

# Thermal Treatment of a Novel Saponin-Cholesterol Nanoparticle Vaccine Adjuvant Named NanoQuil F70 Secures a Uniform Morphology and Size Distribution

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## Abstract

Quil-A and cholesterol can, under certain conditions, form nanoparticles, but the conditions for the formation of a homogeneous population of a particular kind of particles with the same morphology and size have remained elusive. However, a well-defined uniformity, as well as a high degree of batch-to-batch consistency, are prerequisites for adjuvant formulations to be used for practical vaccines. To accomplish the above stated tasks in the present study, we demonstrated that Quil-A and cholesterol form well-defined worm-like stable particles with a mean diameter of around 40 nm by dynamic light scattering (DLS) under carefully controlled thermodynamical conditions with little or no Quil-A degradation. The nanoparticles thus prepared possessed a significantly reduced hemolytic effect in comparison to unformulated free Quil-A. However, when the same conditions were applied to QS-21/cholesterol nanoparticle formation the morphology achieved was coil spring-like particles with a mean diameter of around 70 nm. This discrepancy in particle morphology and size was attributed to the differences in hydrophobicity of the Quil-A, being a heterogenic fraction of acylated as well as deacylated saponins, and the QS-21. With the process optimizations leading to a standardized particle size distribution and nanoparticle morphology presented here, NanoQuil F70 holds the potential as a well-tolerated vaccine adjuvant for veterinary use raising a Th1/Th2 balanced immune response.

## Keywords

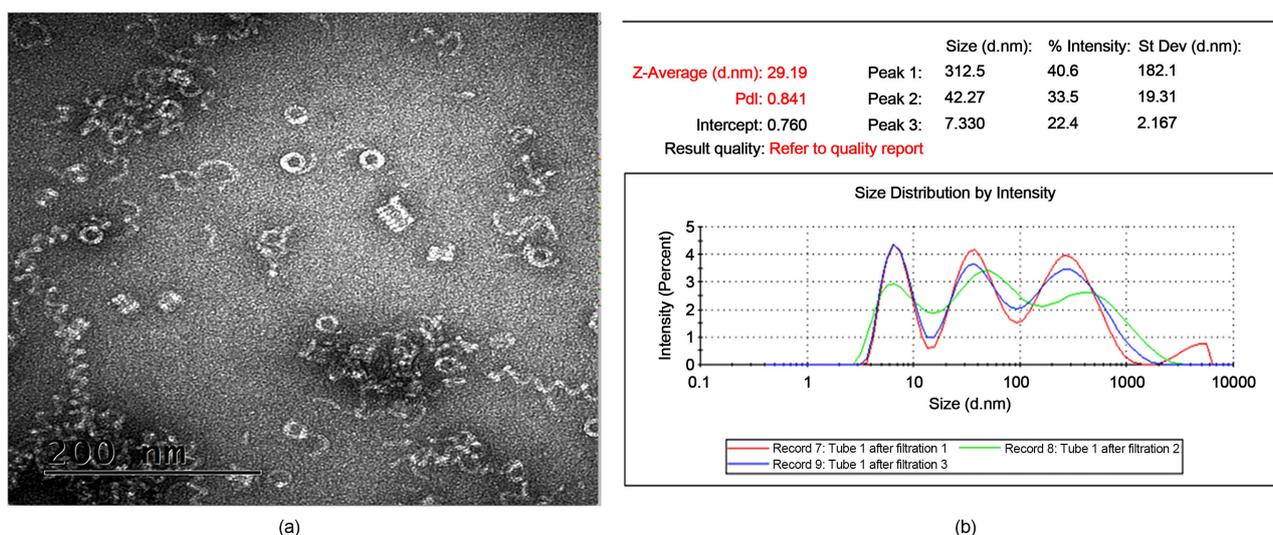
Adjuvant Formulation, G3, NanoQuil, Quil-A, QS-21

## 1. Introduction

The triterpene saponin mixture Quil-A has been used as vaccine adjuvant in animals for many years, but its lytic and surfactant activity introduce limitations for the dose to be applied in order to balance the adjuvant effect against the local adverse reactions at the injection site [1] [2] [3]. This problem was at least partially overcome by Morein *et al.* in 1984 by incorporating the Quil-A saponin into cage-like ISCOM particles when combined with cholesterol and phospholipids (PC) [4] and later with the ISCOMATRIX formulation [5]. Lately, the ability of triterpene saponins to interact with cholesterol alone was used by Morein, Hu *et al.* to design a novel Quil-A containing nanoparticle, originally referred to as G3 and later re-named NanoQuil.

