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# Preparation and Characterization of Nanoliposomes for the Entrapment of Bioactive Hydrophilic Globular Proteins

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| **Corresponding Author:** | Vânia Margaret Flosi Paschoalin<br>Universidade Federal do Rio de Janeiro Instituto de Quimica<br>Rio de Janeiro, RJ BRAZIL |
| **Corresponding Author's Institution:** | Universidade Federal do Rio de Janeiro Instituto de Quimica |
| **Corresponding Author E-Mail:** | paschv@iq.ufrj.br |
| **Order of Authors:** | Anna C. N. T. F. Corrêa |
| | Patricia R. Pereira |
| | Vânia Margaret Flosi Paschoalin |
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Rio de Janeiro, July 10th, 2019.

Xiaoyan Cao
Editor-in-Chief:
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Dear Sir

Please find enclosed the revised manuscript entitled **"Preparation and characterization of nanoliposomes for the entrapment of bioactive hydrophilic globular proteins".** The authors are Anna C. N. T. F. Corrêa, Patricia R. Pereira and Vania M. F. Paschoalin.

We believe we have fully addressed reviewer concerns. The text was modified according to the editor's suggestions, with all modifications highlighted in green. The narration text was highlighted in yellow. All video concerns were addressed. After all text modifications, language was revised by a specialized editing company in order to improve English grammar and syntax.

I hope that the manuscript and video in its revised form will be found suitable for publication as a research article in this reputable journal.

Best regards,

**Dr. Vânia M. Flosi Paschoalin**
Departamento de Bioquímica – Instituto de Química
Universidade Federal do Rio de Janeiro
e-mail: paschv@iq.ufrj.br
tel: 55 21 3938 7362  fax: 55 21 3938 7266

**TITLE:**

# Preparation and Characterization of Nanoliposomes for the Entrapment of Bioactive Hydrophilic Globular Proteins

**AUTHORS AND AFFILIATIONS:**

Anna C. N. T. F. Corrêa[1], Patricia R. Pereira[1], Vania M. F. Paschoalin[1]

[1]Chemistry Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Email addresses of co-authors**:**

Anna C. N. T. F. Corrêa (annac.correa@pos.iq.ufrj.br)
Patricia R. Pereira (patriciarp@iq.ufrj.com)

Corresponding author:
Vania M. F. Paschoalin (paschv@iq.ufrj.br)

**KEYWORDS:**

nanoparticles, size, stability, entrapment efficiency, morphological characterization, biotechnology, tarin, 47 kDa tetrameric protein

**SUMMARY:**

This study describes classical hydration using the thin lipid film method for nanoliposome preparation followed by nanoparticle characterization. A 47 kDa-hydrophilic and globular protein, tarin, is successfully encapsulated as a strategy to improve stability, avoid fast clearance, and promote controlled release. The method can be adapted to hydrophobic molecules encapsulation.

**ABSTRACT:**

Liposome nanocapsules have been applied for many purposes in the pharmaceutical, cosmetic, and food industries. Attributes of liposomes include their biocompatibility, biodegradability, non-immunogenicity, non-toxicity, and ability to entrap both hydrophilic and hydrophobic compounds. The classical hydration of thin lipid films in an organic solvent is applied herein as a technique to encapsulate tarin, a plant lectin, in nanoliposomes. Nanoliposome size, stability, entrapment efficiency, and morphological characterization are described in detail. The nanoliposomes are prepared using 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt; DSPE-MPEG 2000), and cholesterylhemisuccinate (CHEMS) as the main constituents. Lipids are first dissolved in chloroform to obtain a thin lipid film that is subsequently rehydrated in ammonium sulfate solution containing the protein to be entrapped and incubated overnight. Then, sonication and extrusion techniques are applied to generate nanosized unilamellar vesicles. The size and polydispersity index of the nanovesicles are determined by dynamic light scattering, while nanovesicle morphology is assessed by scanning electron microscopy. Entrapment efficiency is determined by the ratio of the amount of unencapsulated protein to original amount of initially loaded protein. Homogeneous liposomes are obtained with an average size of 155 nm and polydispersity index value of 0.168. A high entrapment efficiency of 83% is achieved.

