International Journal of Pharmaceutics 376 (2009) 123–133

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Synthesis of cationic derivatives of Quil A and the preparation of cationic immune-stimulating complexes (ISCOMs)

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ARTICLE INFO

Article history:
Received 28 December 2008
Received in revised form 6 April 2009
Accepted 7 April 2009
Available online 16 April 2009

Keywords:
ISCOMs
Immune-stimulating complexes
Quil A
Quil A derivatives

ABSTRACT

The aim of the study was to prepare cationic ISCOMs using cationic derivatives of the saponin Quil A. The polyamines ethylenediamine, spermidine and spermine were conjugated with the glucuronic acid moiety of Quil A. The aqueous solubility of the derivatives increased with decreasing pH, and the pK_a values were between 6 and 7. The CMCs of the ionised derivatives were around 0.5–1.0 mg/mL. Using the method of hydration of freeze-dried monophase systems, the interaction of each of the Quil A derivatives with phosphatidylcholine and cholesterol, at a mass ratio of 4:4:2 and a pH of 3 and 7.4, was investigated. A few ISCOM-like structures were present in the systems prepared at pH 7.4, hence the ternary system of Quil A spermine derivative, phosphatidylcholine and cholesterol was further investigated at pH 7.4 using a variety of mass ratios. A relatively high number of cationic ISCOM-like structures were observed at the mass ratio of 6:2:2. These ISCOM-like structures were less homogeneous and more irregular in shape than ISCOMs prepared from unmodified Quil A. Colloidal particles with positive zeta potential were produced and may find application in the delivery of nucleic acids or anionic proteins.

1. Introduction

Quil A is a mixture of triterpenoid saponins extracted from the bark of the South American tree, *Quillaja saponaria* Molina (Kensil, 1988). Within this mixture, four prominent saponins have been identified, namely QS7, QS17, QS18 and QS21 (Kensil, 1988). Quil A has received much attention due to its adjuvant activity and ability to stimulate both humoral and cell-mediated immune responses (Robson et al., 2003). The use of Quil A as an adjuvant has been further extended by combining it with phospholipids and cholesterol (Chol) to form *i*mmune-stimulating *com*plexes or *ISCOMs*. ISCOMs are colloidal spherical particles with an open cage-like structure of about 40 nm in diameter (Ozel et al., 1989). Like Quil A, ISCOMs also possess adjuvant activity but are reportedly less haemolytic than Quil A (Rönnberg et al., 1995). Consequently, ISCOMs have received considerable attention as adjuvants for subunit vaccines and are currently approved for use in veterinary vaccines and ongoing human clinical trials (Kersten and Crommelin, 2003; Morein et al., 2004).

Structurally, Quil A saponins comprise an aglycone triterpene nucleus to which is attached a number of substituents at positions 3 and 28 (Fig. 1) to afford a bipolar structure (Barr et al., 1998). The various Quil A saponins differ in the sugar chains attached to the aglycone core. The major saponins (QS7, QS17, QS18 and QS21) all possess a glucuronic acid as a component of the sugars linked at position 3, rendering Quil A (and ISCOMs) with a negative charge at physiological pH. These major saponins also contain an aldehyde functional group at position 4 of the aglycone. Both the carboxylic acid and aldehyde have been used as sites for the chemical modification of Quil A or its fractions, either to better understand the structure–activity relationships of Quil A or to enhance properties such as adjuvant activity and chemical stability (Marciani, 1998; Marciani et al., 2000). Cationic derivatives of Quil A have also been synthesised with the aim of developing carriers for nucleic acids that can also induce an immune response (Marciani, 2004). The cationic saponins were able to bind to the polyanionic nucleic acids and the complexes were reportedly able to stimulate an immune response *in vivo* to a greater extent than free DNA encoding antigen following intramuscular administration. However, the Quil A derivatives were synthesised from deacylated Quil A (the fatty acid domain attached to the fucose residue of Quil A was cleaved, Fig. 1), and required at least a 3-day reaction.

Cationic ISCOMs or ISCOM-like structures have also been investigated as potential delivery systems for proteins and nucleic acids utilising charge–charge interactions to incorporate the actives into the colloidal structures (Kirkby and Dalsgaard, 2002; Kirkby and Samuelsen, 2004; Lendemans et al., 2005b). A cationic cholesterol derivative (3β-[*N*-(*N*′, *N*′-dimethylaminoethane)-carbamoyl]-cholesterol) was used as a replacement for cholesterol for the formation of cationic ISCOMs. Cage-like structures having a size

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doi:10.1016/j.ijpharm.2009.04.011

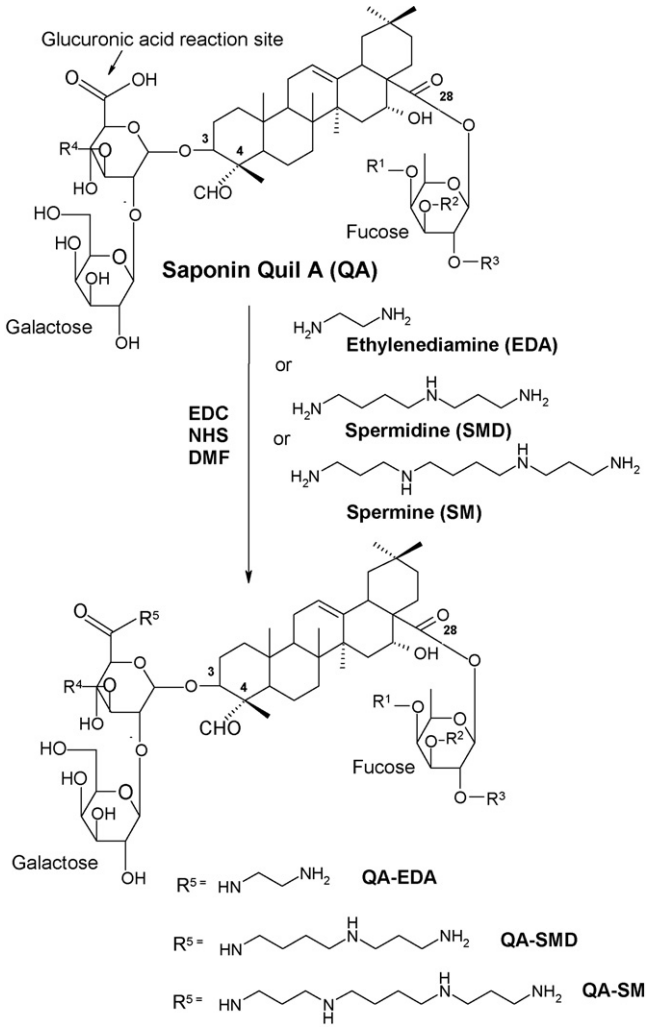

Fig. 1. Schematic showing the synthesis of the cationic Quil A derivatives. The difference between specific saponins (QS17, QS18 and QS21) lies in different substituents attached to R^1, R^2, R^3, and R^4.

of around 40 nm were reportedly formed in studies by Lendemans et al. (2005b), although their formation was highly dependent on the buffer used and the structures formed were less numerous and often more aggregated and heterogeneous than preparations of conventional ISCOMs. Cationic ISCOMs are reported to bind DNA, protect it from degradation by DNase I, and transfect cells (Kirkby and Dalsgaard, 2002). These cationic ISCOMs (termed PosIntro™) were subsequently investigated for their ability to deliver polynucleotide encoding vaccine antigens across the skin and initiate an immune response (Kirkby and Samuelsen, 2004).

The aims of the present investigations were: (1) to develop a simple, one-step method for the synthesis of cationic derivatives of purified but unmodified Quil A saponins and (2) to investigate the colloidal structures formed when these cationic derivatives were combined with phospholipid and cholesterol using our recently reported method for ISCOM preparation based on hydration of freeze-dried monophase systems (Liang et al., 2008).