This formulation of Quil-A + cholesterol, when used as a vaccine adjuvant, generated an immune response overall similar to that of the traditional ISCOM formulation with a balanced Th-1/Th-2 profile. In addition, this new adjuvant formulation when supplemented with the diterpene, Stevia glycoside, known as a commonly used and GRAS food additive (sweetener) [6], could even induce cross-protective immunity against a range of influenza virus strains and stimulated a vast array of innate immune response parameters *in vitro*.

However, G3-nanoparticles made in accordance with the original method gave lead to preparations with a heterogeneous particle size distribution and morphology (Figure 1) which turned out to be the main obstacle for transforming such a concept into a credible and standardized product with a uniform and reproducible particle morphology and particle size distribution.



**Figure 1.** The original method of preparation involving incubation of Quil-A and cholesterol at 37°C overnight, demonstrated various morphologies and a heterogeneous particle size distribution of NanoQuil particles when analyzed by TEM (a) and DLS (b).

Although triterpene saponins, and QS-21 in particular, are known to be heat labile [7], we here present data that identified thermal treatment as the single most important factor for obtaining a uniform morphology and particle size distribution and the ability to maintain an acceptable low tissue lytic effect.

## 2. Materials and Methods

### 2.1. Materials for NanoQuil Formation

Quil-A: lyophilized manufactured by CRODA Denmark A/S.

QS-21: AB 801/QS-21, 5 mg/ampule, Batch No. A010-023, Manufacturing Date: 18 July 2018.

Stock solutions of Quil-A (100 mg/mL) and QS-21 (10 mg/mL) in water were prepared and stored at  $-25^{\circ}\text{C}$ .

Cholesterol. Lot. SCOL129-2, material code: C1231, Sigma-Aldrich.

PBS: pH 7.4, D8662-500ML, Sigma-Aldrich.

Acetone: 32201-2.5L-M. Ph. Eur.,  $\geq 99.5\%$ , Sigma-Aldrich.

Safe-lock Eppendorf tubes: Eppendorf AG, Hamburg, Germany.

Vortex Mixer: VELP wizard, advanced IR, VELP scinetifica, Italy.

Air pump: Eheim 200, type 3702010.

Thermoshaker and block thermostats with smart control: MKR13 from Hettich Lab Technology, The Netherlands.

Sorvall ST16R centrifuge: Thermo Scientific.

Vacuum filtration: 1000 mL, SFCA, 0.22  $\mu\text{M}$  sterilized, VWR European article No 514-1058.

### 2.2. Formation of NanoQuil Particles at $70^{\circ}\text{C}$

1.25 mg of plant-derived cholesterol was dissolved in 1 mL acetone. 0.1 mL of the cholesterol solution was transferred to a 1.5 mL Eppendorf tube. The acetone was removed while rotating the tube on a vortex mixer at 300 rpm, gradually forming a cholesterol membrane covering the V-bottom part of the Eppendorf tube. 1 mL or 0.9 mL of  $70^{\circ}\text{C}$  PBS was added quickly to the tubes with the cholesterol deposit, followed by 10  $\mu\text{L}$  Quil-A stock solution (100 mg/mL) or 100  $\mu\text{L}$  QS-21 stock solution (10 mg/mL) respectively, resulting in a final saponin concentration of 1 mg/mL, and cholesterol concentration of 0.125 mg/mL (the weight ratio of Quil-A: cholesterol at this stage is 8:1). The tubes were incubated at  $70^{\circ}\text{C}$  for 60 minutes, then cooled down to  $4^{\circ}\text{C}$  followed by storing the tubes at  $4^{\circ}\text{C}$  overnight. The tubes were centrifuged at 10,000 x G for 20 minutes followed by filtration through a 0.22  $\mu\text{M}$  filter next day. The Quil-A + cholesterol complexes, named NanoQuil F70, were stored at  $4^{\circ}\text{C}$  until use.

### 2.3. Negative Staining Electron Microscopy (TEM) for Morphology

The TEM was commercially run at BioVis Platform of Uppsala University.