## INTRODUCTION:

The number of studies investigating efficient drug delivery systems has risen in recent years. However, limitations such as rapid clearance, poor biodistribution, and solubility at physiological pH and insufficient cellular uptake still need to be surpassed. The use of nanosystems has emerged as recent progress in cancer therapeutics, applied to increase the intracellular concentration of drugs inside cancerous cells while minimizing toxicity in healthy cells. Moreover, nanoparticles obtained from a different range of materials (i.e., polymers, dendrimers, liposomes, viruses, carbon nanotubes, and metals such as iron oxide and gold) are currently being applied to enhance anticancer effects and reduce systemic toxicity[1]. Liposome nanocapsules in particular have been applied for many purposes in the pharmaceutical, cosmetic, and food industries. In recent years, various nutraceutical products such as vitamins, enzymes, and herbal extracts have been formulated using liposome technology[2].

Liposomes are spherical vesicles consisting of one or more concentric lipid bilayers spontaneously formed by the dispersion of phospholipids in aqueous media[3,4]. The polar heads of the phospholipids are located on the outer and inner surfaces of the membranes, in contact with the aqueous environment. In contrast, fatty acid chains form the hydrophobic core of the membranes and are protected from water[5]. Some attributes of liposomes that make them attractive drug delivery systems include their biocompatibility, biodegradability, non-immunogenicity, non-toxicity, and ability to entrap both hydrophilic and hydrophobic compounds[6].

Liposomes can be prepared using various processes steps such as agitation, sonication, extrusion, lyophilization, freezing, and thawing. Classical methods include reverse phase evaporation, solvent injection, and detergent dialysis. The most applied method is thin lipid film hydration, also known as Bangham's method, which is used to obtain vesicular-lipid forms[7-11]. Lamellarity (the number of phospholipid bilayers) and particle size are classical parameters used to characterize liposomes as either 1) unilamellar vesicles (ULVs), formed by a unique phospholipid bilayer and varying in size as follows: i) small unilamellar vesicles (SUVs, ~0.02–0.20 µm), ii) large unilamellar vesicles (LUVs, ~0.2–1.0 µm), and iii) giant unilamellar vesicles (GUVs, >1 µm); or 2) multilamellar vesicles (MLVs, >0.1 µm)[3,12]. Vesicle size is an important parameter when considering for therapeutic use, such as in cancer treatment, in which sizes of <200 nm are ideal to allow nanovesicles to cross the endothelial barrier and reach tumoral tissues[4].

Herein, the encapsulation procedure following the classical hydration of a thin lipid film technique[7] using tarin, a plant lectin described and characterized as a hydrophilic globular protein[13-15]. Nanosized vesicles are produced by including sonication and extrusion steps in the main technique, resulting in stable liposomal nanovesicles with high entrapment efficiency[16].

## PROTOCOL:

### 1. Preparation of tarin liposomal nanocapsules[16]

NOTE: All preparations should be prepared in triplicate in order to obtain a larger volume (7 mL) and enable the sample to be centrifuged in an ultracentrifuge (see details below).

1.1. Weigh the liposome components using an analytical balance, as shown in **Table 1**.

1.2. Dissolve the lipid components in chloroform using a 250 mL volumetric flask that fits in a rotary evaporator to avoid loss of material.

1.3. Stir the mixture at 150 rpm for 15 min.

1.4. Remove the chloroform using a rotary evaporator under the following conditions:

1.4.1. Adjust the volumetric flask mouth to the standard position (25°) for optimal efficiency, while in contact with the water from the heating bath.

NOTE: The equipment arm should be inclined at 25° to maintain contact between the volumetric flask and water bath, while not affecting evaporation efficiency or damaging the sample. The standard position can vary according to the equipment brand.