2. Materials and methods

2.1. Materials

Quil A was purchased from Brenntag Biosector, Frederikssund, Denmark. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), ethylenediamine (EDA), spermine (SM), spermidine (SMD), 3-hydroxypicolinic acid (HPA), 1,6-diphenyl-1,3,5-hexatriene (DPH), tetrahydrofuran (THF), trifluoroacetic acid (TFA), cholesterol (Chol) (purity approximately 95%), L-α-phosphatidylcholine (PC) from egg yolk (purity approximately 99%) and sucrose were purchased from Sigma–Aldrich Pty Ltd. (St. Louis, MO, USA). Amberlite® IRC-50 resin was obtained from Supelco (Bellefonte, PA, USA). *N*-Hydroxysuccinimide (NHS) was purchased from Auspep Pty Ltd. (Parkville, Victoria, Australia). *N*,*N*-Dimethylformamide (DMF), acetonitrile (HPLC grade), glacial acetic acid and sulfuric acid were obtained from Lab-Scan Analytical Sciences (Bangkok, Thailand). Acetic anhydride, methanol, ethanol, hydrochloric acid 32% (w/v), ammonia solution 28% (w/w) and ammonium acetate (AA) were purchased from Ajax Finechem (Seven Hills, NSW, Australia). 1-Naphthol was purchased from Merck (Schuchardt, Hohenbrunn, Germany). Sodium hydroxide 0.2N was purchased from Prolabo (Paris, France). All chemicals and solvents were at least analytical grade. DMF was dried over 3 Å molecular sieves. Distilled water was used throughout the study.

2.2. Methods

2.2.1. Synthesis of Quil A derivatives

Quil A (150 mg) was dissolved in 3 mL of DMF. EDC (150 mg) and NHS (150 mg) were sequentially added to the solution and the mixture was stirred for 10 min. EDA (150 mg) was dissolved in DMF (0.5 mL) and added dropwise into the stirred mixture over a period of 5 min. The mixture was stirred for a further 3 h and then centrifuged at 5000 rpm for 5 min (Laborzentrifugen, Sigma). The clear supernatant was collected and evaporated to near dryness using a rotary evaporator and a 65 °C water bath. The residue was dissolved in distilled water prior to purification by HPLC as described below. Derivatives of Quil A with SM and SMD were synthesised as described, except 450 mg of SMD and 550 mg of SM were used instead of 150 mg of EDA to maintain equivalent molar ratios of reactants. The structures of the cationic derivatives are shown in Fig. 1 (QA-EDA, QA-SMD and QA-SM).

2.2.2. Purification of Quil A derivatives by semi-preparative HPLC

The Quil A derivatives were purified by reverse-phase HPLC using a Shimadzu HPLC system (SCL-10A VP controller, LC-10AT VP pump, DGU-14A degasser, FCV-10AL VP solvent mixer and a SPD-10A VP UV detector). The mobile phase was 0.15% TFA in water (solvent A) and 0.15% TFA in acetonitrile (solvent B) which was run at a flow rate of 6 mL/min using a linear gradient from 0 to 50% solvent B over 120 min. The crude reaction mixture (500 μL of an approximately 100 mg/mL solution) was separated on a semi-preparative column (LiChrospher® 100, RP-18e, 250 mm × 10 mm, 10 μm, Merck KGaA, Darmstadt, Germany). Compounds were detected at 210 nm. Fractions were collected, freeze-dried, and analysed for the presence of the desired Quil A derivatives using qualitative tests and mass spectrometry (see below for details). All four prominent Quil A saponins (QS7, QS17, QS18 and QS21) have a carboxylic acid group that can react with polyamines. However, we have previously reported that only three of the purified saponins (QS17, QS18 and QS21) extensively form ISCOMs (Pham et al., 2006). Therefore, derivatives of QS17, QS18 and QS21 were the desired saponins.

2.2.3. Qualitative tests and mass spectrometry for identification of Quil A derivatives

The qualitative Liebermann–Burchard (LB) and Molisch (ML) tests were used to identify the presence of triterpenoid structures and carbohydrate components respectively. For the LB test, 1 mg of a purified fraction was placed a test tube with 1 mL of acetic anhydride. After mixing, 1 mL of concentrated sulfuric acid was slowly added to form a two-phase mixture. A red colour at the interface

indicated the presence of a triterpenoid (Tapan and Basu, 1963). For the Molisch test, 3 drops of Molisch reagent (10% solution of 1-napththol dissolved in 96% ethanol) were added to 1 mg of the purified fraction in 1 mL of water. One mL of concentrated sulfuric acid was then slowly added to form a two-phase system. A purple colour at the interface indicated the presence of carbohydrates (Keusch, 2003).

The ninhydrin (NHD) test was used to identify the presence of free primary amine groups in the Quil A derivatives. One mg of a purified fraction was placed in a test tube and the reagents were added sequentially as follows: 2 drops of reagent A (76% (w/w) phenol in ethanol), 4 drops of reagent B (0.2 M potassium cyanide in pyridine) and 2 drops of reagent C (0.28 M ninhydrin in ethanol). The resulting mixture was incubated at 100 °C for 5 min in a dry block heater (Ratek Instruments, Victoria, Australia). A blue-purple colour indicated the presence of free primary amine groups (Yemm et al., 1950).

The molecular weights (MWs) of components in the HPLC fractions were determined using Matrix Assisted Laser Desorption/Ionisation—Time of Flight mass spectrometry (MALDI-TOF) using a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA). One μL of a solution of purified fraction (0.5 mg/mL in 50% aqueous methanol) was mixed with 1 μL of 3-hydroxypicolinic acid solution and spotted onto a stainless steel MALDI-plate. The HPA was prepared by dissolving 50 mg HPA in 1 mL of 1:1 (v/v) acetonitrile:water and diluting this 9:1 (v/v) with 50 mg/mL diammonium citrate in water. The air-dried plate was then loaded into the spectrometer and the sample ionised in positive reflector mode using an accelerating potential of 200,000 V with grid potential set to 66% and a delay time of 300 ns. All mass spectra were collected over the range of 1000–5000 amu.

2.2.4. Further purification of Quil A derivatives by ion-exchange chromatography

Freeze-dried HPLC fractions (Fig. 2, F3.1, F3.2 and F3.3), containing the desired derivatives, were further purified by cation-exchange chromatography using Amberlite® IRC-50 resin (Supelco, Bellefonte, PA, USA). Fifteen grams of resin was mixed with 50 mL of water under magnetic stirring, and aqueous ammonia (28%, w/w) was added until a pH of 7.8 was maintained for at least 1 h. The resin was then transferred to a glass column with an internal diameter of 1 cm and a length of 30 cm. The packed column was washed with 20 mL of 0.02 M ammonium acetate (AA) buffer of pH 7.8 at a flow rate of approximately 1 mL/min and the pH of the eluent was checked to ensure that it remained at 7.8.

Approximately 50 mg of Quil A (control) or a lyophilised fraction of a Quil A derivative [from semi-preparative HPLC (Fig. 2, F3.1, F3.2 and F3.3)] was dissolved in 0.02 M AA buffer (5 mL, pH 7.8) and adjusted to pH 7.8 with aqueous ammonium (28%, w/w). Half of the washed resin was transferred from the packed column to this solution, and the mixture was stirred for 30 min before being returned to the top of the packed column of resin. Fifteen mL of 0.02 M AA buffer (pH 7.8) was passed through the column and collected. Thirty mL of 0.2 M AA buffer (pH 3.8) was then passed through the column at a flow rate of 1 mL/min to elute the adsorbed cationic saponin derivatives. The two fractions were separately lyophilised three times (the powder was redissolved in water for repeat lyophilisations) to remove any trace of ammonia. The purified samples were tested by LB, ML, NHD, MALDI-TOF (as described in Section 2.2.3) to identify the presence of Quil A and Quil A derivatives in each fraction.

Following ion-exchange chromatography, the Quil A derivatives were characterised by analytical reverse-phase HPLC and their chromatograms were compared to that of underivitised Quil A. HPLC was carried out on a C4 column (Vydac, Hesperia, CA, USA, 250 mm × 4.6 mm, 5 μm, 300 Å) using a linear gradient of 15–45% solvent B over 45 min at a flow rate of 1 mL/min with solvent A being 0.15% TFA in water and solvent B being 0.15% TFA in acetonitrile. Two hundred and fifty μL of a 2 mg/mL solution of purified derivative or Quil A was injected onto the column.

