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Micellar aggregates of saponins from *Chenopodium quinoa*: characterization by dynamic light scattering and transmission electron microscopy

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Entire seeds of *Chenopodium quinoa* Willd are a rich protein source and are also well-known for their high saponin content. Due to their amphiphily quinoa saponins are able to form intricate micellar aggregates in aqueous media. In this paper we study the aggregates formed by self-association of these compounds from two quinoa saponin fractions (FQ70 and FQ90) as well as several distinctive nanostructures obtained after their complexation with different ratios of cholesterol (CHOL) and phosphatidylcholine (PC). The FQ70 and FQ90 fractions were obtained by reversed-phase preparative chromatography. The structural features of their resulting aggregates were determined by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). Novel nanosized spherical vesicles formed by self-association with mean diameter about 100–200 nm were observed in FQ70 aqueous solutions whereas worm-like micelles an approximate width of 20 nm were detected in FQ90 aqueous solutions. Under experimental conditions similar to those reported for the preparation of Q*uillaja saponaria* ISCOM matrices, tubular and ring-like micelles arose from FQ70:CHOL:PC and FQ90:CHOL:PC formulations, respectively. However, under these conditions no cage-like ISCOM matrices were observed. The saponin composition of FQ70 and FQ90 seems to determine the nanosized structures viewed by TEM. Phytolaccagenic acid, predominant in FQ70 and FQ90 fractions, is accountable for the formation of the nanosized vesicles and tubular structures observed by TEM in the aqueous solutions of both samples. Conversely, ring-like micelles observed in FQ90:CHOL:PC complexes can be attributed to the presence of less polar saponins present in FQ90, in particular those derived from oleanolic acid.

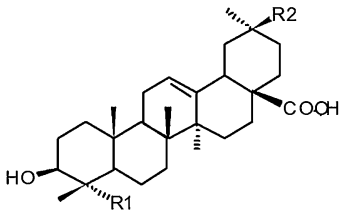
1. Introduction

Seeds of *Chenopodium quinoa* Willd (quinoa or quinua) have been harvested for centuries in several South American countries due to their valuable nutritional properties (Dini et al. 2001; Spehar and Santos 2002; Zhu et al. 2002). Besides their rich content in proteins, carbohydrates, oils, minerals and vitamins, they also comprise a fair amount of a complex mixture of triterpenic saponins that confer to the seeds a strong bitterness so they must be removed before used for food (Kuljanabhagavad et al. 2008; Madl et al. 2006; Zhu et al. 2002).

Chemically, quinoa saponins are triterpene glycosides derived from β-amirin, being phytolaccagenic, oleanolic and serjanic acids, hederagenin, 3β,23,30 trihydroxy olean-12-en-28-oic acid, 3β-hydroxy-27-oxo-olean-12-en-28-oic acid and 3β,23,30 trihydroxy olean-12-en-28-oic acid the most common aglycones (Fig. 1) found in seeds (Kuljanabhagavad et al. 2008; Kuljanabhagavad and Wink 2009; Madl et al. 2006). The glycosidic chains are predominantly composed of arabinose, glucose and galactose; along with glucuronic acid and xylose in a minor extent (Dini et al. 2001; Madl et al. 2006; Mizui et al. 1988, 1990).

Many biological and pharmacological activities, comprising antimicrobial, antifungal and antiviral activities (Stuardo and San Martín 2008; Woldemichael and Wink 2001), brine shrimps toxicity (Ma et al. 1989), cholesterol lowering effects, drug absorption enhancement and potential adjuvant activity for mucosal-administered vaccines (Estrada et al. 1998) have been reported for quinoa saponins.

Many triterpenic and steroidal saponins in aqueous solutions give place, by self association, to the formation of intricate micellar aggregates (Chapagain and Wiesman 2006), to which special attention has been paid as subunit vaccine delivery systems (Demana et al. 2004). Also, mixed micelles composed of saponin, cholesterol and phospholipids, either containing antigen (ISCOM) or not (ISCOM matrix), have been under intensive development and included in clinical trials in recent years due to their ability to act as antigen presenting-carriers with remarkable immunostimulating properties (Song et al. 2009; Sun et al. 2009). A former study on the immunoadjuvant activity and



Aglycone	R_1	R_2
Phytolaccagenic acid	CH_2OH	$COOCH_3$
Serjanic acid	CH_3	$COOCH_3$
Hederagenin	CH_2OH	CH_3
Oleanolic acid	CH_3	CH_3

Fig. 1: Aglycones of main triterpenoid saponins present in *Chenopodium quinoa*

ISCOM preparation using quinoa saponins described the presence of pore-sheets structures rather than the typical spherical ISCOM's micellar aggregates (Bomford et al. 1992). Except for that, the formation of ISCOM or other clearly defined micellar structures with quinoa saponins remained uncorroborated. In this paper, we describe the aggregates formed by self-association in aqueous solutions by two quinoa saponin fractions, as well as several distinctive nanostructures formed after their complexation with cholesterol and phospholipids at different ratios. Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) was performed.