1.4.2. Set the condenser temperature to a minimum of 3 °C.

1.4.3. Set the heating bath temperature to 40 ± 1 °C.

1.4.4. Set the rotation to 120 rpm.

1.4.5. Adjust the vacuum to 207 mbar and boiling point to 20 °C.

1.4.6. After ~25 min, remove the flask and discard the evaporated solvent, remaining in the condenser, appropriately.

NOTE: A thin and opaque film, consisting of the liposome components, is formed in this step and can be easily visualized. The evaporated solvent remaining in the condenser must be stored into disposal recipients (chlorinated) to be handled by a specialized company for appropriate discarding.

1.5. Hydrate the lipid film to reach a 0.01 M lipid concentration in 0.3 M ammonium sulfate solution (pH = 7.4) containing tarin at 1 mg/mL to a final volume of 10 mL.

1.5.1. Stir the mixture for 40 min and incubate overnight at 4 °C.

NOTE: This step can be considered a stop point. Overnight incubation is not obligatory.

1.6. After incubation, sonicate the suspension for 1 min at 25 °C (room temperature; RT) to reduce vesicle size and avoid aggregation.

NOTE: Size reduction was performed in an ultrasonic sonicator under the following conditions: 130 W and 40 kHz.

1.7. Perform a 12-cycle extrusion through a 0.2 µm polycarbonate pore membrane.

NOTE: Before the extrusion process, test the mini-extruder assembly using water to avoid sample leakage. A 0.1 µm pore membrane is also suitable. In this case, pre-heat the mini-extruder holder above the lipid transition temperature to facilitate extrusion, while maintaining the physicochemical characteristics of both lipids and proteins.

1.7.1. Fit the parts of the mini extruder, as described in the manufacturer's manual.

1.7.2. Place the polycarbonate membrane between two pre-wet filter supports and place it into the holder.

1.7.3. Insert a 1 mL empty syringe into the device, fill the other syringe to its total volume with the liposomal suspension, and insert it on the opposite side.

1.7.4. Perform a 12-cycle extrusion through a 0.2 µm polycarbonate pore membrane. Push the sample from one syringe to another, slowly. Collect the extruded suspension in a pre-cooled tube.

NOTE: The polycarbonate membrane should be replaced only when sample transference from one syringe to another becomes difficult. The liposomal suspension should become clear during the extrusion process as a result of size reduction due to the formation of SUVs. About 0.2 mL of the sample can be lost during this step.

1.8. Separate liposomes by ultracentrifugation.

NOTE: Maintain the samples in an ice bath until the ultracentrifuge is ready to be used. Separate SUVs from the remaining components and ammonium sulfate by ultracentrifugation using an ultracentrifuge with a swing-bucket rotor (see details below).

1.8.1. Weigh the sample in the titanium tube that fits the rotor and balance the tubes. Check the minimum volume required according to the rotor used to avoid damaging the tubes, and adjust the liposomal suspension volume with ammonium sulphate, if necessary.

NOTE: The swing bucket should always be supported on the stand when outside of the centrifuge to avoid scratching the "zebra stripes" (i.e., the black and white stripes on the bottom), which are used by the centrifuge to determine rotation speed.

1.8.2. Turn on the vacuum before using the centrifuge to allow it to refrigerate.

1.8.3. Fit the titanium tubes into the swing bucket.

NOTE: Lift the tubes to the position they assume when running to ensure that they are perfectly fitted.

1.8.4. Release the vacuum, open the centrifuge door, and place the rotor inside.

NOTE: Pay attention to the circle mark on the bottom of the rotor, which must fit in the opposite direction of the same circle mark into the centrifuge itself.

1.8.5. Close the centrifuge door, press **Vacuum** and wait until the vacuum reaches from 200 to <20 microns or from 26 Pa to <3 Pa.

