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QS-21 adjuvant: laboratory-scale purification method and formulation into liposomes

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Running head: "QS-21 lab-scale purification"

Summary

QS-21, a saponin extracted from the tree *Quillaja saponaria* Molina, is a vaccine adjuvant which has been shown to elicit robust antibody and cell-mediated immune responses in a variety of preclinical and clinical studies [1]. Its purification from the natural source is a lengthy and difficult process. The commercially available saponin mixture Quil-A® is a fraction of the bark extract containing a variety of saponins, including QS-21. In order to facilitate access to QS-21 at laboratory-scale amounts, we propose here a method of purification of QS-21 starting from Quil-A®. In addition, we describe a protocol to appropriately formulate QS-21 into cholesterol-containing, neutral liposomes which are known to decrease QS-21's hemolytic activity while retaining the adjuvant effect. Methods for the physico-chemical characterization of purified QS-21 and of the QS-21 / liposome formulations are also described.

Key Words

QS-21, saponin, vaccine adjuvant

1. Introduction

The adjuvant effect of extracts of plants from the genus Saponaria has been known for more than 90 years [2]. In particular, bark extracts of the South American tree *Quillaja Saponaria* (QS) Molina have been prepared by Dalsgaard and used as adjuvants in veterinary vaccines [3]. Brenntag Biosector (Frederikssund, Denmark) further developed and validated a process for the preparation of Quil-A®, a fraction of the bark extract containing various saponins. However, Quil-A® has been shown to be too reactogenic for inclusion in human prophylactic vaccines, and subsequent efforts were made to further purify the mixture. In 1991, Kensil et al. [4] described the successful isolation of individual saponin fractions. The saponin fractions named QS-7, QS-17, QS-18 and QS-21 all proved to display strong adjuvanticity. QS-17 and QS-18, the most abundant saponin in Quil-A®, were shown to be

highly reactogenic in mice, however QS-7 and QS-21 were both less reactogenic while maintaining adjuvant properties. As QS-21 was more concentrated than QS-7 in the *Quillaja Saponaria* extracts, it was selected for further development as vaccine adjuvant.

QS-21 is currently being tested in various clinical studies on adjuvanted vaccines. In July 2015, the European Medicines Agency's Committee for Medicinal Products for Human Use gave a positive scientific opinion for the malaria vaccine Mosquirix® for use in children (6 weeks to 17 months old) in areas where malaria is endemic [5]. Mosquirix™, developed by GlaxoSmithKline, and also known as RTS,S, contains the AS01 adjuvant which is a combination of QS-21 saponin together with monophosphoryl lipid A and liposomes. Other vaccines containing QS-21 are currently being evaluated against cancer, HIV and Alzheimer's [1].

The use of QS-21 poses certain challenges such as the difficulty of extraction and purification from its natural source and an associated low yield [4,6,7], hemolytic properties resulting in a dose-limiting toxicity [4,8], an instability to hydrolysis at physiological pH [9], and mechanism of action not yet elucidated.

We propose here a laboratory-scale method to isolate QS-21 from the commercially available Quil-A®, with equipment and resources which are accessible by a large number of research groups. The yield of QS-21 resulting from this method, with respect to the starting amount of Quil-A®, is about five times higher than that previously reported in the literature [4,6]. We also propose a procedure for the preparation of liposomes (containing cholesterol) and their formulation with QS-21 in order to neutralize the hemolytic effect of the saponin. It has been described that saponins bind to cholesterol in membrane lipid bilayers of cells including erythrocytes, resulting in the formation of pores [10-13] and subsequent hemolysis. Liposomes containing cholesterol have the potential to abolish the hemolytic effect of QS-21 while retaining the adjuvant effect [14,15]. In addition, incorporation of QS-21 in the membrane of liposomes protects the saponin from hydrolysis at physiological pH [15]. Finally, in light of the importance of performing systematic quality control of

adjuvant formulations before their use in preclinical models, we provide analytical methods for the characterization of QS-21 and its liposomal formulation.

2. Materials

2.1. Preliminary purification of QS-21 by Liquid Chromatography (LC) on silica gel

1. Rotary evaporator
2. Nitrogen canister with manometer and pressure regulator
3. Glass column (30 x 460 mm) with sintered glass filter and two-way plastic stopper at the bottom
4. Glass adaptor with stopper and tubing to connect the top of the column to the nitrogen bottle
5. 23-mL glass tubes (13 x 160 mm) to collect fractions (see **Note 1**)
6. Quil-A® (Brenntag Biosector)
7. Silica gel (particle size 220 - 240 mesh)
8. Sand (Merck)
9. Eluent: $CHCl_3$/MeOH/H_2O/CH_3COOH 270:139:25:1 v/v/v/v; in a 2-L glass cylinder, add 640 mL of methanol (MeOH) to 1240 mL of chloroform ($CHCl_3$), pour into a 2-L glass bottle, close the bottle and hand shake until homogeneous. In a 200-mL glass cylinder, add 40 mL of ultrapure water (H_2O), then 4.6 mL of acetic acid (CH_3COOH) measured with a graduated glass pipette, add H_2O up to 120 mL, and hand shake. Add the CH_3COOH/H_2O solution to the MeOH/$CHCl_3$ solution, hand shake until homogeneous (see **Note 2**)

2.2. Purification of QS-21 by preparative High Pressure Liquid Chromatography (HPLC)

1. HPLC (Waters) composed of two pumps, pump control module, manual injector, UV absorbance detector, 5 mL injection loop, and Empower™ software
2. C18 column of 21.2 x 250 mm with particle size 10 µm and pore size 100 A° (Interchim)
3. Eluents: A) 0.1 % trifluoroacetic acid (TFA) in H_2O, B) 0.1 % TFA in HPLC-S gradient grade acetonitrile (CH_3CN)

