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# A comparison of pseudo-ternary diagrams of aqueous mixtures of Quil A, cholesterol and phospholipid prepared by lipid-film hydration and dialysis

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## Abstract

Pseudo-ternary diagrams for Quil A, phospholipid (phosphatidylcholine (PC) or phosphatidylethanolamine (PE)) and cholesterol were established in order to identify combinations that result in the formation of immune-stimulating complex (ISCOM) matrices and other colloidal structures produced by these three components in aqueous systems following lipid-film hydration or dialysis (methods that can be used to produce ISCOMs). In addition, the effect of equilibration time (1 month at 4 °C) on the structures formed by the various combinations of the three components was investigated. Depending on the ratio of Quil A, cholesterol and phospholipid, different colloidal particles, including ISCOM matrices, liposomes and ring-like micelles, were found irrespective of the preparation method used. In contrast, worm-like micelles were only observed in systems prepared by lipid-film hydration. For samples prepared by dialysis, ISCOM matrices were predominantly found near the Quil A apex of the pseudo-ternary diagram (> 50% Quil A). On the other hand, for samples prepared by lipid-film hydration, ISCOM matrices were predominantly found near the phospholipid apex of the pseudo-ternary diagram (> 50% phospholipid). The regions in the pseudo-ternary diagrams in which ISCOM matrices were observed increased following an extended equilibration time, particularly for samples prepared by lipid-film hydration. Differences were also observed between pseudo-ternary diagrams prepared using either PE or PC as phospholipids.

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## Introduction

Immune-stimulating complexes (ISCOMs) are symmetrical colloidal particles composed of cholesterol, phospholipid, the saponin mixture Quil A and antigen, mostly in the form of an amphipathic protein (Höglund et al 1989; Rimmelzwaan & Osterhaus 1997). A complex without incorporated antigen is called an ISCOM matrix (Kersten & Crommelin 1995; Barr et al 1998; Copland et al 2000). ISCOMs have a characteristic open cage-like morphology that appears to consist of about 20 ring-like regularly ordered subunits (Höglund et al 1989). ISCOMs are in the size range of 40–100 nm and can be best observed by negative staining transmission electron microscopy (Kersten et al 1991; Rimmelzwaan & Osterhaus 1997).

A liberal definition of the term ISCOMs was suggested by Kersten et al (1990) as "all saponin–lipid complexes prepared for use as an adjuvant or an antigen vehicle". However, this definition has not been generally adopted (Barr & Mitchell 1996; Kersten & Crommelin 2003). Rather, the term ISCOMs generally only applies to a specific colloidal particle with a particular morphology as described above. ISCOMs have received particular attention as delivery systems for sub-unit vaccine antigens as they combine the advantages of a particulate carrier with the presence of a built-in adjuvant, the saponin mixture Quil A (Özel et al 1989; Barr et al 1998; Kersten & Crommelin 2003).

Two methods have traditionally been used for the preparation of ISCOMs, based on either centrifugation or dialysis (Höglund et al 1989). Both methods require the use of an additional surfactant to solubilize the components necessary to form ISCOMs. In the dialysis method, ISCOMs are formed during removal of additional surfactant by extensive dialysis (Höglund et al 1989; Rimmelzwaan & Osterhaus 1997; Kersten & Crommelin 2003). Octylglucoside, a surfactant having a relatively high critical

micelle concentration (CMC), and Mega 10, a surfactant having a much lower CMC, are the surfactants that are mostly used in the dialysis technique (Kersten et al 1991; Sjölander et al 1998). For the centrifugation method, the protein, cholesterol and phospholipid are solubilized using a surfactant such as Triton X-100 in the absence of Quil A (Höglund et al 1989). This micellar solution is then centrifuged through a sucrose gradient containing Quil A. During the centrifugation process ISCOMs are formed. The dialysis technique, however, is by far the most extensively used method for the preparation of ISCOMs. A much simpler process for the preparation of ISCOM matrices was recently developed in our laboratory (Copland et al 2000). This method is based on the classical Bangham method used for the preparation of liposomes and involves the hydration of dried films of phospholipid and cholesterol using an aqueous solution of Quil A. We have since refined this methodology by introducing a freeze-drying step in order to promote intimate mixing of Quil A, cholesterol and phospholipids, and furthermore have identified the optimal weight ratios of the three components that form ISCOMs by this method by constructing pseudo-ternary phase-diagrams (Demana et al 2004).