1.8.6. Adjust the parameters in the ultracentrifuge display to 150,000 x *g* (the equivalent to 29,600 rpm for the aforementioned swing bucket) for 90 min at 4 °C (acceleration: max, deceleration: max).

NOTE: Always convert the speed to x *g* if the specific centrifuge is set in rpm. Use the centrifuge website to convert the unit according to the rotor used.

1.8.7. Press **Recall**, check the conditions, and press **Start** to run.

NOTE: Wait until the centrifuge reaches the desired speed.

1.8.8. After 90 min, release the vacuum by pressing the **Vacuum** button and open the centrifuge door when the vacuum reaches 200–700 microns (equivalent to 26–93 Pa).

1.8.9. Switch off the centrifuge, remove the rotor from the inside, and leave it on the bench with the buckets to dry.

1.8.10. Maintain the ultracentrifuged samples on ice.

1.9. Carefully, separate supernatant from the pellet by turning the tube upside down into a disposable 15 mL centrifuge tube to separate the supernatant and pellet.

NOTE: Store the supernatant containing the unencapsulated protein at 4 °C. It will be used to determine the encapsulation efficiency. The pellet appears as a translucent jelly.

1.10. Suspend the pellet containing the encapsulated protein in HEPES buffered saline (3 mL of 1x HBS).

NOTE: The HBS 2x (stock solution) is prepared by diluting the following amounts of reagents in distilled water: 140 mM NaCl, 1.5 mM $Na_2HPO_4$, 50 mM HEPES, then adjusting the pH to 7.4 and final volume to 100 mL. $Na_2HPO_4$ can be replaced by $NaHCO_3$ or omitted to avoid interference if liposome concentration is to be determined. The HBS 2x stock solution should be diluted in distilled water to obtain HBS 1x before use.

**2. Encapsulation efficiency**

NOTE: Determine the encapsulation efficiency using Peterson's protocol[17] in order to avoid lipid interference in protein quantification. All samples (BSA standards and

liposome supernatant) should be analyzed in triplicate. Also prepare a blank tube.

2.1. Preparation of stock reagents and working solutions[17]

2.1.1. For stock reagents, prepare copper-tartrate-carbonate (CTC) by mixing 10 mL of 20% sodium carbonate, 200 µL of 0.1% copper sulfate, 200 µL of 0.2% potassium tartrate with 9.6 mL of distilled water. Prepare 100 mL of 10% sodium dodecyl sulfate (SDS) and 0.8 N sodium hydroxide (NaOH).

2.1.2. For working solutions, prepare 10 mL of 0.15% sodium deoxycholate (DOC) and 72% trichloroacetic acid (TCA). Dissolve 10 mg of bovine serum albumin (BSA) in 10 mL of distilled water to obtain a 1 mg/mL standard solution. Prepare reagent A by adding equal parts of CTC, NaOH, SDS, and $H_2O$. Prepare reagent B by diluting Folin-Ciocalteu phenol reagent 1:5 in distilled water.

NOTE: Reagent A requires 1 mL for each reaction tube, while reagent B requires 0.5 mL. To determine the final volumes of reagents A and B, first define the number of reaction tubes to be used, considering three distinct concentrations of BSA, blank, and samples in triplicate. Reagent A must be well-homogenized before use and can be stored at 25 °C (RT) for 2 weeks. Reagent B is also stable at 25 °C (RT) if stored in an amber bottle.

2.2. Precipitation

NOTE: This step is performed in microcentrifuge tubes.

2.2.1. Dilute the liposome supernatant with water to a final volume of 1 mL containing 5–100 µg of protein.

NOTE: The blank tube should be filled with 1 mL of distilled water.

2.2.2. Add 0.1 mL of 0.15% DOC, homogenize by vortexing, and incubate for 10 min at RT.

2.2.3. Add 0.1 mL of 72% TCA, mix well, and centrifuge at 3,000 x *g* and RT for 15 min.

NOTE: DOC-TCA promotes protein precipitation, forming two distinct phases. The target protein can be recovered by centrifugation, avoiding lipid interference.