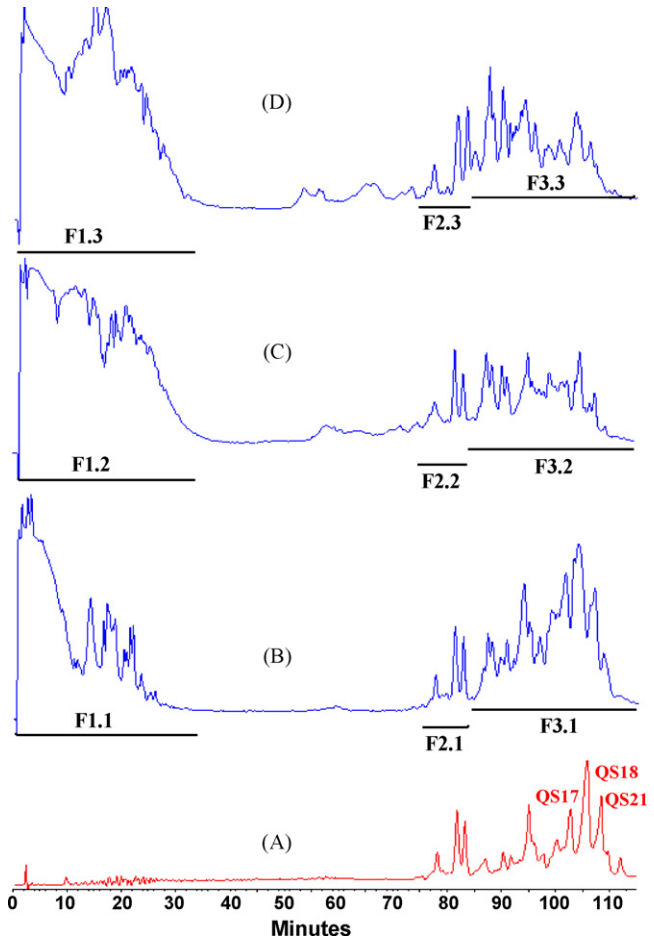

Fig. 2. Semi-preparative reverse-phase HPLC profile of Quil A (A), QA-EDA (B), QA-SMD (C) and QA-SM (D) derivatives showing collected fractions. Only fractions 3.1, 3.2 and 3.3 were strongly positive for the presence of triterpenoids (LB test), carbohydrate (ML test) and amino (NHD test). These fractions were purified further to obtain the QA derivatives.

2.2.5. pH–solubility determination of Quil A derivatives and estimation of pK_a

Five mg of Quil A derivative was dispersed in 1 mL of 5% hydrochloric acid in a 5 mL glass vial and stirred for 10 min at room temperature to allow for equilibration and to obtain a saturated solution of Quil A derivatives at a pH of about 0.5 (as measured by a micro-pH electrode). Aqueous sodium hydroxide (5%, w/v) was then added dropwise to adjust the solution to a stable pH of 1, and stirring was maintained for 5 min. A sample (120 μL) was taken and centrifuged at 5000 rpm for 10 min (Sigma Laboratory Centrifuge 3-15, Rotor 11133). An aliquot (100 μL) of the clear supernatant was diluted with 5% hydrochloric acid to a volume of 1 mL and the absorbance at 210 nm measured. This process was repeated with pH being adjusted in increments of 0.5 or 1 pH units from pH 1 to 11 using aqueous sodium hydroxide solution (0.05%, w/v). The absorbance of the supernatants of the centrifuged samples were converted to concentration of Quil A derivatives (mg/mL) using established absorbance vs. concentration calibration curves constructed using triplicate standard stock solutions of each Quil A derivative. The pH–solubility profile was used to estimate the solubility of the unionised form of the derivative (S_o) and then a plot

of $\log S_o/(S - S_o)$ vs. pH was constructed to estimate the pK_a of each derivative. The chemical stability of each of the derivatives under the conditions and times used for solubility determination had previously been confirmed by HPLC.

2.2.6. Determination of the critical micelle concentration (CMC) of Quil A and Quil A derivatives

The CMC of Quil A and Quil A derivatives was estimated by the method of Zhang et al. (1996) and Nakahara et al. (2005). Briefly, a solution of 1,6-diphenyl-1,3,5-hexatriene (10 mM in tetrahydrofuran) as the fluorescence probe was mixed with solutions of Quil A or Quil A derivatives of various concentrations. The final concentration of DPH in each aqueous solution was 5 μM. These samples were equilibrated for 30 min in the dark at room temperature (~24 °C) (Nakahara et al., 2005). The fluorescence of the resulting solutions was measured using a Shimadzu RF 1501 spectrofluorophotometer with emission and excitation wavelength of 428 and 355 nm respectively (Zhang et al., 1996). The CMC of Quil A and Quil A derivatives were estimated from discontinuities observed in the fluorescence–concentration profile as the environment of the fluorescent probe changed from being aqueous to that of the interior of the micelles formed (Zhang et al., 1996; Nakahara et al., 2005).

2.2.7. Investigation of the colloidal structures formed by Quil A and Quil A derivatives in the presence of phospholipid and cholesterol

A total mass of 10 mg of phosphatidylcholine (PC), fractionated Quil A or Quil A derivative and cholesterol was used to prepare colloidal structures by the method of hydration of freeze-dried monophase (Liang et al., 2008). The phospholipid and cholesterol were first dissolved in 4 mL of *tertiary*-butanol which was added to 4 mL of 0.005 M phosphate buffer (PB) having a pH of 3 or 7.4 containing 100 mg of sucrose. These solutions were added to 4 mg of fractionated Quil A [a mixture of QS17, QS18 and QS21 (Pham et al., 2006)] or Quil A derivative (QA-EDA, QA-SMD or QA-SM). The samples were sonicated for 10 min, and stirred overnight under a nitrogen atmosphere prior to freeze-drying. For samples prepared at pH 7.4, these were additionally stirred at 50 °C for 30 min and briefly sonicated to promote the formation of the monophase solution just prior to freeze-drying. The resulting freeze-dried solid matrices were hydrated with 2 mL of distilled water and sonicated to facilitate dispersion. The resulting dispersions were characterised in terms of size and zeta potential by photon correlation spectroscopy and electrophoretic mobility respectively using a Zetasizer 3000 (Malvern, UK) following dilution in 0.01 M PB pH 3.0 and pH 7.4, and 0.01 M sodium acetate buffer (SAB) pH 5. The morphology of the structures formed was visualised by transmission electron microscopy (TEM) using a JEOL-JEM 1010 electron microscope (Japanese Electron Optics Limited, Japan). For TEM, samples were placed on carbon coated, discharged copper grids and negatively stained using an aqueous solution of ammonium molybdate 1% (w/v), pH 6.5. Samples were viewed at an acceleration voltage of 80 kV and at a magnification varying from 80,000× to 200,000×. Characterisation studies were performed in duplicate for all derivatives and also fractionated Quil A.

3. Results and discussion

3.1. Purification of Quil A derivatives by semi-preparative HPLC

Crude reaction products were purified using a semi-preparative C18 column and Quil A was also analysed using this column to compare the retention time (RT) of the compounds. As can be seen from Fig. 2, the group of peaks with a RT of less than 30 min observed in the chromatograms of Quil A derivatives (Fig. 2B–D) are absent in the chromatogram of Quil A (Fig. 2A). These compounds were collected as a single fraction (Fig. 2, F1.1–F1.3), lyophilised and the resulting powders tested negative using the qualitative assays LB, ML and NHD for the presence of triterpenoids, carbohydrates and amino groups respectively. Therefore, there is no evidence for the presence of Quil A or Quil A derivatives in fraction 1 eluting over the first 30 min. Reactants such as EDC, NHS and DMF may be responsible for these peaks because these reactants eluted at a RT of less than 10 min when analysed using the same HPLC conditions (data not shown). EDA, SMD and SM also had retention times of less than 30 min under the conditions used but there was no evidence of unreacted amines in fractions 1.1–1.3 as determined by the NHD test.

A second fraction was collected over the time period 75–85 min (Fig. 2, F2.1–2.3). As can be seen in Fig. 2, the group of peaks with a RT of 75–85 min appear in the chromatograms of both Quil A and Quil A derivatives. It is therefore postulated that the saponins in these fractions did not react with EDA, SMD and SM. The LB, ML and NHD tests of fraction 2, in all cases, were only mildly positive for the presence of triterpenoid, carbohydrate and amine functional groups. The MALDI-TOF mass spectra of the fractions (F2.1–F2.3) showed two prominent $[M+H]^+$ ions with m/z values of 1247 and 1379 which is well below the molecular weight of the prominent saponins present in Quil A and which contain glucuronic acid residues (van Setten et al., 1995).

A third HPLC fraction was collected over the time period 85–115 min. As illustrated in the chromatogram (Fig. 2A), this fraction contains QS17, QS18, and QS21 which are the three most prominent hydrophobic, glucuronic acid containing saponins in Quil A (Pham et al., 2006). After reaction with EDA, SMD and SM, the retention times of these three peaks were slightly reduced and the profile of the chromatogram over this time period was altered (Fig. 2B–D). This indicates that compounds eluting in this period have been modified, and the formation of compounds that are more hydrophilic. Fraction 3 for all derivatives tested strongly positive for the presence of triterpenoids (LB test), carbohydrate (ML test) and amino groups (NHD test). When examined by MALDI-TOF, the mass spectra of fraction 3 contained molecular ions corresponding to that expected for derivatives of QS17, QS18 and QS21 when conjugated to EDA, SMD and SM (Table 1).

