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Preparation of immuno-stimulating complexes (ISCOMs) by ether injection

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Abstract

This study investigated the application of the solvent dispersion technique, specifically ether injection, which has been successfully used in the preparation of liposomes, as a new, continuous and potentially scaleable method for the preparation of ISCOMs. Phosphatidylcholine (PC) and cholesterol (Chol) were dissolved in ether, which was injected into an aqueous solution, maintained at 55 °C, containing Quil A. The influences of the following variables on ISCOM formation were investigated: ratio of PC:Quil A:Chol used, pumping rate, total lipid mass and concentration of buffer salts and Quil A in the aqueous phase. All samples were characterized by negative stain transmission electron microscopy, photon correlation spectroscopy and sucrose ultracentrifugation gradient. It was demonstrated that ISCOMs could be produced by this method but the homogeneity of the preparation was influenced by the conditions used. Homogeneous ISCOM preparations were consistently produced only when the weight ratio of PC:Quil A:Chol was 5:3:2 with a total lipid mass of 20 mg, the Quil A dissolved in a 0.01 M phosphate buffer at a concentration of 6 mg in 4 ml, and the ether solution injected into the warmed buffer solution at a rate of 0.2 ml/min. Changing any of these variables resulted in more heterogeneous preparations in which ISCOMs typically co-existed with other colloidal structures such as worm-like and helical micelles, liposomes, lamellae and lipidic particles.

Keywords: ISCOM; Immuno-stimulating complex; Quil A; Adjuvant; Ether injection

1. Introduction

Immuno-stimulating complexes (ISCOMs) have received much attention as delivery systems for sub-unit vaccines as they combine the advantages of a particulate carrier with the presence of a built-in adjuvant (Kersten and Crommelin, 2003). Their effectiveness as adjuvants has resulted in two ISCOM-based vaccines being registered for veterinary use and several clinical trials being carried out in humans (Kersten and Crommelin, 2003; Morein et al., 2004). ISCOMs are colloidal spherical particles with an open cage-like structure in the size range 40–100 nm (Ozel et al., 1989). They consist of the saponin Quil A (or one of its purified components), phospholipids, cholesterol and an antigen (Kersten et al., 1991). When the antigen is omitted, the colloidal structures formed between Quil A, phospholipid and cholesterol and which have the same morphology as ISCOMs are termed ISCOM matrices (Barr et al., 1998).

Two methods have traditionally been used for the preparation of ISCOMs, one based on centrifugation and the other on dialysis (Hoglund et al., 1989). In the centrifugation method, the protein, cholesterol and phospholipids are first solubilised using a surfactant such as Triton X-100 in the absence of Quil A. This micellar solution is then centrifuged through a sucrose gradient containing Quil A. During the centrifugation process, ISCOMs are formed and can be collected as a band in the sucrose gradient. The centrifugation method appears quite simple but is rarely used due to the limited quantities of ISCOMs that can be prepared in each run and the tedious nature of preparing gradients and collecting sucrose bands containing ISCOMs. In the dialysis method, the lipid components and Quil A are first solubilised in a surfactant system such as octylglucoside or Mega 10, which is subsequently removed by dialysis. This method is more commonly used than the centrifugation method and produces very pure and well-defined ISCOM preparations. However, it is a batch process and a time consuming procedure requiring at least 3 days dialysis against a suitable buffer (Reid, 1992; Johansson and Lovgreen-Bengtsson, 1999; Da Fonseca et al., 2000). There is also the possibility of residual surfactant

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remaining in the preparation when ISCOMs are prepared by either of these methods (Demana et al., 2004b). More recently, a surfactant-free procedure for the formation of ISCOMs was reported involving the hydration of dried lipid films of phospholipid and cholesterol using an aqueous solution of Quil A (Copland et al., 2000) and subsequently incorporating a freeze-drying step (Demana et al., 2004b,c). Despite the simplicity of this procedure, pure ISCOM preparations are only obtained following prolonged incubation times of several weeks.