2.2.4. Carefully discard the supernatant by verting the tube downwards and laying it on an absorbent paper. Save the pellet for the subsequent step.

NOTE: The pellet can be very difficult to see, but the tube should be turned upside down even if it is not visible.

2.3. Spectrophotometry

2.3.1. Suspend the pellet obtained from step 2.2.4 in 1 mL of distilled water. Mix

thoroughly to make sure the pellet is dissolved and transfer the sample to a new test tube.

2.3.2. Prepare dilutions of albumin (BSA) standards to a final volume of 1 mL.

NOTE: Protein standards must be prepared between 5–100 µg/mL.

2.3.3. Add 1 mL of reagent A to the tubes from step 2.3.1 and 2.3.2 without exception, mix well, and incubate for 10 min at RT.

NOTE: SDS can relieve possible lipid interferences while aiding in protein solubilization.

2.3.4. Add 0.5 mL of reagent B to the tubes from step 2.3.1 and 2.3.2, mix well, and incubate for 30 min at RT while protected from the light.

NOTE: The Follin-Ciocalteu phenol reagent is a mixture of phosphomolybdate and phosphotungstate used for colorimetric assays of some nitrogen-containing compounds, such as proteins. Copper complexation increases the reactivity of phenols towards this reagent, producing a blue/purple complex according to protein concentration.

2.3.5. Determine absorbances at 750 nm using a spectrophotometer.

2.3.6. Calculate the protein concentration in the supernatant based on the standard curve as follows.

2.3.6.1. Plot absorbance value (Abs) *vs.* BSA concentration (mg/mL) to obtain the angular coefficient (*k*) considering a linear tendency line.

2.3.6.2. Determine the supernatant protein concentration (*C*) by the ratio between the absorbance value and angular coefficient (*k*), then multiply by the total volume as follows:

$$C = \left[\frac{\text{Abs}}{k}\right] \times \ supernatant\ volume\ (mL)$$

2.4. Determine encapsulation efficiency according to the following formula:

$$Encapsulation\ Efficiency\ (\%) = \left[\frac{\text{Loaded Protein} \ - \ \text{Nonencapsulated Protein (mg)}}{Loaded\ Protein\ (mg)}\right] \times 100$$

where loaded protein = 10 mg, nonencapsulated protein = value of *C* obtained in step 2.3.6.2.

NOTE: In this case, a total of 10 mg tarin dissolved in ammonium sulfate solution (1 mg/mL) is used to perform the encapsulation procedure, since this concentration is sufficient to obtain satisfactory in vitro effects[13,16,18].

**3. Size and stability determination**

NOTE: Size distribution and polidispersity index (PdI) of the liposomal preparations are evaluated by dynamic light scattering (DLS). A PdI close to 0.1 indicates a homogeneous preparation. Nanoliposomes are stored at 4 °C and analyzed for 180 days, indicating stability during this time period. For stability determination, store liposomes at 4 °C and check size distribution and size average regularly.

3.1. Turn on the DLS equipment 30 min before use to warm up the laser lamp.

3.2. Transfer the liposomal preparation obtained in step 1.10 to a disposable sizing cuvette.

3.3. Set the equipment parameters as follows: dispersant type = water (RI = 1.33); material = lipids (RI = 1.45); and RT.

3.4. Press **Start** and wait while the equipment finishes its reading.

3.5. Remove the cuvette and turn off the equipment.

NOTE: Either transfer the sample from the cuvette back to the disposable 15 mL centrifuge tube for subsequent analyses, or discard it if there is a sufficient amount for new reads.

**4. Morphological characterization**

NOTE: Liposome characterization is performed according to Murtey and Ramasamy[19]. Samples containing nanoliposomes obtained in step 1.10 are prepared in triplicate.

4.1. Fix the glass coverslips (13 mm diameter) in the bottom of a Petri dish with double-sided tape. Cut the tape into small pieces (appropriate size to fix the coverslips), remove the protective paper underneath, and fix it on the bottom of the Petri dish. With the aid of tweezers, remove the protective paper on top of the tape and fix the coverslips on it.

