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Incorporation of ovalbumin into ISCOMs and related colloidal particles prepared by the lipid film hydration method

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Abstract

The aim of this study was to investigate the incorporation of a model antigen, fluorescently labelled ovalbumin (FITC-OVA), into various colloidal particles including immune stimulating complexes (ISCOMs), liposomes, ring and worm-like micelles, lamellae and lipidic/layered structures that are formed from various combinations of the triterpene saponin Quil A, cholesterol and phosphatidylethanolamine (PE) following hydration of PE/cholesterol lipid films with aqueous solutions of Quil A. Colloidal dispersions of these three components were also prepared by the dialysis method for comparison. FITC-OVA was conjugated with palmitic acid (P) and PE to produce P-FITC-OVA and PE-FITC-OVA, respectively. Both P-FITC-OVA and PE-FITC-OVA could be incorporated in all colloidal structures whereas FITC-OVA was incorporated only into liposomes. The incorporation of PE-FITC-OVA into all colloidal structures was significantly higher than P-FITC-OVA ($P < 0.05$). The degree of incorporation of protein was in the order: ring and worm-like micelles < liposomes and lipidic/layered structures < ISCOMs and lamellae. The incorporation of protein into the various particles prepared by the lipid film hydration method was similar to those for colloidal particles prepared by the dialysis method (provided both methods lead to the formation of the same colloidal structures). In the case of different colloidal structures arising due to the preparation method, differences in encapsulation efficiency were found ($P < 0.05$) for formulations with the same polar lipid composition. This study demonstrates that the various colloidal particles formed as a result of hydrating PE/cholesterol lipid films with different amounts of Quil A are capable of incorporating antigen, provided it is amphipathic. Some of these colloidal particles may be used as effective vaccine delivery systems.
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Keywords: Ovalbumin; Quil A; ISCOMs; Liposomes; Micelles; Vaccine delivery

1. Introduction

Lipid particles have received particular attention in the immunological field due to their ability to function as adjuvant systems for vaccines and carriers for subunit antigens (Rimmelzwaan and Osterhaus, 1997; Barr et al., 1998; Kersten and Crommelin, 2003). The development of subunit vaccines instead of attenuated or inactivated whole pathogens for vaccination offers many advantages. These advantages include lowering the likelihood of adverse and allergic reactions, or the production of so called "blocking antigens" as an undesired immune response upon administration (Rimmelzwaan and Osterhaus, 1997). It has been shown that isolated subunit antigens, whilst immunogenic as part of a microorganism, are almost

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non-immunogenic in their purified form (Kersten et al., 1988; Morein et al., 1990; Bungener et al., 2002). The immunogenicity of purified antigens can sometimes be enhanced to that of the whole pathogen by the creation of defined multimeric forms, e.g. the formulation of antigens in liposomes or as protein micelles (Kersten and Crommelin, 1995; Kensil, 1996). It is thought that the colloidal size of these delivery systems mimics the submicroscopic particulate nature of a pathogen carrying several copies of the antigenic epitope (Höglund et al., 1989; Morein and Bengtsson, 1998; Sjölander et al., 1998). However, typically additional adjuvants have to be added to liposomes or protein micelles to elicit a protective immune response (Höglund et al., 1989; Kersten and Crommelin, 1995, 2003).

As such, immune stimulating complexes (ISCOMs) have received much attention in the immunological field (Mowat and Donachie, 1991; Morein et al., 1995; Barr and Mitchell, 1996; Sjölander et al., 1998; Kersten and Crommelin, 2003). ISCOMs are symmetrical colloidal particles composed of phospholipid, cholesterol, the saponin mixture Quil A and an antigen mostly in the form of an amphipathic protein (Barr et al., 1998; Morein and Bengtsson, 1999; Sjölander et al., 2001). A formulation without the incorporated antigen is called ISCOM matrix (Rimmelzwaan and Osterhaus, 1997; Barr et al., 1998; Ekström et al., 1999). ISCOMs have a characteristic open cage-like morphology that appears to consist of about 20 ring-like regularly ordered subunits (Özel et al., 1989; Kersten and Crommelin, 2003), and a size in the range of 40–100 nm. They can be best observed by negative staining transmission electron microscopy (Kersten et al., 1991; Rimmelzwaan and Osterhaus, 1997). Due to the presence of Quil A in their structure, which in itself is a strong adjuvant, ISCOMs have been reported to be about ten times more immunogenic than other colloidal particulate delivery systems such as liposomes or protein micelles (Sundquist et al., 1988; Kersten and Crommelin, 1995; Barr and Mitchell, 1996; Osterhaus and Rimmelzwaan, 1998). ISCOMs are able to boost both humoral and cellular responses (Barr and Mitchell, 1996; Morein and Bengtsson, 1999; Sjölander et al., 2001; Kersten and Crommelin, 2003). Further, in addition to use in parenteral immunisation, ISCOMs have also been used for both oral and mucosal delivery of antigens (Rimmelzwaan and Osterhaus, 1997; Barr et al., 1998; Morein and Bengtsson, 1998; Mowat et al., 1999; Sjölander et al., 2001; Kersten and Crommelin, 2003). The applicability of ISCOMs in vaccine delivery is therefore diverse.