ISCOMs can be prepared from different phospholipids and it has been reported that the use of phosphatidylethanolamine (PE) produces ISCOMs with more uniform morphology and size than those produced using phosphatidylcholine (PC) (Devries et al 1990; Kersten et al 1991). ISCOMs prepared from PC tend to be more heterogeneous in size and irregular in morphology (Kersten et al 1991).

The work described in this paper extends our previous investigations in which structures produced in different regions of the pseudo-ternary diagrams of Quil A, cholesterol and PC prepared by hydration were identified to investigate the effect of the phospholipid used and also compare the pseudo-ternary diagram produced by hydration with that produced when the ternary systems are processed according to the dialysis method used for the preparation of ISCOMs.

The aim of this study was to identify combinations that result in the formation of ISCOM matrices and other colloidal particles produced by these three compounds in aqueous systems following lipid-film hydration or the dialysis method. A further aim was to characterize colloidal structures found in the vicinity of ISCOM regions of the pseudo-ternary diagrams and structures that transform into ISCOMs on extended equilibration times, in order to get further insight into the possible mechanism of ISCOM formation and structure.

## Materials and Methods

### Materials

Quil A was obtained from Superfos Biosector, Denmark. Octylglucoside (purity approx. 98%), cholesterol (purity approx. 95%), L-$\alpha$-phosphatidylethanolamine from bovine brain (purity approx. 98%), and L-$\alpha$-phosphatidylcholine from egg yolk (purity approx. 99%) were purchased from Sigma-Aldrich (Missouri, MO). Distilled deionized water having a conductivity of less than 0.1 $\mu$S was used throughout (Milli-Q Water system, Millipore Corporation, MA). All other chemicals and solvents were of at least analytical grade.

### Preparation of dispersions

*Lipid-film hydration method*

Various amounts of PE or PC and cholesterol were dissolved in 0.5 mL chloroform and evaporated to dryness at 45 °C for 1 h (Rotavapor R110, Büchi, Switzerland). The lipid films formed were hydrated for 5 h at room temperature with 3 mL Tris buffer (140 mM, pH 7.4) containing various amounts of Quil A. The total polar lipid concentration in each sample (Quil A, cholesterol and PE or PC) was 6.7 mg mL$^{-1}$. The samples were subsequently freeze-dried for 24 h (Freezone 6, Model 79340, Labconco, Missouri, MO) at a condenser temperature of $-82$ °C and pressure of less than $10^{-1}$ mbar. The freeze-dried samples were rehydrated with 3 mL water and stirred using a magnetic stirrer for 5 h.

*Dialysis method*

The dialysis method was performed as described by Kersten et al (1991) and other research groups for the preparation of ISCOMs (Andersson et al 2000; Blomqvist et al 2003). Various amounts of phospholipids (PE or PC) and cholesterol were dissolved in 0.5 mL chloroform and evaporated at 45 °C for 1 h (Rotavapor R110, Büchi, Switzerland). To the dried lipid film, different amounts of Quil A together with octylglucoside (40 mg mL$^{-1}$) in 3 mL Tris buffer (140 mM, pH 7.4) were added so that the total polar lipid (Quil A, cholesterol and PE or PC) concentration in each sample was 6.7 mg mL$^{-1}$. The resulting micellar solutions were subsequently stirred using a magnetic stirrer for 5 h at room temperature before placing in dialysis tubing (molecular weight cut-off of 1000 Da). The samples were dialysed against seven changes of 1 L Tris buffer (140 mM, pH 7.4) at 4 °C for 3 days to remove octylglucoside.

### Characterization of colloidal dispersions

All samples were investigated within a day of preparation and after storage in a fridge (4 °C) for a period of 1 month by transmission electron microscopy (TEM) and polarized light microscopy (PLM).