2. Investigations, results and discussion

2.1. CMC calculation

Despite the chemical structure of quinoa saponins is well described as nonionic triterpenes (Madl et al. 2006), little is known about their behavior as amphiphilic compounds and their ability to led to the formation of micelles by self-association mechanisms.

In the assay for determination of the CMC, both FQ70 and FQ90 solutions showed a typical CMC inflexion plotting absorbance versus the saponin fractions concentrations (data not shown). The CMC values were calculated as 0.39 ± 0.01 g/L and 0.37 ± 0.01 g/L for FQ70 and FQ90, respectively. Since quinoa saponins behave as nonionic surfactants, their CMC values are expected to be lower than those of ionic surfactants. Accordingly, the measured CMC value determined for sodium dodecyl sulphate (2.4 ± 0.02 g/L) using the same method was markedly higher than the CMC values measured for FQ70 and FQ90. Even though the measured CMC values may depend on sample purity and the assay method, the data recorded for both quinoa saponin fractions closely agree with those reported for other saponins. CMC values from nonionic saponins from *Panax notoginseng* (Xiong et al. 2008) and soya saponins (Decroos et al. 2007) were very close to the ones found for quinoa saponins, namely 0.339 and 0.56 g/L, respectively. The influence of sample purity can be appraised in *Q. saponaria* bark saponins. For a high purified saponin fraction it was reported a CMC value of 0.13 g/L (Mitra and Dungan 1997), while for a lower grade fraction of the same saponins CMC values of 0.51 g/L and 0.56 g/L were reported by the dye solubilization method.

2.2. Characterization of FQ70 and FQ90 micelles by TEM and DLS

TEM-micrographs of a FQ70 solution with a concentration above its CMC (0.4% w/v) revealed a spontaneous self-association of saponins to give spherical-shaped nanosized vesicles (Fig. 2A). The DLS mean diameters of the same FQ70 solution measured in two different days were very close, namely, 156.1 ± 1.67 nm and 144.8 ± 2.99 nm. Both data are consistent with the apparent diameter observed in Fig. 2A.

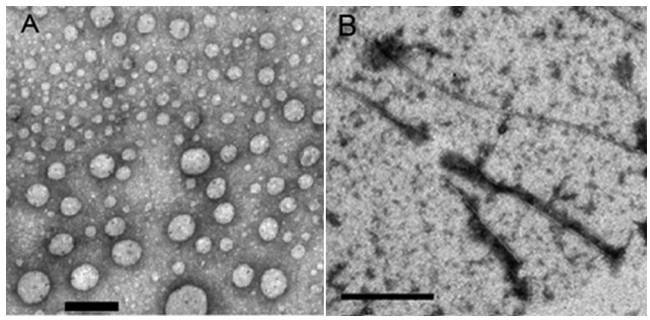


Fig. 2: (A) Transmission electron micrographs of FQ70 at 0.4% (w/v) showing nanosized vesicles; magnification of 30,000×; Bar = 200 nm.
(B) Transmission electron micrographs of FQ90 at 0.4% (w/v) showing formation of worm-like micelles; magnification of 50,000×; Bar = 500 nm

Usually, vesicle-like micelles as those observed in FQ70 were previously described for triterpenoid and steroidal saponins (Chapagain and Wiesman 2006). However, FQ90 above its CMC (0.4% w/v aqueous solution) revealed a different self-association pattern which leads to the arrangement of worm-like micelles of about 700 nm length and 20 nm width (Fig. 2B). Noteworthy, worm-like micelles formed by self-association were former noticed for *Ilex paraguariensis* saponins. Moreover, both mate and quinoa worm-micelles are very similar regarding size and shape (Peixoto et al. 2011).