Thus, from the data gathered, there was strong evidence to suggest that fraction 3 of the semi-preparative HPLC contained the Quil A derivatives but contamination with unreacted Quil A saponins was also possible, because the RT of the Quil A derivatives and Quil A are such that they would co-elute in fraction 3 (Fig. 2). Fraction 3 was therefore subjected to cation-exchange chromatography to separate the cationic Quil A derivatives from any unreacted Quil A.

3.2. Further purification of Quil A derivatives by ion-exchange chromatography

In a control experiment, when Quil A was loaded onto the column and eluted with 0.02 M AA buffer (pH 7.8) followed by 0.2 M AA

Table 1
Molecular weight of Quil A derivatives.

Samples	Molecular ions $[M+H]^+$ (m/z)	Quil A derivatives
QA-EDA	2340	QS17-EDA
	2194	QS18-EDA
	2032	QS21-EDA
QA-SMD	2425	QS17-SMD
	2279	QS18-SMD
	2117	QS21-SMD
QA-SM	2482	QS17-SM
	2336	QS18-SM
	2174	QS21-SM

buffer (pH 3.8), the 0.02 M fraction was strongly positive for the LB and ML tests, and no compound eluted in the 0.2 M AA fraction. This indicated that all saponins in Quil A were eluted with the 0.02 M, pH 7.8 AA buffer. When ~50 mg of fraction 3 (collected from the semi-preparative HPLC of the crude reaction mixture over the time period 85–115 min) was loaded onto the column, the 0.02 M fraction yielded ~20 mg of powder after lyophilisation, and this powder was positive for the LB, ML tests but negative with the NHD test. The 0.2 M AA fraction gave approximately 30 mg of powder following lyophilisation and this was strongly positive with LB, ML and NHD tests. These tests were conducted following repeated lyophilisation of the samples which was previously found to be necessary to remove any traces of ammonia (data not shown). Molecular ions $[M+H]^+$ corresponding to the derivatives of QS17, QS18 and QS21 were found in the mass spectra of this fraction (Fig. 3 and Table 1). Thus it was demonstrated that the derivatives, which would be expected to contain cationic amino groups, were bound to the resin in the presence of 0.02 M AA buffer (pH 7.8), and were eluted by increasing the ionic strength and decreasing the pH of the buffer (0.2 M AA buffer, pH 3.8).

It is interesting to note that clustered around most $[M+H]^+$ ions, there are peaks with a mass of 14 mass units greater. For example, in Fig. 3C, aside from the QS21-SM $[M+H]^+$ peak at m/z 2174, a peak with m/z of 2188 is apparent. Likewise, peaks with m/z of 2350 and 2496 appeared alongside the QS18-SM and QS17-SM $[M+H]^+$ peaks at m/z 2336 and 2482 respectively. Ions with the m/z difference of 14 were also observed in the MALDI-TOF mass spectra of Quil A (data not shown). A mass difference of 14 is characteristic of the presence

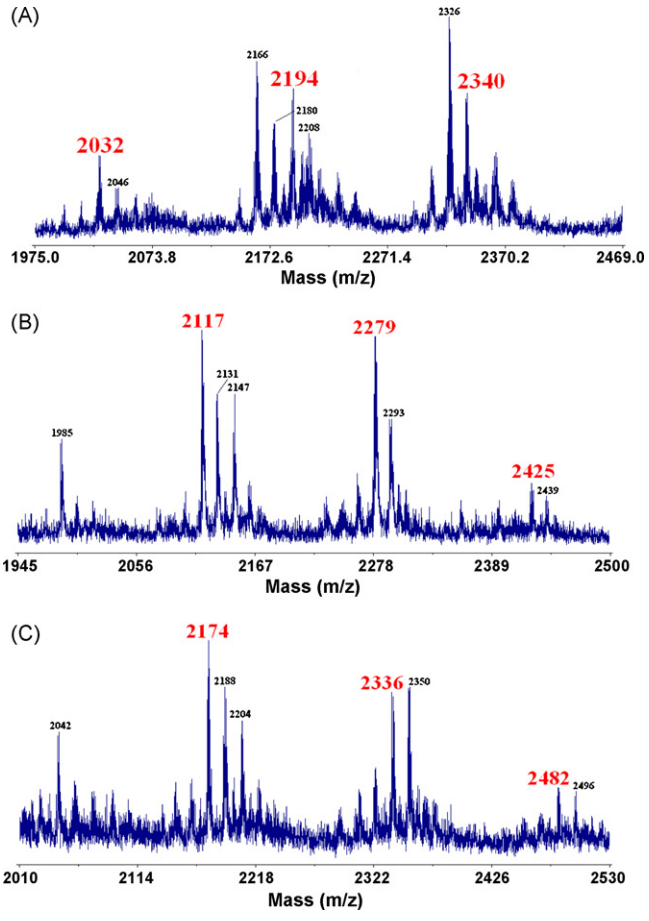

Fig. 3. Mass spectrum of isolated fraction of QA-EDA (A), QA-SMD (B), and QA-SM (C) derivatives after purification by cation-exchange chromatography. See Table 1 for assignments.

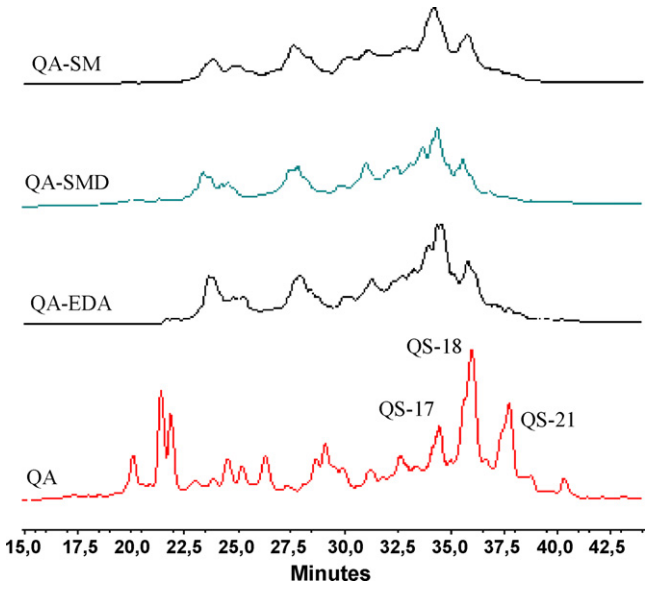

Fig. 4. C4 analytical HPLC profile of crude Quil A and cationic QA-EDA, QA-SMD and QA-SM derivatives following purification by cation-exchange chromatography.

of rhamnose (MW = 164.16 Da) at R^4 instead of the more common xylose (MW = 150.14 Da) (Fig. 1). Therefore, the peaks 14 mass units higher most likely represent molecular ions $[M+H]^+$ for rhamnose-containing Quil A derivatives. This variation in the sugar moieties at position R^4 was previously reported by Bankefors (2006). Ions with m/z 132 less than the xylose-containing $[M+H]^+$ ions, and m/z 146 less than the rhamnose-containing $[M+H]^+$ ions, were also observed. For example, in Fig. 3C, the QS21-SM $[M+H]^+$ peaks at m/z 2174 (xylose-derivative) and 2188 (rhamnose-derivative) are associated with a peak at m/z 2042. This may correspond to fragmentation of the $[M+H]^+$ ions via loss of the xylose or rhamnose unit. Similar fragmentation of saponins was observed by Fang et al. (1998). Alternatively, these peaks may represent the molecular ions $[M+H]^+$ for Quil A derivatives that lack xylose or rhamnose at R^4 (i.e. R^4 = H). This feature was also seen in the MALDI-TOF mass spectra of Quil A (data not shown).

Following cation-exchange chromatography, the purified Quil A derivatives, were characterised by analytical reverse-phase HPLC. The chromatograms for each derivative and unfractionated Quil A are shown in Fig. 4. The chromatograms clearly show a difference in elution profiles between Quil A and Quil A derivatives further confirming derivatisation of Quil A.