### Transmission electron microscopy

Carbon-coated copper grids were glow-discharged (Edwards E306A Vacuum Coater, UK) and 10 $\mu$L of sample adsorbed onto these grids. The samples were negatively stained using 10 $\mu$L of filtered 2% phosphotungstic acid, pH 5.2, as a contrast agent. Samples were viewed using a Phillips CM100 transmission electron microscope at an acceleration voltage of 100 kV and typically at a

magnification of ×135 000. The size of the colloidal structures was determined using AnalySIS software (Soft Imaging Systems, Reutlingen, Germany). At least 1000 particles per sample were observed and measured, and from this the prevalence of different colloidal structures was estimated and expressed as a percentage of all the colloidal particles present in the sample. Pseudo-ternary diagrams were established and the estimated prevalence of different colloidal structures in the samples investigated, based on TEM investigation, is presented in the diagrams. Colloidal particles that comprised less than 5% of the total colloidal particles in a specific sample were classed as minor colloidal structures. These minor colloidal structures are not represented in the pseudo-ternary diagrams.

### Polarized light microscopy

All formulations were examined using a phase contrast light microscope (Model 218502, Nikon, Japan) equipped with a polarizer and analyser (Nikon Optiphot, Nikon, Japan) to determine the presence of cholesterol crystals.

## Results and Discussion

Pseudo-ternary diagrams of aqueous mixtures of Quil A, cholesterol and PE or PC prepared by the lipid-film hydration method and characterized within 1 day of preparation and following 1 month of storage at 4 °C are shown in Figure 1. Similar pseudo-ternary diagrams of aqueous mixtures of Quil A, cholesterol and PE or PC prepared by the dialysis method and characterized within 1 day of preparation and following 1 month of storage at 4 °C are shown in Figure 2. Both the lipid-film hydration and dialysis methods produced ISCOM matrices, liposomes, ring-like micelles and lipidic particles as predominant colloidal structures. However, differences between the two preparation methods were observed with regard to the prevalence and distribution of some of these colloidal structures.

