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Pseudo-ternary phase diagrams of aqueous mixtures of Quil A, cholesterol and phospholipid prepared by the lipid-film hydration method

Patrick H. Demana [a], Nigel M. Davies [b], Uwe Vosgerau [a], Thomas Rades [a,*]

[a] *Drug Delivery Solutions, New Zealand National School of Pharmacy, University of Otago, Dunedin, New Zealand*
[b] *School of Pharmacy, University of Queensland, Brisbane, Australia*

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Abstract

Pseudo-ternary phase diagrams of the polar lipids Quil A, cholesterol (Chol) and phosphatidylcholine (PC) in aqueous mixtures prepared by the lipid film hydration method (where dried lipid film of phospholipids and cholesterol are hydrated by an aqueous solution of Quil A) were investigated in terms of the types of particulate structures formed therein. Negative staining transmission electron microscopy and polarized light microscopy were used to characterize the colloidal and coarse dispersed particles present in the systems. Pseudo-ternary phase diagrams were established for lipid mixtures hydrated in water and in Tris buffer (pH 7.4). The effect of equilibration time was also studied with respect to systems hydrated in water where the samples were stored for 2 months at 4 °C. Depending on the mass ratio of Quil A, Chol and PC in the systems, various colloidal particles including ISCOM matrices, liposomes, ring-like micelles and worm-like micelles were observed. Other colloidal particles were also observed as minor structures in the presence of these predominant colloids including helices, layered structures and lamellae (hexagonal pattern of ring-like micelles). In terms of the conditions which appeared to promote the formation of ISCOM matrices, the area of the phase diagrams associated with systems containing these structures increased in the order: hydrated in water/short equilibration period < hydrated in buffer/short equilibration period < hydrated in water/prolonged equilibration period. ISCOM matrices appeared to form over time from samples, which initially contained a high concentration of ring-like micelles suggesting that these colloidal structures may be precursors to ISCOM matrix formation. Helices were also frequently found in samples containing ISCOM matrices as a minor colloidal structure. Equilibration time and presence of buffer salts also promoted the formation of liposomes in systems not containing Quil A. These parameters however, did not appear to significantly affect the occurrence and predominance of other structures present in the pseudo-binary systems containing Quil A. Pseudo-ternary phase diagrams of PC, Chol and Quil A are important to identify combinations which will produce different colloidal structures, particularly ISCOM matrices, by the method of lipid film hydration. Colloidal structures comprising these three components are readily prepared by hydration of dried lipid films and may have application in vaccine delivery where the functionality of ISCOMs has clearly been demonstrated.

Keywords: Quil A; ISCOMs; ISCOM matrix; Liposomes; Micelles; Pseudo-ternary phase diagrams; Vaccine delivery

1. Introduction

There is a growing interest in the use of colloidal particles (i.e. structures in the nanometer size range)

* Corresponding author. Tel.: +64-3-479-5410; fax: +64-3-479-7034.
E-mail address: thomas.rades@stonebow.otago.ac.nz (T. Rades).

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as subunit vaccine delivery systems. Liposomes, for example, allow for the encapsulation of labile antigenic proteins and peptides in a multimeric particulate form. However, liposomes due to the lack of sufficient inherent immunogenicity usually require the use of additional adjuvants if they are to be effective in stimulating an immune response (Kersten and Crommelin, 1995). The addition of soluble adjuvants, which can be added to liposomes in order to increase their immunogenicity are not, however, linked to the colloid containing the antigen and may thus be suboptimal for immune stimulation or even result in undesirable side effects (Kersten and Crommelin, 1995). Colloidal lipidic structures known as immune-stimulating complexes (ISCOMs) have been developed as improved lipidic vaccine delivery systems (Morein et al., 1984). ISCOMs are symmetrical colloidal particles with an open cage-like structure in the size range of 40–100 nm (Kersten et al., 1991). They are composed of the saponin adjuvant Quil A, cholesterol, phospholipid and the subunit antigen (usually an amphipathic protein). ISCOMs also form in the absence of protein and these structures are termed ISCOM matrices, indicating that the polar lipids are the structural elements in these colloidal particles. ISCOMs combine the advantages of a particulate carrier system with the presence of an in-built adjuvant (Quil A). ISCOMs induce a strong immunogenic response, as the adjuvant is part of the colloidal structure. Consequently, ISCOMs have been found to be more immunogenic than other colloidal systems such as liposomes and protein micelles (Kersten and Crommelin, 1995; Barr and Mitchell, 1996).