In many respects, particularly their composition, ISCOMs resemble liposomes because they are both comprised of phospholipids and cholesterol, but ISCOMs additionally contain the bipolar saponin, Quil A. As ISCOMs are considered to be thermodynamically stable, they should spontaneously form in systems containing the above three components in a similar manner to the formation of liposomes when bilayer-forming phospholipids are dispersed in aqueous systems. Indeed, the dialysis and lipid film hydration methods of preparing ISCOMs are based on previously reported methods for the preparation of liposomes (Bangham et al., 1965; Rhoden and Goldin, 1979). Very recently, Lendemans et al. (2005) investigated the use of another method of liposome preparation, ethanol injection, as a possible method for the preparation of ISCOMs. These authors reported that the rapid injection of an ethanolic solution of phospholipid and cholesterol into an aqueous solution of Quil A led to the formation of ISCOMs in high numbers after 48 h of stirring but the preparation was not as homogeneous as an ISCOM preparation prepared by dialysis containing larger aggregates, ring-like micelles and helices. The removal of ethanol from the resulting preparation was also highlighted as a limitation of the technique, possibly requiring the use of dialysis in a similar manner to that required for the removal of surfactant in the dialysis method of ISCOM preparation. Further, the concentration of ethanol in the resulting dispersion was restricted to less than 7.5% in order that the components of ISCOMs could self-assemble by hydrophobic interactions and as such the lipid titre and hence concentration of ISCOMs was also limited by this parameter.

With consideration to the limitations of these published methods for ISCOM preparation, we investigated the possibility of using ether injection, another technique used for preparation of liposomes, as a possible scaleable and continuous method of ISCOM preparation. The ether injection method has the advantages that phospholipids and cholesterol have a high solubility in ether and as such, the final lipid titre can be high and, since ether is volatile, it can be readily removed from the system by gentle heating (New, 1990). The volatility of ether and the possibility of continuous removal from the system potentially allow the preparation of systems with elevated lipid titres. A further aim of this study was to investigate the effects of pumping rate (the technique used for solvent dispersion), lipid, Quil A and buffer concentration used on the formation of ISCOMs.

2. Materials and methods

2.1. Materials

Quil A was purchased from Brenntag Biosector, Frederikssund, Denmark. Cholesterol (purity approximately 95%), L-α-phosphatidylcholine from egg yolk (purity approximately 99%) and diethyl ether were purchased from Sigma–Aldrich Pty. Ltd., St. Louis, Missouri, USA. All other chemicals and solvents were of at least analytical grade.

2.2. Preparation of ISCOMs by ether injection

The mass ratio of phosphatidylcholine (PC), Quil A and cholesterol (Chol) used in the investigations was based on the pseudo-ternary phase diagram of these components as reported by Demana et al. (2004c). Twelve ratios were investigated which were reported by Demana et al. (2004c) to form ISCOMs to varying degrees when dried lipid films were hydrated with an aqueous solution of Quil A. The ratios of the three components, which were investigated are reported in Table 1. In the initial studies, used to identify the preferred mass ratio of the components, PC and Chol were dissolved in 1 ml of diethyl ether and the Quil A was dissolved in 4 ml phosphate buffer (0.01 M, pH 7.4). The total mass of lipids and Quil A was kept constant at 20 mg/5 ml-combined volume of ether and aqueous solution.

The aqueous solution of Quil A was placed into a 20 ml glass bottle fitted with a silicone rubber injection cap and this bottle was placed in a water jacket connected to a circulating water bath maintained at 55 °C. The ether solution containing the lipids was loaded into a syringe fitted via 1/16″ tubing to a 19 gauge needle. The syringe was placed in a syringe pump (PHD 2000 Infusion, Harvard Apparatus, USA) and the needle inserted through the silicone rubber injection cap into the aqueous Quil A solution such that the tip of the needle was under the surface of the aqueous solution. A second needle was inserted through the cap to act as a gas release vent. One millilitre of lipid in ether solution was then pumped at a rate of 0.2 ml/min into the aqueous solution, which was magnetically stirred and maintained at 55 °C for 2 h. Finally, the resulting preparation was allowed to equilibrate at room temperature with stirring for between 1 and 7 days prior to characterization of the resulting dispersion. Following identification of the preferred mass ratio of the three components for ISCOM formation, the effect of the rate at which the organic lipid solution was introduced into the aqueous phase (pumping rate varied from 0.2 to 1 ml/min), concentration of Quil A in the aqueous phase (6 mg dissolved in 2, 4 and 8 ml), total mass of lipid used (20, 50 and 100 mg) and the concentration of the buffer in the aqueous phase (0.001, 0.01 and 0.1 M) were investigated in order to identify the optimal conditions for ISCOM formation as prepared by ether injection.