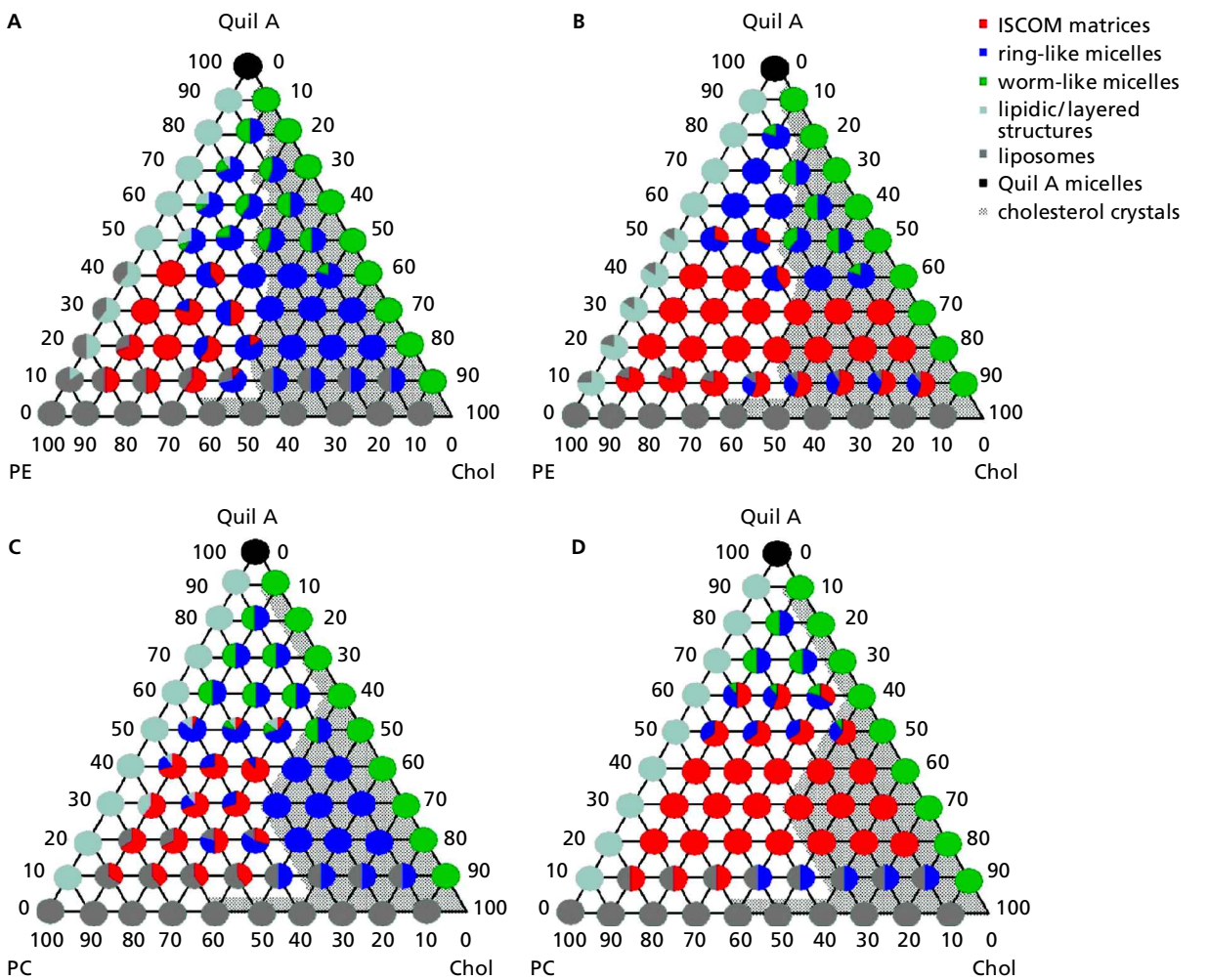


**Figure 1** Pseudo-ternary diagrams of aqueous mixtures of Quil A, cholesterol (Chol) and PE or PC within 1 day of preparation (A and C) and after equilibration for 1 month at 4 °C (B and D). Samples were prepared by lipid-film hydration.