A 5  $\mu\text{L}$  drop of the sample was placed on a formvar and carbon coated 200-mesh copper grid. After 30 seconds, the excess solution was removed by blotting with filter paper. The sample was then directly stained for 10 seconds with 2% uranyl acetate. Excess of uranyl acetate was removed by blotting on filter paper. Dried grids were examined by TEM (FEI Tecnai G2 operated at 80 kV).

## 2.4. Dynamic Light Scattering (DLS) for Particle Size Distribution

A sample of NanoQuil colloid (100  $\mu\text{L}$ ) was transferred to a Quartz  $\mu$ -cuvette (Art. No. 105-203-1085-40, Hellma Analytics) and put into the instrument (ZetaSizer Nano ZS90, Malvern Instruments). Measurements were performed at 25°C after a period of equilibration of 2 minutes. Instrumental settings: 173° backscatter detection, 3 measurements per sample, a refractive index of 1.4 and absorption coefficient of 0.001; for the dispersant, the default settings for 1 x PBS buffer. Data are presented as intensity distributions as function of particle size (d.nm).

## 2.5. Quil-A Quantification

The Quil-A content of the Quil-A/cholesterol nanoparticles was determined by orcinol test, which is based on a color reaction caused by both pentose and hexoses as parts of Quil-A molecules. A Quil-A standard curve was created ranging from 1.75 mg/mL, 1.50 mg/mL down to 0.00 mg/ml (0.25 mg/mL per step, 8 steps in total). 100  $\mu\text{L}$  sample + 200  $\mu\text{L}$  orcinol reagent per well were incubated at 100°C for 20 minutes. The result was read spectrophotometrically at 570 nm. The Quil-A content in the unknown samples was extrapolated from the standard curve according to their OD values.

## 2.6. Cholesterol Quantification

Cholesterol content in the Quil-A + cholesterol products was determined using a commercially available cholesterol assay kit (ab65359 cholesterol/cholesteryl ester quantitation kit) from ABCAM according to its instruction manual.

## 2.7. Hemolysis Test

160  $\mu\text{L}$  5 times diluted sheep whole blood (Statens Serum Institute, Denmark) were mixed with 40  $\mu\text{L}$  Quil-A standard or NanoQuil F70 sample and incubated at 37 °C for 45 minutes. After centrifugation at 500 x G for 5 minutes. The supernatant of 100  $\mu\text{L}$ /well x 3 wells for each sample was analyzed spectrophotometrically at 540 nm. The degree of hemolysis reduction was calculated as the percentage of untreated Quil-A at the same concentration according to their respective OD values.