The current standard method for ISCOM preparation is the dialysis method (Kersten and Crommelin, 1995; Barr et al., 1998; Sjolander et al., 2001). This method requires the use of additional surfactant (e.g. MEGA 10 or octylglucoside), which is removed by extensive dialysis using a membrane with a molecular weight cut-off of typically 1000 (Lovgren and Morein, 1988). 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, whereas the dialysis method is comparable to the detergent-removal-technique to produce liposomes. We have since refined this methodology by introducing a freeze-drying step in order to promote intimate mixing of Quil A, cholesterol and phospholipid.

The aim of this study was to construct a pseudo-ternary phase diagram for Quil A, phospholipid and cholesterol in order to identify combinations which result in the formation of ISCOM matrices, identify other colloidal particles produced by these three compounds in aqueous systems and identify structures formed in the vicinity of the ISCOM matrix region of the phase diagram to further understand ISCOM matrix structure and formation. A further aim was to investigate the effects of buffer salts and equilibration time on the nature of the pseudo-ternary phase diagram as these two factors are known to influence the swelling behavior of phospholipid films (Gulik-Krzywicki et al., 1969; Hauser, 1984; New, 1990).

2. Methods

2.1. Materials

Quil A was purchased from Superfos Biosector, Denmark. Cholesterol (purity approx. 95%) and L-α-phosphatidylcholine from egg yolk (purity approx. 99%) were purchased from Sigma-Aldrich Pty Ltd., Missouri, USA. Distilled demonized water having a conductivity of less than 0.1 μS (Milli-Q Water system, Millipore Corporation, MA, USA) was used throughout the study. All other chemicals and solvents were of at least analytical grade.

2.2. Preparation of colloidal dispersions

Various amounts of phosphatidylcholine (PC) and cholesterol (Chol) 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 2 h at room temperature with 3 ml water or Tris buffer, 140 mM, pH 7.4 containing various amounts of Quil A. The total polar lipid concentration in each sample (Quil A, Chol and PC) was 6.7 mg/ml. The samples were subsequently freeze-dried for 24 h (Freezone 6, Model 79340, Labconco, Missouri, USA) at a condenser temperature of −82 °C and a pressure of less than 10^{-1} mbar.

The freeze-dried samples were rehydrated with 3 ml of water and stirred using a magnetic stirrer for 2 h.

2.3. Characterization of colloidal dispersions

Dispersions were either investigated within a day of preparation or after storage in a fridge (4 °C) for a period of 2 months (samples prepared in water only).

2.3.1. Transmission electron microscopy (TEM)

Carbon-coated copper grids were glow-discharged (Edwards E306A Vacuum Coater, England) and 10 μl of sample adsorbed on to these grids. The samples were negatively stained using 10 μl of filtered 2% phosphotungstic acid, pH 5.2 as a contrast agent. Although it cannot be excluded that the staining agent might affect the morphology of the structures viewed in the TEM, reproducible visualization of the morphology of ISCOMs, liposomes, and other colloidal structures has been demonstrated for negatively stained samples by various groups (Ozel et al., 1989; Kersten et al., 1991). The effect of phosphotungstic acid on the morphology of the structures observed in this study was therefore considered to be minimal.

Samples were investigated using a Phillips CM100 transmission electron microscope at an acceleration voltage of 100 kV and typically viewed 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 percentage of all the colloidal particles present in the sample. Colloidal particles that comprised less than 10% of the total colloidal particles counted in a specific sample were classed as minor colloidal structures.

2.3.2. Polarized light microscopy (PLM)

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

3. Results and discussion

3.1. Pseudo-ternary phase diagrams for Quil A, Chol and PC dispersed in water

The pseudo-ternary phase diagrams of mixtures of Quil A, Chol and PC dispersed in water and characterized within 1 day of preparation, following 2 months of storage at 4 °C and dispersed in Tris buffer pH 7.4 as well as the type and propensity of the structures observed therein are shown in Figs. 1–3,

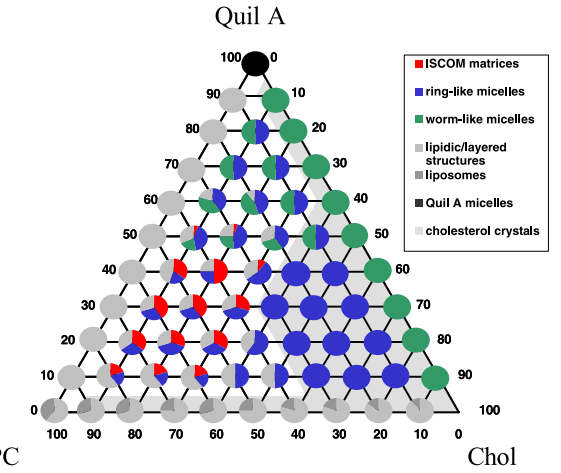

Fig. 1. Pseudo-ternary phase diagram for mixtures of Quil A, Chol and PC hydrated in water and characterized within 1 day of preparation.