2.3. Characterization of resulting dispersions

The resulting dispersions were characterized by photon correlation spectroscopy (PCS, Zetasizer 3000, Malvern) to measure size (*z*-average) and polydispersity of the particles present in the system. Samples were also visualized by transmission electron microscopy (JEOL-JEM 1010 electron microscope, Japanese Electron Optics Limited). For electron microscopy, samples were placed on carbon coated, discharged copper grids and negatively stained using an aqueous solution of ammonium molybdate 2% (w/v), pH 6.5. Samples were viewed at

Table 1
Mass ratios of PC, Quil A and Chol of the various formulations used for ether injection and the resulting particle size and polydispersity of samples

Formulation	PC (%)	Quil A (%)	Chol (%)	Mean size ($n = 2$)	Polydispersity ($n = 2$)
1	70	20	10	165.5	0.30
2	60	30	10	168.4	0.30
3	50	40	10	116.8	0.40
4	40	50	10	85.1	0.38
5	60	20	20	165.9	0.22
6	50	30	20	61.9	0.12
7	40	40	20	136.1	0.33
8	30	50	20	122.1	0.32
9	50	20	30	197.5	0.31
10	40	30	30	171.1	0.39
11	30	40	30	114.8	0.36
12	20	50	30	111.6	0.20

an acceleration voltage of 80 kV and at a magnification varying from 100,000× to 150,000×. The morphology of colloidal structures was observed and the diameter of structures resembling ISCOMs was measured using AnalySiS® software (Soft Imaging Systems, Megaview III, Munster, Germany). Subsequently, samples were subjected to sucrose gradient ultracentrifugation further to confirm the presence of ISCOMs (band at 30% (w/v) sucrose) and to separate ISCOMs from any other colloidal systems present in the systems. Sucrose solutions (3 ml each) having concentrations of 10, 20, 30, 40 and 50% (w/v) in phosphate buffer (pH 7.4) were sequentially layered into a 26 ml Beckman ultracentrifuge tube (top layer 10% (w/v) and bottom layer 50% (w/v) sucrose solution). The sample was then gently loaded onto the top of the sucrose gradient and the tubes centrifuged at 200,000 × *g* for 6 h at 10 °C (Beckman, L-70 ultracentrifuge, Rotor Type 70Ti).

3. Results and discussion

3.1. Mass ratio of PC:Quil A:Chol

Twelve formulations prepared using varying mass ratios of PC, Quil A and Chol as reported in Table 1 at a total lipid mass of 20 mg were investigated as to their ability to form ISCOMs upon injection of 1 ml of an ether solution of PC and Chol into 4 ml of a 0.01 M, pH 7.4 phosphate buffer maintained at 55 °C. The colloidal systems formed were investigated after 1, 3 and 7 days of stirring and despite some differences being apparent between samples taken at Day 1 and Day 3, no differences were observed between samples taken at Day 3 and Day 7. Indeed, even after 2 months of storage, the nature of the structures formed did not change further, suggesting that the samples had reached their equilibrium state 3 days following preparation. This is a much shorter equilibration time than that required for ISCOM preparation by lipid film hydration where even with the inclusion of a freeze-drying step to promote intimate mixing, an equilibration period of at least 1 week is typically required (Demana et al., 2004a). As equilibration was considered to have been reached within 3 days, the results relating to the type of colloidal structures formed in the 12 formulations as investigated by TEM and reported below are for the samples taken 3 days after mixing.

For systems containing 10% (w/w) of Chol (40–70% (w/w) PC and 20–50% (w/w) Quil A), ISCOM-like structures were observed in samples but they were not the predominant colloidal structure. At a relatively high weight ratio of PC (70% (w/w)), ISCOMs were found together with bilayer structures (liposomes) (Fig. 1(A)). Upon increasing concentration of Quil A the occurrence of liposomes decreased as worm-like and helical micelles increasingly appeared (similar to those shown in Fig. 1(B) and (C)). At 50% Quil A, the predominant structures appeared to be aggregates of these micelles with few ISCOMs being observed in this system.