2.3. Preparation of neutral liposomes

1. 10-mL Thermobarrel extruder (Northern Lipids)
2. Heating circulator with water bath (Julabo)
3. Drain disks (Whatman)
4. Polycarbonate filter membranes with pores of 0.4 µm, 0.2 µm and 0.1 µm (Whatman)
5. Rotary evaporator
6. Nitrogen canister of 200 bar with gas regulator (Linde).
7. 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids) stock solution: 40 mg/mL DOPC in $CHCl_3$.
8. Cholesterol stock solution: 10 mg/mL cholesterol in $CHCl_3$.
9. Dulbecco's PBS 1x, without Ca^{2+} and Mg^{2+} pH 7.0 -7.3 (DPBS -/-)

2.4. Quality control

1. Thin Layer Chromatography (TLC) plates (Merck, Kieselgel 60 Alufolien 20 x 20 cm)
2. TLC glass development chamber
3. Glass capillaries
4. Hair dryer
5. Developing agent: $CHCl_3$/MeOH/H_2O 60/40/10 v/v/v
6. Revealing agent: 0.1 % orcinol in 5% concentrated sulfuric acid (H_2SO_4)/EtOH
7. HPLC (Waters) composed of two pumps, pump control module, manual injector, UV absorbance detector, 50 µL injection loop, and Empower™ software
8. C18 column of 4.6 x 100 mm with particle size 3.5 µm and pore size 300 A° (Waters, XBridge BEH300)
9. Eluents: A) 0.1% TFA in H_2O, B) 0.1% TFA in CH_3CN
10. Micromass QTOF Ultima (Waters), ionization modes: positive and negative electrospray, infusion of 20 µL/min, sample dilution in appropriate eluent to 0.1 mg/ml (just before injection)

11. Positive mode eluent: H_2O/CH_3CN/formic acid (HCOOH) 49.9/50/0.1 v/v/v
12. Negative mode eluent: H_2O/CH_3CN 59/50 v/v, infusion: 20 µL/min, sample dilution: 0.1 mg/mL (just before injection)
13. Horizontal shaker for 96 well plate
14. Plate reader for UV-visible detection (TECAN, Sunrise)
15. Minicentrifuge (e.g. VWR Galaxy Ministar, max speed 2000 g)
16. Centrifuge for 96 well plates
17. U-bottomed 96 well plates
18. Flat-bottomed 96 well plates
19. Sheep blood
20. Water for injection (WFI)
21. Dynamic light scattering particle sizer (e.g. Malvern ZetaSizer® Nano ZS)
22. Disposable microcuvettes (Malvern)
23. Dulbecco's PBS without Ca^{2+} and Mg^{2+} pH 7.0 – 7.3 (DPBS -/-)
24. Transmission electron microscopy (TEM) microscope (FEI, Tecnai Spirit-Bio)
25. Glow discharge device
26. Carbon coated cupper grids 400 mesh (Canemco-Marivac)
27. TEM staining solution: 2% (w/v) Uranyl acetate dehydrate
28. Pliers (Dumont)

3. Methods

3.1. Preliminary purification of QS-21 by Liquid Chromatography (LC) on silica gel

1. Weigh 50 g of silica gel in a 500 mL glass beaker, add 100 mL of the $CHCl_3/MeOH/H_2O/CH_3COOH$ eluent (prepared as described in the materials section), and hand shake until homogeneous.

2. Pour the suspension into the 30 x 460 mm glass column, leaving the two ways stopper open at the bottom, collect the eluent into a recipient and discard the flow-through. Recover the rest of silica gel from the 500 mL beaker with about 50 mL of fresh eluent, pour into the column, and discard the flow-through. Wash carefully silica out of the walls of the column with fresh eluent using a Pasteur pipette, then fill column up with fresh eluent.
3. With the two ways bottom stopper always open, apply pressure from the nitrogen bottle until the silica level does not go down any more (see **Note 3**). The silica should fill the glass column up to 15.5 cm from the sintered glass filter, and the top of the silica layer should be flat.
4. Having about 10 cm of eluent above the top of the silica layer, add sand slowly to form a layer of about 5-8 mm on top of the silica column (see **Note 4**). Using nitrogen pressure, push down the eluent until its level is just above the top of the sand layer. Let about 3-4 mL of eluent flow down by gravity. When the level of the eluent is in the sand layer, close the two ways stopper at the bottom of the glass column. The column is now ready to be loaded with the sample to be purified.
5. Weigh 800 mg of Quil-A® in a glass tube. Add 2 mL of eluent and vortex three times for 10 s, or until obtaining a viscous, dark yellow solution. Add this solution on top of the sand layer with a 5 mL glass pipette by allowing the mixture to flow down along the walls of the column, as close as possible to the top of the sand layer. Open the two ways stopper at the bottom of the glass column and leave it open for the rest of the manipulation.
6. Recover the rest of sample from the glass tube with 0.5 mL of fresh eluent and pour carefully into the column as just described. Let the sample go through the sand layer and adsorb on top of the silica layer. A yellow ring should be visible just under the sand layer.
7. Make sure that there is no sample solution left on the walls of the column by washing these walls twice with 2 mL of eluent, using a long stem Pasteur pipette. Wait for all the eluent to flow under the sand layer, then add five times 2 mL of eluent with a long stem Pasteur pipette. Add gently more eluent to fill the column without destroying the sand layer