However, the benefits of ISCOMs as an adjuvant and in the delivery of antigens appear best if the antigen or protein can be incorporated into the ISCOM matrix (Barr and Mitchell, 1996; Ekström et al., 1999). For incorporation into ISCOMs, it is desirable that the antigen has an amphiphilic character (Kersten and Crommelin, 1995; Barr and Mitchell, 1996). If the antigen is hydrophilic there is no driving force for its incorporation into the lipid matrix (Morein et al., 1990; Kersten et al., 1991; Rimmelzwaan and Osterhaus, 1997; Barr et al., 1998), while if it is too lipophilic the protein tends to aggregate, thus preventing its incorporation into a colloidal structure (Kersten and Crommelin, 1995; Morein et al., 1995). The protein ovalbumin is often utilised as a model antigen (Morein et al., 1990; Sjölander et al., 1997; Ekström et al., 1999; Johansson and Lövgren-Bengtsson, 1999; Mowat et al., 1999; Könnings et al., 2002). Because of its hydrophilic nature, it is not possible to incorporate ovalbumin into ISCOM matrices unless it is modified (Höglund et al., 1989; Morein et al., 1990; Akerblom et al., 1993). Some researchers have attempted to overcome this by exposing hydrophobic regions of the protein by partial denaturation at low pH (Morein et al., 1990), at high temperatures (Höglund et al., 1989) or by using chemical agents such as urea and mercaptoethanol (Akerblom et al., 1993). For bovine serum albumin, these procedures were successful as the pH-modified material was shown to partly incorporate into ISCOM matrices (Morein et al., 1990). Preliminary experiments in our laboratory utilising acid or chloroform treatment to incorporate ovalbumin into ISCOM matrices did not, however, result in inclusion of protein into the matrix (Könnings et al., 2002). Furthermore, exposure of protein to conditions of low pH, high temperatures or chemical agents may disrupt sensitive antigenic determinants (epitopes) (Reid, 1992; Barr et al., 1998), compromising the immunogenicity of the antigen. One of the most common methods to modify soluble proteins for incorporation into ISCOM matrices is the conjugation of fatty acids to the protein under mild conditions (Reid, 1992; Sjölander et al., 1997; Barr et al., 1998; Ekström et al., 1999; Mowat et al., 1999).

This method has been successfully used to incorporate proteins into ISCOMs prepared by the dialysis method (Kersten et al., 1991; Reid, 1992; Sjölander et al., 1997; Ekström et al., 1999; Johansson and Lövgren-Bengtsson, 1999; Mowat et al., 1999).

Recently, a simpler process than the dialysis method for the preparation of ISCOM matrices was developed in our laboratory (Copland et al., 2000). This method is based on the classical Bangham method used for the preparation of liposomes (Bangham et al., 1965), whereas the dialysis method is comparable to the detergent removal technique to produce liposomes (Weder and Zumbuehl, 1983). We have since refined this methodology by introducing a freeze-drying step in order to promote intimate mixing of Quil A, cholesterol and phospholipids (Demana et al., 2004). The freeze-drying step may be particularly important for hydrophobically modified proteins or antigens which are often difficult to disperse in an aqueous medium (Huang et al., 1983; Kersten and Crommelin, 1995; Morein et al., 1995), but are required to be incorporated into ISCOM matrices (and possibly other colloidal particles) by hydrophobic interactions. Using the lipid film hydration method, we have constructed a pseudo-ternary diagram of aqueous mixtures of Quil A, cholesterol and phospholipid describing the various types of colloidal particles, including ISCOM matrices, liposomes and micelles that were observed in different regions of the diagram (Demana et al., 2004). However, little is known about the incorporation of antigen or protein in ISCOM matrices and related colloidal particles containing Quil A prepared by the lipid film hydration method. Moreover, the impact of incorporation of antigen on the type of colloidal particles produced by aqueous mixtures of Quil A, cholesterol and phospholipid in the pseudo-ternary diagram is not known.

The objectives of this study therefore were:

- to modify ovalbumin by two different approaches to render the model antigen amphipathic, with the aim of investigating which modified protein is better able to incorporate into the various colloidal particles formed by hydration of dried lipid films with an aqueous solution of Quil A;
- to investigate which of the various colloidal particles prepared by the lipid film hydration method are capable of incorporating the modified protein and to rank the various types of colloidal particles in terms of their ability to incorporate modified protein;
- to assess the impact of incorporation of modified protein on morphology, size and prevalence of the various colloidal structures;
- to compare the incorporation of antigen into particles prepared by the lipid film hydration method and particles prepared by the dialysis method.

2. Methods

2.1. Materials

Quil A was obtained from Superfos Biosector, Denmark. Octylglucoside (purity approximately 98%), cholesterol (purity approximately 95%), L-α-phosphatidylethanolamine from bovine brain (purity approximately 98%), ovalbumin (Grade V, purity approximately 98%), fluorescein isothiocynate (purity approximately 90%), *N*-(palmitoyloxy) succinimide (purity approximately 98%), 1-ethyl-3′-(3-dimethylaminopropyl)carbodiimide hydrochloride (purity approximately 99%), triton-X 100 (purity approximately 99%) and *N*-hydroxysuccinimide (purity approximately 98%) were purchased from Sigma–Aldrich Pty Ltd., Missouri, USA. Sodium deoxycholate (purity approximately 90%) was purchased from Koch-Light Laboratories, England. Distilled deionised water having a conductivity of less than 0.1 μS (Milli-Q Water system, Millipore Corporation, Massachusetts, USA) was used throughout the study. All other chemicals and solvents were of at least analytical grade.