For systems containing 20% Chol (30–60% (w/w) PC and 20–50% (w/w) Quil A), ISCOMs were observed in all systems. At the highest ratio of PC (60% (w/w)), however, ISCOMs were observed together with liposomes in a similar manner to the system containing 10% (w/w) Chol and high weight ratios of PC (Fig. 1(A)). However, when the Quil A concentration was increased from 20 to 30% (w/w), liposomes were not formed, being replaced by ISCOMs as the only colloidal structure formed by this combination of the three components (Fig. 1(D)). Thus, a homogeneous dispersion of ISCOMs was formed within a few days of equilibration when PC, Quil A and Chol were combined by ether injection in a ratio of 5:3:2. Further, the structures of the ISCOMs formed under these conditions as observed by TEM were similar to those reported for ISCOM matrices prepared by the dialysis method (Kersten et al., 1991) and the hydration of dry lipid films (Demana et al., 2004c). When the concentration of Quil A was increased to 40% (w/w), worm-like and helical structures became apparent in the samples in co-existence with ISCOMs. The predominance of these structures increased while that of ISCOMs decreased upon increasing the concentration of Quil A further to 50% (w/w).

As the weight fraction of Chol was increased to 30% (w/w), lamellae structures appeared in the samples as the prevalence of ISCOMs decreased (Fig. 1(E)). The formation of lamellae structures at higher Chol weight ratios was explained by Kersten and Crommelin (1995) to be a result of the increased hydrophobicity of the ring-like micelles imparted by Chol, leading to their association. At a high PC concentration, these lamellae structures were observed together with liposomes (Fig. 1(F)). However, as the concentration of Quil A was increased, liposomes were again