NOTE: Be careful in the following steps not to release the coverslips, and use a strong tape.

4.2. Coat the coverslips with poly-L-lysine. Place wet filter papers inside the Petri dish to maintain moisture and incubate for 1 h at RT (25 °C).

4.3. After coating, rinse the coverslips with distilled water.

4.4. Fill the coverslips with a drop of the sample from step 1.10 and allow them to dry for 1 h at RT.

4.5. To fix the samples, cover them with 4% glutaraldehyde prepared in 0.1 M phosphate buffer, pH = 7.2. Place wet filter papers inside the Petri dish and seal the dish to maintain moisture levels. Incubate at 4 °C for 48 h.

4.6. Rinse the coverslips 3x for 5 min with the same phosphate buffer.

4.7. Dehydrate samples as follows: 35% ethanol 1x for 15 min, 50% ethanol 1x for 15 min, 75% ethanol 1x for 15 min, 95% ethanol 2x for 15 min, and absolute ethanol 3x for 20 min.

4.8. Chemically dry the samples by immersion 2x in 1−2 mL of hexamethyldisilazane (HMDS) for 10 min.

NOTE: The HMDS should be manipulated carefully inside a fume hood. Samples should be allowed to dry overnight inside a desiccator or inside the fume hood at RT.

4.9. Mount the dried samples on a stub with a carbon conductive adhesive tape.

4.10. Sputter the surface of the coverslip in a vacuum with an electrically conductive layer (20 nm thickness) of gold-palladium.

4.11. Record images with a scanning electron microscope (SEM) at low vacuum mode and low voltage (20 kV).

**REPRESENTATIVE RESULTS:**

**Figure 1** describes the nanoliposome preparation[16,20,21]. Phospholipids, 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt; DSPE-MPEG 2000), and cholesterylhemisuccinate (CHEMS), the main liposome constituents, were first dissolved in chloroform to obtain the lipid film. The lipid film was then rehydrated in ammonium sulfate solution containing the hydrophilic protein (tarin) to be entrapped, and the incubation was performed overnight. Then, sonication and extrusion techniques were applied to generate small unilamellar vesicles. The ultracentrifugation step separated the liposomal preparation from free lipids and unencapsulated protein, while the supernatant was used for the determination of entrapment efficiency.

Nanoliposomes produced using the aforementioned methodology exhibited a size distribution ranging from 51−396 nm and an average size of 155 nm (**Table 2**). The preparation was homogeneous, since the polydispersity index was 0.168. A high entrapment efficiency of 83% can be reached if the liposomes are extruded through a 0.2 µm pore size membrane (**Table 2**).

Morphological nanoliposome characteristics were evaluated by SEM. **Figure 2A,B** displays round-shaped liposomal vesicles in the range of 121 nm and analyzed at 20 kV, whereas **Figure 2C,D** displays inadequately prepared samples. Nanoliposomes were simply air dried without previous fixation or any other treatment described in this study. As a result, larger and damaged vesicles in the range of 332 µm and analyzed at 5 kV were observed.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Schematic representation of nanoliposome preparation.** DOPE, PEG, and CHEMS, the main liposome constituents, were first dissolved in chloroform to obtain the lipid film (**1, 2, 3**). The lipid film was then rehydrated in a saline buffer containing the hydrophilic protein (tarin) to be entrapped, and the incubation was performed overnight (**4**)**.** Then, sonication and extrusion techniques were applied to generate SUVs (**5, 6**)**.** The ultracentrifugation step separated the liposomal preparation from free lipids and unencapsulated protein, while the supernatant was used for the determination of entrapment efficiency (**7**). This figure has been modified from Correa et al.[16].