2.2. Conjugation of fluorescein isothiocynate (FITC) to ovalbumin (OVA)

The conjugation of FITC to OVA was performed as described previously (Könnings et al., 2002). Briefly, 20 mg FITC was dissolved in 10 ml of carbonate buffer (220 mM, pH 9.5) and 100 mg OVA was added. The mixture was gently stirred in the dark at 4 °C for 18 h. Buffer salts and unbound FITC were removed by repeated dilution with water and ultrafiltration using a 10,000-molecular weigh cut-off membrane and a 50 ml filtration cell (Amicon, Beverly, Massachusetts, USA) pressurised to 200 kPa. The resulting FITC-OVA solution was freeze-dried

and subsequently stored protected from light at 4 °C. Yield, determined gravimetrically, of FITC-OVA was >90%.

2.3. Conjugation of palmitic acid (P) to FITC-OVA

The conjugation of P to FITC-OVA was performed as described previously (Könnings et al., 2002). Briefly, 30 mg FITC-OVA was dissolved in 30 ml of carbonate buffer (50 mM, pH 9) containing 30 mg sodium deoxycholate. 470 μl of a solution of 5 mg *N*-(palmitoyloxy) succinimide (NPS) in 500 μl dimethylsulfoxide was added. The mixture was protected from light and gently stirred for 15 h at 37 °C. Sodium deoxycholate and unbound NPS were then removed by washing and ultrafiltration as described above. The resulting solution was centrifuged at 30,000 × *g* for 30 min and the clear supernatant was freeze-dried. Yield, determined gravimetrically, of P-FITC-OVA was >95%.

2.4. Conjugation of phosphatidylethanolamine (PE) to FITC-OVA

The conjugation of PE to FITC-OVA was performed according to the method described by Sjölander et al. (1997), modified for ISCOM preparation by the lipid film hydration method. 1-Ethyl-3′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was used to link the amino group of PE to the carboxyl groups in FITC-OVA. 100 mg FITC-OVA dissolved in 10 ml of water was mixed with 100 mg PE dissolved in 20 ml of 10% w/v aqueous octylglucoside solution. 100 mg of N-hydroxysuccinimide was then added to the mixture. The conjugation was initiated by addition of 2 g of EDC, and the total volume was adjusted to 80 ml with water. The mixture was protected from light and gently stirred for 30 h at 4 °C. After this incubation period, the PE-FITC-OVA formed was purified by repeated washing and ultrafiltration as described above. The resulting solution was freeze-dried. Yield, determined gravimetrically, of PE-FITC-OVA was >90%.

2.5. Mass spectroscopy

Molecular weight determinations of OVA and modified OVA were carried out using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) as described by Hubbard and McHugh (1996). Mass spectra were collected on a De-Pro mass spectrometer (PerSeptive Biosystems, Inc., Massachusetts, USA) fitted with a nebulisation assisted atmospheric pressure ionisation source. Masses were calibrated with respect to the expected molecular weight of ovalbumin of 42,700 Da (Nisbet et al., 1981). All samples were injected into the spectrometer using a fused silica capillary tube (i.d. 60 μm), at a rate of 5 μl/min using a syringe pump (Harvard Apparatus, model 2400-001, Massachusetts, USA). A coaxial spray of nitrogen at 0.9 l/min assisted nebulisation of the liquid sample.

2.6. Preparation of colloidal dispersions

Colloidal dispersions were prepared using the lipid film hydration method as previously described (Copland et al., 2000). The dialysis method, as described by Kersten et al. (1991), was also used to prepare colloidal dispersions of Quil A, cholesterol and phospholipid for comparison. The formulations investigated were chosen as representative samples of the different regions of the pseudo-ternary diagram (Fig. 1, formulations A–F). The composition of formulations A–F are presented as percentage mass ratios of Quil A:cholesterol:PE and they were prepared in the presence of protein or modified protein as described below.

2.6.1. Lipid-film hydration method

Various amounts of PE and cholesterol were dissolved in 0.5 ml chloroform and evaporated to dryness at 45 °C for 1 h (Rotavapor R110, Büchi, Switzerland). The resulting lipid films were hydrated for 5 h at room temperature with 3 ml Tris buffer (140 mM, pH 7.4) containing various amounts of Quil A and 3 mg of protein or modified protein. The total polar lipid concentration in each sample (Quil A, cholesterol and PE) 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 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 5 h. All samples were prepared in triplicate and equilibrated for 1 week at 4 °C before analysis.