2.3. Characterization of micellar structures by TEM

2.3.1. Quil A micellar structures

Typical ISCOM matrices of *Q. saponaria* were obtained through quick injection of an ethanolic solution containing CHOL and PC to a Quil A aqueous solution (Fig. 3A). Numerous ring-like micelles were also observed along with worm-like micelles and helices (Fig. 3B), very similar to those already reported for these mixtures and technique (Lendemans et al. 2005). This observation supports the fact that the experimental conditions used in this case are adequate for the preparation of the observed mixed micelles structures when using *Q. saponaria* saponins, and were used therefore to compare the relative capacity for different saponins to form such structures.

The saponin fractions FQ70 and FQ90 were investigated with respect to the formation of typical ISCOM matrices applying the same method used to prepare the micelles from Quil A.

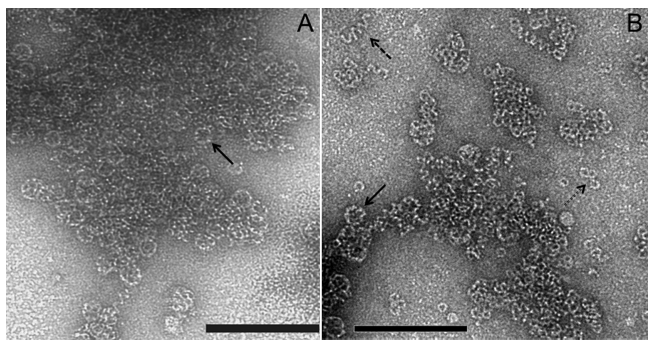


Fig. 3: TEM micrographs of samples prepared with Quil A by ethanol injection. A – ISCOM matrices. B-ring-like micelles (dotted arrow) and worm-like micelles (dashed arrow) and few ISCOM matrices (solid arrow). Bar = 200 nm

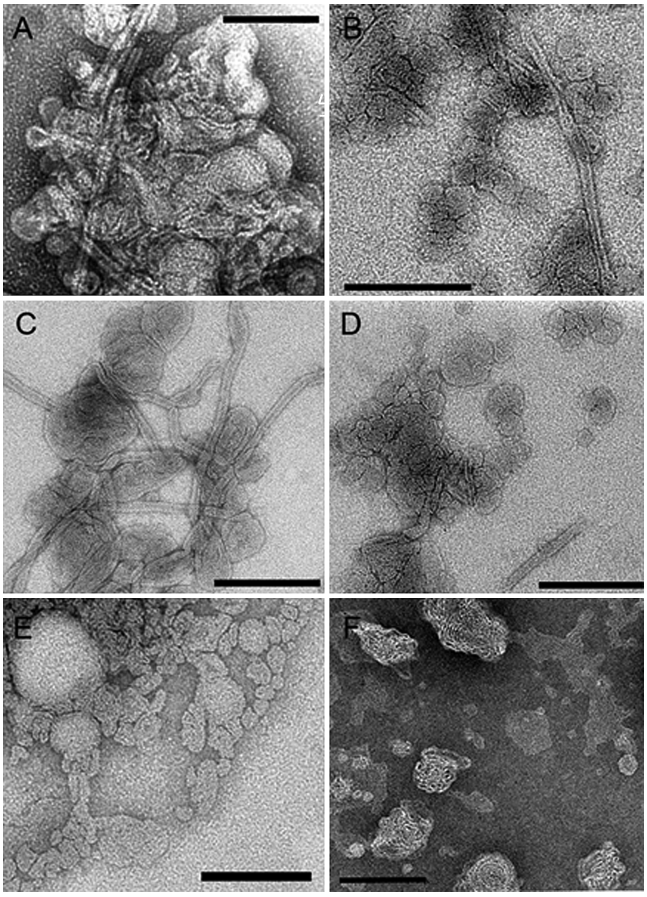


Fig. 4: TEM micrographs of selected formulations prepared with FQ70 fraction by ethanol injection. (A) Formulation 1; (B) Formulation 2; (C) Formulation 4; (D) Formulation 5; (E) Formulation 6; (F) Formulation 7. Bar = 200 nm

2.3.2. *FQ70 micellar structures*

Despite pore-sheet structures arising from quinoa saponins: CHOL:PC complexes previously described (Bomford et al. 1992), the formation of typical ISCOM matrices based on quinoa saponins was not observed in the experimental conditions used. The TEM analysis of all FQ70:CHOL:PC formulations (Formulations **1–10**, Table) showed that no typical cage-like ISCOM were formed for any of them (Fig. 4), despite this, a wide variety of different micellar structures were observed. Nonetheless, the tubular-shaped micelles were more noticeable in formulations **1**, **2** and **4,** and closely resembled nanosized structures described for saponins of *Q. saponaria* (Demana et al. 2004; Lendemans et al. 2005) and some mannosylated derivatives of oleanolic acid