# 3. Results

## 3.1. Morphology and Size of NanoQuil Particles

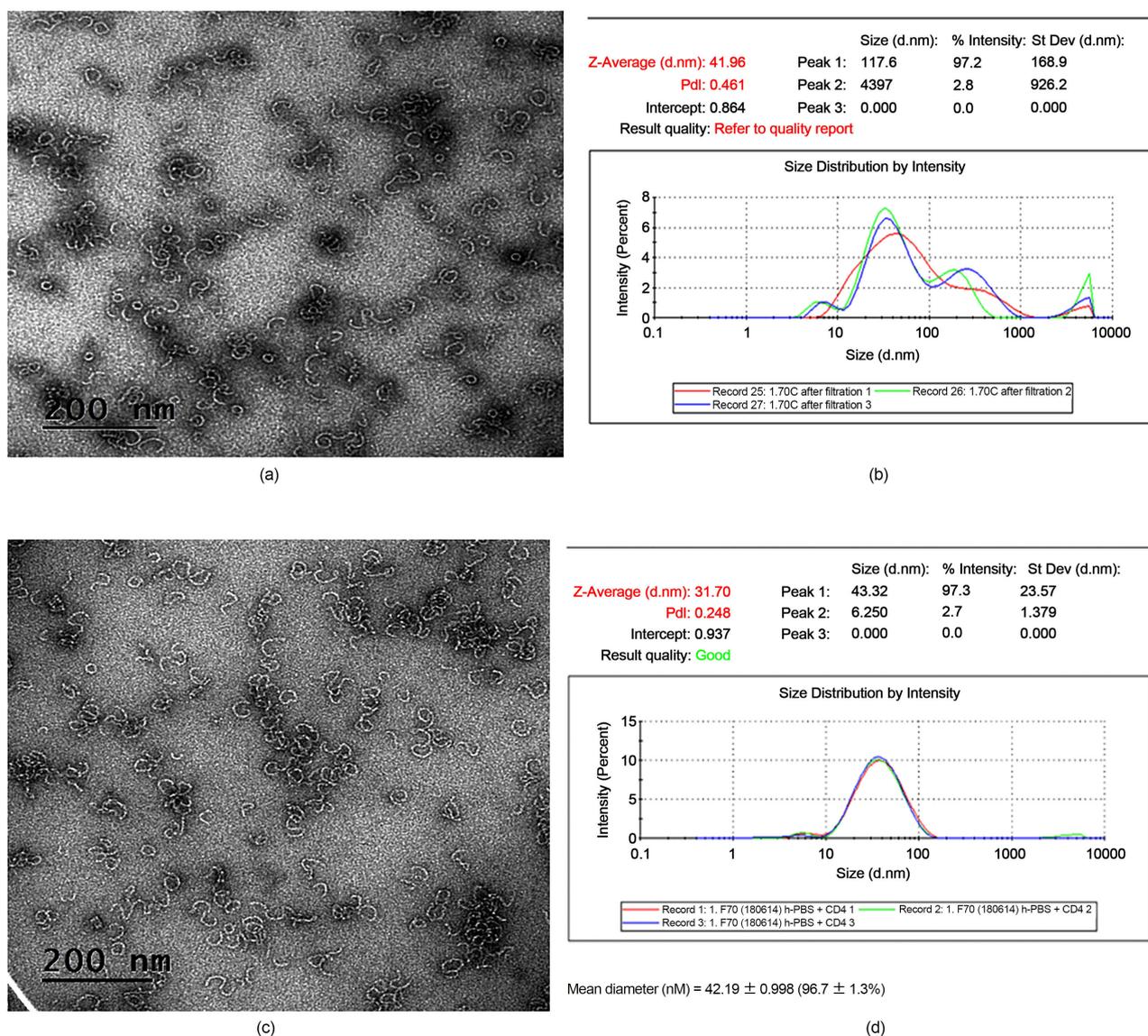
### 3.1.1. Quil-A + Cholesterol Particles Prepared in Accordance with the Original Method

Initially, Quil-A + cholesterol particles were formulated during overnight incubation at 37°C, corresponding to body temperature, which was the highest temperature to think of due to concerns of saponin degradation. Under these conditions, various particle morphologies were generated, including worm-like, helices/double helices, coil spring-like etc., as shown by TEM (**Figure 1(a)**), and

three major peaks were seen in DLS (**Figure 1(b)**), demonstrating a shortage of homogeneity and reproducibility

### 3.1.2. Optimization of F70 Protocol

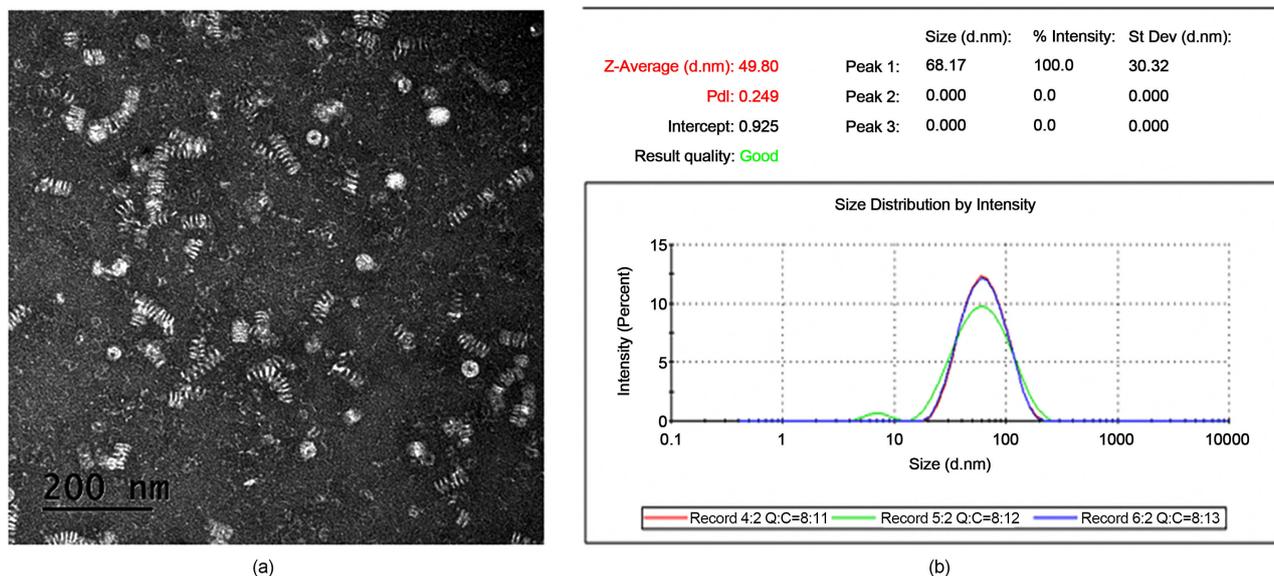
When Quil-A and cholesterol were incubated at 70°C for 30 minutes, an identical population of worm-like particles was formed (**Figure 2(a)**, **Figure 2(b)**). At temperatures lower than 70°C, it was not possible to form such a homogeneous population. Further studies showed that prolonging the time of incubation to 60 minutes, an optimized result was achieved judging from TEM (**Figure 2(c)**) and DLS (**Figure 2(d)**). After this, we settled for a protocol referred to as NanoQuil F70 (incubation at 70°C for 60 minutes).