8. Collect the first 100 mL in a beaker by gravity (without applying pressure), then 200 mL with pressure from nitrogen bottle at a flow rate of 4 - 5 mL/min (see **Note 5**). After having collected these 300 mL, start collecting fractions of 22 mL using glass tubes, at the same flow rate. QS-21 is expected to be found in fractions 8 to 14.
9. Spot a QS-21 reference (see **Note 6**) and the fractions on the TLC plate, dip the bottom of the plate into the developing agent (prepared as described in the materials section), allow the migration, dry the plate with a hair-dryer at low heat, dip the plate completely in the revealing agent (prepared as described in the materials section), retrieve it and dry with the hair dryer at high heat until brown or black spots appear on the plate. Select the fractions that have a similar profile to that of the QS21 and less contaminants (should be fractions 9 – 13).
10. Pool the fractions containing QS-21 in a 200-mL glass flask (one neck, round bottom) and recover the residues by washing tubes with 2 mL of MeOH. Evaporate using a rotary evaporator at 30 mbar, setting the water bath at 25°C. Stop evaporation when no more condensation of solvent in the collecting flask is visible. An aqueous residue should remain in the flask (see **Note 7**).
11. Transfer the aqueous residue from the flask into a pre-weighed 15 mL polypropylene tube. Wash the flask twice with 1 mL of ultrapure water and add into the tube. Proceed with the freeze-drying during at least 48 hours at -90°C and 50-100 µbar. Retrieve the tube from the freeze-dryer and weigh. Approximately 55 to 60 mg of white powder should be recovered (see **Note 8**). Store at 2-8°C until proceeding with next steps.

3.2. Purification of QS-21 by preparative HPLC

1. Dissolve the freeze-dried product into 1 mL of CH_3CN/H_2O 45/55 v/v and vortex 10 s (see **Note 9**).
2. Inject into the preparative HPLC system (with 21.2 x 250 mm column, as described in the materials section), in two injections of 0.5 mL each. Apply the following gradient (using eluent A and B as described in the materials section): 5 to 45 % of eluent B in 3 min, then 45 to 53 % in 20

min, then 100% for 6 min, and back to 5%. Apply the following settings during the procedure: flow rate: 10 mL / min, UV detection at 210 or 220 nm, column temperature: 20 - 22 °C. Start collecting fractions at around 15.5 min and stop at around 18.5 min before appearance of the "shoulder" at the end of the peak (**Figure 1**) using pre-weighed tubes.

3. Pool the fractions collected during both injections. Freeze-dry at least 48 hours at -90°C and 50-100 µbar. Weigh the freeze-dried product. Approximately 15 to 18 mg of white powder should be recovered (see **Note 10**). Store at 2-8°C
4. Dissolve the powder into Dulbecco's PBS without Ca^{2+} and Mg^{2+} (pH 7.0 - 7.3) at 1 mg / mL (see **Note 11**) and aliquot into polypropylene tubes of desired volume. Perform quality control analyses (as described in section 3.3 below) and store at -20°C until use.

3.3. Quality control of purified QS-21

1. TLC: spot QuilA®, a QS-21 reference (**see Note 6**) and the purified QS-21 (all samples at 1 mg/mL in CH_3CN/H_2O 30/70 v/v) on the TLC plate, dip the bottom of the plate into the developing agent ($CHCl_3/MeOH/H_2O$ 60/40/10 v/v/v), allow the migration, dry the plate with a hair-dryer at low heat, dip the plate completely in the revealing agent (0.1% orcinol in 5% H_2SO_4-EtOH), retrieve it and dry with the hair dryer at high heat until brown or black spots appear on the plate.
2. Analytical HPLC**:** weigh 0.5 mg of freeze-dried product into a polypropylene tube, dissolve with 500 µL of CH_3CN/H_2O (30/70 v/v) and vortex for a few seconds to obtain a clear, foaming solution. Inject 20 µL of the solution and elute with the following gradient of eluent B: 30 to 55% in 15 min (flow rate 1 mL/min, UV detection at 210 or 220 nm). QS-21's peak should be detected at around 12 min (see **Note 12**) (**Figure 2**).
3. Electrospray Ionization Time of Flight Mass Spectrometry (ESI-TOF-MS): weigh about 0.1 mg of freeze-dried product into a polypropylene tube, dissolve with CH_3CN/H_2O (50/50 v/v). Inject immediately into the mass spectrometer with negative mode, infusion at 20 µL/min, source

temperature: 80°C, voltage: 35 volts (see **Note 13**). An example of a mass spectrum of purified QS-21 is shown in **Figure 3.**

3.4. Preparation of cholesterol-containing neutral liposomes

1. In a 50 mL glass round bottom flask, add 1 mL of the DOPC stock solution, 1 mL of the cholesterol stock solution and 2 mL of $CHCl_3$. Evaporate with the rotary evaporator, setting the water bath at 40°C: start with a vacuum of 700 mbar and decrease the pressure slowly down to 50 mbar (see **Note 14**) to obtain a thin, transparent, homogeneous lipid film on the wall of the flask. The film can be stored at - 20°C up to 72 hours before hydration.
2. Prewarm the DPBS -/- buffer at 40°C. Transfer 4 mL of buffer into the flask containing the lipid film. Vortex 15 min or until all the film is resuspended. Incubate for 30 min at room temperature. During this time, set up the extruder as described in the following step.
3. Assemble the extruder according to the manual instructions [16] and insert a drain disk and a polycarbonate filter of pore size of 0.40 µm. Connect the water bath to the thermobarrel of the extruder and set the temperature at 40°C.
4. Pre-wet the filter with 1 drop of buffer and insert the hydrated lipid film in the thermobarrel and apply pressure slowly and gradually. Stop increasing pressure when the solution starts dropping from the outlet tube, without going over 500 psi as indicated by the manometer on the regulator of the nitrogen bottle. Then adjust the pressure to maintain a dropwise flow and collect the output liquid in a 12-mL polypropylene tube.
5. When all buffer is ejected from the system, reintroduce the collected liposome suspension in the extruder and repeat the manipulation. Perform 10 cycles of extrusion with the 0.4 µm filter, replace the 0.4 µm filter with a 0.2 µm filter and perform 10 cycles of extrusion with the 0.2 µm filter, replace the 0.2 µm filter with a 0.1 µm filter and perform 10 cycles of extrusion with the 0.1 µm filter (see **Note 15**).

