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On the dilution behaviour of immuno-stimulating complexes (ISCOMs)

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Immunostimulating complexes (ISCOMs) are used as potent vaccine delivery systems. However, the mechanisms by which ISCOMs work are mostly unknown. The immunological potency of ISCOMs has often been associated with their characteristic cage-like structure. Since ISCOMs can be regarded as composite mixed micelles that contain *Quillaja* saponin as the surfactant, we postulated a micelle to vesicle transition of the particles upon dilution with aqueous solutions. The dilution behaviour of ISCOMs is not only an important preparative aspect, but may also be of high relevance for both cell culture and *in vivo* applications in which dilutions occur. Crude and purified preparations of ISCOM matrices were prepared by dialysis. Methods used to analyse the micelle to vesicle transitions were transmission electron microscopy and dynamic light scattering. Significant morphological changes occurred upon dilution with TRIS buffered saline and non-buffered saline, and a step-wise transition from the typical cage-like structure via both less well defined ISCOM-like structures and helical micelles to small lipidic particles was observed. Aggregation of the resulting small lipidic particles was noted. The results obtained by dynamic light scattering complemented these findings. With increasing dilution factors, increase in particle size and polydispersity was observed. Zeta-potentials showed a trend towards less negative values upon dilution, indicating that saponins were not retained within the lipid matrix. The results indicated that at least partial separation of *Quillaja* saponins from the remaining colloidal species occurred upon dilution with aqueous solutions. The release of saponins upon dilution may be an important aspect of how ISCOMs work.

1. Introduction

Immuno-stimulating complexes (ISCOMs) are spherical, cage-like colloids composed of antigen, cholesterol, phospholipid and *Quillaja* saponins and are used as particulate subunit vaccine delivery systems (Barr and Mitchell 1996). *Quillaja* saponins are surface-active triterpenoids and act as built-in immuno-potentiators (Lovgren Bengtsson and Sjolander 1996). A semi-purified fraction of *Quillaja* saponins, termed Quil-A, is mostly utilised in the preparation of ISCOMs. Quil-A is relatively toxic and hemolytic, making it unsafe for use in humans (Barr et al. 1998). However, further purification by HPLC has led to the identification of saponin sub-fractions with high adjuvant activity, but lower toxicity (Ronnberg et al. 1995; Sjolander et al. 1997). The saponins are critical in the formation of the characteristic ISCOM structure, but not all saponins in the sub-fractions form ISCOMs equally well (Ronnberg et al. 1995; Stittelaar et al. 2000). One of the earliest findings in the study of ISCOMs was that they are obtained only when formulated above the critical micellar concentration (CMC) of the *Quillaja* saponins (Morein et al. 1984). The fate of pre-formed ISCOMs upon dilution below the CMC of the saponins has never been reported. ISCOMs have a micellar character due to their surfactant load, and are regarded as aggregates of inverted ring-like micelles held together by hydrophobic interactions, hydrogen bonds and steric factors (Kersten and Crommelin 1995; Kersten et al. 1991). The strongly negative zeta-potential due to ionised saponin molecules confers further colloidal stability. Because of the strong interaction between saponins and cholesterol, ISCOMs are said to be stable structures (Lovgren Bengtsson and Sjolander 1996) and formulation shelf-lives of over a year have been reported (Pearse and Drane 2004; Sjolander et al. 2001).

We recently described the formation of cationic, cage-like structures containing the cationic cholesterol derivative DC-cholesterol, phospholipid and Quil-A (Lendemans et al. 2005). These had a size and morphology very similar to classical ISCOM matrices and thus were also regarded as composite micelles. We made the observation that their morphology changed towards liposomes upon dilution with buffer (unpublished results) and concluded that the surface-active saponins were not retained within the lipid matrix at lower saponin concentrations. We postulated that the same phenomenon may apply to classical ISCOMs. Dilution of ISCOMs occurs during dose adjustment, at the site of administration and in cell culture experiments (Behboudi et al. 1996b; Demana et al. 2004b; Villacres-Eriksson 1995; Villacres-Eriksson et al. 1993) and may lead to the release of saponins with a concomitant change in particle morphology. This release of saponins may in fact be an important aspect of their mechanism of action. Instability of micelles subjected to strong

dilution is a problem recognized in some pharmaceutical applications and has led to the design of polymeric surfactants (Torchilin 2001). However, in other applications the dilution of stable mixed micellar systems is utilised to achieve the intentional transition into vesicles (Alkan-Onyuksel et al. 1994; Alkan-Onyuksel and Son 1992; Son and Alkan 1989). The reverse vesicle to micelle transition has been described for a variety of surfactants and lipids (Almgren 2000; Edwards et al. 1993; Gustafsson et al. 1997; Silvander et al. 1996). In this study we investigated the behaviour of ISCOM matrices as composite mixed micelles upon dilution with aqueous solutions. The aim of this study was to observe morphological changes by transmission electron microscopy and changes in colloid size by dynamic light scattering.