Thus, it was concluded that Quil A was successfully derivatised to form the cationic derivatives: QA-EDA, QA-SMD and QA-SM; each containing QS17, QS18 and QS21 as listed in Table 1. In terms of yield for the various conjugates, around 40 mg of QA-EDA, and 30 mg of QA-SMD and QA-SM was obtained from 150 mg of Quil A (unfractionated) following semi-preparative and cation-exchange chromatography and repeated lyophilisations.

3.3. *pH–solubility determination of Quil A derivatives and estimation of pK_a*

To determine the suitability of the Quil A derivatives for formulation, the aqueous solubility of each derivative was measured. The pH–solubility profiles for the Quil A derivatives are shown in Fig. 5. As would be expected for the cationic derivatives, the solubility decreased with increasing pH. The rank order of solubility of the derivatives was also as expected with the solubility increasing with increasing the number of amino groups present on the derivatives (i.e. QA-SM > QA-SMD > QA-EDA). The intrinsic solubility of the

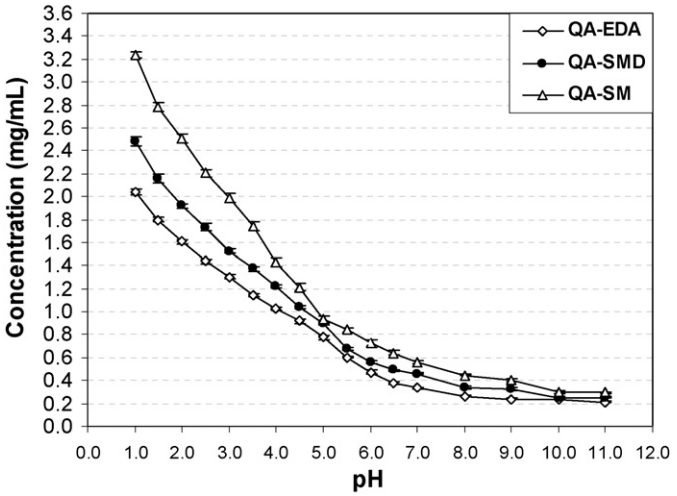

Fig. 5. pH–solubility profile for QA-EDA, QA-SMD and QA-SM derivatives. Values represent the mean ± SD of three experiments. The intrinsic solubility of the QA-EDA, QA-SMD and QA-SM was estimated to be 0.21, 0.25 and 0.29 mg/mL respectively. The pK_a values of the QA-EDA, QA-SMD and QA-SM were estimated to be 6.2, 6.4 and 6.8 respectively.

derivatives was estimated by extrapolation of the data obtained at pH greater than 10 and was 0.29, 0.25 and 0.21 mg/mL for QA-SM, QA-SMD and QA-EDA respectively.

The estimated intrinsic solubilities of the derivatives were then used to construct a plot of $\log S_o/(S - S_o)$ vs. pH. Linear relationships ($r^2 \geq 0.98$) for the plots were observed, particularly over the pH range of 4–8 which was used for the estimation of the pK_a from the intercept of the *x*-axis (when pH = pK_a). It was estimated that the pK_a of the derivatives was 6.2, 6.4 and 6.8 for the QA-EDA, QA-SMD and QA-SM respectively. The estimated pK_a values are consistent with the nature of the pH–solubility profiles where the solubility is seen to increase sharply below pH of 6.

3.4. Critical micelle concentration determination of Quil A derivatives

The fluorescence intensity vs. concentration profiles of Quil A and the Quil A derivatives are shown in Fig. 6 and were used for the estimation of the CMC of the compounds according to the method of Zhang et al. (1996) and Nakahara et al. (2005). This is based on the fluorescence of the lipophilic probe DPH changing according to its surrounding environment and hence a discontinuity in the fluorescence intensity vs. concentration profile is observed upon passing through the CMC. The CMC of Quil A was estimated at both pH 3.0 (where ionisation is suppressed) and also at pH 8 (where Quil A is ionised). At pH 3.0, a discontinuity was observed at a concentration of around 0.4–0.5 mg/mL for Quil A and this was observed to shift to a concentration of around 0.8 mg/mL at pH 8. The value measured at pH 8 is in agreement with literature values previously reported for Quil A (Mitra and Dungan, 1997). Discontinuities were also observed in the fluorescence intensity vs. concentration profiles of all cationic derivatives when measured at pH 3.0. These discontinuities were observed in the concentration range 0.5–1.0 mg/mL and indicate that the derivatives of Quil A, when ionised, form micelles in this concentration range. At pH 8, the solubility of the Quil A derivatives is low, being less than 0.5 mg/mL. No discontinuities were observed in the fluorescence intensity vs. concentration when measured below a concentration of 0.5 mg/mL and the lack of any discontinuity in the profile probably indicates that the CMCs of the derivatives at pH 8 are above the solubility limit of the compounds.

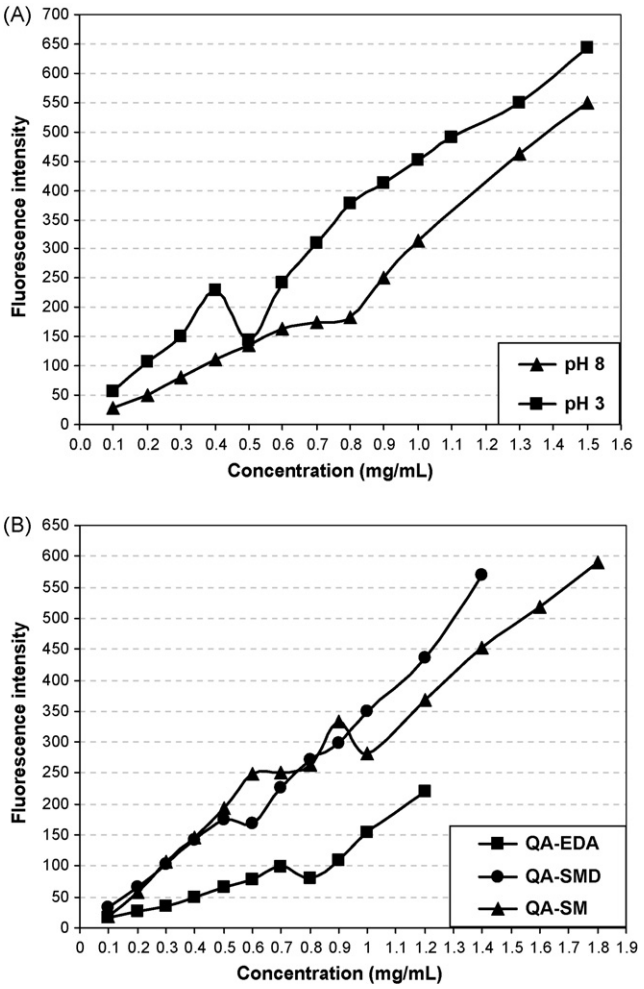

Fig. 6. Fluorescence intensity vs. concentration profile of Quil A (unfractionated) (A) and Quil A derivatives (B) in the presence of 10 mM DPH. Values represent means, $n = 2$. All experimental values were within 15% of the mean value. The CMC values of Quil A were estimated to be ~0.4–0.5 mg/mL at pH 3.0, and ~0.8 mg/mL at pH 8.0. The CMC values of the Quil A derivatives were estimated to be between 0.5 and 1.0 mg/mL.

3.5. Investigation of the colloidal structures formed by Quil A derivatives in the presence of phospholipid and cholesterol, at pH 3.0 and pH 7.4, using a mass ratio which typically produces ISCOMs

The colloidal structures formed by Quil A derivatives in the presence of phosphatidylcholine and cholesterol were investigated by the method of hydration of freeze-dried monophase as described by Liang et al. (2008). To prepare by this method, PC, Quil A and Chol in a ratio of 4:4:2 are dissolved along with sucrose in an aqueous solution of *tertiary*-butanol (50%, v/v). Typically 20 mg of total lipid and 200 mg of sucrose are used and are dissolved in a volume of 4 mL of co-solvent. The monophase is freeze-dried to yield a sugar matrix in which the ISCOM components are dispersed. Upon hydration of the sucrose matrix, homogeneous ISCOM dispersions are formed. It was observed in preliminary investigations that the Quil A derivatives had limited solubility in the aqueous *tertiary*-butanol (50%, v/v) co-solvent but could be dissolved at a concentration of 4 mg in 8 mL. As such, all studies were carried out using 8 mL of solvent, a total lipid mass of 10 mg with a ratio of PC:Quil:Chol of 4:4:2 and 100 mg of sugar as the matrix forming excipient. When fractionated Quil A (QS17, QS18 and QS21) was used, dissolved in a co-solvent of *tertiary*-butanol and PB pH 7.4 and processed as described, ISCOM