6. Collect the final suspension into a 12-mL polypropylene tube then filter with a 0.22 µm membrane into another polypropylene tube (see **Note 16**)**.** Flush the tube with nitrogen (see **Note 17**) and store at 4°C up to six months. Clean the extruder (see **Note 18**).

3.5. Quality control of cholesterol-containing neutral liposomes

1. Average particle size and polydispersity index (PdI): add 10 µL of liposomes to 90 µL of DPBS (-/-), vortex and transfer 70 µL of the mix into a micro cuvette for size measurement. Software parameters for the measurement are as follows: material--polystyrene latex, dispersant--water, temperature--25°C, equilibration time--120 s, backscatter--173°, 3 measurements of 11 runs each. Expected average particle size of liposomes is between 80 and 160 nm and expected PdI lower than 0.2.
2. Transmission electron microscopy by negative staining with 2% uranyl acetate: adsorb the sample on glow discharged, carbon coated, cupper grids, during 30 s. Rinse the surplus by dipping the grid in Milli-Q water during 1-2 min. Dry the grids with filter paper, then place the grid over a drop of 2% (w/v) aqueous uranyl acetate for exactly 30 sec. Remove the surplus of uranyl acetate solution with a filter paper, and analyze samples at an acceleration voltage of 80 kV under low-dose conditions and a magnification of 43,000. Digital images are recorded on an Eagle CCD camera using FEI TIA software (**Figure 4**).

3.6. Formulation of QS-21 with cholesterol-containing neutral liposomes

1. Bring the liposome suspension (prepared as described in Section 3.3) from 4°C to room temperature and bring the QS-21 solution (1 mg/mL in DPBS -/-, prepared as described in Sections 3.1 and 3.2) from -20°C to room temperature.
2. Add 100 µL of the liposome suspension and 100 µL of the QS-21 solution into a polypropylene tube. Invert the tube gently five times (vortexing is not required).

3.7. Quality control of QS-21 / liposome formulation

1. Average particle size and PdI: proceed as described in Step 1 of Section 3.5 (see **Note 19**).
2. Transmission electron microscopy by negative staining with 2% uranyl acetate: proceed as described in Step 2 of Section 3.5. A cage-like structure should be visible, similar to what is found for ImmunoStimulatory Complex (ISCOM) formulations of liposomes with QuilA® [12]
3. Hemolysis assay: add 200 µL of sheep blood to 1 mL of DPBS (-/-) in a polypropylene tube and invert the tube gently five times. Centrifuge with minicentrifuge at max speed for 10 s and discard the supernatant. Repeat two more times to obtain a clear supernatant.
4. Resuspend the erythrocyte pellet in 1 ml of DPBS (-/-) and transfer the suspension into a tube containing 11 mL DPBS (-/-). Store the suspension at 4°C while preparing the 96 wells plate (see below) but use it within 1 hour.
5. For preparing plate, use a U-bottomed 96 wells plate, distribute 50 µL of DPBS (-/-) in all wells except for 6 wells (e.g. A7-A12) where 50 µL of WFI per well are added. In one column (e.g. column 1), add 100 µL of QS-21 in DPBS (-/-) solution at 0.2 mg/mL in DPBS -/- (e.g. well B1), 100 µL of one QS-21/liposome formulation to be tested with QS-21 at 0.2 mg/mL (e.g. well C1), 100 µL of a second QS21/liposome formulation (e.g. in well D1) and so on.
6. Transfer 50 µL from all wells of column 1 (B1-H1) into the next column (B2-H2) with a multipipette, mixing 3 times up and down. Transfer 50 µL from column 2 to column 3 and so on until column 12, then discard the last 50 µL.
7. Shake plate for 10 min at room temperature in order to obtain a homogenous suspension. Add 50 µL per well of sheep erythrocytes suspension and incubate for 30 min at room temperature with shaking.
8. Centrifuge the plate at 1800 g, 20°C, 5 min. In a flat bottom 96 wells plate, distribute 250 µL EtOH per well. Transfer 50 µL of supernatant from the centrifuged plate into the plate containing ethanol, mix up and down 7 times with a multipipette and read absorbance of the plate at 412 nm (reference at 700 nm) shaking 30 s.

9. The average absorbance of water is the value for 100% lysis of red blood cells and the average absorbance of PBS is the value of 0% lysis. To calculate the % of lysis for the samples use the following formula: (absorbance of sample - absorbance of PBS) / absorbance of water x 100. Results can be displayed in a graph of % lysis of red blood cells as a function of QS-21 concentration in the formulation (**Figure 5**).

3.8. Use of QS-21 / liposome formulation in preclinical studies

1. Add 300 µL of an antigen solution to 200 µL of the QS-21 / liposome formulation.
2. Use the final formulation within the following six hours. As an example, 50 µL of formulation are recommended to be used for vaccination per mouse, by intramuscular route (see **Note 20**).

4. Notes

1. To minimize the presence of endotoxin, we recommend that all the glassware is kept overnight in an oven at 190°C before use.
2. Keep the bottle closed to avoid modification of the mixture by evaporation and water intake.
3. Packing of silica in the column may be facilitated by gently tapping on the column with a piece of rubber tube. Make sure that there is always enough eluent above the sedimentation of silica gel during all the manipulation, as the silica should never dry. The eluent will flow through, be collected in a recipient and discarded. It is recommended not to reuse it, as its composition changes during the manipulation.
4. Take care to have a layer of sand of homogeneous thickness. If some sand sticks to the walls of the column, wash it out with some eluent. Never let the level of eluent go below the layer of sand to avoid drying and cracking of the silica.