2. Investigations and results

2.1. Quantification of Quil-A saponins and lipids by HPLC

Crude dispersions of ISCOM matrices were prepared by a dialysis procedure and subsequently purified by centrifugation through a sucrose cushion in order to remove unincorporated *Quillaja* saponins. The Table gives an overview of saponin and lipid concentrations in crude and purified preparations of ISCOM matrices. The initial concentration chosen prior to dialysis was 2 mg/mL, a value well above the reported CMC of the semi-purified saponin fraction Quil-A (0.3 mg/mL). The dialysis procedure led to a loss of all components and purification of samples decreased the saponin and phosphatidylcholine (PC) concentrations further (Table). However, cholesterol (CHOL) could be fully recovered following purification. This may indicate that excess saponins and PC formed colloidal structures of lower density that were separated from ISCOM matrices during the purification process. The finding is in agreement with an earlier report (Lovgren and Morein 1988), however is in contrast to a publication by Kersten et al. (1988) in which ISCOM matrices with a lower CHOL content were obtained. Crude and purified preparations of ISCOM matrices were then diluted for analysis by Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS).

2.2. Detection of n-octylglucoside (OG) by ESI-MS

Crude and purified ISCOM matrix formulations were analysed for the presence of *n*-octylglucoside (OG), the "helper detergent" used in the dialysis process. Analysis of OG dissolved in TBS by negative ESI-MS yielded in a peak with an m/z of 327 (OG-chloride). This peak was also found in the crude formulations (data not shown). The analysis of the purified formulations was not conclusive as neither lipid peaks nor OG peak were detected. Hence it can be concluded that residual *n*-octylglucoside was a contaminant in at least the crude preparations.

Table: Saponin and lipid compositions (concentrations in mg/mL)

	Saponins	Cholesterol	Phosphatidylcholine
Initial mixture	2	0.5	0.5
Crude preparations following dialysis	1.39 ± 0.09	0.38 ± 0.01	0.16 ± 0.01
Purified preparations	0.52 ± 0.07	0.38 ± 0.02	$0.09 + 0.01$

2.3. Effect of dilution on particle size and morphology

Purified ISCOM matrices were diluted with saline to saponin concentrations of 0.01, 0.02, 0.05 and 0.1 mg/mL, values below the reported CMC of Quil-A in water. Samples were stirred for 12 h at 4 °C and then analysed by TEM. Coated grids were viewed on several fields in order to avoid under-sampling. All experiments were repeated twice with very similar results. The undiluted, purified samples showed mostly well-defined particles with the characteristic cage-like morphology and a narrow size distribution (41.5 ± 4.2 nm, $n = 800$, Fig. 1a). "Fragments" could be observed among the cage-like particles and these had an either worm-like or helical morphology (arrow in Figure 1a). At a saponin concentration of 0.1 mg/mL, the majority of particles had an ISCOM-like morphology with significantly increased diameter (47.8 ± 5.0 nm, $n = 450$; $p < 0.001$, t-test; Fig. 1b) and a broader size distribution. The pores in these ISCOM-like particles appeared to be fewer in number and were significantly bigger in diameter compared to undiluted ISCOM matrices (8.50 ± 0.85 nm, $n = 75$ versus 5.94 ± 0.98 nm, $n = 75$; t-test, $p < 0.001$). Small lipidic particles which showed a complete loss of the cage-like morphology (no visible pores) constituted the second predominant particle species at a saponin concentration of 0.1 mg/mL. These may have a vesicular structure and nature. The lipidic particles seemed to be derived from ISCOM-like structures as they were either observed to bud off or be adjacent to ISCOM-like particles (Fig. 1c). Occasionally, the lipidic particles were observed to fuse into bigger aggregates (Fig. 1e). This situation could also be observed at a saponin concentration of 0.05 mg/mL. Helices appeared to "grow out" of ISCOM-like particles (arrow in Fig. 1d). A physical overlap of two separate colloidal structures is unlikely as this frequent observation was made at high dilution factors. At saponin concentrations of 0.01 and 0.02 mg/mL, small lipidic particles (18.2 ± 3.6 nm, $n = 270$) were the dominant structure with very few ISCOM-like structures remaining (Fig. 1e).