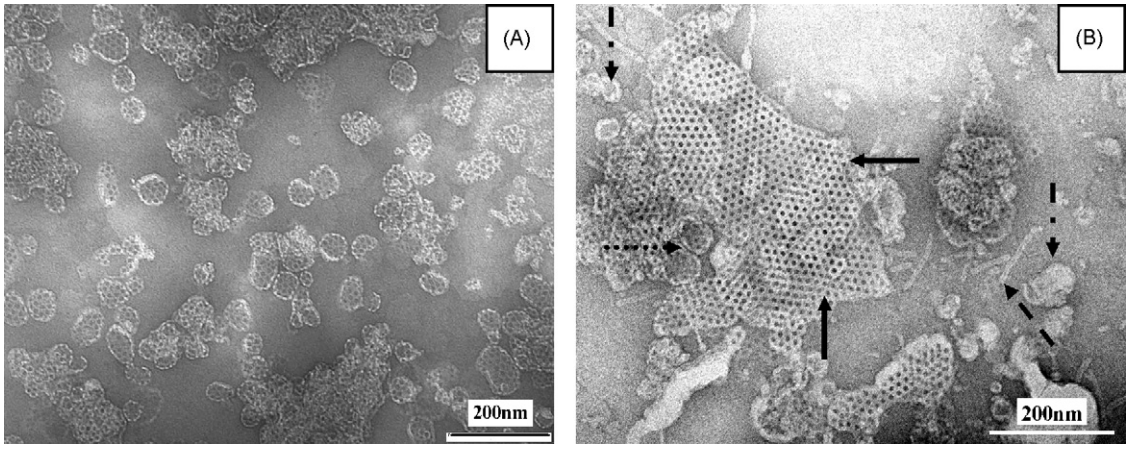

Fig. 7. Electron micrographs showing various colloidal structures formed by 4:4:2 (Quil A or QA-SM/PC/Chol) at pH 7.4. (A) ISCOM structures formulated from fractionated Quil A. (B) Lamellae structures (solid arrows) together with lipid particles (dotted–dashed arrows), ISCOM-like structures (dotted arrow) and rod-like structures (dashed arrow) formed from Quil A derivatives.

structures were found as the predominant colloidal structures in the dispersion (Fig. 7A). The morphology of the ISCOMs formed, as observed by TEM, is consistent with those reported for ISCOM matrices prepared from unfractionated Quil A and as prepared by this method (Liang et al., 2008). The zeta potential of the ISCOMs formed from fractionated Quil A, as measured in 0.01 M pH 7.4 phosphate buffer, was −23.6 mV which is in agreement with that previously reported using a similar buffer concentration (Kersten et al., 1991).

When the cationic Quil A derivatives were used, dissolved in a co-solvent of *tertiary*-butanol and PB pH 7.4 and processed as described, lamellar sheets were observed as the most prevalent structure (Fig. 7B). These lamellar sheets had a similar appearance to those observed by Demana et al. (2004) in the pseudo-ternary phase diagram of Quil A, PC and Chol but occurred at higher ratios of Chol in their study. These lamellar structures are believed to form because of micelle fusion caused by the lipophilicity of Chol (Demana et al., 2004). The lamellar sheets observed in samples prepared from the Quil A derivatives were noted to co-exist with other lipidic particles and a few ISCOM-like structures (Fig. 7B). Lipidic particles similar to those observed in systems prepared from the cationic Quil A derivatives, have previously been reported by Lendemans et al. (2005b) when cationic ISCOMs were prepared from the cationic cholesterol derivative, DC-cholesterol (DC-Chol), using a similar ratio of Quil A, DC-Chol and PC (4:3:3) (Lendemans et al., 2005b). For the SMD and SM derivatives, rod-like structures were also observed in the samples (similar to those shown in Fig. 7B). These structures were not found in samples formulated with the EDA derivatives. The size of the particles in dispersions prepared from the Quil A derivatives at pH 7.4, when the derivatives are largely unionised, was between 220 and 250 nm and the polydispersity index confirmed the heterogeneous nature of the dispersion, as was also evident in the TEM micrographs (Table 2). The zeta potential of these colloids measured at pH 7.4 was near neutral for all derivatives but became increasingly positive at lower pH values and below the estimated pK_a of the derivatives (Table 2). The zeta potential of these colloids also became increasingly positive with increases in the number of amine functional groups present on the derivative (Table 2).

Table 2
Mean size and zeta potential of colloidal structures prepared in PB pH 7.4.

Samples	Size (nm, $n=2$)	Polydispersity ($n=2$)	Zeta potential (mV, $n=2$)		
			PB pH 7.4	SAB pH 5	PB pH 3
QA-EDA	219	0.29	−2.1	+4.8	+8.5
QA-SMD	245	0.41	−0.5	+11.1	+10.3
QA-SM	255	0.45	0.9	+16.7	+16.8

The types of colloidal structures formed by the Quil A derivatives in the presence of PC and Chol was also investigated at pH 3.0, such that the amino groups of the derivatives were ionised. The structures formed by fractionated Quil A at this pH were also investigated and the micrographs are shown in Fig. 8A and B. It can be seen that at pH 3.0, homogeneous ISCOM dispersions are not formed. Rather, ring-like micelles are the predominant colloidal structures which exist either alone or aggregated to form lamellae or large ISCOM-like structures (Fig. 8A and B). The existence of ring-like micelles together with lamellae is consistent with the hypothesis of Kersten and Crommelin (1995) who proposed that lamellae formed as a result of the association of ring-like micelles resulting from increased lipophilicity imparted by Chol and observed at higher Chol ratios. It can be speculated that the ring-like micelles, which co-exist with lamellae at pH 3.0, occurred as a result of Quil A losing its negative charge and thereby its increased lipophilicity results in micelle aggregation. The zeta potential of the dispersion formed from Quil A at pH 3.0 was +2 mV. It would thus appear that ionisation of Quil A is critical for the formation and stabilisation of ISCOMs. Some worm-like micelles were also observed in the micrographs (Fig. 8A).

When Quil A derivatives were used at pH 3.0, bilayer structures were observed in the TEM micrographs which are typical of liposomes (Fig. 8C). A few lamellar structures were also observed in the samples (similar to those shown in Fig. 7B). It is unusual for liposomes to exist at such a high ratio of Quil A as reported in both the phase diagrams of Quil A/PC/Chol or Quil A/PC/DC-Chol (Demana et al., 2004; Lendemans et al., 2005b). In samples prepared from QA-SMD and QA-SM, some ring-like micelles (similar to those shown in Fig. 8A) and helical structures (Fig. 8D) respectively were also observed. It has previously been reported that these micellar structures could be precursors to ISCOM-like structures (Demana et al., 2004). Hence, it was postulated that Quil A derivatives may be able to form discrete colloidal or micellar structures but may only do so at higher ratios of the Quil A derivatives. The size and polydispersity of the dispersions formed by Quil A derivatives in the presence of PC and Chol are reported in Table 3. The sizes of the dispersions were larger than those measured at pH 7.4, as might be expected for dispersions containing predominantly liposomes. The zeta potential of the colloids was positive in all cases with the magnitude of the positive zeta potential increasing with increasing the number of amine functional groups present on the derivative (Table 3).