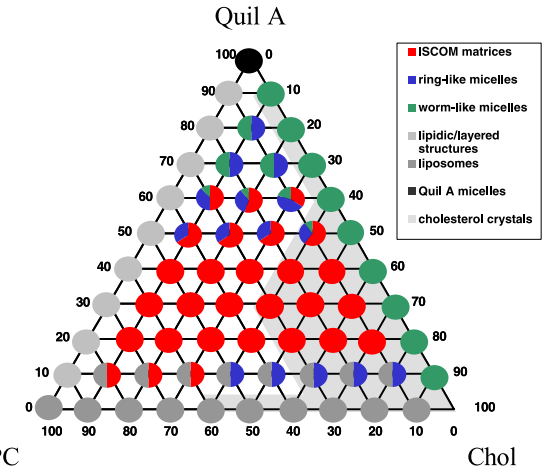

Fig. 2. Pseudo-ternary phase diagram for mixtures of Quil A, Chol and PC hydrated in water and equilibrated for 2 months at 4 °C.

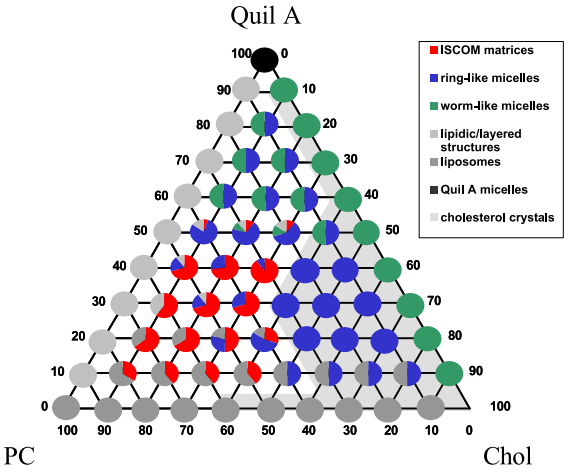

Fig. 3. Pseudo-ternary phase diagram for mixtures of Quil A, Chol and PC hydrated in Tris buffer (140 mM, 7.4) and characterized within 1 day of preparation.

respectively. Depending on the mass ratio of Quil A, Chol and PC in the systems, various colloidal particles including ISCOM matrices, liposomes, lipidic particles, ring-like and worm-like micelles were observed. Other colloidal particles were also observed as minor structures in the presence of these predominant colloids including helices, layered structures and lamellae (hexagonal pattern of ring-like micelles). The prevalence and distribution of these structures in the samples used for the construction of the pseudo-ternary phase diagrams was influenced by the presence of buffer salts and equilibration time and is reported and discussed below. Chol crystals were also observed in some systems particularly in areas of high Chol ratios or in the absence of PC.

3.1.1. *Systems containing only Quil A, Chol and PC*

Fig. 4A and B show electron micrographs of aqueous dispersions of Quil A and PC, respectively. Fig. 4C shows an electron micrograph of PC hydrated