In order to further investigate the speed of this described transition, samples were diluted to a saponin concentration of 0.02 mg/mL and then immediately prepared for TEM by negative staining. The vast majority of particles had lost their cage-like morphology and the aforementioned lipidic particles were obtained (Fig. 1f). These findings indicate a fast transition process.

These results demonstrate a significant change in particle morphology upon dilution of ISCOM matrices. ISCOM matrices appear to undergo a transition to lipidic particles in a saponin concentration dependent manner with ISCOM-like particles and helices as intermediate colloidal species. Bigger aggregates appeared to form by fusion or aggregation of either lipidic particles or ISCOM-like structures.

2.4. Effect of dilution on particle size and zeta-potential

Crude and purified preparations of ISCOM matrices were diluted with TBS or 136 mM NaCl, respectively, and equilibrated at 25 °C for 24 h. The particle size in each dilution was subsequently analysed by DLS. As described above, the dilution of ISCOMs resulted in the formation of helices and bigger aggregates which are non-spherical in shape. However, spherical particles are assumed with this technique and sizes reported must thus be regarded as "apparent" sizes. Crude and purified formulations slightly differed in their individual profiles, but some general

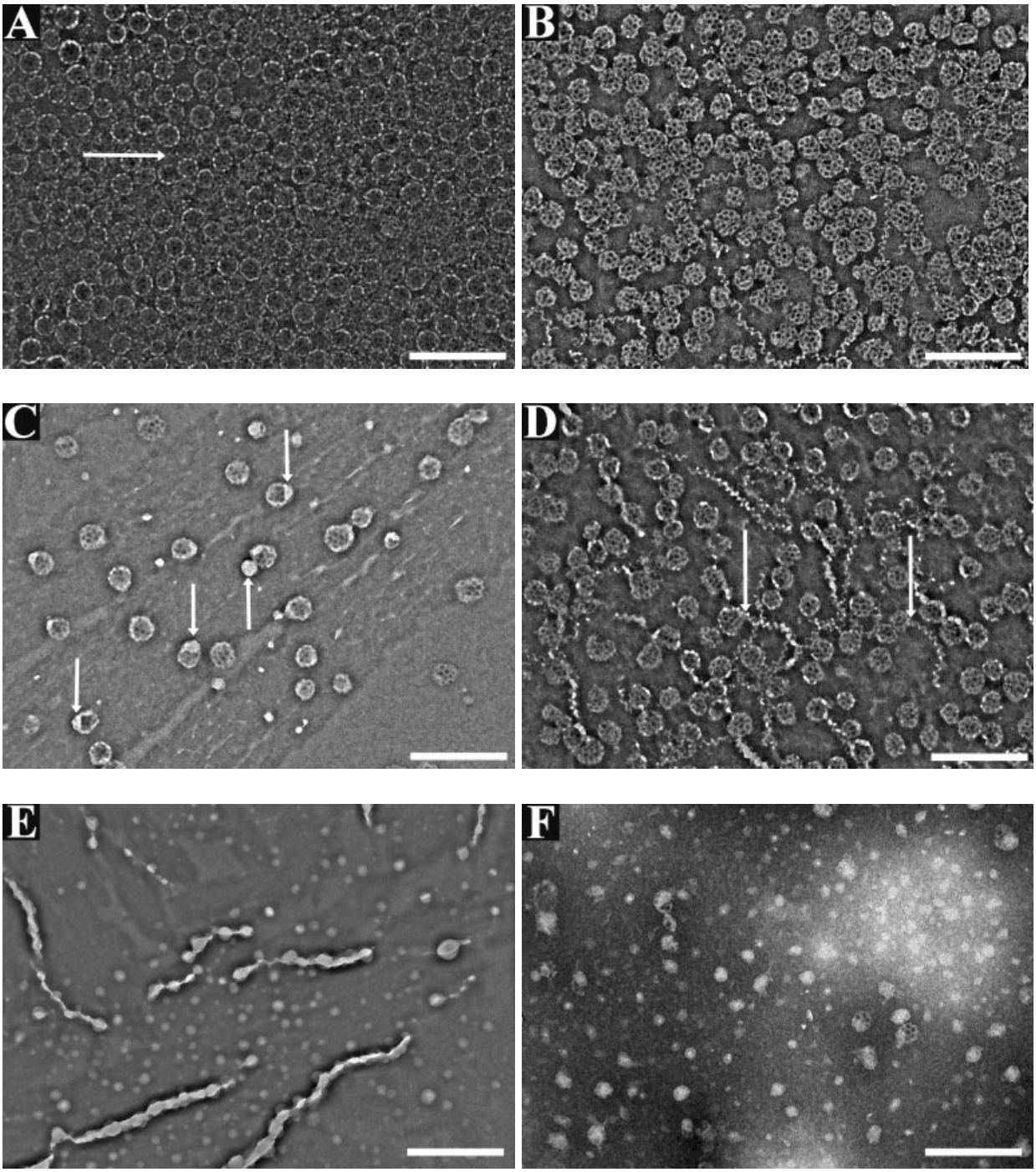

Fig. 1: Morphology of preformed ISCOM matrices diluted to various concentrations (c) of *Quillaja* saponins. Micrographs were obtained by TEM. **A**: ISCOM matrices, purified undiluted formulation, at c = 1 mg/mL; **B**: ISCOM-like structures at c = 0.1 mg/mL; **C**: ISCOM-like structures with adjacent lipidic particles and loss of cage-like morphology at c = 0.02 mg/mL; **D**: ISCOM-like structures with helices at c = 0.05 mg/mL; **E**: Lipidic particles and aggregates thereof, complete loss of typical ISCOM morphology at c = 0.01 mg/mL; **F**: Lipidic particles and some remaining ISCOM-like structures at c = 0.02 mg/mL obtained immediately following dilution (multiple coatings on carbon grid). Bar = 200 nm