Table: Composition of FQ70:CHOL:PC and FQ90:CHOL:PC micellar formulations (expressed as percent mass ratios) prepared by the ethanol injection method

Formulation	FQ70	PC	CHOL	Formulation	FQ90	PC	CHOL
1	63	24	12	11	63	24	12
2	72	18	10	12	72	18	10
3	80	13	7	13	80	13	7
4	84	11	5	14	84	11	5
5	40	40	20	15	40	40	20
6	30	60	10	16	30	60	10
7	20	70	10	17	20	70	10
8	40	20	40	18	40	20	40
9	30	10	60	19	30	10	60
10	20	10	70	20	20	10	70

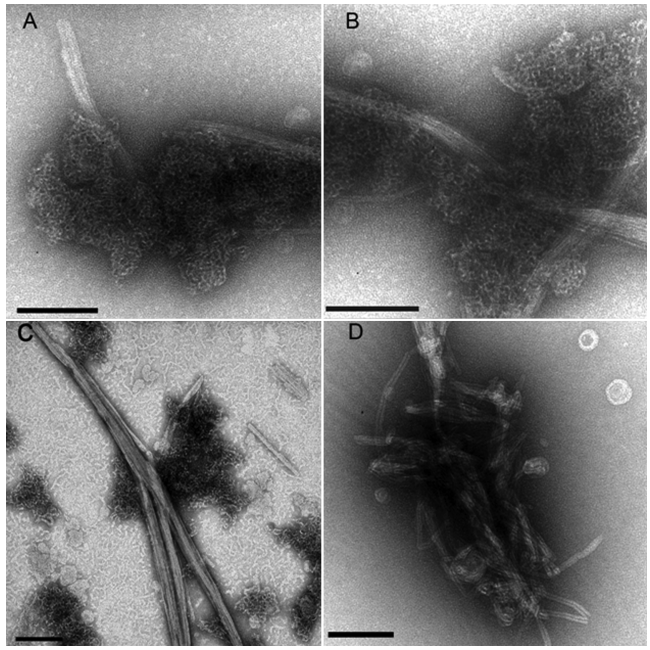


Fig. 5: TEM micrographs of selected formulations prepared with FQ90 fraction by ethanol injection. (A) formulation 11; (B) formulation 11 after 30 days; (C) formulation 14; (D) formulation 18. Bar = 200 nm

(Daines et al. 2009), among other types of compounds, including bile salts, cholesterol and steroids (Zastavker et al. 1999). Thus, tubular micelles became evident after the initial increase of FQ70 concentration (Formulations **1** to **4**) with predominance over other micellar aggregate forms (Fig. 4 A, B and C). The fact is worth mentioning because most of the current interest in helical and tubular nanostructures is driven by technological and medical applications (Polidori et al. 2005).

In formulations **5** to **7** (Table) the PC concentration was increased (40–70%) while the FQ70 concentration was diminished (40–20%), and CHOL concentration was kept at 10–20 %. In these systems only a few tubular structures (Fig. 4D) and lipid particles became noticeable (Fig. 4E and 4F). Conversely, ternary systems of Quil A, PC, and CHOL with similar mass ratio as those in Formulations **5** to **7,** prepared by the lipid film hydration method, afforded typical cage-like ISCOM (Demana et al. 2004).

Regarding the TEM observations for Formulations **8** to **10** it can be concluded that when the proportion CHOL is above a given level, only lipidic particles and bilayer structures are obtained. In systems containing a relative proportion of 70% of CHOL, lipid particles coexist with cholesterol crystals (data not shown).

2.3.3. *FQ90 micellar structures*

Regarding the second quinoa saponin fraction studied, the TEM analysis of FQ90:CHOL:PC formulations yielded basically ring-like micelles associated with few tubular structures (Fig. 5A and 5B) as those above described to FQ70 formulations. Again, no typical ISCOM matrices was noticed in FQ90:CHOL:PC formulations. The ring-like micelles observed in this work was previously described to mannosylated saponins based on oleanolic acid (Daines et al. 2009). Notwithstanding that the term pore-sheet was initially applied to describe similar nanosized structures derived from quinoa saponins (Bomford et al. 1992), it seems preferable to consider them as actual ring-like structures. In pseudo-ternary systems containing quinoa saponins no typical ISCOM matrices were observed even after a 4 weeks-equilibration time, but instead ring-like micelles did (Fig. 5B).