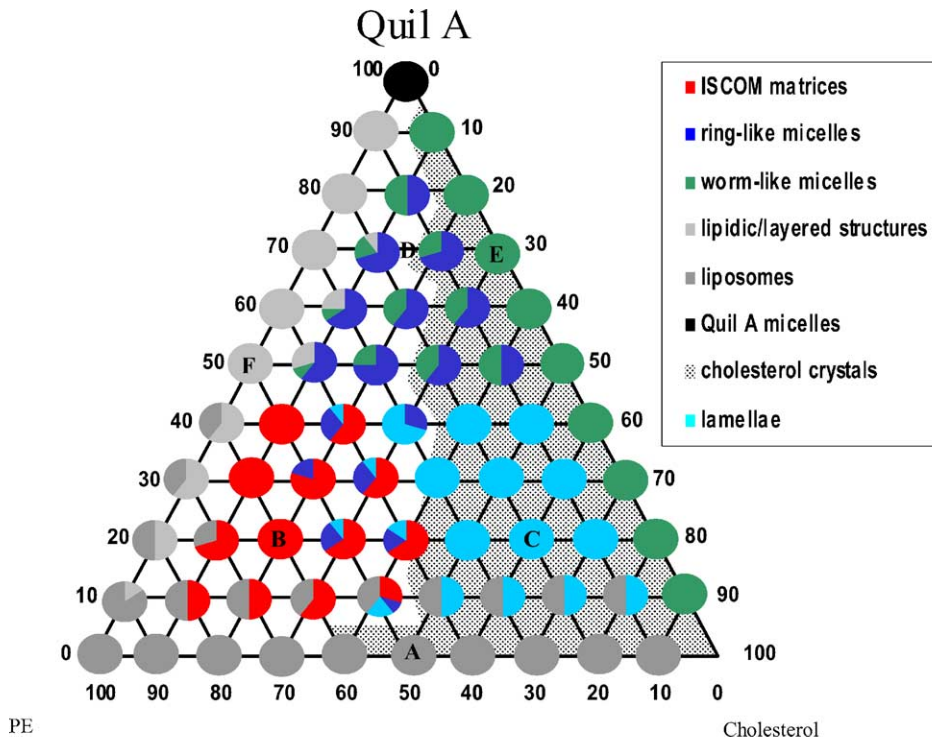

Fig. 1. Pseudo-ternary diagram for aqueous mixtures of Quil A, cholesterol and PE.

2.6.2. Dialysis method

Various amounts of PE and cholesterol were dissolved in 0.5 ml chloroform and evaporated at 45 °C for 1 h (Rotavapor R110, Büchi, Switzerland). Different amounts of Quil A in Tris buffer (140 mM, pH 7.4) was added to the dried lipid film so that the total polar lipid concentration in each sample (Quil A, cholesterol and PE) was 6.7 mg/ml. The surfactant octylglucoside at a concentration of 40 mg/ml was added to solubilise the polar lipids. Protein or modified protein at a concentration of 1 mg/ml was added to the mixture. The micellar mixture was stirred using a magnetic stirrer for 5 h at room temperature. The samples were placed in a dialysis tubing (Regenerated Cellulose Tubing, molecular weight cut-off of 1000 Da, Spectrum Laboratories Inc., California, USA) and dialysed against seven changes of 1 l Tris buffer (140 mM, pH 7.4) at 4 °C for 3 days to remove the octylglucoside. All samples were prepared in triplicate and equilibrated for one week at 4 °C before analysis.

2.7. Characterisation of colloidal dispersions

2.7.1. Transmission electron microscopy (TEM)

Carbon-coated copper grids were glow-discharged (Edwards E306A Vacuum Coater, England) and 10 μl of sample adsorbed onto these grids. The samples were negatively stained using 10 μl of filtered 2% phosphotungstic acid (pH 5.2) as a contrast agent. Samples were investigated using a Philips 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.

2.7.2. Polarised light microscopy (PLM)

All formulations were examined using a phase contrast light microscope (Model 218502, Nikon, Japan)

equipped with polariser and analyser (Nikon Optiphot, Nikon, Japan) to determine the presence of cholesterol crystals.

2.7.3. Sucrose density gradient ultracentrifugation (SGU)

Colloidal particles were separated from non-incorporated protein or modified protein by sucrose density gradient ultracentrifugation (10–60% (w/w), 18 h at $200{,}000 \times g$, 10 °C) as previously described (Sjölander et al., 1997; Johansson and Lövgren-Bengtsson, 1999; Könnings et al., 2002). Visible bands of colloids entrapping fluorescently labelled proteins were recovered (Könnings et al., 2002) and dispersed in 1 ml Tris buffer before fluorescence spectroscopy measurements to quantify for protein incorporation.

2.7.4. Determination of entrapment efficiency

Quantification of incorporated protein into the colloidal particles was carried out by fluorescence spectroscopy (Könnings et al., 2002). Colloidal structures were disrupted by addition of 50 μl of the dispersion to 750 μl Tris buffer (pH 6.6) containing 5% Triton X-100. Fluorescence of the resulting solution was measured (Shimadzu FR 540; ex. 495 nm, em. 518 nm) and entrapment efficiency of protein into various colloidal particles was estimated based on standard curves constructed for each protein. All measurements were performed in quadruplicates. The amount of protein or modified protein incorporated into the colloidal particles was calculated and expressed as a percentage of the original amount of protein or modified protein used per μmol of lipid as described by Allen (Allen, 1983) and Kirby and Gregoriadis (1983). The molecular weight of Quil A was taken as 2000 g/mol (Barr et al., 1998), and the molecular weight of PE and cholesterol as 745 and 386.7 g/mol (Knight, 1981; Windholz et al., 1983), respectively.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons was used to assess statistical significance between the molecular weights estimated for the various proteins. The same tests were also used to assess statistical significance between the levels of protein incorporation into the various colloidal structures. P-values of less than 0.05 were considered statistically significant.