5. It is important to start the purification slowly, using only gravity, to obtain a good separation. To measure the flow rate, use a tube previously calibrated with 22 ml of water. Flow rate should be 4-5 mL/min.
6. A QS-21 reference can be made available upon request at our laboratory (Vaccine Formulation Laboratory, University of Lausanne, Switzerland).
7. This residue should be frozen with liquid nitrogen as soon as possible to minimize the time of QS-21 in acidic environment which could degrade the product.
8. This intermediate product can be checked by analytical HPLC as described in Step 2 of Section 3.3.
9. The freeze-dried product is soluble up to 60 mg/mL in this solvent.
10. The yield of QS-21 is around 2% with respect to the weight of Quil-A®.
11. This solubilization process is well described in reference [17].
12. It is possible to confirm the presence of QS-21, by spiking the product purified as described in this method into a solution of Quil-A® and comparing the HPLC profile of Quil-A® before and after spiking (Figure 2). An alternative quantification method by HPLC is described in reference [17].
13. This analysis can be run also in positive mode. In this case the sample is dissolved into H_2O/CH_3CN/formic acid (49.9:50:0.1 v/v/v). All other parameters are the same as for the analysis in negative mode.
14. During evaporation it is necessary to avoid formation of bubbles that would break the lipid film. If the film is not homogeneous, suspend it in 2 mL of $CHCl_3$ and repeat all the described steps.
15. The correct progress of the extrusion process can be monitored by measuring the average particle size (see Step 1 of Section 3.5) after each series of cycles on the same filter. Typically, the particle size should be 600 - 900 nm after 10 cycles on 0.4 µm filter, 140 - 160 nm after 0.2 µm filter and 80 - 120 nm after 0.1 µm filter. The extrusion process converts the Multi

Lamellar Vesicles (MLVs), obtained by hydration and resuspension of the lipid film, into Small Unilamellar Vesicles (SUVs). Transmission electron microscopy indicates that the shape of these SUVs is not spherical but oblate and sometimes tubular.

16. At the end of the process 3.5 mL of liposome suspension are recovered. The process can be scaled up to obtain 8.5 mL of final suspension, starting with 10 mL of hydrated lipid film suspension. An alternative manufacturing method employs mini-extruders and is described on the web site http://www.avantilipids.com, see page about “Avanti Mini-Extruder”.
17. Storage under nitrogen is recommended to avoid oxidation of double bonds in DOPC. It is recommended to place a sterile, 1000 µL pipette tip with 0.22 µm filter (e.g. ART 1000E, self-sealing barrier pipette tip) at the exit of the tubing that brings the nitrogen from the bottle to the tube to be flushed.
18. After disassembling the extruder, rinse all the parts with warm water. Rinse all metal parts with ethanol using a syringe. O-rings and outlet tube must be immediately rinsed with demineralized water, then with ethanol 70% (always rinse immediately the O-rings and outlet tube) using a syringe. Finally, rinse all parts with demineralized water and let dry in a laminar flow hood.
19. No significant difference in the average particle size should be found with respect to the liposome without QS-21.
20. Administration routes other than intramuscular for this formulation have not been tested in our laboratory.

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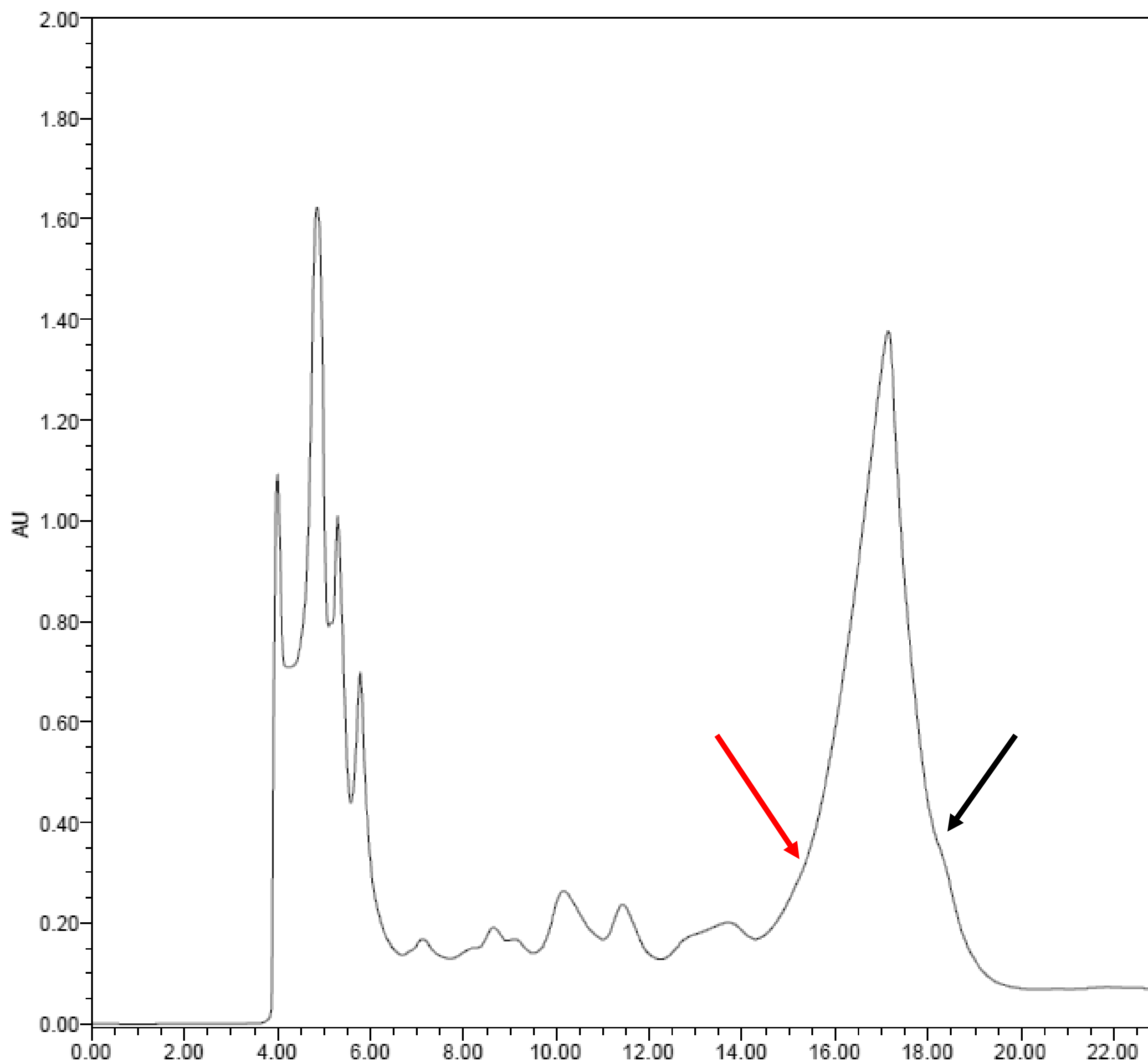