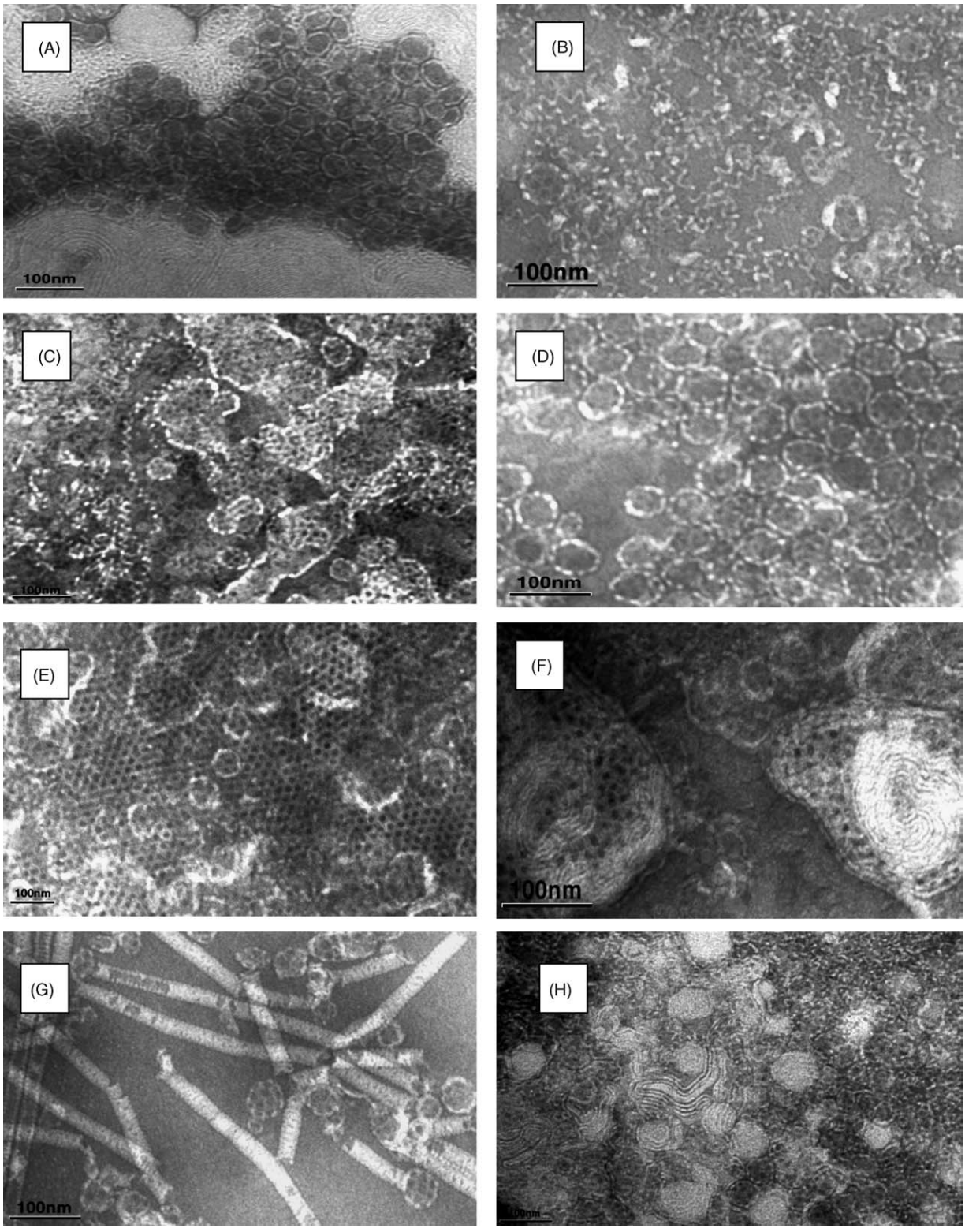

Fig. 1. Electron micrographs depicting various colloidal structures produced upon injection of ether solutions of phospholipid and cholesterol into aqueous solutions of Quil A: (A) ISCOM matrices together with liposomes, (B) worm-like micelles together with few ISCOM matrices, (C) lamellae and helical structures together with few ISCOM matrices, (D) ISCOM matrices, (E) lamellae structures together with ISCOM matrices, (F) liposomes and lamellae structures together with few ISCOM matrices, (G) spiral structures together with ISCOM matrices, and (H) lipidic particles together with liposomes and ISCOMs.

not formed, being replaced by worm-like and helical micelles. Additionally, the extent of association of the ring-like micelles (or size of the lamellae) was seen to decrease upon increasing Quil A concentration suggesting that association is being disrupted upon increasing presence of this hydrophilic component. The prevalence of distinct ISCOM structures in systems containing 30% (w/w) Chol was less than in systems containing 10% (w/w) and particularly 20% (w/w) Chol suggesting that the increased hydrophobicity imparted by increasing the amount of Chol in the system promotes the association of colloidal structures present in the system.

The TEM observations of the pseudo-ternary systems prepared by ether injection are not dissimilar to those reported by Demana et al. (2004a) who constructed pseudo-ternary phase diagrams for PC, Quil A and Chol as prepared by dialysis and lipid film hydration. Also in agreement with Demana et al. (2004a,b), ISCOMs were observed together with liposomes at higher concentrations of PC. In addition, the presence of liposomes decreased, being replaced by worm-like micelles at higher Quil A concentrations. Increasing the concentration of Chol promoted the existence of hexagonal lamellae resulting from association of ring-like micelles. In contrast, however, to the observations of Demana et al. (2004a,b), the formation of lipidic/layered structures was not evident in systems prepared by ether injection. The lipidic/layered systems reported by Demana et al. (2004a,b) were only observed in tertiary systems equilibrated for 1 day and were noted to disappear following a 1-month equilibration period. The similarity of the results for ether injection with systems prepared by hydration of lipid films and equilibrated for prolonged periods suggests that tertiary systems of these components reach equilibrium faster following mixing by ether injection compared to the hydration of a lipid film using an aqueous solution of Quil A.

The TEM observations regarding the type and homogeneity of the colloidal structures produced by the different ratios of the three components were further confirmed by particle size analysis as conducted by photon correlation spectroscopy (Table 1). The system prepared using a PC, Quil A and Chol ratio of 5:3:2 which was observed by TEM to produce a homogeneous ISCOM preparation had a size of approximately 60 nm and a relatively low polydispersity index of just over 0.1. This size is consistent with previous reports for the size of ISCOM matrices as prepared by dialysis (Kersten et al., 1991; Copland et al., 2000) but slightly larger than those reported by Lendemans et al. (2005) following preparation of ISCOMs by ethanol injection. According to Kersten et al. (1991), ISCOMs have a size of between 40 and 100 nm with their size being dependent on the nature of the Quil A used and whether they contain antigen or not. When measured by PCS, size of colloidal structures is also influenced by thickness of the hydration shell, which will be dependent on the nature of the vehicle used for dispension. The large size reported in this manuscript as compared to that reported by Lendemans et al. (2005) for ISCOMs prepared by ethanol injection may be a result of the different buffer used for analysis by PCS. The narrow size distribution confirms the homogeneity of the particles present. When other colloidal structures were observed by TEM to be present in samples, the mean particle size typically increased to above 100 nm and the polydispersity index was also noted to increase. Generally, mean particle size was largest when either liposomes or lamellar structures were present in the system (i.e. at high weight ratios of PC) and decreased as these structures were replaced by micellar structures as formed upon increasing weight ratio of Quil A in the system.