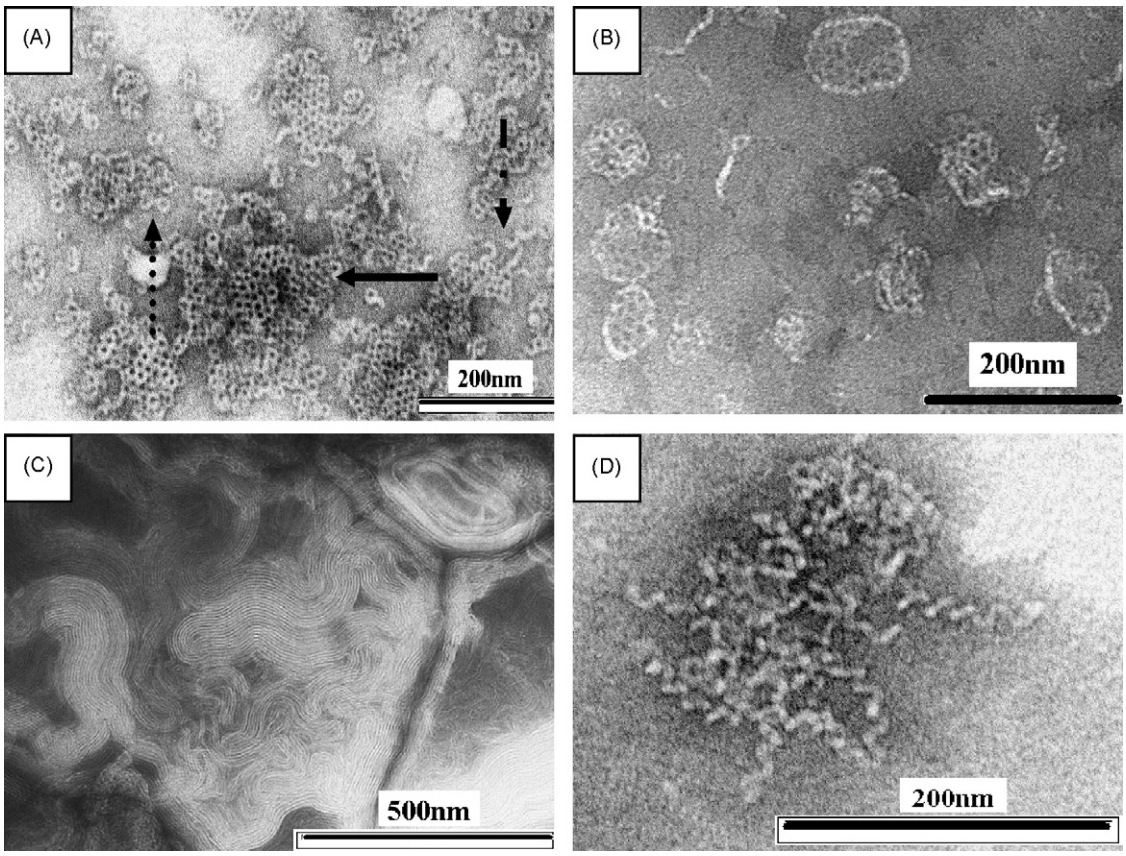

Fig. 8. Electron micrographs showing various colloidal structures formed by 4:4:2 (Quil A or QA-SM/PC/Chol) at pH 3. (A) Lamellae structures (solid arrow) existing with ring-like (dotted arrow) and worm-like micelles (dashed arrow) formed from fractionated Quil A. (B) ISCOM-like structures formed from fractionated Quil A. (C) Liposome and (D) helical structures formed from QA-SM.

3.6. Investigation of the colloidal structures formed by QA-SM in the presence of phospholipid and cholesterol in PB pH 7.4, using various mass ratios

As described in the above section, a few ISCOM-like structures were evident in the system containing 40% (w/w) Quil A derivatives, 40% (w/w) PC and 20% (w/w) Chol prepared at pH 7.4. However, no ISCOM-like structures were found in the corresponding systems prepared at pH 3.0. This suggests that PB pH 7.4 is a more suitable medium for Quil A derivatives to form ISCOM structures but the ratio of 4:4:2 (QA derivative/PC/Chol) may not be optimal. In the following paragraphs, we describe a preliminary investigation of the phase diagram with QA-SM and PB pH 7.4, to examine what colloidal structures exist at other ratios. QA-SM was chosen because it is the most soluble derivative as revealed in Fig. 5.

Seventeen formulations were prepared using various mass ratios of QA-SM, PC, and Chol as reported in Table 4. The colloidal systems formed were examined within 1 day (3 h post-preparation), and after 3 and 7 days of stirring. Despite some differences being apparent between samples observed at day 1 and day 3, no differences were apparent between samples observed at day 3 and day 7. Therefore, equilibrium was considered to have been reached within 3 days. Hence, the types of colloidal structures formed in the 17 formulations were investigated by TEM and polarized light microscopy (PLM) using samples following 3 days equilibration.

Table 3
Mean size and zeta potential of colloidal structures prepared in PB pH 3.0.

Samples	Size (nm, $n = 2$)	Polydispersity ($n = 2$)	Zeta potential (mV, $n = 2$)
QA-EDA	284.7	0.59	+6.1
QA-SMD	418.5	0.18	+10.3
QA-SM	474.1	0.17	+11.2

Binary systems containing QA-SM and PC (Table 4, samples 1–4) produced mainly bilayer structures together with other lipidic particles (Fig. 9A). The size of the lipidic particles decreased upon increasing the concentration of the Quil A derivative and the zeta potential of the colloids was observed to increase. Very few lamellae or ISCOM-like structures were observed in these binary systems.

Addition of Chol (10%) (Table 4, samples 5–8) resulted in formation of lamellae sheets which were predominant structures at 40–50% QA-SM (Fig. 9B). At the QA-SM concentration of 40% (w/w), the hexagonal lamellae structures existed together with liposomes. Indeed, these lamellae structures appeared to be associated with, or at least superimposed on the liposomes (Fig. 9B). A small number of cage-like structures were also present in this system; however, these structures were irregular in shape, varied in size and contained less subunits then the classical ISCOMs. The subunits are the hydrophilic pores accessible to ammonium molybdate reagent and thus appearing as dark pores on TEM micrographs (Fig. 9C). At higher weight ratios of QA-SM (50–70%, w/w), irregular cage-like structures of varying size were observed (similar to those shown in Fig. 9C) in addition to ring-like and worm-like micelles (similar to those shown in Fig. 8A). The conversion of lamellae sheets to colloidal cage-like structures and micelles was associated with a decrease in mean particle size and an increase in the zeta potential of the particles (Table 4). Lipid particles (similar to those shown in Fig. 7B) were also found in systems containing 10% (w/w) Chol, and these particles became smaller in size towards the lower weight ratios of PC.

Table 4
Mass ratios of QA-SM, PC and Chol of the various formulations used for hydration of freeze-dried monophase system method (PB pH 7.4) and the resulting particle size, polydispersity and zeta potential of the samples. Values represent means ± SD ($n = 3$).

Sample	QA-SM (%)	PC (%)	Chol (%)	Mean size nm ($n = 3$)	Polydispersity ($n = 3$)	Zeta (mV) SAB pH 5 ($n = 3$)
1	40	60	0	276 ± 5	0.38 ± 0.01	17.5 ± 2.0
2	50	50	0	232 ± 9	0.34 ± 0.04	18.1 ± 1.5
3	60	40	0	205 ± 6	0.34 ± 0.03	22.3 ± 0.6
4	70	30	0	186 ± 7	0.29 ± 0.03	24.0 ± 1.2
5	40	50	10	246 ± 7	0.37 ± 0.02	15.3 ± 1.0
6	50	40	10	221 ± 9	0.45 ± 0.03	18.2 ± 0.9
7	60	30	10	190 ± 5	0.30 ± 0.05	23.4 ± 1.2
8	70	20	10	150 ± 5	0.23 ± 0.02	22.6 ± 1.4
9	40	40	20	255 ± 9	0.45 ± 0.04	16.7 ± 2.2
10	50	30	20	198 ± 6	0.41 ± 0.02	18.5 ± 0.9
11[a]	60	20	20	156 ± 3	0.30 ± 0.03	22.9 ± 1.4
12	70	10	20	143 ± 4	0.31 ± 0.03	21.8 ± 1.8
13	40	30	30	236 ± 6	0.42 ± 0.04	13.9 ± 0.9
14	50	20	30	349 ± 8	0.55 ± 0.06	16.0 ± 1.5
15	60	10	30	306 ± 7	0.38 ± 0.02	17.5 ± 0.8
16	40	20	40	317 ± 10	0.67 ± 0.04	10.8 ± 0.7
17	50	10	40	285 ± 8	0.63 ± 0.04	12.5 ± 1.1

[a] This sample formed a relatively high number of cationic ISCOM-like structures.