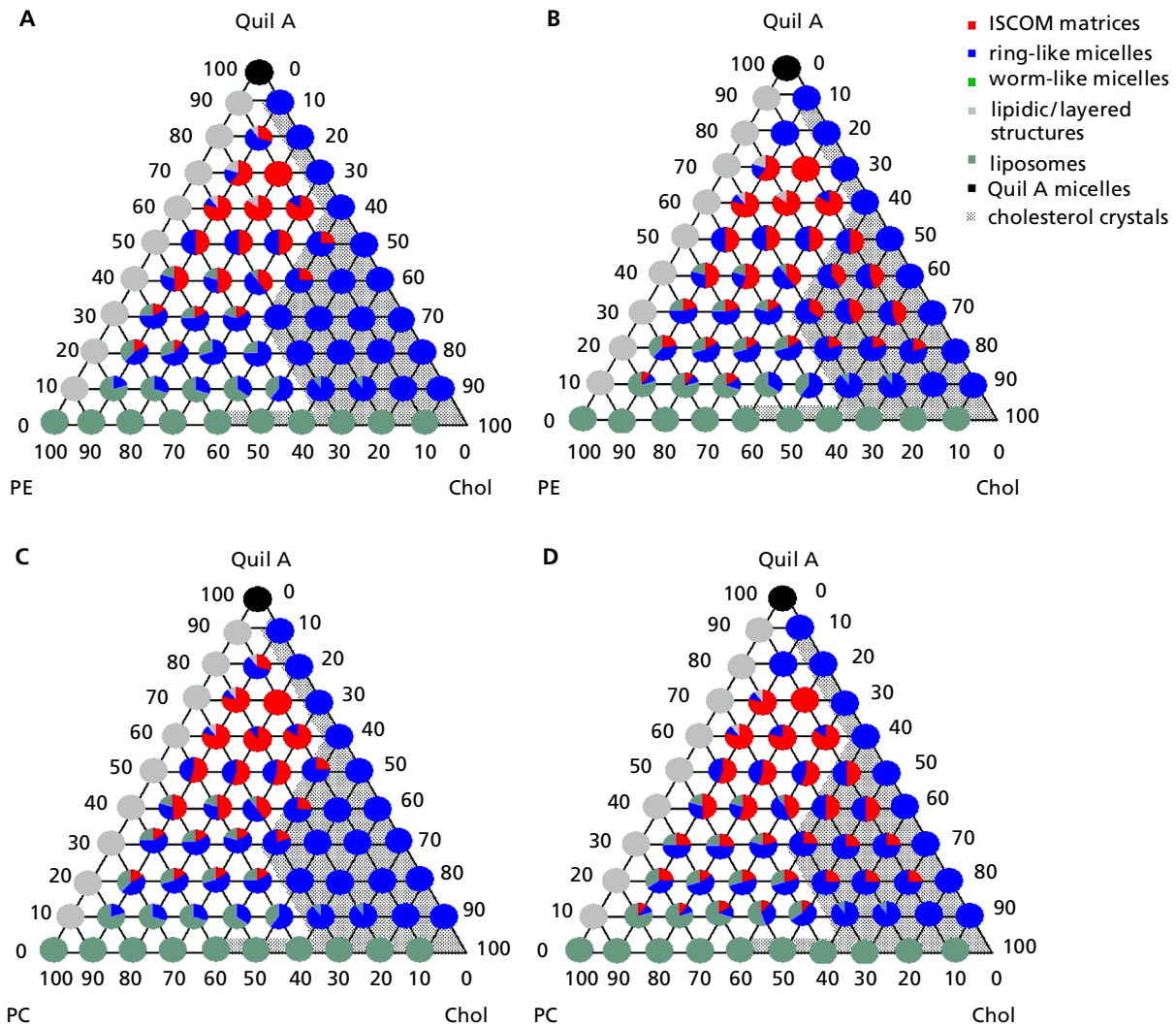


**Figure 2** Pseudo-ternary diagrams of aqueous mixtures of Quil A, cholesterol (Chol) and PE or PC within 1 day of preparation (A and C) and after equilibration for 1 month at 4 °C (B and D). Samples were prepared by dialysis.

In aqueous pseudo-binary systems containing only Quil A and cholesterol, and prepared by lipid-film hydration, worm-like micelles were observed as the only colloidal structure (Figure 3A). In contrast, the same systems prepared by dialysis produced predominantly ring-like micelles (Figure 3B). In addition to ring-like micelles, ISCOM matrices as minor colloidal structures were observed, especially at higher Quil A:cholesterol mass ratios. The presence of ISCOM matrices in pseudo-binary systems of Quil A and cholesterol prepared by dialysis that were not observed in these systems prepared by hydration is interesting. This is in agreement with results reported by Morein et al (1984), who proposed that ISCOMs could form in the absence of phospholipids. However, the formation of ISCOMs in pseudo-binary systems containing only Quil A and cholesterol is in contrast to reports by other investigators who have suggested that the presence of phospholipid together with cholesterol and Quil A is essential for ISCOM formation (Devries et al 1990; Kersten et al 1991; Copland et al 2000). In the dialysis method octylglucoside as an additional surfactant is used, whereas the hydration method does not require the use of any additional surfactant. It has been suggested that formation of ISCOM matrices can occur in the absence of a phospholipid due to a residual amount of surfactant left with the samples during the dialysis process (Kersten & Crommelin 1995). It would seem clear from the present investigation using the lipid-film hydration method, which does not involve the use of additional surfactants, that ISCOM formation does require the presence of an additional polar lipid component to Quil A and cholesterol.

As stated above, the existence of worm-like micelles in pseudo-binary systems containing only Quil A and cholesterol prepared by lipid-film hydration that were not found in samples prepared by dialysis is a major difference