**Figure 2.** A homogenous population of NanoQuil particles was created after incubation of Quil-A and cholesterol at 70°C for 30 minutes (a) for TEM and DLS result for the same formulation (b). (c) and (d) show TEM and DLS results for a NanoQuil F70 Quil-A formulated with the optimized protocol (at 70°C for 60 minutes).

### 3.1.3. NanoQuil Particles Generated with QS-21 at 70°C for 60 Minutes

After incubation with the optimized protocol for Quil-A *i.e.*, at 70°C for 60 min, QS-21 and cholesterol formed coil spring-like particles (**Figure 3(a)**, **Figure 3(b)**), distinctly different from the worm-like particles seen with Quil-A + cholesterol under the same conditions.



**Figure 3.** Coil spring-like NanoQuil particles were formed after incubation of QS-21 and cholesterol at 70°C for 60 minutes. (a) For TEM and (b) for DLS result for the same formulation (b).

### 3.2. Quil-A Recovery in the Quil-A + Cholesterol Products

The Quil-A content in the Quil-A containing NanoQuil F70 products was measured by the orcinol test, which gave, on average, a recovery rate around 97% in comparison to that of the starting materials used (**Table 1**) *i.e.*, only 3% of the Quil-A was lost in the production process. The 3% Quil-A was trapped and detected (data not shown) in the pellets of cholesterol aggregates after the centrifugation step.

**Table 1.** Quil-A recovery in five different batches of NanoQuil F70 products.

F70 Batch #	Quil-A recovery (% of starting material)
180301	92.3
180326	97.3
180508	100.0
180614	100.0
190126	94.0
<b>Mean ± SD</b>	<b>96.7 ± 3.5</b>

### 3.3. Cholesterol Recovery in the Quil-A + Cholesterol Products

The cholesterol recovery rate was low *i.e.*, only about 19% (**Table 2**).

**Table 2.** Cholesterol recovery in five different batches of NanoQuil F70 products.

F70 Batch #	Cholesterol recovery rate (% of starting material)
180301	19.2
180326	18.4
180508	17.6
180614	16.0
190126	24.0
<b>Mean ± SD</b>	<b>19.0 ± 3.0</b>

### 3.4. Quil-A/Cholesterol Ratios in the Products

On average, a Quil-A/cholesterol ratio in the F70 preparations is about 42:1 on weight basis (Table 3) in comparison to that of the starting ratio between Quil A/cholesterol 8:1, resulting a 5-fold reduction in cholesterol content in the final F70 products.

**Table 3.** Quil-A/cholesterol ratio in NanoQuil F70 products.

F70 Batch #	Quil-A/cholesterol (ratio)
180301	38.5
180326	42.3
180508	45.5
180614	49.5
190126	31.3
<b>Mean ± SD</b>	<b>41.2 ± 6.9</b>

### 3.5. Reduction of Hemolysis Effect after Formulating Quil-A with Cholesterol into F70

A significant reduction of hemolysis was recorded when Quil-A was formulated into F70 nanoparticles with cholesterol as demonstrated with 5 batches of F70 with a mean hemolysis reduction value of around 31% (Table 4) in comparison to the hemolysis effect of the same concentrations of Quil A in unformulated form.