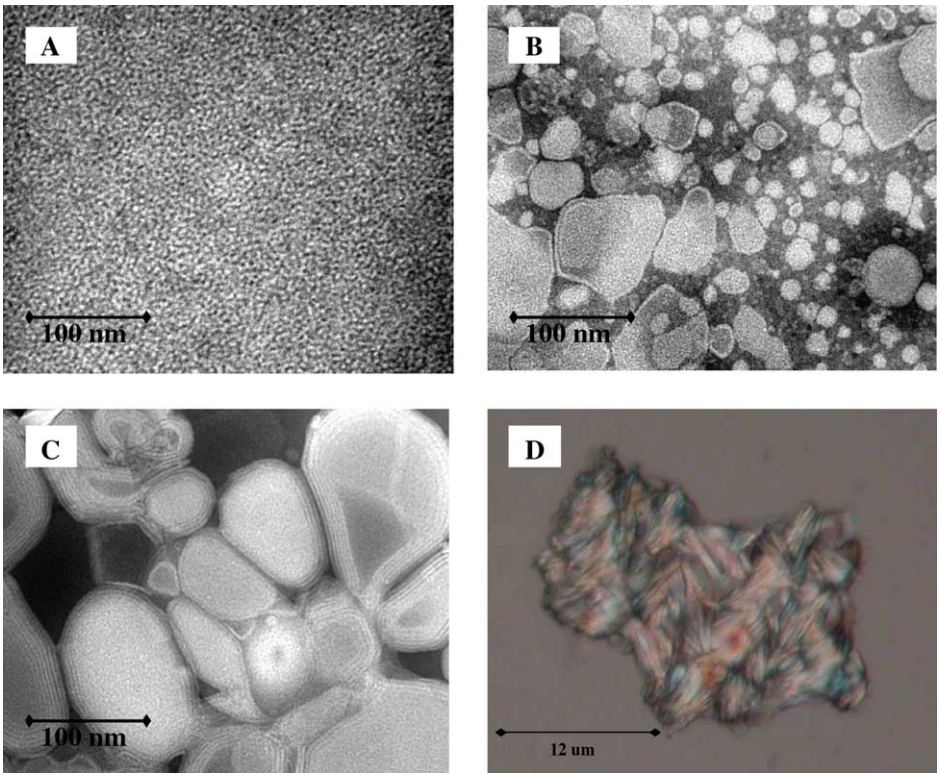

Fig. 4. Electron micrographs of single lipid component systems. (A) Quil A (Quil A micelles), (B) PC, hydrated in water (lipidic particles), (C) PC, hydrated in buffer (liposomes) and PLM micrograph of cholesterol crystal (D).

in buffer, and Fig. 4D shows a polarized light micrograph of a crystal of Chol dispersed in water. Quil A forms micelles in the absence of other components in water or buffer. It has a reported CMC in water of 0.03% (Ozel et al., 1989). In aqueous systems of PC alone, a mixture of lipidic particles (predominantly) and some bilayered structures (liposomes, typically <40%) were observed whereas PC in buffer formed predominantly liposomes. In systems containing only Chol, crystals were observed, which were birefringent when viewed under polarized light.

The nature of the structures formed in these single lipid component systems did not change with incubation time or when buffer was used as the medium for lipid-film hydration except for samples containing PC. In these samples, the presence of buffer salts or prolonged storage time promoted the formation of bilayered structures, which is in agreement with previous results (Gulik-Krzywicki et al., 1969; Hauser, 1984; New, 1990). It is well known that phospholipids in excess water swell and form multilamellar vesicles (Szoka and Papahadjopoulos, 1981; Kersten and Crommelin, 1995). However, various investigators have reported limited or no swelling at short equilibration times when PC or other neutral or isoelectric lipids such as phosphatidylethanolamine, sphingomyelines, glycolipids, and mono- and diacylglycerols are dispersed in excess water (Chapman et al., 1967; Reiss-Husson, 1967; Small, 1967; Gulik-Krzywicki et al., 1969; Shipley et al., 1969; Shipley, 1973; Hauser, 1984). It is now generally accepted that phospholipids with a net charge show continuous swelling and uncharged or isoelectric phospholipids show no or limited swelling when they are dispersed in excess water (Reiss-Husson, 1967; Small, 1967; Gulik-Krzywicki et al., 1969; Shipley et al., 1969; Shipley, 1973; Hauser, 1984). The reason for this is the swelling process itself, which is a purely electrostatic phenomenon (Hauser, 1984), which may be influenced by the addition of buffer salts.

3.1.2. Pseudo-binary Quil A/Chol samples (worm-like micelles)

In systems containing Quil A and Chol, worm-like micelles, as shown in Fig. 5A, were observed which are very different in appearance from Quil A micelles. These worm-like Quil A/Chol micelles were observed in all systems containing these two components. However, in systems where the ratio of Chol:Quil A was greater than 1:9, these worm-like micelles were found together with Chol crystals indicating that the solubilization of Chol by Quil A was not complete. The predominance of Chol crystals increased on descending the Quil A–Chol axis of the pseudo-ternary phase diagram. Neither the presence of buffer salts nor an extended equilibration time influenced the nature of the structures formed in binary systems of Quil A and Chol.