The results of both TEM and particle sizing indicated that the optimal ratio of PC:Quil A:Chol for ISCOM formation as prepared by ether injection was 5:3:2. The formation of homogenous ISCOM preparations at this component ratio was further confirmed by sucrose gradient ultracentrifugation (SGU). Following SGU, only a single opaque band at 30% (w/v) sucrose was observed for this system, consistent with previous reports for the SGU characterization of ISCOMs as prepared by dialysis (Sjolander et al., 1997) and hydration of dried lipid films (Demana et al., 2004c). The preferred ratio of 5:3:2 for PC, Quil A and Chol, respectively, is in accord with the results of Demana et al., (2004a) who characterized the pseudo-ternary phase diagram for these three components in aqueous systems. It is, however, in contrast to the preferred ratio of the components when using dialysis as the method of ISCOM formation, which appears to require higher concentrations of Quil A (Demana et al., 2004a). As proposed by Demana et al., (2004a), this difference in the preferred ratio of components required for ISCOM formation as prepared by the different methods may indeed be the result of the additional surfactant used in dialysis which may take the place of some of the phospholipid, hence ISCOMs are preferentially formed at apparently higher Quil A concentrations.

3.2. Effects of pumping rate, concentration of buffer and Quil A, and total lipid mass on ISCOM formation

Having identified the preferred ratio of PC, Quil A and Chol for ISCOM formation as prepared by ether injection, the influence of the rate of addition of the lipids/ether solution, buffer concentration/ionic strength of the Quil A solution, lipid and Quil A concentration in the ether and aqueous phases, respectively, on ISCOM formation were investigated. Again, all systems were characterized after 3 days of equilibration.

The rate of addition of the ether solution was investigated at 0.2, 0.5 and 1 ml/min. As discussed above, the samples prepared at 0.2 ml/min had a mean size of 61.9 nm and a narrow size distribution (polydispersity of 0.12) in which ISCOMs were the only observed colloidal structures (Fig. 1(D)). The mean size and polydispersity index of the systems, however, were noted to increase with increasing flow rate (Table 2). When viewed by TEM, a reduction in the prevalence of ISCOMs and homogeneity of the sample was observed, consistent with the results of PCS. With increasing flow rate, an increasing abundance of worm-like micelles and lamellar structures were observed (similar to those shown in Fig. 1(B) and (E)). It would thus appear that the rate of introduction of the organic solution, containing lipids, must be sufficiently slow to allow for the concomitant evaporation of the ether, thereby maintaining the polarity of the aqueous phase so as to promote ISCOM formation by hydrophobic interactions. This result is in agreement with that reported for the preparation of

Table 2
Effects of pumping rate, buffer and Quil A concentration, and total lipid mass on mean size and polydispersity of particles produced upon ether injection when the ratio of PC, Quil A and Chol was 5:3:2

	Pumping rate (ml/min)			Concentration of buffer (M)			Total mass of lipid (mg)			Volume of aqueous phase (ml)		
	0.2	0.5	1.0	0.001	0.01	0.1	20	50	100	2	4	8
Mean size ($n = 2$) (nm)	61.9	75.9	86.3	79.6	61.9	102.8	61.9	106.3	122.7	106.7	61.9	87.0
Polydispersity ($n = 2$)	0.12	0.15	0.17	0.14	0.12	0.17	0.12	0.32	0.34	0.31	0.12	0.14

Unless otherwise specified, systems were prepared at a pumping rate of 0.2 ml/min, a buffer concentration of 0.01 M, total lipid mass of 20 and 6 mg of Quil A dissolved in 4 ml of aqueous phase.

liposomes by the ether injection technique wherein a flow rate of less than 0.2 ml/min was recommended for optimal results (New, 1990).