Similar results were observed with formulations containing mannosylated saponins, CHOL and PC (Daines et al. 2004), although ring-shaped micelles are hypothetically considered as the building blocks of *Q. saponaria* ISCOMs (Kersten and Crommelin 2003).

As the FQ90 concentration was increased (Formulations **12** to **14**), ring-like micelles were detected along with some tubular structures (Fig. 5C).

When the PC concentration was increased (Formulations **15** to **17**) the prevalent lipid structures observed were ring-like micelles and tubular structures (data not shown). Tubular structures (Fig. 5D) resembling those viewed with FQ70 were observed at higher CHOL concentrations (Formulation **18**). In contrast to what was observed with FQ70 micelle formation, in the case of FQ90, clouding due to cholesterol crystallization in Formulations **19** and **20** avoided further analysis by TEM.

In a previous work (Verza et al. unpublished data) ten triterpenic saponins were characterized in FQ70 and FQ90. These saponins were characterized as bidesmosides and derived from five different aglycones moieties linked to different sugar side chains. The predominant peaks, in FQ70 and FQ90 were derived from phytolaccagenic acid (Verza et al. unpublished data). In that occasion two less polar saponins were observed in FQ90 derived from hederagenin and oleanolic acid. Besides FQ90 contained four additional saponins but neither the anomeric configurations of their glycoside linkages nor the identity of the monosaccharide residues were defined. However, these triterpenic saponins, in FQ90, were characterized as serjanic and oleanolic acid derivatives (Verza et al. unpublished data).

The spontaneous self-organization of surfactants to form nanosized structures, e.g. membranes, tubules and layers, is driven by specific molecular features (Kersten and Crommelin 2003). Regarding saponins specifically, studies on micellization-structure relationship are rather scarce. In that context it is worth mentioning the ability of QS-17, QS-18 and QS-21, saponins of *Q. saponaria*, to form typical ISCOM matrices, whereas the saponin QS-7 produces predominantly lamellae structures and liposomes (Pham et al. 2006). Structurally speaking quinoa saponins differ from those of *Q. saponaria* regarding the triterpene aglycone moiety (the later are derived from quillajic acid mainly), aglycone-sugar linkage, sugar chain composition, molecular weight and hydrophobic/hydrophilic domains, that may explain the preferential occurrence of tubular micelles and pore-sheet structures in quinoa formulations, instead of cage-like ISCOMs.

Additional evidence concerning the role played by the saponin sugar side chain and triterpene aglycone in the formation of such tubular structures could be inferred from the relative compositions of FQ70 and FQ90, and the micellar structures they form.

The major peaks in the quinoa saponin fractions (Verza et al. unpublished data) were both ascribed to phytolaccagenic acid saponins, in agreement with Madl et al. (2006). It suggests a possible relationship between those saponins and the tubular structures viewed in FQ70:CHOL:PC and FQ90:CHOL:PC complexes by TEM.

Theoretically, bidesmosidic saponins derived from oleanolic acid are unable to build ring-like micelles formulated with cholesterol and phospholipids (Daines et al. 2009). Given that both FQ70 and FQ90 fractions contain oleanolic acid bidesmosides (Verza et al. unpublished data), the occurrence of ring-like and tubular nanostructures in the latter fraction must be ruled by other factors as the oligosaccharide sequence, for instance. Further studies will be needed to enlighten these observations, since the possible monodesmosides in FQ90 cannot be completely discarded.

3. Experimental

3.1. Materials

Cholesterol (CHOL), 1-(2-pyridylazo)-2-naphthol (PAN), sodium dodecyl sulphate, analytical grade ethanol, methanol and pentane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphatidylcholine (Lipoid® S 100) was purchased from Lipoid (Ludwigshafen, Germany) and Quil A from Breentag Biosector (Frederikssund, Denmark). TRIS buffered saline solution pH 7.4 (TBS) consist of 65 mM TRIS (Sigma-Aldrich) and 80 mM NaCl (Sigma-Aldrich). Purified Water was prepared using a Milli-Q system (Millipore®, Bedford, MA, USA).