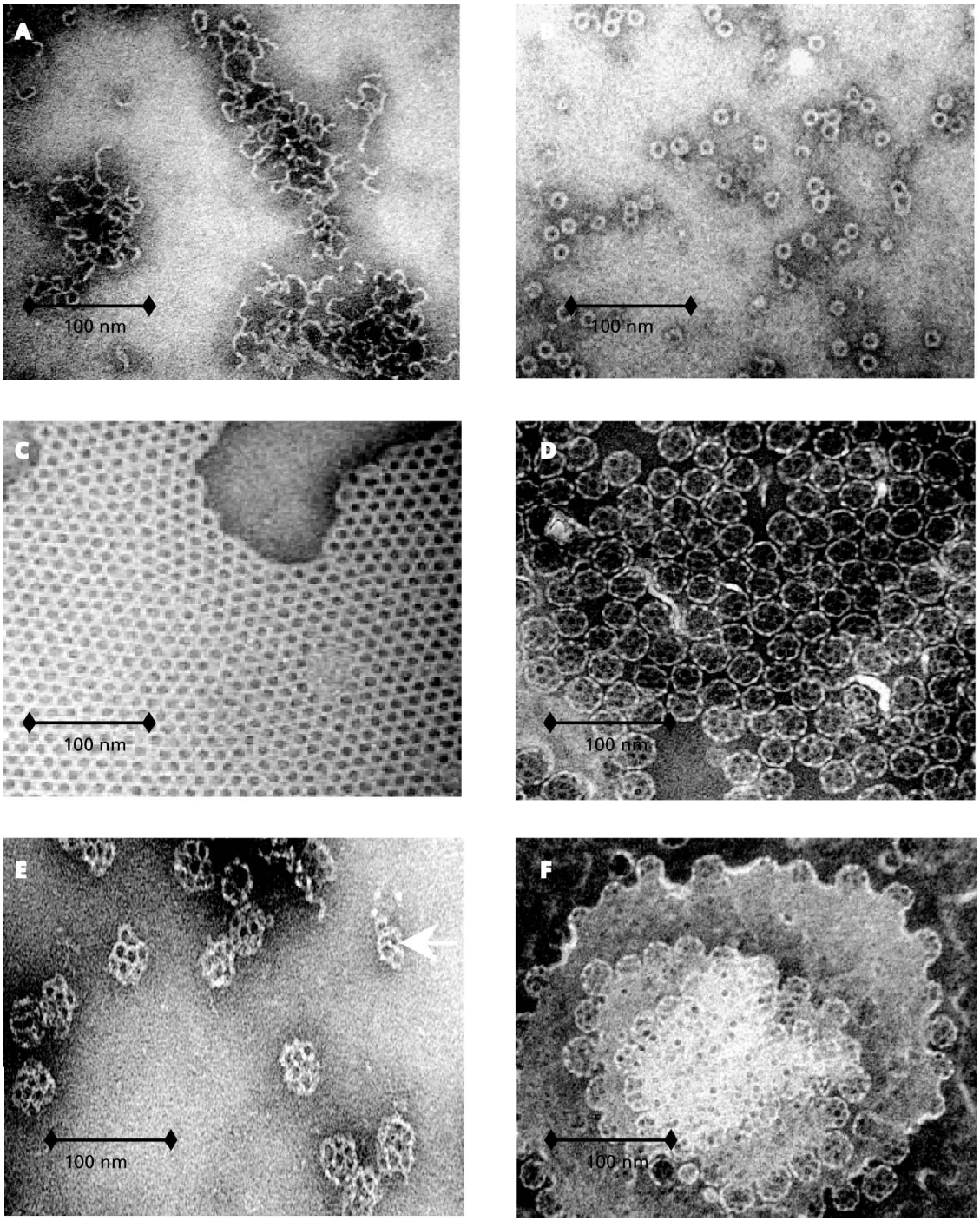


**Figure 3** Electron micrographs of pseudo-binary and pseudo-ternary systems. (A) Quil A:cholesterol mass ratio of 4:1 (HM); (B) Quil A:cholesterol mass ratio of 4:1 (DM); (C) Quil A:cholesterol mass ratio of 1:4 (DM); (D) Quil A:cholesterol:PE mass ratio of 1:1:3 (HM); (E) Quil A:cholesterol:PE mass ratio of 2:1:2 (HM); (F) Quil A:cholesterol:PE mass ratio of 1:2:7 (HM). HM, hydration method; DM: dialysis method.

between the two methods of preparation. PLM showed cholesterol crystals in all pseudo-binary systems containing Quil A and cholesterol prepared by hydration. Fewer cholesterol crystals were observed in similar samples prepared by dialysis, and in some of these pseudo-binary mixtures crystals were not observed (Figure 2). These observations suggest that cholesterol is solubilized into Quil A/octylglucoside micelles and on removal of octylglucoside promotes the formation of ring-like micelles. Increasing the cholesterol:Quil A mass ratio for

pseudo-binary systems prepared by dialysis resulted in ring-like micelles associating into a hexagonal pattern previously described as lamellae structures (Kersten et al 1991) (Figure 3C). Lamellae structures only form if a high concentration of cholesterol is solubilized into Quil A micelles and most likely results from the increased hydrophobicity of the ring-like micelles (Kersten et al 1995). In contrast only worm-like micelles and cholesterol crystals were observed in pseudo-binary systems of Quil A and cholesterol prepared by the hydration method, suggesting that in the absence of an additional surfactant, the ability of Quil A to solubilize cholesterol is limited. As the prevalence of cholesterol crystals increased on increasing the cholesterol:Quil A ratio, it would also appear that these worm-like micelles comprise a defined Quil A/cholesterol composition.