trends could be observed. Fig. 2 shows representative data obtained with a crude preparation, Fig. 3 shows representative data of a purified preparation.

With the crude formulations, no significant increase in particle size was observed up to dilution factors of 14–18 ($p = 0.240$, modelled as a linear response). This corresponds to saponin concentrations of approx. 0.08–0.1 mg/mL (Table). However, higher dilution factors resulted in a significant increase in particle size (Fig. 2a) and polydispersity index (Fig. 2b). The increase in particle size could be modelled as a linear response with a slope of 2.18 nm/dilution factor ($p = 0.002$). This increase could represent either formation of helices or aggregation of lipidic particles. A second peak = 1000 nm could sometimes be detected at higher dilution factors (indicated with a "+" in Fig. 2a). This peak is the result of dilution and not due to contamination as formulations were filtered (pore size 200 nm) prior to dilution. Compensation of the count rate with the respective dilution factors always showed an increase in particle numbers between dilution factors of 6–8 (Fig. 2c). This may indicate that upon dilution ISCOM matrices fall apart into several fragments. At higher dilution factors, a slight decrease in compensated count rate could sometimes be observed, which may again indicate aggre-

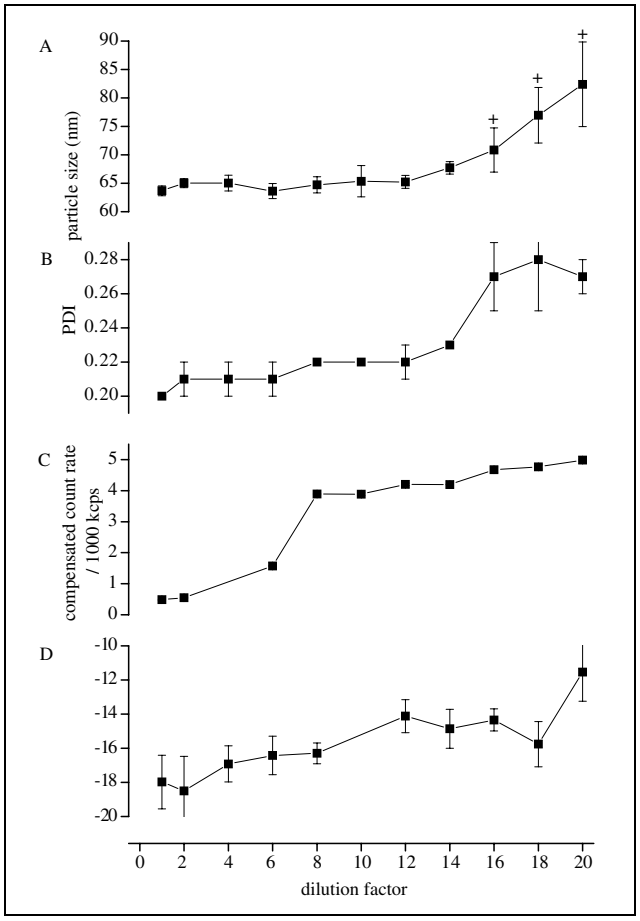

Fig. 2: Change of particle size (**A**), polydispersity (**B**), compensated count rate (**C**) and zeta-potentials (**D**) upon dilution of a crude preparation. "+" in **A** indicates detection of a second peak $\geq$ 1000 nm