3.1.3. Pseudo-binary Quil A/PC samples (lipidic particles and layered structures)

The addition of Quil A to systems containing PC resulted in the formation of some layered structures which appeared together with a predominance of lipidic particles. These lipidic particles were similar to those observed in systems containing only PC and those which have been previously reported in Quil A/PC systems (de Vries et al., 1990). An electron micrograph of lipidic particles with few layered structures is shown in Fig. 5B. The sample has a Quil A:PC mass ratio of about 2:1. The layered structures appear as stacks (Fig. 5B or C, arrow) having a layer thickness similar to that observed for liposomes (∼6 nm). This suggests that these layered structures might be bilayer fragments of vesicular structures. Interestingly, hardly any liposomes were observed in samples containing Quil A even at high ratios of PC:Quil A. Thus, Quil A would appear to hinder vesicle formation despite forming bilayer structures with PC possibly by altering the lipid packing. The existence of these layered structures in systems containing Quil A and PC has not previously been reported. Upon increasing the ratio of Quil A:PC, both layered and lipidic structures became fewer and smaller in size being replaced by micelles of a similar appearance to those observed in the system containing only Quil A.

The nature of the structures formed in these pseudo-binary systems did not change with incubation time or when buffer was used as the medium for lipid-film hydration. However, the prevalence of layered structures appeared to be higher in the presence of buffer salts or following storage in water systems (Fig. 5C). This is again likely due to the effect of ions and time on the swelling behavior of PC (Gulik-Krzywicki et al., 1969; Hauser, 1984; New, 1990).

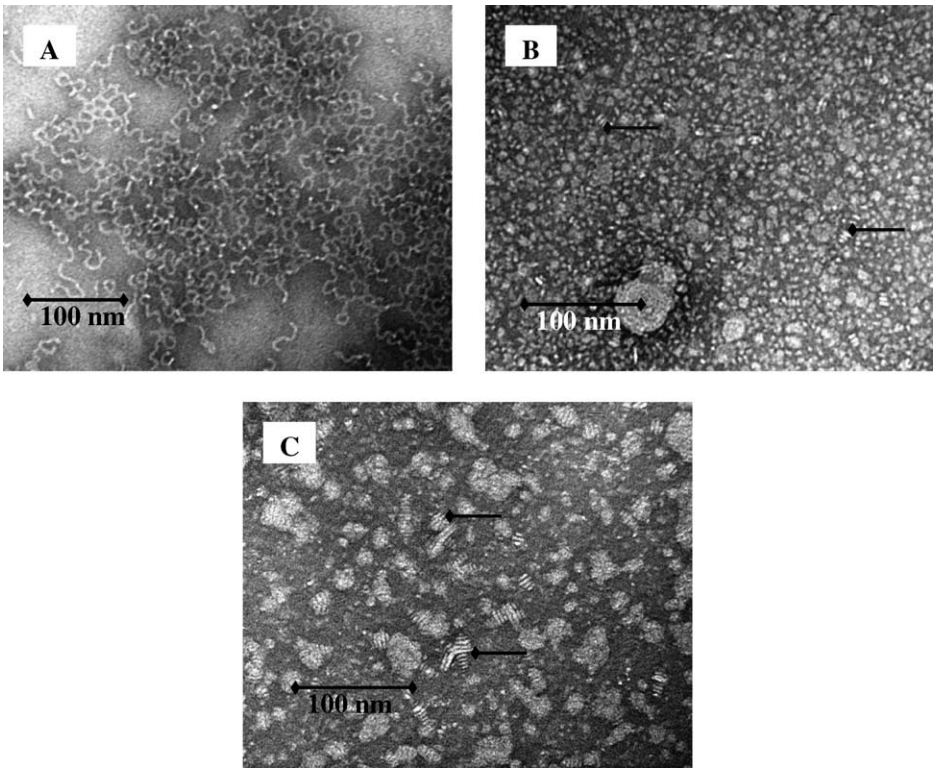

Fig. 5. Electron micrographs of pseudo-binary systems. (A) Quil A:Chol, mass ratio of 1:1 (worm-like micelles), (B) Quil A:PC, mass ratio of 2:1, within one day of preparation (lipidic/layered structures), (C) Quil A:PC, mass ratio of 2:1, after 2 months storage at 4 °C (lipidic/layered structures).

3.1.4. Pseudo-binary PC/Chol samples (liposomes, lipidic particles and Chol crystals)

At high PC concentrations the predominant structures were lipidic particles with some liposomes. The occurrence of liposomes in the systems decreased with increasing concentration of Chol resulting from less PC being present in the systems. Likewise, the prevalence of Chol crystals increased with increasing concentration of Chol. Furthermore, crystals were evident in all samples, which had been prepared in water and analyzed within 1 day.

Hydration of the dried lipid films using buffer or an extended equilibration time again promoted the formation of liposomes (multilamellar vesicles) at the expense of lipidic particles which were no longer evident in the samples. Chol crystals only appeared in PC/Chol binary systems hydrated with buffer or equilibrated for a prolonged period at a Chol:PC weight ratio of about 40:60 or higher. This is in agreement with previous literature reporting the maximum solubility of Chol in PC bilayers to be about 33% (w/w) (Bourges et al., 1967; Freeman and Finean, 1975; Collins and Phillips, 1982).