**Table 4.** Reduction of hemolysis effect achieved by F70.

F70 Batch #	Hemolysis reduction (% of raw Quil-A)
180301	25.8
180326	31.9
180508	46.0
180614	25.0
190126	26.0
<b>Mean ± SD</b>	<b>30.9 ± 8.9</b>

## 4. Discussion

NanoQuil particles consisting of Quil-A and cholesterol, originally referred to as G3 particles (indicating that they constituted the third generation of saponin-containing nanoparticles after the ISCOMs [4] (as the 1st generation) and ISCOMATRIX [5] [6] (as the 2nd generation), have shown interesting and promising results in several studies since 2014. Van de Sandt *et al.* [8], Hjertner *et al.* [9] and Hellmann *et al.* [10] [11] demonstrated that this new adjuvant formulation supplemented with DT could induce cross-protective immunity against influenza virus and *in vitro* stimulated a vast array of innate immune response respectively, including Th1 immunity [9]. Initially, Quil-A + cholesterol nanoparticles gave lead to mixed populations of several different kinds of particles after overnight incubation at 37°C. Incubation temperatures higher than that had not yet been tested before the present study, largely due to the risk for saponin hydrolytic degradation. This is particularly true with QS-21 since it is highly sensitive to environmental changes such as pH, temperature etc. **Table 5** summarizes all the particles that could be generated by ISCOMs (Quil-A + cholesterol + PC), Quil-A + cholesterol and QS-21 + cholesterol.

**Table 5.** Structures created with Quil-A/QS-21 + Cholesterol (CHO) with/without Phospholipid (PC).

Morphologies created	ISCOM method		NanoQuil method	
	Quil-A + CHO + PC		Quil-A + CHO	QS-21 + CHO
1. Ring-like	X		F37	
2. Worm-like	X		F37, F70	
3. Helices	X		F37	
4. Double helices	X		F37	
5. Coil spring-like			F37	F70
6. ISCOM	X			
7. Cage-like	X			
8. Lamellae	X			

In the present study, to generate a homogeneous particle population, we did the unthinkable by incubating Quil-A or QS-21 with saturated amounts of cholesterol at 70°C for 60 minutes. Two distinct populations of particles were generated *i.e.*, worm-like and coil spring-like particles for Quil-A + cholesterol and QS-21 + cholesterol complexes, respectively. This approach solved once and for all both homogeneity in terms of particle morphology and size, and reproducibility problems while changes of other parameters such as pH, ion strength, NaCl concentration and addition of detergents etc. did not result in any significant improvement (results for these parameters are not shown). Quil-A alone, as a control, was also treated under the same conditions and result showed that its

HPLC profile was largely unchanged.

Producing a homogeneous population of such complexes in a reproducible manner is a prerequisite for transforming such a concept into a standardized product.

After optimizing the preparation protocol, we saw a recovery rate of approx. 20% of cholesterol, indicating around 80% loss was seen when particles were made according to the current protocol. Part of this may have been lumps of cholesterol retained on the sterility filter. Solubility of cholesterol in water/PBS is very low (in water, 0.095 mg/L at 30°C [12]), and only solubilized cholesterol can be utilized to form the Quil-A + cholesterol complexes, cholesterol in excess is the key to providing enough solubilized cholesterol molecules to feed the interaction between Quil-A and cholesterol.

Hemolysis effect has been used for *in vitro* evaluation of potential tissue lysis after injection of saponin products. NanoQuil alone can significantly reduce such an effect in comparison to Quil-A alone. Addition of DT to NanoQuil reduced hemolysis even further (unpublished data). This is of interest because when using Quil-A saponin in veterinary vaccines the dosing of Quil-A constitutes a balance between the adjuvant effect and the local tissue reactogenicity. Consequently, a formulation like NanoQuil F70 with reduced tissue lytic effect will allow increasing the dose of Quil-A with the aim of achieving a higher degree of immunostimulation.

This is the first detailed study to characterize the morphology and particle size distribution of the cholesterol-containing dual component nanoparticles made from either Quil-A saponin or QS-21 with cholesterol. We have identified thermal treatment as the decisive parameter for particle morphology and size. We have devised a method for obtaining a high degree of standardization, comprising a homogenous particle morphology and size distribution for NanoQuil F70, by thermal treatment, without causing heat-derived degradation of the saponin molecules.