3. Results and discussion

Table 1 shows the molecular weights of OVA and modified OVAs. The molecular weight of FITC-OVA was on average more than 1500 g/mol higher than that of OVA suggesting that about 4 mol of FITC were bound to each mole of OVA. For P-FITC-OVA and PE-FITC-OVA the molecular weights were also significantly higher than that of FITC-OVA (1547 and 6548 g/mol, respectively) representing an average conjugation of approximately 6 mol of P per mole FITC-OVA and approximately 9 mol of PE per mole FITC-OVA. These results confirm the successful conjugation of FITC to OVA and conjugation of P or PE to FITC-OVA. Although FITC-OVA, P-FITC-OVA and/or PE-FITC-OVA have been prepared in previous studies (Sjölander et al., 1997; Johansson and Lövgren-Bengtsson, 1999; Könnings et al., 2002), the extent of conjugation for these proteins was not known. It is important to determine the extent of conjugation because it is known that if the antigen is too hydrophilic it cannot be incorporated into ISCOM matrices (Morein et al., 1990; Kersten et al., 1991; Rimmelzwaan and Osterhaus, 1997; Barr et al., 1998), and if it is too lipophilic the protein tends to aggregate, thus also preventing its incorporation (Kersten and Crommelin, 1995; Morein et al., 1995). In previous studies it was speculated that conjugation had occurred because P-FITC-OVA or PE-FITC-OVA could be incorporated into ISCOM matrices whereas FITC-OVA could not be incorporated into these

Table 1
Molecular weight of OVA and modified OVAs determined from MALDI-TOF-MS

Protein	Molecular weight (Da)
OVA	43,683 ± 150
FITC-OVA	45,256 ± 80
P-FITC-OVA	46, 803 ± 92
PE-FITC-OVA	51,804 ± 172

Values are mean ± S.D. ($n = 4$). Molecular weights of FITC and palmitic acid are 389.4 g/mol (Sigma–Aldrich Pty Ltd.) and 256.4 g/mol (Windholz et al., 1983), respectively. Molecular weight of PE was taken as 745 g/mol (Knight, 1981).

colloids (Sjölander et al., 1997; Johansson and Lövgren-Bengtsson, 1999; Könnings et al., 2002).

Initially, the pseudo-ternary diagram for aqueous mixtures of Quil A, cholesterol and PE in the absence of protein as prepared by the lipid-film hydration method was constructed (Fig. 1) and found to be similar to the pseudo-ternary diagram we have reported earlier (Demana et al., 2004) in which phosphatidylcholine was used as the phospholipid instead of PE. Depending on the ratio of Quil A:cholesterol:PE, various types of colloidal particles were identified including ISCOM matrices, liposomes, ring-like micelles, lamellae (hexagonal array of ring-like micelles), worm-like micelles and lipidic/layered structures as previously reported by our group (Demana et al., 2004). A lamellae is defined as a sheet-like structure (often of micron size) composed of a hexagonal pattern of ring-like micelles (Kersten et al., 1991; Kersten and Crommelin, 1995). Systems were prepared for protein incorporation studies with compositions which yielded typical structures noted in different areas of the diagram i.e. liposomes (A), ISCOMs (B), lamellae (C), ring- and worm-like micelles (D), worm-like micelles (E) and lipidic/layered structures (F) (Fig. 1). The structures formed in the presence of FITC-OVA, PE-FITC-OVA and P-FITC-OVA were similar in appearance (as visualised by electron microscopy) to the structures formed in the absence of modified protein as exemplified in Fig. 2 for systems containing PE-FITC-OVA at a lipid:protein ratio of 6.7:1. Thus, incorporation of modified protein did not appear to influence the size, morphology and prevalence of colloidal structures noted in the samples investigated. This suggests that the nature of the modified protein investigated and the amount of protein incorporated into the various colloidal structures does not have a significant influence on the various regions of the pseudo-ternary diagram shown in Fig. 1.

Fig. 3 shows the entrapment efficiency of FITC-OVA, P-FITC-OVA and PE-FITC-OVA into various colloidal particles prepared by the lipid-film hydration method, and for ISCOMs and micelles prepared by the dialysis method. These results demonstrate that all colloidal structures are capable of incorporating both P-FITC-OVA and PE-FITC-OVA to varying degrees. Furthermore the results show that incorporation of FITC-OVA into colloidal particles is only possible in the absence of Quil A, i.e. when liposomes are present in the dispersion. This implies that the non-modified, hydrophilic FITC-OVA could be encapsulated into the aqueous compartments of liposomes but could not be incorporated into non-liposomal structures containing Quil A such as ISCOMs, lamellae, ring- and worm-like micelles.

Incorporation of PE-FITC-OVA or P-FITC-OVA was possible with all colloidal structures investigated and was particularly evident when ISCOMs and lamellae were the predominant colloids with incorporation efficiencies reaching 20%/μmol lipid or above for PE-FITC-OVA. The encapsulation efficiency of PE-FITC-OVA was significantly higher ($P < 0.05$) than that of P-FITC-OVA for all colloids except liposomes implying that PE-FITC-OVA was relatively more hydrophobic compared to P-FITC-OVA. This is because of the more lipophilic nature of PE having two fatty acids compared to palmitic acid having a single chain fatty acid. The higher lipophilicity of PE-FITC-OVA was confirmed by MALDI-TOF-MS analysis as approximately 9 mol of PE were conjugated per mole of FITC-OVA compared with about 6 mol of palmitic acid per mole of FITC-OVA. The higher lipophilicity of PE-FITC-OVA also resulted in lower aqueous solubility of the compound compared to P-FITC-OVA which has been found to be freely soluble in water. In addition, upon sucrose gradient ultracentrifugation a precipitate of PE-FITC-OVA at the bottom of the centrifuge tube was frequently observed in contrast to samples containing P-FITC-OVA or FITC-OVA where no sediment of modified protein was found. A precipitate is often observed with proteins due to aggregation of protein molecules as a result of increased hydrophobic interactions which may have been brought about by the conjugation of PE (Huang et al., 1982).