3.1.5. Pseudo-ternary Quil A/Chol/PC samples

3.1.5.1. Ring-like micelles. Excess Chol was present as crystals in all systems where the weight fraction of cholesterol was greater than 60% and in systems containing more than 40% Chol if the concentration of PC and Quil A was less than 30% respectively. Chol crystals were also apparent in systems containing up to 50% of Quil A, but only if the weight fraction of PC was small (Fig. 1). Thus, the presence of both PC and Quil A promoted the solubilization of Chol or its incorporation into colloidal structures.

In ternary systems containing >50% of Quil A, worm-like micelles, as observed in the pseudo-binary Quil A/Chol systems, were present together with

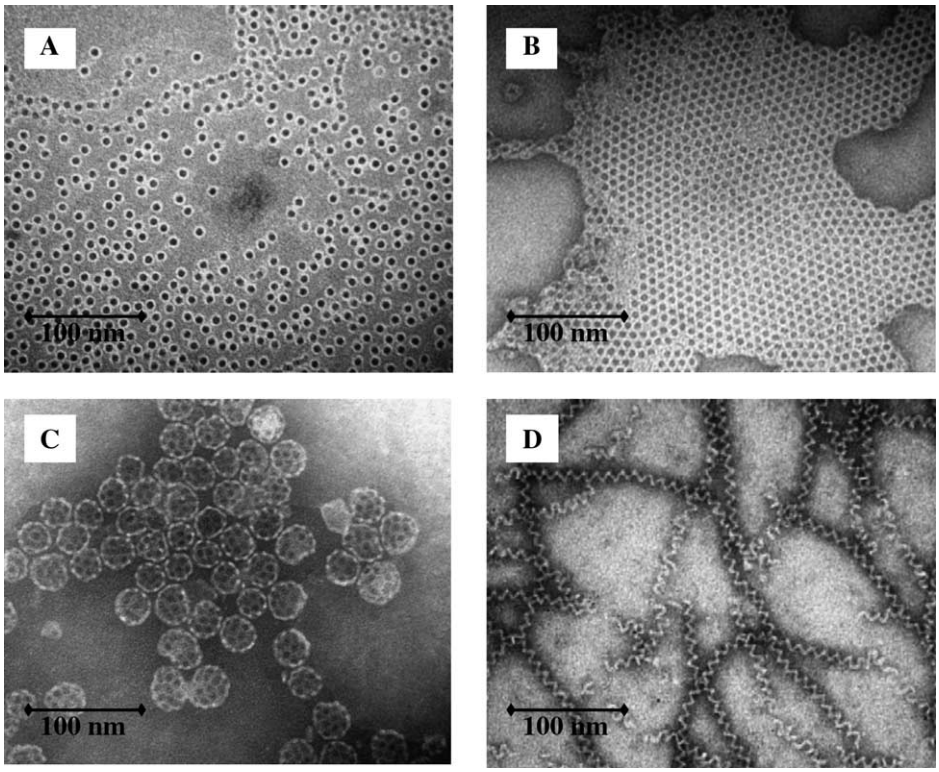

Fig. 6. Electron micrographs of pseudo-ternary systems. (A) Quil A:Chol:PC, mass ratio of 1:3:1 (ring-like micelles), (B) Quil A:Chol:PC, mass ratio of 1:3:1, as minor colloidal structure (lamellae), (C) Quil A:Chol:PC, mass ratio of 2:1:2 (ISCOM matrices), (D) Quil A:Chol:PC, mass ratio of 2:1:2, as minor colloidal structure (helices).

ring-like micelles. Lipidic and layered structures were also observed in some samples with relatively high percentages of PC (20–40%). In ternary systems containing >40–50% of Chol, ring-like micelles having a diameter of about 10 nm were the dominant colloidal structure, sometimes appearing exclusively and sometimes appearing together with lamellae (hexagonal pattern of ring-like micelles which present a minor structure, having a size greater than 1 μm). The TEM micrographs (Fig. 6A and B) show the ring-like micelles and associated lamellae structures from a sample with a Quil A:Chol:PC mass ratio of 1:3:1. The ring-like micelles are similar in size and morphology to those described by other groups and result from the solubilization of Chol by the Quil A micelles (Kersten and Crommelin, 1995). Lamellae were previously observed in systems having a high concentration of Chol and are believed to be a result of the increased lipophilicity imparted onto the mixed micelles by Chol, thereby promoting micelle association in aqueous systems (Kersten and Crommelin, 1995).