Taken together, NanoQuil F70 formulated with Quil-A with or without DT could be used as a more cost-friendly, simplified version of ISCOMs for use as a veterinary vaccine adjuvant. On the other hand, the same adjuvant formulated with QS-21 needs to be further investigated before being considered for human use.

## Acknowledgements

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

## References

- [1] Dalsgaard, K. (1974) III. Isolation of a Substance from *Quillaja saponaria molina* with Adjuvant Activity in Foot-and-Mouth Disease Vaccines in Saponin Adjuvants. *Arch Gesamte Virusforsch*, **44**, 243-254. <https://doi.org/10.1007/BF01240612>
- [2] Reed, S., *et al.* (2013) Key Roles of Adjuvants in Modern Vaccines. *Nature Medicine*, **19**, 1597-1608. <https://doi.org/10.1038/nm.3409>
- [3] Singh, M. and O'Hagan, D.T. (2003) Recent Advances in Veterinary Vaccine Adjuvants. *International Journal for Parasitology*, **33**, 469-478. [https://doi.org/10.1016/S0020-7519\(03\)00053-5](https://doi.org/10.1016/S0020-7519(03)00053-5)
- [4] Morein, B., Sundquist, B., Höglund, S., Dalsgaard, K. and Osterhaus, A. (1984) Iscom, a Novel Structure for Antigenic Presentation of Membrane Proteins from Enveloped Viruses. *Nature*, **308**, 457-460. <https://doi.org/10.1038/308457a0>
- [5] Madhun, A.S., Haaheim, L.R., Nilsen, M.V. and Cox, R.J. (2009) Intramuscular Matrix-M-Adjuvanted Virosomal H5N1 Vaccine Induces High Frequencies of Multifunctional Th1 CD4+ Cells and Strong Antibody Responses in Mice. *Vaccine*, **27**, 7367-7376. <https://doi.org/10.1016/j.vaccine.2009.09.044>
- [6] Arumugam, B., Subramaniam, A. and Alagaraj, P. (2020) Stevia as a Natural Sweetener: A Review. *Cardiovasc Hematol Agents Med Chem*, **18**, 94-103. <https://doi.org/10.2174/1871525718666200207105436>
- [7] Kensil, C.R. (2000) QS-21 Adjuvant. In: O'Hagan, D.T., Ed., *Vaccine Adjuvants. Methods in Molecular Medicine*<sup>™</sup>, Springer, Totowa, 259-271. <https://doi.org/10.1385/1-59259-083-7:259>
- [8] van de Sandt, C.E., Kreijtz, J.H., Geelhoed-Mieras, M.M., Vogelzang-van Trierum, S.E., Nieuwkoop, N.J., van de Vijver, D.A., Fouchier, R.A., Osterhaus, A., Morein, B. and Rimmelzwaan, G.F. (2014) Novel G3/DT Adjuvant Promotes the Induction of Protective T Cells Responses after Vaccination with a Seasonal Trivalent Inactivated Split-Virion Influenza Vaccine. *Vaccine*, **32**, 5614-5623. <https://doi.org/10.1016/j.vaccine.2014.08.003>
- [9] Hjertner, B., Bengtsson, T., Morein, B., Paulie, S. and Fossum, C. (2018) A Novel Adjuvant G3 Induces Both Th1 and Th2 Related Immune Responses in Mice after Immunization with a Trivalent Inactivated Split-Virion Influenza Vaccine. *Vaccine*, **36**, 3340-3344. <https://doi.org/10.1016/j.vaccine.2018.04.054>
- [10] Hellman, S., Tydén, E., Hjertner, B., Nilfors, F., Hu, K. and Morein, B. (2021) Cytokine Responses to Various Larval Stages of Equine Strongyles and Modulatory Effects of the Adjuvant G3 *in Vitro*. *Parasite Immunology*, **43**, e12794. <https://doi.org/10.1111/pim.12794>
- [11] Hellman, S., Hjertner, B., Morein, B. and Fossum, C. (2018) The Adjuvant G3 Promotes a Th1 Polarizing Innate Immune Response in Equine PBMC. *Veterinary Research*, **49**, Article No. 108. <https://doi.org/10.1186/s13567-018-0602-2>
- [12] Dannenfeller, R. and Yalkowsky, S.H. (1989) Database for Aqueous Solubility of Nonelectrolytes. *Computer Applications in the Biosciences*, **5**, 235-236. <https://doi.org/10.1093/bioinformatics/5.3.235>