All modified proteins (FITC-OVA, P-FITC-OVA and PE-FITC-OVA) could be incorporated into liposomes (Formulation A). However, the lowest encapsulation efficiency was found with the hydrophilic FITC-OVA. Thus conjugation of fatty acids to FITC-OVA promotes incorporation even into liposomes that have an associated aqueous volume. The liposomes obtained by the preparation method used were observed to be multilamellar and hence the relative aqueous volume of these is small compared to other forms of liposomes such as unilamellar liposomes (Kirby and Gregoriadis, 1984; New, 1990). Hence lipid modifi-

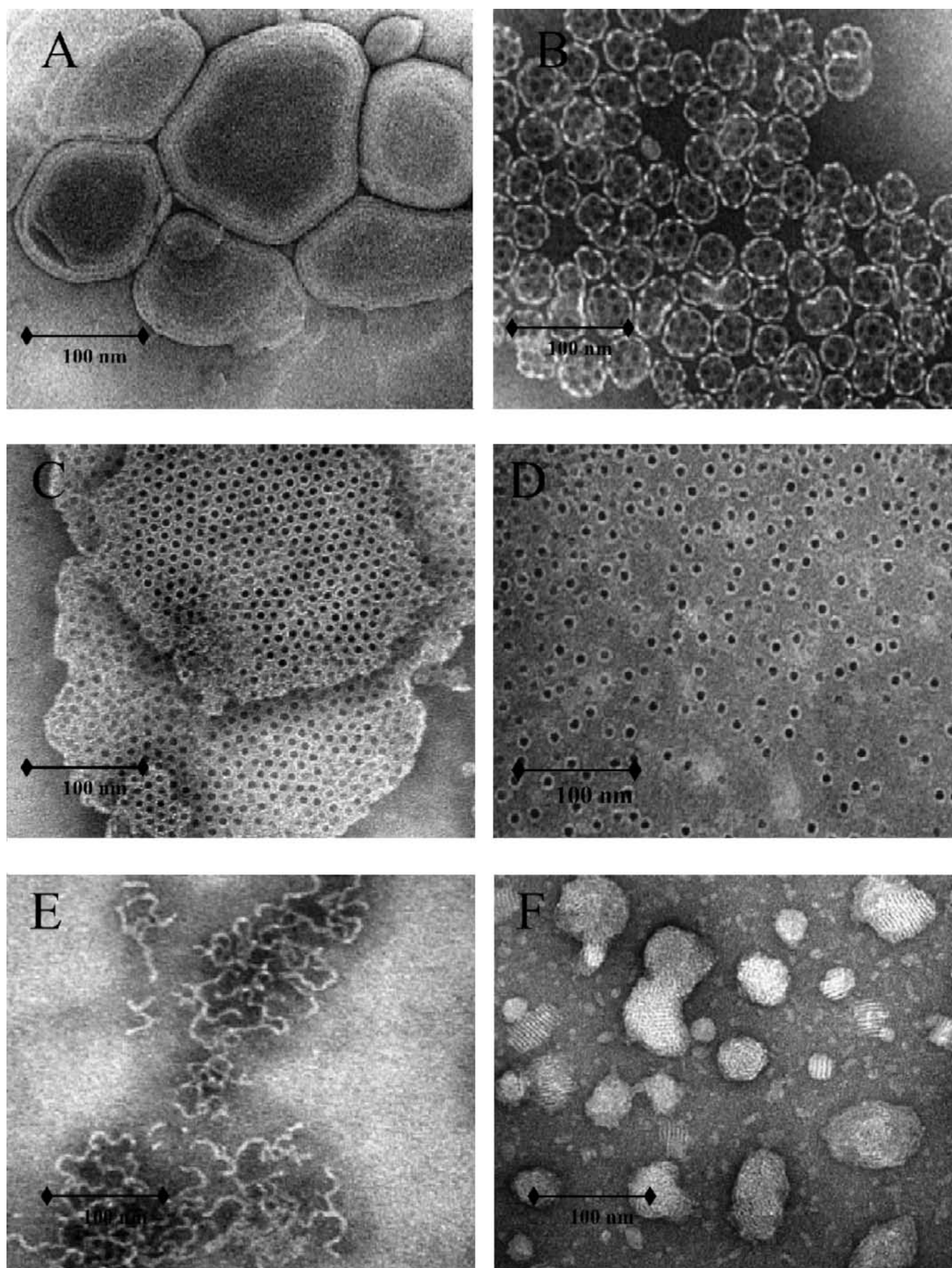

Fig. 2. Electron micrographs of pseudo-binary and pseudo-ternary systems prepared using the lipid film hydration method. All systems contained a total lipid:PE-FITC-OVA mass ratio of 6.7:1. A–F represent the samples investigated for protein incorporation studies, the composition of which is given in Fig. 1.