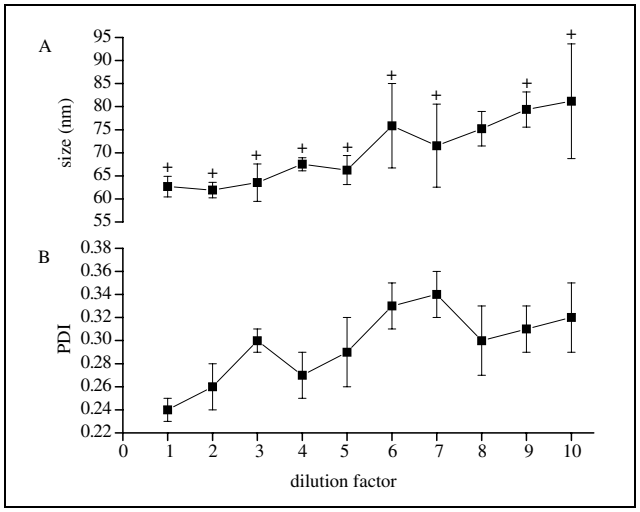

Fig. 3: Change of particle size (**A**) and polydispersity (**B**) upon dilution of a purified ISCOM preparation. "+" in **A** indicates detection of a second peak $\geq$ 350 nm

gation of lipidic particles. Zeta-potentials of colloids became less negative towards higher dilution factors, which may indicate a separation of the anionic saponins from the remaining particles (Fig. 2d).

With the purified preparations, a significant increase in particle size was observed from the lowest dilution factors onwards (Fig. 3a). A linear response could be modelled with a slope of 2.1 nm/dilution factor ($p < 0.0001$). The immediate increase can be explained by the lower saponin content in the purified preparations (Table), leading to changes at lower dilution factors. The increase in particle size was accompanied by an overall increase in polydispersity (Fig. 3b). These results are in agreement with a previous report in which an increase in particle size was observed with the loss of Quil-A when individual "ISCOM populations" were separated on a sucrose gradient (Kersten et al. 1991). A second peak $\geq$ 350 nm was detected at almost all dilution factors and even in the undiluted samples ("+" in Fig. 3a). This indicates a significant change of the system upon removal of unincorporated Quil-A from the crude samples. The percentage intensity contribution of these bigger particles grew with higher dilution factors, potentially indicating aggregation of lipidic particles upon dilution (not shown). Taken together, these results confirm the observations made by TEM.

3 Discussion

The change in the morphology of ISCOM matrices observed upon dilution is not an unexpected result given the fact that these contain surface-active saponins which are essential in the formation of the characteristic cage-like structure. ISCOMs could thus be regarded as aggregates of mixed micelles containing surfactant, cholesterol and phospholipid. Using TEM, we could show a step-wise transformation of the colloidal structures, and a concomitant change in particle size, polydispersity and count rate was measured by DLS. Zeta-potentials of crude samples shifted towards less negative values and small lipidic particles, potentially vesicles, constituted the final products of the transition. However, these appeared to be short-lived transient species as aggregation of these then occurred. A similar transition upon dilution was previously reported with mixed micelles containing bile salts and phospholipid, which resulted in the formation of liposomes (Alkan-Onyuksel et al. 1994; Alkan-Onyuksel and Son 1992).

The interpretation of the transition observed upon dilution is complicated by the fact that the saponins in Quil-A constitute a highly complex mixture (Kensil 1996; Kensil et al. 1991; Nord and Kenne 1999). The presence of two different lipids (cholesterol and phospholipid) solubilised by the saponins further adds to the complexity of the system. Nevertheless, we propose the following model based on the observations made in this work and on comparison with other colloidal systems containing surfactants and lipids. For the sake of comparison with vesicle solubilisation by other surfactants, the process is described in reversed order, starting from small vesicles and ending with mixed micelles. According to Lichtenberg, vesicle solubilisation with surfactants occurs in three stages (Lichtenberg 1985). At low ratios of surfactant to lipid, the surfactant merely inserts into vesicle bilayers. At a critical ratio of surfactant to lipid, mixed micelles start to form. In this transition stage surfactant-saturated vesicles co-exist with mixed micelles, and depending on the nature of the surfactant, transition species with different morphologies are found. At a second critical ratio of surfactant to lipid, bilayered species disappear completely and mixed micelles are the only colloidal species observed. This three-stage transition has been observed for a variety of nonionic and ionic surfactants and was reviewed by Almgren (2000).