Increasing the weight fraction of Chol to 20% (Table 4, samples 9–12) resulted in formation of ISCOM-like structures in all systems. However, they were not the predominant colloidal structures. At the lowest ratio of QA-SM (40%, w/w) as described in Section 3.5, a few ISCOM-like structures were observed. However, when the QA-SM concentration was increased from 40 to 50% (w/w), more ISCOM-like structures were seen together with small size lamellae and lipid particles. The appearance of more ISCOM-like structures may due to the division of rigid lamellae structures into small fragments. When the concentration of QA-SM was increased to 60% (w/w), ISCOM-like structures were found in relatively high numbers (Fig. 9D), although they were still not as well defined and homogeneous as the conventional ISCOMs (which are open cage-like structures of around 40 nm in diameter) (Ozel et al., 1989). Some small size lamellae sheets, lipidic colloidal particles (similar to those shown in Fig. 7B) and ring-like micelles (similar to those shown in Fig. 8A) also co-existed with ISCOMs in the system using a ratio of 6:2:2 (QA-SM, PC and Chol). An increase in QA-SM concentration (70%, w/w) did not lead to a further prevalence of ISCOM-like structures but instead resulted in a predominance of worm-like

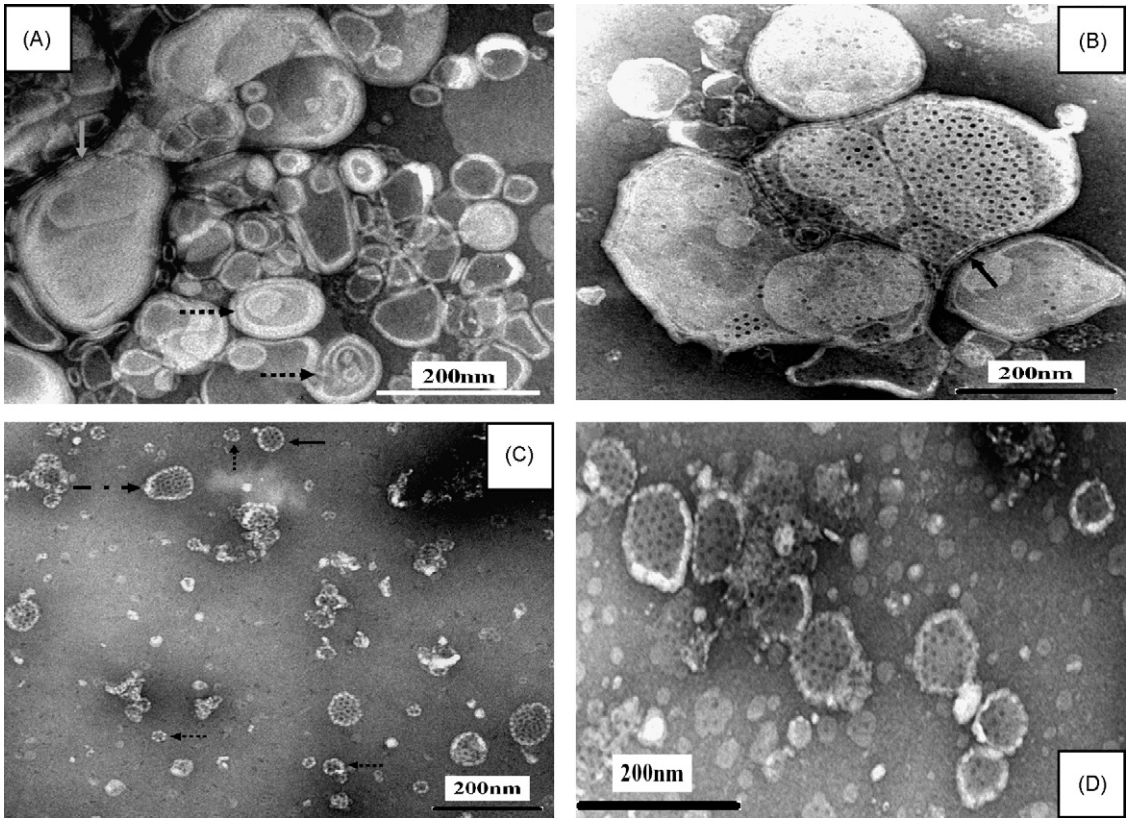

Fig. 9. Electron micrographs depicting various colloidal structures: (A) liposomes (solid arrow) together with layer lipidic particles (dotted arrows) formed by 4:6:0 (QA-SM/PC/Chol); (B) lamellae structures superimposed on the liposomes formed by 4:5:1 (QA-SM/PC/Chol); (C) ISCOM structures (solid arrow), ISCOM-like structures which are less subunit than ISCOM structures (dotted arrows) and irregularly shaped ISCOM-like structures (dotted–dashed arrow) formed by 5:4:1 (QA-SM/PC/Chol); (D) larger open cage-like and ISCOM-like structures formed by 6:2:2 (QA-SM/PC/Chol).

structures and ring-like micelles (similar to those shown in Fig. 8A) which sometimes aggregated into lamellae structures. Only a few irregularly shaped ISCOM-like structures were observed in the system containing highest concentration of QA-SM. The increase in ISCOM-like structures when QA-SM was increased from 40 to 60% (w/w), and the appearance of ring-like and worm-like micelles at 70% of QA-SM, was associated with a decrease in mean particle size and an increase in the zeta potential of the particles (Table 4). Specifically the system prepared using QA-SM, PC and Chol at ratio of 6:2:2, which was observed by TEM to produce relatively high number of ISCOM-like structures (Fig. 9D), had a size of approximately 156 ± 3 nm and a fairly low polydispersity index of around 0.3 ± 0.03 (Table 4, sample 11). According to Kersten et al. (1991), homogeneous 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. The slightly large size of system 6:2:2 (QA-SM/PC/Chol) can be explained by the heterogeneous nature of dispersion as depicted by TEM characterisation.

Further increasing cholesterol to 30% (Table 4, samples 13–15) resulted in formation of lamellae (similar to those shown in Fig. 7B). Upon increasing the concentration of QA-SM in this system, the number and size of lamellae structures were noted to increase. However, some fragments of lamellae were also found and some of them were of the same size as classical ISCOMs (similar to those shown in Fig. 9C). At high QA-SM concentration (60%, w/w), these lamellae structures were observed together with liposomes (similar to those shown in Fig. 7B) and worm-like micelle structures (similar to those shown in Fig. 8A). Lipidic particles (similar to those shown in Fig. 7B) were still found; especially in the system containing 20–30% PC (w/w).

In systems containing 40% Chol (Table 4, samples 16–17), cholesterol crystals as observed by PLM were observed in all samples indicating that Chol was in excess and could not be completely solubilised by the polar lipids present in the system. Systems containing greater than 40% cholesterol were therefore not investigated.

For colloidal structures using a higher Chol ratio ($\geq$30%, w/w), the mean particle size typically was around 300 nm and the polydispersity index was 0.38–0.67 (Table 4, samples 13–17). Generally, mean particle size was largest when big lamellar sheets or lipidic particles (which were prevalent at high weight ratios of PC) were present in the systems and decreased as these structures were replaced by micellar structures formed upon increasing weight ratio of QA-SM in the system. The polydispersity was dramatically increased (up to 0.6) when excess Chol was used (40%, w/w) due to the presence of Chol crystals.

4. Conclusion

Cationic derivatives of Quil A were successfully synthesised and purified by semi-preparative reverse-phase HPLC and ion-exchange chromatography. The molecular masses of QS17, QS18 and QS21 conjugated with EDA, SMD and SM were observed in the mass spectra of the purified products which were positive in LB, ML and NHD tests, thus confirming successful synthesis. Solubility measurements illustrated the cationic nature of the derivatives, which had pK_a values of around 6.5. There was some indication that the derivatives formed micelles in aqueous solution at pH 3.0. The ternary system of QA-SM/PC/Chol was investigated via the method of hydration of freeze-dried monophase systems, in PB at pH 7.4. The ratio of 4:4:2, (QA-SM/PC/Chol) showed predominantly lamellar structures which were observed to co-exist with other lipidic particles and a few ISCOM-like structures. Increasing concentration of saponin derivative in the ternary system of QA-SM/PC/Chol did not lead to the formation of homogeneous cationic ISCOMs as hoped. However, a high number of cationic ISCOM-like structures were observed in the system containing QA-SM/PC/Chol at a ratio of 6:2:2 respectively. This indicated that higher amounts of Quil A derivative appears to be required to form ISCOM-like structures than unmodified Quil A, at least when prepared by hydration of freeze-dried monophase. Further, the ISCOM-like structures formed were less homogeneous and more irregular in shape than ISCOMs prepared from unmodified Quil A. Hence, at appropriate ratio, colloidal particles having positive zeta potential were produced and may find application in the delivery of nucleic acids or anionic proteins.

Acknowledgements

We acknowledge the Ministry of Education and Training (MOET) in Vietnam and The University of Queensland for a scholarship for HLP. The authors also thank the Centre for Microscopy and Microanalysis (CMM), The University of Queensland, particularly Robyn Webb for assistance with electron microscopy. We also appreciate the assistance of Chris Wood, School of Chemistry and Molecular Biosciences, The University of Queensland, in MALDI-TOF analysis.

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