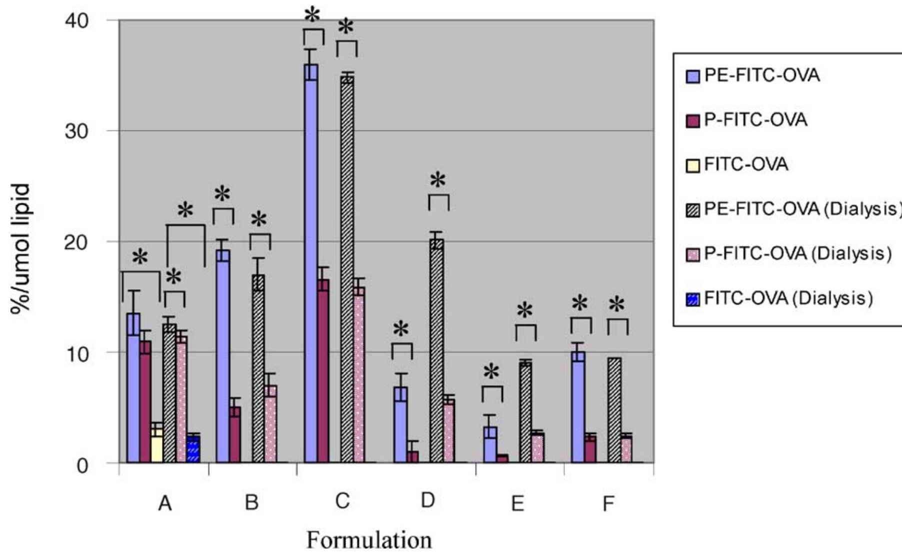

Fig. 3. Encapsulation efficiency of protein in different colloidal dispersions (amount entrapped as a percentage of original amount of protein used per micromole lipid, type of colloidal dispersion described in Fig. 1). Results are mean ± S.D., $n = 4$. The asterisks represent statistically significant differences between specified samples. Unless otherwise stated, samples were prepared by the lipid film hydration method.

cation of the protein would have increased interaction with the lipid bilayers leading to a more efficient encapsulation of the modified protein within liposomes having a relatively low aqueous volume (Knight, 1981; Kirby and Gregoriadis, 1984; New, 1990).

The addition of Quil A to a binary mixture containing PE and cholesterol resulted in the formation of other colloidal structures, depending on the mass ratio of Quil A:cholesterol:PE (Fig. 1B–D (E and F are binary systems)). Formulations having a Quil A:cholesterol:PE mass ratio of 1:1:3 produced predominantly ISCOM matrices having a diameter of approximately 40 nm (Formulation B). The incorporation of modified protein into ISCOM matrices was higher than for liposomes especially with PE-FITC-OVA (Fig. 3). FITC-OVA could not be incorporated into ISCOM matrices. ISCOMs have been characterised as "cage-like structures" and hence do not have a defined entrapped aqueous volume (Kersten et al., 1991). Hence, ISCOMs can only associate with proteins which have sufficient amphipathic character that enable them to interact via hydrophobic interactions with the lipidic ISCOM matrix. This may also explain the higher incorporation of PE-FITC-OVA compared with P-FITC-OVA or FITC-OVA. Further, due to their open, cage-like structure, ISCOMs may provide a larger accessible surface area for the modified protein compared to liposomes, leading to a higher encapsulation efficiency of PE- and P-FITC-OVA.

When a high amount of cholesterol was used in the presence of comparatively small amounts of Quil A and PC to yield lamellae structures (Formulation C), incorporation of protein was even higher than for incorporation into ISCOM matrices for both P- and PE-FITC-OVA. Lamellae form as a result of the aggregation of the ring-like micelles formed at higher concentrations of cholesterol to yield an hexagonal pattern (Kersten and Crommelin, 1995). A few individual ring-like micelles were also observed in this system further supporting the idea that these micelles are the precursors of lamellae. A high entrapment of the lipid-modified protein in this system probably arises due the increased lipophilicity of the lamellae compared to ISCOMs and liposomes as a result of the high ratio of cholesterol present in the lamellae. Both PE-FITC-OVA and P-FITC-OVA were incorporated into lamellae to a greater extent than into any other colloidal structure investigated in this study with the efficiency of entrapment again being greater for the more lipophilic PE conjugated protein.

At high concentrations of Quil A, the predominant structures were ring-like micelles (Formulation D). An electron micrograph of a formulation with a Quil A:cholesterol:PE mass ratio of 5:1:1 shows these ring-like micelles, having a size of approximately 10 nm (Fig. 2D). These micelles were capable of incorporating both PE-FITC-OVA and P-FITC-OVA (Fig. 3, Formulation D). FITC-OVA on the other hand was not incorporated into these ring-like micelles. The incorporation of PE-FITC-OVA or P-FITC-OVA was lower than into ISCOM matrices, lamellae or liposomes possibly because the ring-like micelles are less lipophilic in comparison to these other colloidal structures due to their high Quil A concentration. Interestingly, the incorporation of the more lipophilic PE-FITC-OVA was still higher into the ring-like micelles than P-FITC-OVA.