At low concentrations of saponins (i.e. high dilution factors), small lipidic particles, $\sim$20 nm in diameter, were observed (Fig. 1e). These particles lacked cage-like morphology and may constitute vesicles. Although they are probably a transient species and only obtained due to dilu-

tion, the transition is assumed to start from these particles. An increase in saponin concentration (i.e. intermediate dilution factors) led to insertion of these into the vesicle bilayers with a resulting expansion of the particles and pores becoming visible. The ISCOM-like structures had a TEM diameter of ~48 nm. Both the increase in size and the formation of pores or "defective structures" have been described with other surfactant/lipid systems, for example sodium dodecyl sulfate (SDS)/lecithin (Silvander et al. 1996), cetyltrimethylammonium chloride (CTAC)/lecithin (Edwards et al. 1993) and cetyltrimethylammonium bromide/glycerol monooleate (Gustafsson et al. 1998). It is difficult to define the borderline between bilayered structures and mixed micellar structures in the systems investigated. It is possible that the observed ISCOM-like structures (Fig. 1b) with less well defined morphology still constitute species with bilayered sections. It was discussed previously that within "defective structures" a local segregation of amphiphile components with different geometry occurs. However, for this to happen the tail groups of the amphiphiles must be "similar enough" (Gustafsson et al. 1997). In ISCOMs, cholesterol and saponins indeed share tail group similarities.

The more regular ISCOM matrices (Fig. 1a), however, must be somewhat different from the ISCOM-like structures as the size of particles decreases from ~48 nm to ~40 nm in this transition step. Fluorescence polarisation experiments by Kersten et al. (1991) also indicated a more rigid molecular arrangement compared to liposomes. Thus, from ISCOM-like structures to the regular ISCOM structure there may indeed be a change in molecular arrangement from bilayered structure to the non-bilayered model suggested by this group. In this model, ISCOMs are regarded as aggregates of inverted ring-like subunits (~10 nm) held together by hydrophobic interactions, hydrogen bonds and steric factors (Kersten et al. 1991). Edwards et al. (1993) believed "mesh structures" to be the most probable model for the perforated particles obtained with CTAC/lecithin. Although not shown in this work, a further increase of the saponin concentration then leads to a separation of these subunits within ISCOMs and ring-like micelles are eventually obtained (Demana et al. 2004a; Kersten and Crommelin 1995). This was also shown in a systematic study by constructing pseudo-ternary phase diagrams (Demana et al. 2004a). This final decrease in size is in agreement with vesicle-micelle transitions of CTAC/lecithin and SDS/lecithin (Edwards et al. 1993; Silvander et al. 1996).

Qualitative detection of OG in crude and purified ISCOM matrices was attempted using ESI-MS. It was previously suggested that the detergent used during dialysis may become partially inserted into the ISCOM matrices (Kersten and Crommelin 1995). Indeed, OG could be detected in the crude formulations, but analyses of the purified formulations were not conclusive. One could argue that the observed changes in particle morphology and size may be partially due to OG separating from ISCOM matrices following dilution. However, we believe that this effect plays only a minor role if any as most of this detergent, which has a relatively high CMC (23 mM), should have been removed during dialysis and subsequent purification. In the current work only the behaviour of "empty" ISCOM matrices was investigated. It is conceivable that ISCOMs loaded with antigen show a different behaviour and stability. This remains to be investigated.