The tendency of pseudo-ternary systems to form ring-like and/or worm-like micelles did not seem to be affected by the presence of buffer salts. However, at low Quil A concentrations, hydration using buffer appeared to promote the formation of liposomes at the expense of lipidic particles resulting in systems containing both liposomes and ring-like micelles. In contrast, an extended equilibration period (2 months at 4 °C) had a significant effect on the pseudo-ternary systems, which tended to form ring-like micelles at a Quil A concentration of less than 60%. In these systems, the predominant colloidal structures were ISCOM matrices, which in some samples appeared together with helices (see Sections 3.1.5.2 and 3.1.5.3 for further discussion on ISCOM matrices and helices). Thus, an extended equilibration time transformed systems containing predominantly

ring-like micelles into systems containing predominantly ISCOM matrices. The disappearance of ring-like micelles and lamellae structures from these pseudo-ternary systems upon storage suggests that these colloids could be involved in the formation of ISCOM matrices. At higher Quil A weight fractions, ISCOM matrices co-existed with ring-like micelles and even worm-like micelles. At low Quil A concentrations and high PC concentrations, again ring-like micelles co-existed with liposomes.

3.1.5.2. ISCOM matrices. In systems containing greater than 30% PC and less than 50% Quil A, ternary systems were found to contain ISCOM matrices. These were particularly evident in systems containing 40–70% PC, 20–40% Quil A and 10–30% Chol. Fig. 6C is an electron micrograph of a sample containing ISCOM matrices observed in a sample containing 40% PC, 40% Quil A and 20% Chol. A close examination of the micrographs showed that the samples comprise spherical ISCOM matrices together with more irregularly shaped ISCOM-like structures. This is probably due to the use of PC as phospholipid instead of phosphatidylethanolamine (PE), another phospholipid commonly used in the preparation of ISCOMs. PE is reported to be better able to promote the formation of ISCOMs with a more uniform size and shape compared to PC (Kersten et al., 1991). The use of PC may result in the formation of a more heterogeneous ISCOM population (de Vries et al., 1990; Kersten et al., 1991). In samples prepared by hydrating the lipid film with water and analyzed within a day, ISCOM matrices were typically observed together with ring-like micelles and lipidic/layered structures. The co-existence of ring-like micelles with ISCOM matrices again suggests the involvement of these micelles in ISCOM matrix formation. In the presence of sufficiently high concentrations of PC, these ring-like micelles are probably aggregated and linked together with PC by hydrophobic interactions to produce ISCOM matrices having the characteristic cage-like structure.

When <30% PC was present in the pseudo-ternary systems, no ISCOM matrices were observed. This is consistent with previous reports (de Vries et al., 1990; Kersten and Crommelin, 1995; Copland et al., 2000), documenting the requirement of phospholipids for ISCOM formation but is contrast to another study (Lovgren and Morein, 1988) in which phospholipids were reported not to be essential for ISCOM formation. These conflicting results possibly arise due to the different methods that are used to prepare ISCOMs. The standard method to prepare ISCOMs is the dialysis method (Kersten and Crommelin, 1995). Using this method, an additional surfactant such as octylglucoside (Kersten and Crommelin, 1995) or MEGA-10 (Andersson et al., 2000) is used to solubilize the lipidic components of ISCOMs. ISCOMs are then formed by the removal of this surfactant by dialysis. It is possible that in the reported phospholipid-free ISCOMs, the role of the phospholipid has been taken over by residual surfactant (Kersten and Crommelin, 1995). It is clear, however, from the present investigation, which does not involve the use of additional surfactants, that ISCOM matrix formation does require the presence of all three components, Quil A, Chol and PC.

When buffer was used to hydrate the dried lipid film, the prevalence of ISCOM matrices in the samples was higher than when water was used. A comparison of the pseudo-ternary phase diagram for samples hydrated in water and samples hydrated in buffer not only shows the increased prevalence of these structures in systems in which ISCOM matrices were evident but also shows that some samples which did not produce ISCOM matrices in water produced ISCOM matrices upon hydration of the samples in buffer. This may be a result of buffer salts being better able to promote the swelling of phospholipid films. It is also known that ISCOMs can only form when Quil A is above its CMC (Ozel et al., 1989). Since buffer salts generally lower the CMC of surfactants and hence promote aggregation of micelles compared to water systems (Mazer et al., 1979), it is possible that the aggregation of micelles was promoted in the presence of buffer resulting in the increased formation of ISCOM matrices.