Figure 1. Preparative HPLC chromatogram of QS-21 after purification on silica gel column. Red (left) and black (right) arrows indicate the time to respectively start and stop the collection of fractions

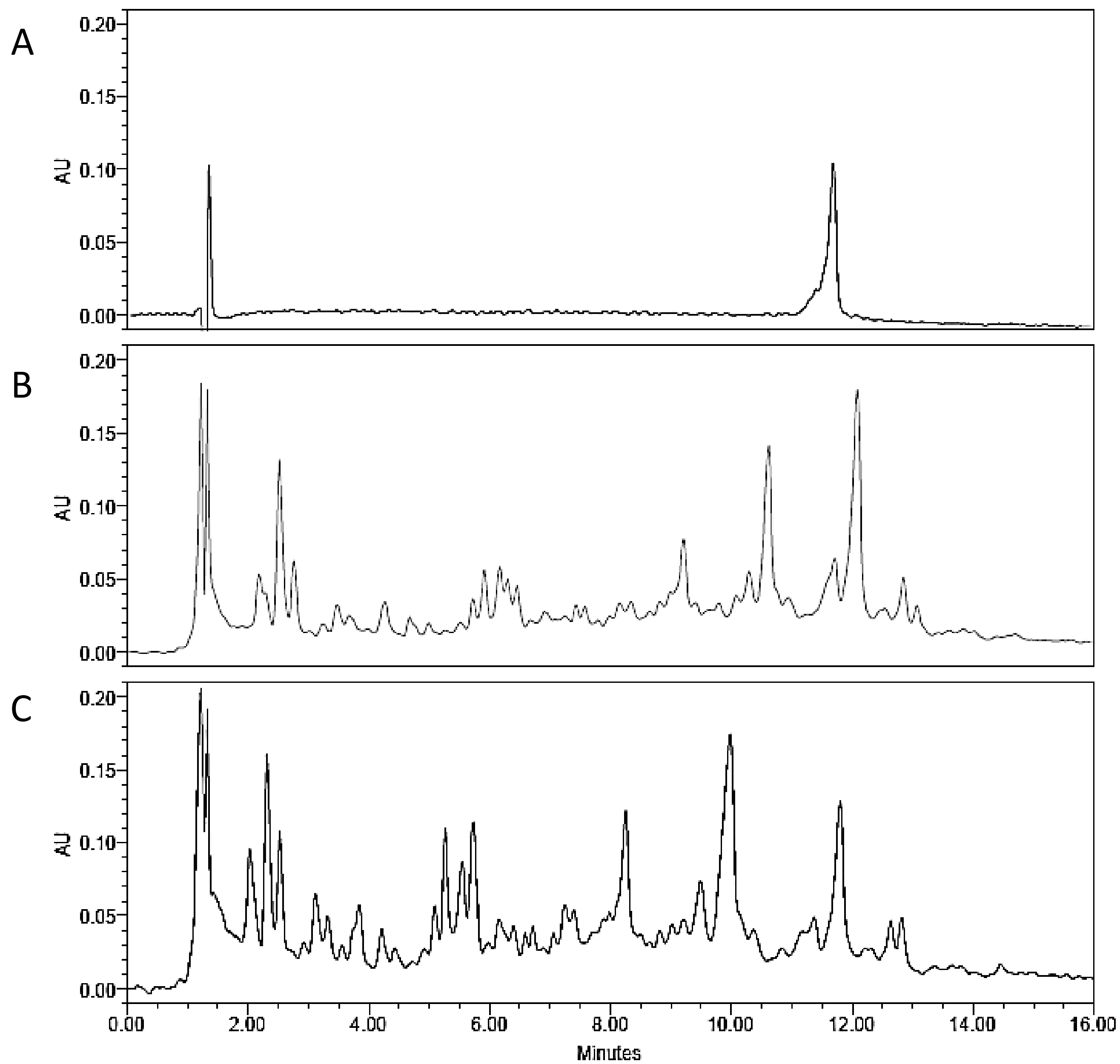
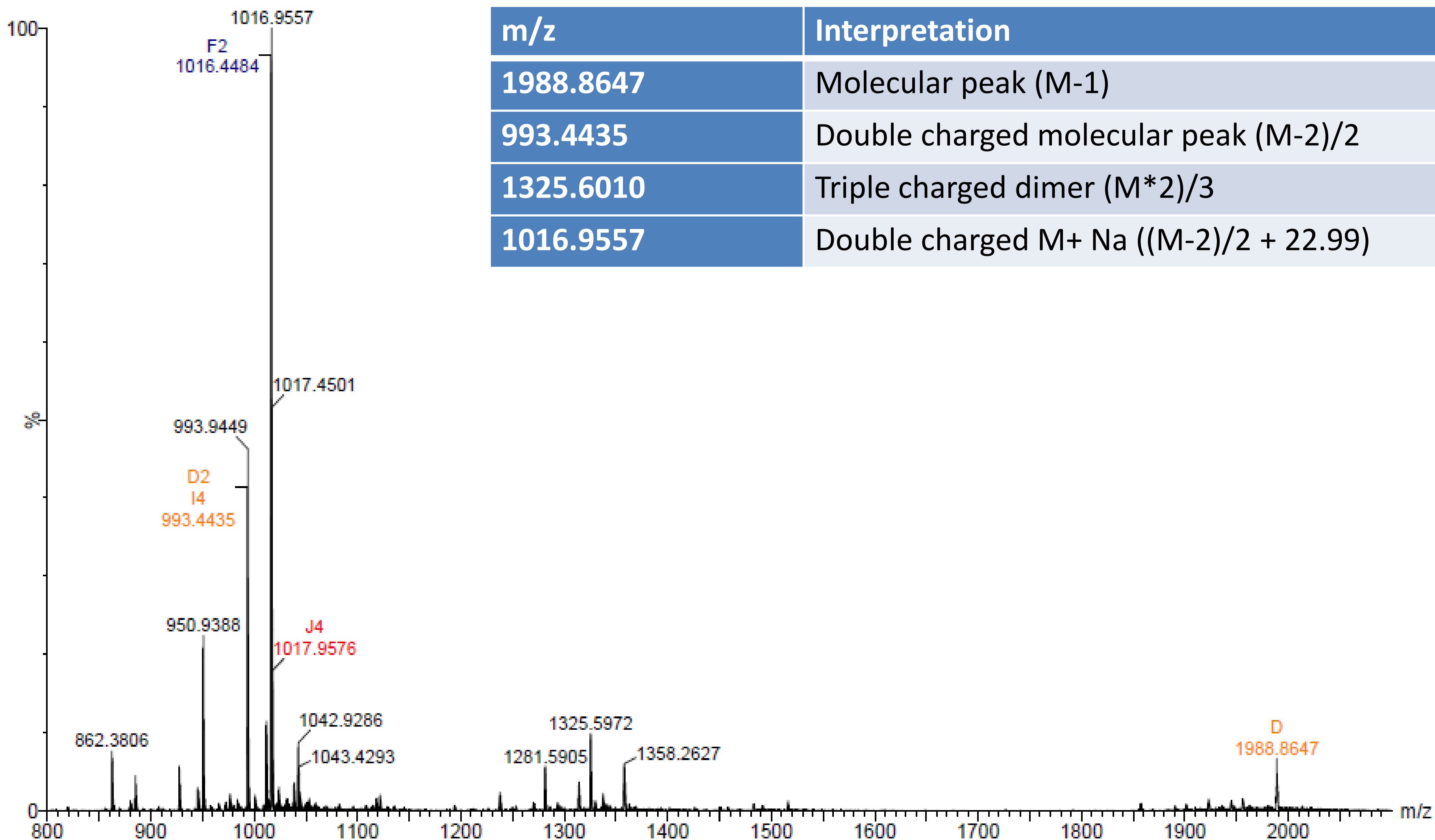