The regions of the pseudo-ternary diagrams in which ISCOM matrices were observed as the predominant colloidal structures also differed between the two methods of preparation. For samples prepared by dialysis, ISCOM matrices were predominantly found near the Quil A apex of the pseudo-ternary diagram ( > 50% Quil A). These results are in agreement with data published by other research groups (Kersten et al 1991; Rimmelzwaan & Osterhaus 1997; Sjölander et al 1997; Mowat et al 1999; Andersson et al 2000). In contrast, for samples prepared by lipid-film hydration, ISCOM matrices were predominantly found near the phospholipid apex of the pseudo-ternary diagram ( > 50% phospholipid). The morphology of the ISCOM matrices prepared by the two methods, however, appeared identical when viewed by TEM. An electron micrograph showing ISCOM matrices observed in a sample containing 20% Quil A, 20% cholesterol and 60% PE prepared by lipid-film hydration is shown in Figure 3D. At this ratio of components no or few ISCOMs but rather liposomes and ring-like micelles were observed in samples prepared by dialysis. It remains to be investigated whether the chemical composition of the ISCOMs prepared by the two methods is similar.

As previously suggested for pseudo-binary Quil A/cholesterol systems, if residual octylglucoside plays (at least partly) the role of phospholipids and thereby promotes ISCOM formation in pseudo-binary systems, then it is equally likely that octylglucoside can play the role of phospholipids in pseudo-ternary systems and hence result in a shift of the region in the pseudo-ternary diagram in which ISCOMs are observed into areas of lower phospholipid concentration, i.e. towards the Quil A apex. Octylglucoside may also play a role in pseudo-ternary systems having a relatively high concentration of phospholipids in promoting the formation of liposomes and ring-like micelles instead of the ISCOMs observed in pseudo-ternary systems prepared by hydration. Liposomes were observed in pseudo-ternary systems prepared by hydration at much higher phospholipid ratios than in pseudo-ternary systems prepared by dialysis, again suggesting that octylglucoside may become involved in structure formation. Alternatively, the difference noted between the two methods of preparation may result from the loss of Quil A during dialysis. Quil A is a heterogeneous mixture containing saponins of different molecular weights (Barr et al 1998). It may be possible that saponins with a low molecular weight can pass through the 1000 molecular weight cut-off membrane and are therefore lost as a partner to form ISCOM matrices. Consequently, this would lead to an insufficient effective concentration of Quil A, especially in formulations with a low weight percentage of the saponin mixture. A low concentration of Quil A would result in the formation of colloidal structures other than the ISCOM matrices, such as liposomes.

The regions in the pseudo-ternary diagrams in which ISCOM matrices were observed increased following an extended equilibration time. This was particularly apparent for samples prepared by lipid hydration. Pseudo-ternary systems originally containing ring-like micelles and liposomes were seen to transform to ISCOM matrices with time, suggesting that these colloidal structures may be precursors to ISCOM formation. The widespread distribution of ISCOM matrices in samples after an extended equilibration period therefore not only implies that this equilibration period is important to prepare ISCOMs by lipid-film hydration but also that ISCOMs are a thermodynamically more stable colloidal structure than the initially detectable colloids from which they have formed. On the other hand, the regions in which ISCOM matrices were the main colloidal structures after dialysis were not much affected by equilibration time, in agreement with reports in the literature (Höglund et al 1989; Kersten et al 1991). Surprisingly, the total area within the pseudo-ternary diagram in which ISCOMs were the predominant colloidal structure was significantly smaller following the dialysis method than following the hydration method (18% for the dialysis method and 31% for the hydration method of the total area of the pseudo-ternary diagrams after 1 month of storage at 4 °C, using PE as phospholipid). These differences may again be attributed to the role of the additional surfactant used in the dialysis method. This suggestion is based on speculation provided by other investigators (Kersten et al 1991; Kersten & Crommelin 1995) that residual surfactant, remaining within the formulation during dialysis, may influence the formation and appearance of ISCOMs. The presence of residual surfactant in the colloidal particles prepared by the dialysis method was not examined in this study nor in that of Kersten et al (1991), and to the best of our knowledge no other group has investigated this aspect. It will be important, however, in future studies to analyse ISCOMs made by the dialysis method for the presence of residual surfactant.

