM structures in the samples decreased and ring-like micelles and sheet-like lamellae structures were also observed in the preparation (Fig. 2B). The existence of such micellar and lamellar (as fused ring-like micelles) structures has previously been reported in the phase diagram of Quil A, phospholipid and cholesterol with ring-like micelles forming at high Quil A ratios and lamellae forming at relatively high ratios of cholesterol [9]. The phospholipids with hydrophobic alkyl chains are thought to be the driving force for the arrangement of the ring-shaped subunits into the cage-like ISCOM structures [10]. However, it is known that disaccharide molecules can interact with phospholipids by the formation of hydrogen bonds [11]. Such an interaction at the relatively high sucrose concentrations present in systems containing greater than 7.5% sugar (>300 mg) may disrupt the driving force for ISCOM formation arising from the phospholipids, resulting instead in the formation

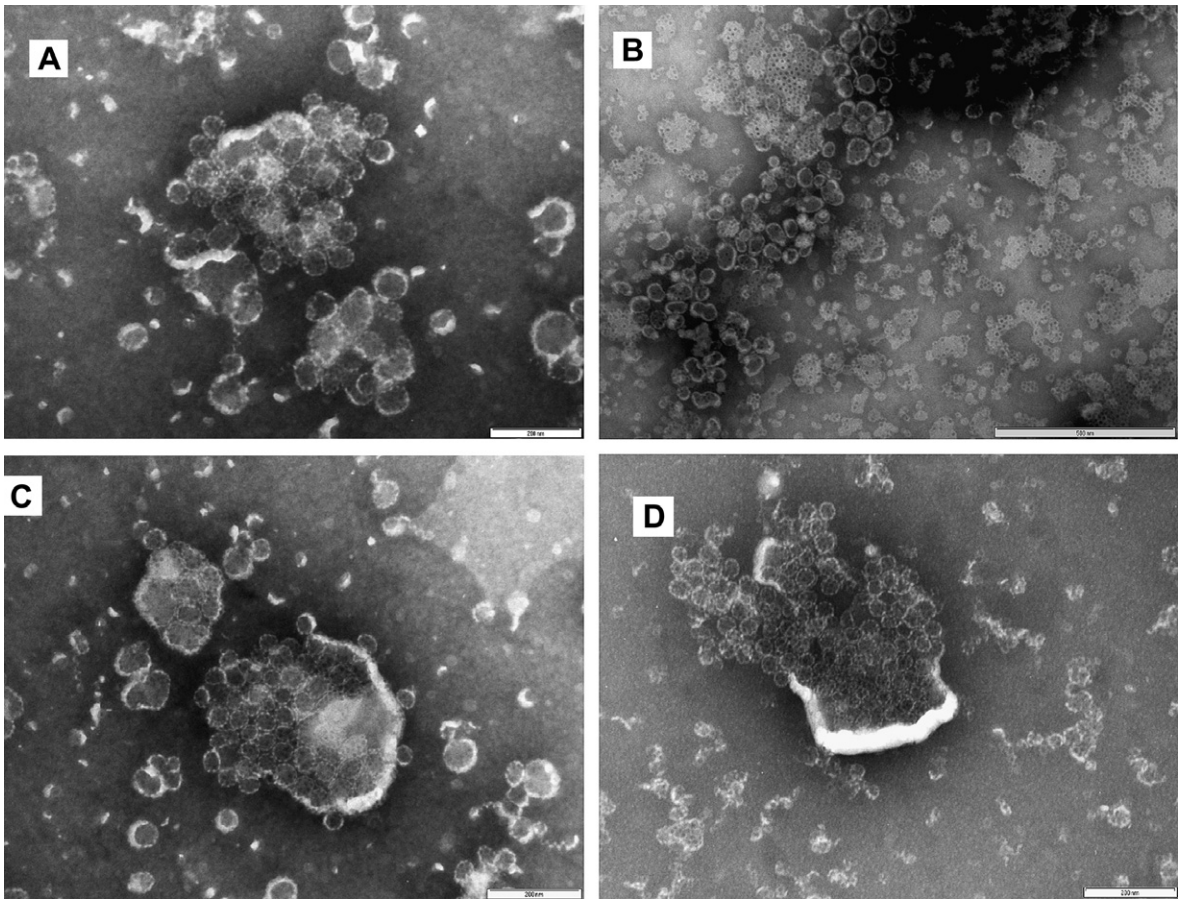


Fig. 2. TEM micrograph of ISCOMs prepared by hydration of freeze-dried matrix: (A) sample was prepared from PC/cholesterol matrix with 200 mg sucrose and hydrating with a Quil A buffer using a ratio of 8:8:4 (PC:Quil:Chol); (B) sample was prepared from PC/cholesterol matrix with 300 mg sucrose and hydrating with a Quil A buffer using a lipid ratio of 8:8:4 (PC:Quil:Chol); (C) sample was prepared from PC/cholesterol matrix with 200 mg sucrose and hydrating with a Quil A buffer using a lipid ratio of 10:6:4 (PC:Quil:Chol); (D) sample was prepared from PC/Quil A/cholesterol matrix with 200 mg sucrose and hydrating with buffer using a lipid ratio of 8:8:4 (PC:Quil:Chol).

of ring-like micelles and lamellae. Further, sucrose may be acting as a competitive replacement molecule for Quil A which also contains numerous saccharide moieties. These interactions could further help explain the formation of ring-like micelles and sheet-like lamellar structures as opposed to ISCOMs at relatively high concentrations of sucrose in the formulations. In addition, it was observed in the investigations relating to the effect of sucrose concentration that the hydration process could be shortened by a brief period of sonication. As such, all subsequent studies included a short period of sonication (<1 min) and used 200 mg of matrix-forming material in 4 mL of solution (i.e. 5% w/v saccharide).