The mixture of Quil A and cholesterol in the absence of PE resulted in the formation of predominantly worm-like micelles (Formulation E). An electron micrograph of these worm-like structures is shown in Fig. 2E. Worm-like micelles were always observed together with cholesterol crystals, irrespective of the Quil A:cholesterol mass ratio used (Fig. 1), suggesting that solubilisation of cholesterol by Quil A micelles in the absence of phospholipid is minimal. Like ring-like micelles, worm-like micelles were capable of incorporating both PE-FITC-OVA and P-FITC-OVA but were not capable of incorporating FITC-OVA. This again would imply that they have accessible hydrophobic regions in their structures. The encapsulation efficiency of lipid-modified protein into these micelles, however was the lowest amongst the various colloidal particles investigated (Fig. 3, Formulation E). The low incorporation is likely to be due to the comparatively low hydrophobicity of worm-like micelles which in turn is due to the high amount of Quil A present in these colloidal particles.

A binary mixture of Quil A:PE at a mass ratio of 1:1 produced predominantly lipidic particles (Fig. 2F). Lipidic particles appeared either as colloids that do not have a detectable bilayer structure when investigated by TEM or as layered structures in the form of stacks (Fig. 2F) having a layer thickness similar to that observed for liposomes (∼6 nm) suggesting that these layered structures might be bilayer fragments of vesicular structures (Demana et al., 2004). Thus, Quil A would appear to prevent vesicle formation despite forming bilayer structures with PE. A closer examination of the lipidic particles seems to indicate that the layered structures and non-bilayer particles are basically the same structures, and their appearance is dependent on their orientation when viewed by TEM. The morphology of lipidic particles is similar to those in previous reports for the interaction between Quil A and PE (Devries et al., 1990) except that the layered morphology has not been previously reported. Both PE-FITC-OVA and P-FITC-OVA could be incorporated into these lipidic particles, PE-FITC-OVA again to a larger extent than P-FITC-OVA (Fig. 3, Formulation F). The lipidic particles probably lack closed aqueous compartments in their structures because FITC-OVA could not be incorporated into these colloids.

The incorporation of modified protein into the various colloidal particles prepared by the dialysis method using the same mass ratios of the polar lipids showed a similar pattern of encapsulation efficiency as for colloids prepared by the lipid film hydration method. Incorporation was essentially similar in samples prepared by the dialysis method to those prepared by hydration, provided the same structures were formed, i.e. in Formulation A (liposomes), Formulation C (lamellae) and Formulation F (lipidic particles) (Fig. 3). In the case where different colloidal structures were formed due to the method of preparation, differences in encapsulation efficiency were found ($P < 0.05$). For example, using the dialysis method for the preparation of the sample comprising Quil A:cholesterol:PE mass ratio of 5:1:1 (Formulation D) produced mainly ISCOMs in agreement with the literature (Kersten et al., 1991; Ekström et al., 1999; Mowat et al., 1999; Sjölander et al., 2001). In contrast, the same formulations prepared using the lipid film hydration method resulted in predominantly ring-like micelles, together with a few worm-like micelles (Fig. 2D). The reasons for the discrepancy between the two methods of preparation in some areas of the pseudo-ternary diagram (Demana et al., 2004) are currently unclear but may be due to residual octylglucoside in the systems prepared by the dialysis method (Kersten and Crommelin, 1995) or due to a very long equilibration time in the formulations prepared by the lipid film hydration method (Demana et al., 2004). Since ISCOMs were formed when Formulation D was prepared by dialysis, encapsulation efficiencies of modified protein

into this formulation resembled the encapsulation efficiencies observed for Formulation B which produced predominantly ISCOMs following both methods of preparation rather than the encapsulation efficiency noted when Formulation D was prepared by lipid film hydration yielding micelles. Similarly for Formulation E, where ring-like micelles were produced by dialysis as compared to worm-like micelles by lipid film hydration, encapsulation of lipid modified protein in Formulation E prepared by dialysis more closely resembled the results obtained for Formulation D prepared by hydration where ring-like micelles were also most prevalent.

4. Conclusions

In this study palmitic acid and phosphatidylethanolamine were successfully conjugated to FITC-OVA resulting in the formation of P-FITC-OVA and PE-FITC-OVA respectively. Consequently, P-FITC-OVA and PE-FITC-OVA could be incorporated into various colloidal structures formed in systems containing Quil A, cholesterol and PE (ISCOM matrices, liposomes, ring-like and worm-like micelles, lamellae and lipidic/layered structures). The highest entrapment efficiency within all colloidal structures was found for PE-FITC-OVA, whereas FITC-OVA could only be incorporated into liposomes. The incorporated protein did not affect the size, morphology or the prevalence of the colloidal structures, and hence the appearance of the pseudo-ternary diagram. The degree of incorporation of protein as a function of the colloidal structure was in the order: worm-like micelles < ring-like micelles < lipidic/layered structures < liposomes < ISCOM matrices < lamellae. The incorporation of protein into various particles prepared by the lipid film hydration method was similar to those for samples prepared by the dialysis method provided the same structures were formed. However, in the case of different colloidal structures arising due to the preparation method, differences in encapsulation efficiency were found. This study has demonstrated that different types of colloids following the simple method of hydrating PE/cholesterol lipid film with aqueous solution of Quil A, are capable of incorporating an amphipathic antigen. These colloids have application as future vaccine delivery systems.

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