A startling difference was observed between the pseudo-ternary systems examined immediately following preparation and following an extended equilibration period (Figs. 1 and 2). The prevalence of ISCOM matrices was markedly increased following prolonged storage to the extent that many samples contained only ISCOM matrices as colloidal particles. These samples not only arose from systems, which contained ISCOM matrices when observed within a day of preparation but also from samples

in which ISCOM matrices were not immediately apparent. Systems producing only ISCOM matrices following extended equilibration time tended to develop from systems, which initially contained either ring-like micelles (with lamellae) or ISCOM matrices together with ring-like micelles and lipidic/layered structures. In systems containing high concentrations of Chol, ISCOM matrices were observed together with Chol crystals, although the amount of Chol crystals appeared to have decreased following prolonged equilibration compared to samples examined immediately.

3.1.5.3. Helices. In samples found to contain ISCOM matrices, helical micelles were also observed as minor colloidal particles (Fig. 6D). Helices have been observed by a number of researchers in aqueous systems of bile salt, cholesterol and phosphatidylcholine (Konikoff et al., 1992, 1994; Tao et al., 1993; Kaplun et al., 1994; Tazuma et al., 1994; Ochi et al., 1996; Wang and Carey, 1996; Ringel et al., 1998). It has recently been shown that helices are not unique to bile salt containing systems, but are a general property of a whole range of multicomponent systems, which contain a micelle-forming surfactant, bilayer-forming amphiphiles, and a sterol (Zastavker et al., 1999). It is therefore not unexpected that helices can form from the mixture of Quil A, PC and Chol.

Helices are believed to be tilted bilayers of chiral amphiphiles, in which the constituent molecules have orientational but no positional long range order (Zastavker et al., 1999). It is believed that the packing of cholesterol (or any sterol molecule) and the micelle-forming surfactant in the bilayer portion of the phospholipid together with the molecular chirality in the system is the driving force behind helix formation (Chung et al., 1993; Nandi and Bagchi, 1996; Selinger et al., 1996; Komura and Zhong-can, 1998). It has also been previously reported that bilayers of PC can have a helical configuration if the PC is mixed with an acidic phospholipid in the presence of Ca^{2+} (Lin et al., 1982), or is mixed with diacetylenic compounds (Thomas et al., 1999). Helical bilayers have also been reported for phospholipid–nucleoside conjugates (Yanagawa et al., 1989). It is now generally accepted that the morphology of the helix assembly is strongly dependent on the chirality of the molecules used (Kaplun et al., 1994; Nandi and Bagchi, 1996; Wang and Carey, 1996; Ringel et al., 1998; Thomas et al., 1999; Zastavker et al., 1999) and therefore the helix itself is a chiral structure (Nandi and Bagchi, 1996). Since Quil A has many chiral centers and is a mixture of many structurally related bisdesmosidic triterpenoid glycosides (Kensil et al., 1991), it is possible that a particular configuration of Quil A molecules, in the presence of PC and Chol, will determine whether ISCOM matrices or helices are formed.

4. Conclusions

The construction of pseudo-ternary phase diagrams for the three components typically used for the preparation of ISCOM matrices, namely Quil A, PC and Chol, has demonstrated that these three components can also form a number of other colloidal structures depending on the mass ratio of the three components present in the system. Interestingly, the relative abundance of specific colloidal particles was affected by the aqueous medium used for lipid film hydration and also by the equilibration period. Buffer solution promoted the formation of ISCOM matrices and liposomes while a prolonged equilibration promoted the formation of ISCOM matrices to an extent that almost all ternary systems containing less than 50% of Quil A only formed ISCOM matrices following a 2-month storage at 4 °C. These investigations have thus further confirmed that ISCOM matrices can be prepared by hydration of dried PC:Chol films, a method that is similar to the Bangham method for the preparation of liposomes, and have identified preferred weight ratios of the components for ISCOM matrix formation by this method. The study has also further demonstrated that ISCOM matrix formation requires the presence of all three components as ISCOM matrices were not observed in any pseudo-binary systems. The existence of ring-like micelles and lamellae in ISCOM matrix containing samples or in samples which formed predominantly ISCOM matrices following a prolonged equilibration suggests that these structures are involved in ISCOM matrix formation, i.e. they may act as ISCOM matrix precursors. The existence of helices as a minor colloidal structure in ISCOM matrix-rich samples may be a result of a the preferred configuration of a component of Quil A having a particular chiral configuration and interacting with the PC/Chol bilayers.

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