The formation of ISCOMs by dialysis involves a different pathway (from mixed micelles to ISCOMs) from that in which ISCOMs are formed by lipid-film hydration (from ring-like micelles or liposomes to ISCOMs). Electron micrographs showing the possible formation of ISCOM matrices from ring-like micelles or liposomes for samples prepared by lipid-film hydration are presented in Figures 3E (arrow) and 3F respectively. Individual ring-like micelles appear to associate and aggregate into ISCOM matrices, possibly as a result of micelle–micelle hydrophobic interactions. On the other hand, ISCOM matrices appear to bud off from the phospholipid bilayers

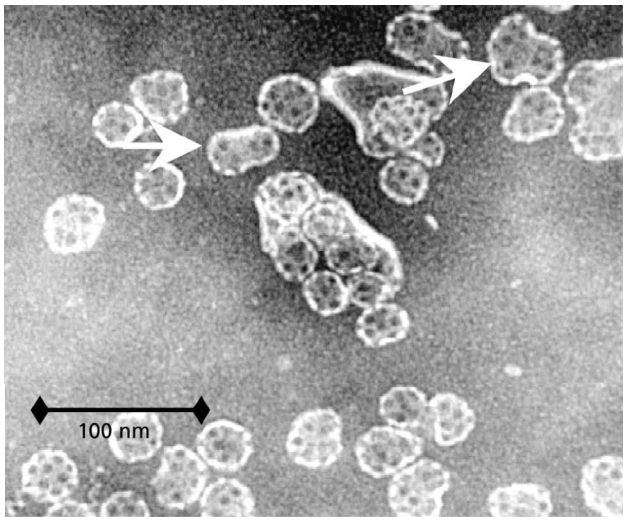


**Figure 4** Electron micrograph of a pseudo-ternary system prepared by lipid-film hydration. Quil A:cholesterol:PC mass ratio of 3:2:5.

(Figure 3F). This phenomenon was frequently observed in ternary systems with high concentrations of phospholipids on increasing the Quil A concentration.

A comparison of the various regions of colloidal structures in the pseudo-ternary diagrams prepared using either PE or PC as phospholipids by both methods of preparation showed only minor differences (Figures 1 and 2). The main difference was that ISCOM-like structures (i.e. morphologically less-defined ISCOM matrices) were observed more frequently in pseudo-ternary systems containing PC than in PE-containing systems (Figure 4, arrows). This result was not unexpected because it has been previously reported that ISCOMs prepared from PC-containing systems tend to be more heterogeneous in size and morphology (Devries et al 1990; Kersten et al 1991). ISCOM regions following the hydration method after 1 month of storage at 4 °C were larger for PC than for PE systems (37 and 31% of the total area of the pseudo-ternary diagram, respectively). Another difference observed was that the hexagonal pattern of ring-like micelles in the cholesterol-rich samples was well formed and more frequently observed in PE-containing pseudo-ternary systems than in the respective PC-containing systems. These findings may be due to differences in the polarity of the head group of the phospholipids (Kersten et al 1991).

## Conclusion

This study has demonstrated that the preparation method and type of phospholipid influence the appearance of colloidal structures in the pseudo-ternary diagram. The study has also further demonstrated that the formation of ISCOM matrices requires the presence of all three components (Quil A, cholesterol and PC or PE) as ISCOM matrices were not observed in the pseudo-binary systems prepared by lipid-film hydration, which does not require the use of an additional surfactant. The existence of worm-like micelles in pseudo-binary systems containing only Quil A and cholesterol prepared by lipid-film hydration, which were not found in samples prepared by dialysis, implied that in the absence of an additional surfactant, Quil A solubilized cholesterol to an extent insufficient to allow the formation of ring-like micelles. The widespread distribution of ISCOM matrices in samples prepared by lipid-film hydration after an extended equilibration period not only implies that equilibration period is important to prepare ISCOMs by this method but also that ISCOMs appear to be the thermodynamically stable colloidal form for the majority of pseudo-ternary aqueous systems of Quil A, cholesterol and phospholipid. Future work needs to look at the chemical composition of the colloidal structures formed, especially using the dialysis method, to investigate further the role of additional surfactants in the formation process of these important sub-unit vaccine delivery systems.

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