Figure 2. A: QS-21 after preparative HPLC; B: QuilA®, spiked into the QS-21 sample used to obtain the chromatogram in A; C: QuilA®



m/z	Interpretation
1988.8647	Molecular peak (M-1)
993.4435	Double charged molecular peak (M-2)/2
1325.6010	Triple charged dimer (M*2)/3
1016.9557	Double charged M+ Na ((M-2)/2 + 22.99)

Figure 3. ESI-QTOF-MS of QS-21 after preparative HPLC, negative mode

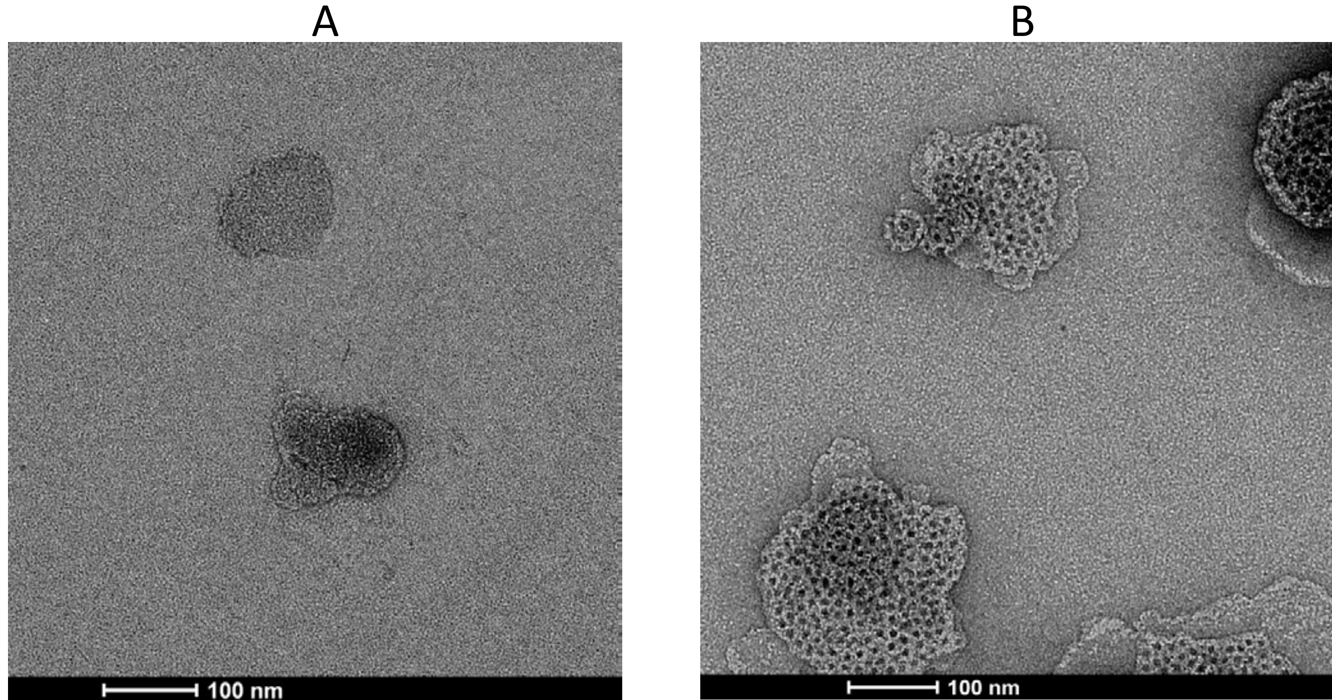


Figure 4. Transmission Electron Microscopy image of A: liposomes 5 times diluted in water B: extemporaneous mixture of liposome and QS-21, cholesterol/QS-21 ratio of 1:2 w/w. = 2.6/1 mol/mol. Negative staining with 2% uranyl acetate.

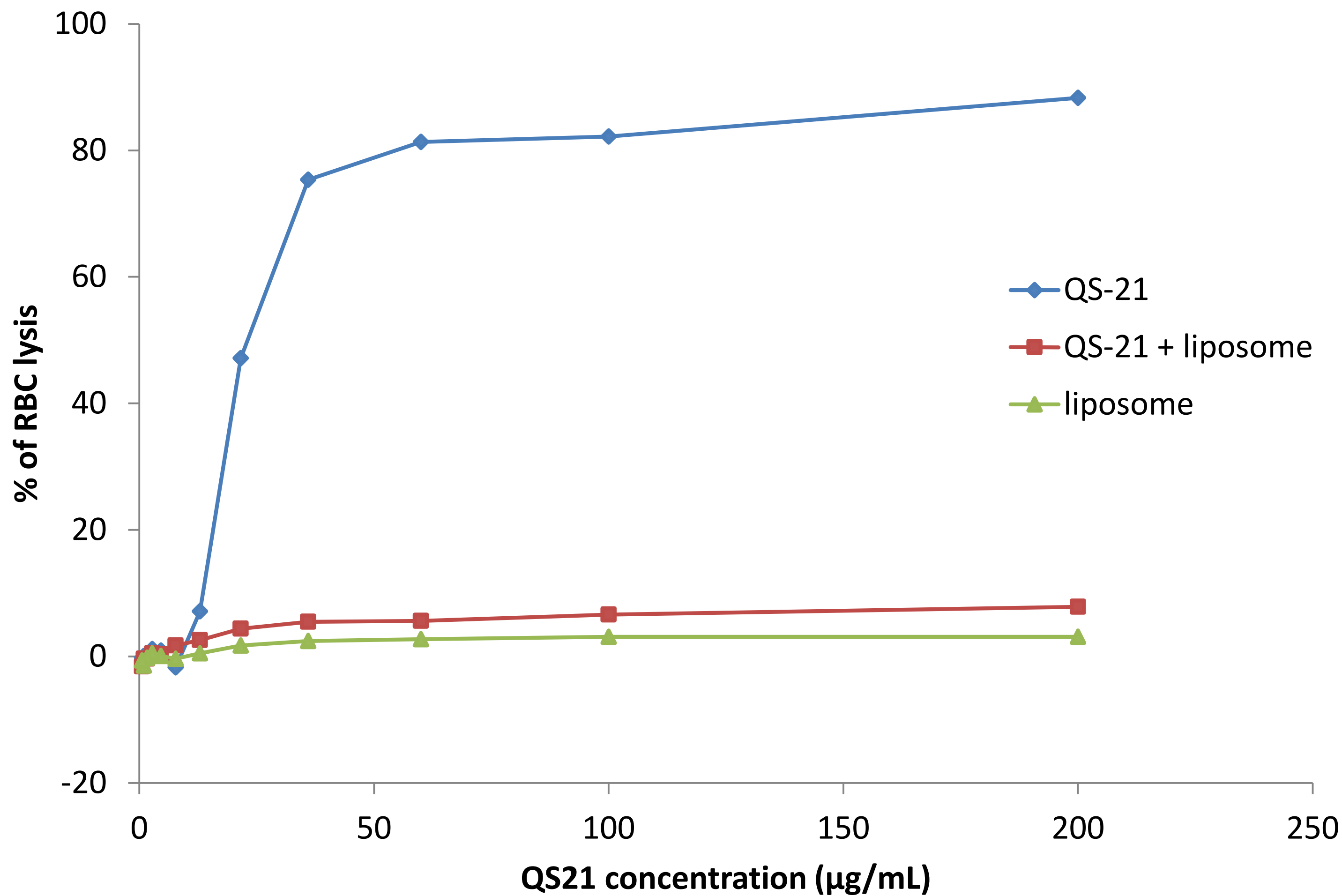


Figure 5. % of red blood cell lysis with respect to water (100% hemolysis) as a function of the concentration of QS-21 in the sample