The effect of including Quil A within the monophase matrices as compared to including Quil A in the hydrating solution was subsequently assessed with the hypothesis that inclusion in the monophase solution would give a better distribution of Quil A within the lipid matrix and hence promote ISCOM formation. A second ratio of components based on the pseudo-ternary phase diagram of Demana et al. [9] of 10:6:4 of PC, Quil A and cholesterol was also investigated in attempt to investigate the susceptibility of ISCOM formation to the ratio of the components used. Samples prepared using the ratio of 10:6:4 were observed to contain fewer individual ISCOMs and more lipid ''clumps'' from which ISCOMs were seen to be budding-from (Fig. 2C). The higher ratio also resulted in a larger mean particle size further confirming the more heterogeneous nature of the preparation and the existence of larger lipid aggregates (samples 2 and 4 in Table 1). A higher ratio of phospholipid was reported by Demana et al. [12] to similarly promote formation of bilayer structures such as liposomes. Hence it was established that the preferred ratio for ISCOM formation as prepared by hydration of freeze-dried monophase systems was 8:8:4. The inclusion of Quil A in the freeze-dried matrix slightly decreased the homogeneity of the system and resulted in samples predominantly containing ISCOM matrices but in combination with ring-like micelles and lipid aggregates (Fig. 2D). The size and polydispersity of the preparation as measured by PCS increased for both the ratio of components investigated (Table 1). Although unclear as to the reason for the increased heterogeneity when including Quil A in the monophase, it is recommended that Quil A be included

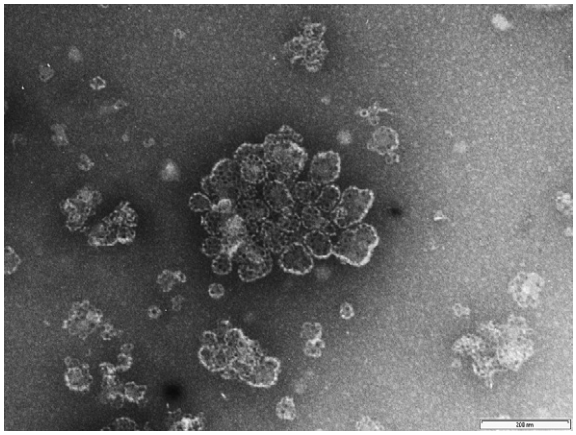


Fig. 3. TEM micrograph of lipopeptide-ISCOMs prepared by hydration of freeze-dried matrix: sample was prepared from PC/cholesterol matrix with 200 mg sucrose and 1 mg C_{12}LCMV and hydrating with a Quil A buffer using a ratio of 8:8:4 (PC:Quil:Chol).

in the solution used to hydrate the freeze-dried matrix rather than including Quil in the matrix itself when preparing ISCOMs by this method. The effect of changing the disaccharide from sucrose to trehalose was also investigated. Despite the critical importance of the ratio of the sugar used, it was noted that the nature of the disaccharide used did not influence ISCOM formation with homogeneity as measured by PCS and TEM being comparable in samples prepared using both sugars.