In an *in vitro* study with peritoneal cells, ISCOM matrices formed by various fractions of saponins were able to induce secretion of IL-6 (Behboudi et al. 1997) and soluble IL-1α; (Behboudi et al. 1996a). Using empty ISCOM matrices, IL-6 and sIL-1α; secretion was most pronounced at saponin concentrations of 2.5 μg/mL. Using loaded ISCOM matrices, IL-6 and sIL-1α; responses peaked at saponin concentrations of 0.34 μg/mL and 0.7 μg/mL, respectively. In a cell culture study performed by our group, activation of bone marrow derived dendritic cells (BMDCs) with empty ISCOM matrices, as measured by the increase in expression of CD86 and CD40, was achieved at saponin concentrations of less than 10 μg/mL (Demana et al. 2004b). Incubation of BMDCs with ovalbumin loaded ISCOMs at saponin concentrations of ~1 μg/mL and 2 μg/mL resulted in specific $CD8^+$ (Robson et al. 2003a) and $CD4^+$ (Robson et al. 2003b) T cell proliferation, respectively. In all these studies, the concentrations of saponins were lower than those investigated in the current study, hinting towards the possibility that the typical cage-like structure did not constitute the active colloid in these studies. Liposomes including small amounts of saponin, formulated at a lower saponin:lipid ratio than ISCOMs, were reported to induce CTL responses (Lipford et al. 1994). The cage-like morphology may thus not be an absolute prerequisite for immunogenicity and there may be no need for the uptake of completely intact particles either. The active form of ISCOMs may thus constitute released non-complexed saponins in combination with a mixture of particulate remainders – regular ISCOMs, ISCOM-like structures, helices and lipidic particles.

4. Experimental

4.1. Materials

Quil-A was purchased from Brenntag Biosector, Denmark. Cholesterol (CHOL), phosphatidylcholine (PC) and n-octylglucoside (OG) were obtained from Sigma, USA. The buffer used for dialysis was TRIS buffered saline pH 7.4 (TBS) and had a composition of 60 mM TRIS (BDH, England) and 85 mM NaCl (Sigma). Stock solutions of Quil-A in TBS and of CHOL and PC in chloroform at a concentration of 10 mg/mL were made prior to formulation and stored at −20 °C. HPLC solvents were supplied by Merck, Germany.

4.2. Preparation and purification of ISCOM matrix

4.2.1. Crude preparations

ISCOM matrices were formed by dialysis as previously described at an initial weight ratio of 4 : 1 : 1 Quil-A : CHOL : PC (Kersten et al. 1988). 1.5 mg of each CHOL and PC were pipetted into glass vials. Chloroform was evaporated under a nitrogen stream and then quantitatively removed under vacuum over night. 6 mg Quil-A and 120 mg OG were added and the volume was adjusted to a total of 3 mL with TBS. The mixture was stirred for 2 h at room temperature until an optically clear micellar solution was obtained. This solution was transferred into a dialysis membrane (Spectrum Laboratories, CA, USA) with a molecular weight cut-off (MWCO) of 1,000 and extensively dialysed against 1 L TBS at 4 °C. The buffer was exchanged every 12 h and dialysis was stopped after 48 h. The concentration of Quil-A in the crude formulations was determined by HPLC as described below.

4.2.2. Purified preparations

Excessive Quil-A was removed from crude preparations as described by Sundquist et al. (1988) with slight modifications by carefully layering 1 mL of the crude preparations on top of 3 mL of a 10% (w/w) sucrose cushion and centrifuging for 5 h at 50,000 g at 4 °C using a Beckman L-80 ultracentrifuge. Following centrifugation, the upper 3.8 mL of the supernatant were discarded by careful pipetting. Particles were resuspended in the original sample volume by adding 0.8 mL of 136 mM NaCl to the pellets which were then resuspended by vortexing. The dispersions were transferred into dialysis membranes (MWCO = 1,000) and dialysed for 6 h at 4 °C against 1 L 136 mM NaCl in order to remove sucrose. The concentration of Quil-A in these purified preparations was determined by HPLC and the dispersions were stored at 4 °C until further use and analysis.