The concentration of buffer used in the aqueous phase containing Quil A was noted to affect the ability of the systems to form ISCOMs when a flow rate of 0.2 ml/min was used to introduce the organic phase into the aqueous phase containing Quil A. When the phosphate buffer concentration (pH 7.4) was reduced from 0.01 to 0.001 M, ISCOMs were observed to be present together with micellar structures and some liposomes. These were not evident in the sample prepared using 0.01 M buffer. The size and polydispersity of the sample was also noted to increase when the buffer concentration was reduced (Table 2). When the buffer concentration was increased to 0.1 M, spiral structures were consistently observed under TEM and the mean size of particles in the system increased to just over 100 nm (Fig. 1(G)). The existence of these structures in pseudo-ternary aqueous systems of Quil A, PC and Chol has not, to our knowledge, previously been reported and the effect of buffer salts, ionic strength and possibly other components present in the systems on the ability of Quil A, PC and Chol to spontaneously form or remain as ISCOMs may warrant further investigation.

It was further noted that the homogeneity of the ISCOM preparation formed when the ether solution of PC and Chol was injected at a rate of 0.2 ml/min into a 0.01 M buffer was influenced by the total amount of lipid present in the system (Quil A, PC and Chol). As described above, when the total mass of lipid was 20 mg with a ratio of 5:3:2 of PC, Quil A and Chol, respectively, a very homogeneous ISCOM preparation was produced (Fig. 1(D)) which had a characteristic mean particle size of 61.9 nm. When the ratio of the components was maintained at 5:3:2 but the total mass of lipid was increased, the resulting preparations became more heterogeneous with other colloidal structures being apparent when viewed by TEM. In addition, both the mean particle size and polydispersity increased with the increasing mass of lipid used. When the total mass of lipid was 50 mg, TEM showed ISCOMs to be present together with worm-like and helical micelles and what appeared as dense lipidic particles. The presence of ISCOMs in the sample decreased further when the lipid mass was increased to 100 mg with a greater quantity of bilayer and lipidic particles being observed in the sample (Fig. 1(H)).

The volume of aqueous phase used to dissolve the Quil A (whilst maintaining the total mass of Quil A in the aqueous at 6 mg and at the preferred mass ratio of 5:3:2 PC:Quil A:Chol) also had an influence on the nature of the colloidal structures formed in the system. When the aqueous volume was reduced to 2 ml (as compared to 6 mg dissolved in 4 ml), the particle size and polydispersity of the resulting preparation increased (Table 2). When viewed by TEM, ISCOMs were present as the predominant structures (albeit appearing quite aggregated and concentrated) but were accompanied by some worm-like micelles. Increasing the volume to 8 ml resulted in a preparation having a particle size of 87.0 nm (Table 2). This system contained predominantly aggregated spherical micelles forming lamellar structures with ISCOM structures being present as a minor component (similar to those shown in Fig. 1(E)). Worm-like micelles were also observed in this system. Thus, the concentration of Quil A in the aqueous phase also influenced the nature of the colloidal structures produced upon ether injection.

4. Conclusions

This study demonstrates that ISCOMs can be prepared by the ether injection technique in a manner analogous to its use for the preparation of liposomes. Ether injection overcomes many of the limitations of the existing methods for ISCOM preparation, which are often time consuming and typically use additional surfactant. Further, ether injection can be considered as a scalable and continuous method of ISCOM preparation while the other reported methods are batch processes. A number of variables, however, were noted to influence ISCOM formation following ether injection: the ratio of the components, the flow rate, the concentration of buffer and Quil A, and the total lipid mass. The preferred conditions for ISCOM formation was a ratio of 5:3:2 for PC:Quil A:Chol with a total lipid mass of 20 mg, the PC and Chol being dissolved in the ether solution and Quil A dissolved in a 0.01 M phosphate buffer at a concentration of 6 mg in 4 ml, and the ether solution injected into the warmed buffer solution at a rate of 0.2 ml/min. Under these conditions, a homogenous preparation of ISCOMs was produced as evidenced by TEM, having a size characteristic of ISCOM matrices and a low polydispersity index. Changing any of these variables resulted in more heterogeneous preparations, in which ISCOMs typically existed together with other colloidal structures such as worm-like and helical micelles, liposomes, lamellae and lipidic particles.

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