In the case of preparing lipopeptide-loaded ISCOMs, lipopeptide together with phospholipid and cholesterol were dissolved completely in the *tert*-butanol–water co-solvent before lyophilization. Upon hydration of the freeze-dried matrices with PBS buffer containing Quil A, the resulting colloidal particles showed a larger size and lower homogeneity as compared to ISCOM matrices, but were observed by TEM to still have an ISCOM-like structure (Fig. 3). The entrapment efficiency of lipopeptides within ISCOMs prepared by this procedure is reported in Fig. 4. As can be seen, incorporation was just over 80% for all

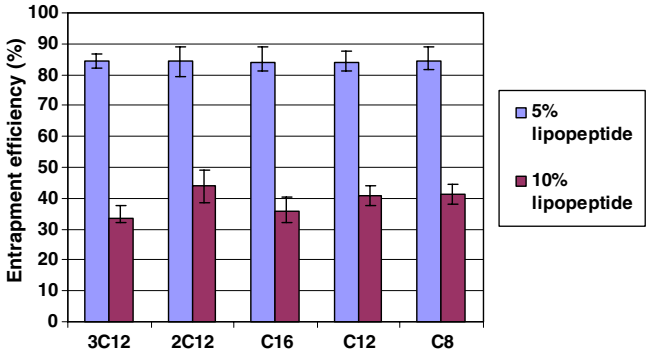


Fig. 4. Incorporation efficiency of lipopeptides within ISCOMs prepared by hydration of freeze-dried matrix: samples were prepared from PC/cholesterol matrix with 200 mg sucrose and 1 mg lipopeptide and hydrating with a Quil A buffer using a ratio of 8:8:4 (PC:Quil:Chol).

of the constructs synthesised at a 5% (w/w, lipopeptide/total lipid) loading. Encapsulation efficiency was not influenced by the length or number of acyl chains of the lipopeptides, however it decreased dramatically with increasing lipopeptide loading with around 40% or less of lipopeptide being incorporated within ISCOMs when loaded at 10% by weight of total lipids. Of interest was also the marked difference in the physical stability of the ISCOM matrices produced by this technique as compared to lipopeptide-loaded ISCOMs. No aggregation of ISCOM matrices was observed following 1 week of storage. In contrast, lipopeptide-loaded ISCOMs were observed to aggregate within days of preparation. Previously, differential scanning calorimetry study revealed the absence of any gel-to-fluid phase transitions for the synthesised lipopeptides in the temperature range 20–300 °C, reflecting highly ordered packing of the lipid chains [8]. Although the lipopeptides may be molecularly dispersed and associated within ISCOM colloidal structures in the early stage of storage, they may have mobility in the lipid membranes of the colloids and may re-organize with time to form systems which are unstable and aggregate. Further, all lipopeptides have an amide group at the C-terminus as a result of cleavage from the MBHA resin, which together with the free amino group present on the lipoamino acid leads to a net positive charge at neutral pH (Fig. 1). Thus, with consideration of the negative charge carried by the Quil A as a result of its glucuronic acid residue, the observed aggregation could also be a result of charge–charge interaction of lipopeptide and Quil A. Hydration of a freeze-dried matrix with a carbonate buffer pH of 8.5 where ionization of the amino groups is suppressed was shown to improve the stability of ISCOMs containing lipopeptide and hence supporting this hypothesis to some extent. However, the peptide-loaded ISCOMs were still observed to aggregate given sufficient time. The mechanism underlying the instability of lipopeptide-loaded ISCOMs is the subject of on-going studies.

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