3.2. Quinoa extract and saponin fractions

Quinoa seeds were harvested in Salta, Argentina. A 100 g-sample was extracted with 1 L of aqueous ethanol (40% v/v) with gentle magnetic stirring (Ikamag RO15, IKA, Staufen, Germany) for 1 h at 50 °C. The mixture was filtered through a Whatman paper filter N° 2 (Kent, UK), the ethanol was evaporated under vacuum at 40 °C (Büchi R114, Switzerland), and the aqueous solution was freeze-dried (Modulyo 4K, Edwards, Crawley, UK). The freeze-dried powder was labeled as EXQ, and stored in amber glass containers until used. A sample of EXQ (700 mg) was dissolved in water (20 mL) and fractioned onto a 50 x 2.8 cm-column containing 40 g of Diaion HP 20 (Supelco, Bellefonte, PA, USA) with methanol:water mixtures of 500 mL each, following a decreasing polarity gradient. The flow rate was 5.5 mL/min. The fractions eluting with 70% and 90% methanol were separately concentrated under vacuum at 40 °C, and freeze-dried to give fractions FQ70 (15.6 % yield) and FQ90 (5 % yield), respectively.

3.3. Determination of critical micelle concentration (CMC)

The CMC values were calculated by the dye solubilization technique, using the water-insoluble dye 1-(2-pyridylazo)-2-naphthol (PAN), after modification of the original method (Hergenhother and Martin 1997). Briefly, 37.5 μL of a 1.6 mM PAN pentane solution was added to 200 μL of aqueous solutions of the assayed fractions at concentrations ranging from 1.53×10^{-5} to 2.0% (w/v), the mixture was shaken vigorously and left stand for 30 min. Aliquots of each aqueous solution (200 μL) were transferred to a 96-well microplate and the absorbance was determined at 470 nm using a EnVision 2104 Multilabel Reader (Perkin Elmer, Massachusetts, USA). The CMC value was determined in each sample plotting the measured absorbance values against the logarithm of the concentration of FQ70 and FQ90. At saponin concentrations below the CMC, the dye is insoluble and the measured absorbance is therefore near to zero. As micellar aggregates start forming the dye becomes solubilized within the micelles and absorbance increases. The onset of solubilization is taken as the CMC. For comparison, sodium dodecyl sulphate was assayed under similar conditions. All measurements were made in triplicate, and the CMC was calculated as the mean value of three experimental data.

3.4. Dynamic light scattering (DLS)

The measurements were performed using a BI-200SM Research Goniometer System standard setup with a BI-200 M goniometer, a BI-9000AT digital correlator (Brookhaven Instruments, NY, USA), and vertically polarized coherent He–Ne Laser as light source ($\lambda = 632.8$ nm). The scattering volume was minimized using a 0.4 mm aperture before the entrance of the photomultiplier. All the measurements were carried out at 20 ± 1 °C and a same final concentration of 0.4% (w/v).

3.5. Transmission Electron Microscopy (TEM)

A 10 μL-sample of a 0.4% (w/v) of FQ70 and FQ90 aqueous solutions were placed on formvar carbon grids (200 Mesh) and negatively stained with uranyl acetate (2% w/v) for 2 min. The analysis was performed on a JEOL Microscope JEM 1200 Ex II (Kyoto, Japan) at an acceleration voltage of 80 kV and magnifications from 30,000 to 150,000×. A similar staining procedure and TEM experimental conditions were used for the observation of FQ70:CHOL:PC and FQ90:CHOL:PC micellar complexes.

3.6. ISCOM matrix preparation

The FQ70:CHOL:PC and FQ90:CHOL:PC complexes were prepared in general, following the ethanol injection method previously described for the preparation of ISCOM matrices (Lendemans et al. 2005). In brief, mixtures mass ratios were defined according to the pseudo ternary-phase diagram (Demana et al. 2004) (Table). The formulations were prepared by addition of 0.225 mL of an ethanol solution of PC and CHOL to 2.775 mL of a TBS solution containing either FQ70 or FQ90 (Table). In all cases the final volume of the micellar preparation was 3 mL.

Aiming a better appraisal of the TEM images recorded for FQ70:CHOL:PC and FQ90:CHOL:PC formulations ISCOM matrices were prepared using Quil A in the proportions indicated for Formulation **1** (Table).
All formulations were magnetically stirred for 48 h at 25 °C and analyzed by TEM. Next, all samples were stored at 4 °C and analyzed again 30 days later. At least three preparations of each formulation were analyzed.

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