4.3. Quantification of Quil-A saponins and lipids in ISCOM matrix formulations by HPLC

Quil-A and lipids were quantified as described previously with slight modifications (Behboudi et al. 1995). ISCOM matrix preparations were first separated into Quil-A saponins and lipid components using a chloroform-methanol-water extraction process. The saponins were recovered from the aqueous phase and lipid components from the chloroform phase. These phases were dried on a Speedvac (Savant Instruments, USA) at reduced pressure and room temperature for 6 and 2 h, respectively. The dried saponins were re-dissolved in 136 mM NaCl and dried lipids in 2-propanol. For the analysis of both saponins and lipids, a Shimadzu LC-10AT VP pump was used in combination with a Shimadzu UV-Vis Detector SPD-10A VP. Saponins and lipids were detected at 206 nm. For the analysis of saponins, a reversed phase column (Phenomenex Luna C18(2), 250 × 4 mm) with a particle size of 5 μm was used. Acetonitrile (ACN) : water 50 : 50 was used as the mobile phase. Injection volume was 20 μL and chromatography was carried out at a flow rate of 0.5 mL/min. Under these conditions only one peak representing the total quantity of Quil-A was obtained at a retention time of approximately 3.8 min. The peak height was used to obtain a standard curve and to calculate the Quil-A content in the various preparations. Quil-A standard solutions were analysed at concentrations ranging from 0.2 mg/mL to 1.0 mg/mL. The resulting standard curve was characterised by $r^2 = 0.983$, $LOD_{Quil\text{-}A} = 0.04$ mg/mL, $LOQ_{Quil\text{-}A} = 0.11$ mg/mL. For the analysis of cholesterol and phospholipid, a normal phase column (Phenomenex Silica Luna (2), 250 × 4.6 mm) with a particle size of 5 μm was used in combination with ACN : MeOH : water 65 : 21 : 14 as mobile phase. Injection volume was 20 μL and chromatography was carried out at a flow rate of 0.5 mL/min. Standard solutions of CHOL and PC dissolved in 2-propanol were analysed at concentrations ranging from 0.05 mg/mL to 0.5 mg/mL. CHOL eluted at a retention time of 6.13 min (peak height was used to calculate concentrations), PC eluted as a double peak at a retention time from ~24.5–27.0 min (total peak area was used to calculate concentrations). The resulting standard curves were characterised by $r^2 = 0.999$, respectively. $LOD_{CHOL} = 0.002$ mg/mL, $LOQ_{CHOL} = 0.005$ mg/mL; $LOD_{PC} = 0.008$ mg/mL, $LOQ_{PC} = 0.023$ mg/mL.

4.4. Electrospray ionisation mass spectrometry (ESI-MS)

Crude and purified preparations were analysed for the presence of n-octylglucoside. A Shimadzu QP8000 alpha with ESI probe was used in combination with a manual Rheodyne injector fitted with a 5 ul loop and a Shimadzu LC10AD HPLC pump to provide direct sample injection. Shimadzu LCMS Solutions R2.01 software was used to save and process the data. Spectra were run using a 90 : 10 ACN:water mobile phase and a controlled desorption line temperature of 250 °C.

4.5. Dilution of ISCOM matrices and analysis by transmission electron microscopy (TEM)

Purified ISCOM matrix was diluted with 136 mM NaCl (4 °C) to saponin concentrations of 0.01, 0.02, 0.05 and 0.1 mg/mL. These concentrations are below the reported CMC of Quil-A in water, 0.3 mg/mL (Morein et al. 1984). Dilutions were stirred for 12 h at 4 °C and subsequently analysed by TEM. An additional experiment was carried out by diluting purified ISCOM matrices to a concentration of 0.02 mg/mL and immediate processing for TEM. Dilutions of ISCOM matrices were coated onto glow discharged, carbon coated copper grids (multiple coating steps were performed for some dilutions), negatively stained with 2% w/v phosphotungstic acid (pH 5.2) and subsequently viewed on a Philips CM 100 at an acceleration voltage of 100 kV and a magnification of 93,000×. Images were enhanced and analysed using AnalySIS software (Soft Imaging Systems, Germany). Diameters of colloidal structures were calculated by drawing area-equivalent circles around the particle projections. Undiluted, purified ISCOM matrix served as a control.

4.6. Dynamic light scattering (DLS) and laser Doppler velocimetry

Crude samples for DLS analysis were passed through 0.2 μm filters prior to dilution or purification to ensure that bigger aggregates detected during measurements were the result of dilution and not of contamination due to sample preparation. Crude and purified samples were then diluted one day after preparation. Crude samples were diluted with TBS and the purified samples with 136 mM NaCl. Dilutions were incubated at 25 °C for 24 h to allow equilibration. Particle size at 25 °C was subsequently determined using a Malvern Zetasizer Nano-ZS with dispersion technology software version 4.1. This instrument detects scattered light at an angle of 173° (backscatter detection). Light source is a 4 mV 633 nm He−Ne laser. Pathlength within the measuring cell and laser light intensity were optimised by the software. Ten sub-runs were performed per measurement, each having a duration of 20 s. Five repetitive measurements were performed per dilution. Three crude and three purified samples were analysed. The instrument was calibrated with a 60 nm polystyrene nanosphere standard (Duke Scientific, USA). Intensity based parameters observed were Z-Average (Z-AVE) and polydispersity index (PDI), resulting from cumulants analysis, and distribution peaks resulting from non-negative least square analysis. Zeta-potentials of crude dilutions were determined using the same instrument. Twenty sub-runs were performed per measurement and five repetitive measurements were made per dilution.

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