**Figure 2: Nanoliposome photomicroscopy by SEM.** (**A**,**B**) Images of round-shaped liposomal vesicles in the range of 121 nm and analyzed at 20 kV. (**C**,**D**) Images of inadequately prepared samples. Mistreated samples allowed for the observation of larger and/or damaged vesicles, which cannot resist vacuum and/or voltage conditions at 5 kV. This figure has been modified from Correa et al.[16].

**Table 1: Preparation of tarin liposomal nanocapsules.**

**Table 2: Size, polidispersity index, and entrapment efficiency of the nanoliposome preparation.**

**DISCUSSION:**

The protocol described herein was tested by Correa et al.[16] to encapsulate tarin, an immunomodulatory and antitumoral lectin purified from *Colocasia esculenta*[22]. The methodology yielded successful results, allowing for the production of stable nanoliposomes of appropriate size for therapeutic applications. The formulation presents controlled release at different pH levels under physiological conditions. It also potentiates tarin pharmacological properties, such as inhibition of human glioblastoma U-87 MG and breast cancer MDA-MB-231 cell lines and stimulation of mice bone marrow cells. The liposomal preparation exhibited no toxic effects in healthy mice cells[16].

The classical method, first described by Bangham et al.[7], allows for the production of large multilamellar liposome vesicles, heterogeneous in size and shape. Adaptations of this method, as reported in the present study, are successfully applied by including additional steps such as sonication and extrusion through a 0.2 µm polycarbonate membrane. This allows production of a more homogeneous dispersion regarding size in the nanometer range[16,23,24]. Therefore, to ensure successful results, the encapsulation protocol and liposomal formulation described here should be strictly followed.

The nanoliposome composition was carefully selected in order to ensure the formation of a bilayer membrane with DOPE, MPEG 2000-DSPE, and CHEMS as the main constituents. These are natural animal membrane bilayer constituents and the latter can confer fluidity to nanoliposome architecture, ensuring broad application for bioactive compound delivery in human beings.

Nanoliposome pegylation is essential to guarantee liposome structure stability. The absence of PEG leads to size enlargement, a high polydispersity index, and low entrapment efficiency. Optimal results can be obtained with DOPE as the main liposome component. However, this is a high-cost phospholipid. The financial costs of nanoliposome production can be achieved by replacing DOPE with other similar lipids such as DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine). CHEMS is a cholesterol molecule naturally found in animal cell membranes, which should not be excluded from the formulation, since it is important to ensure lipid bilayer fluidity and malleability[16].

Other aspects of the encapsulation protocol can also be adapted. The chloroform used to dissolve the liposomal components can easily be replaced by methanol with no effects on size average, homogeneity, and entrapment efficiency. However, some protein leakage can occur at storage under 4 °C[16]. The overnight incubation step with ammonium sulfate solution containing tarin is not mandatory; however, for convenience it can be performed with no damage to nanoliposomal biophysical characteristics, encapsulation, or stability efficiency losses, as demonstrated by Correa et al.[16]. The extrusion step is performed at room temperature, which can decrease flow rate between the syringes if a 0.1 µm pore size membrane is used.

To overcome this issue, use of a 0.2 µm pore size membrane or heating of the extruder holder above the lipid transition temperature should be considered. The analyst must be careful not to damage the lipids or protein that can be inactivated and lose biological activity. Alternatively, liposomal preparation can be dialyzed against HBS instead of ultracentrifugation, using a cut-off membrane according to protein molecular weight. The choice of chemical nature of the buffer in which nanoliposomes are suspended after ultracentrifugation is directly related to its subsequent application. Since perspectives of this study include in vivo and in vitro assays, suspension in HEPES buffered saline was adequate to ensure no cytotoxic effects and a pH range close to physiological conditions.

Liposomes should be finely treated, similar to living cells, to obtain higher quality SEM images. Fixation and drying procedures are important to ensure the visualization of smaller intact vesicles that support values higher than 20 kV under vacuum conditions. **Figure 2A,B** displays nanosized vesicles compatible with the extrusion procedure. Visualization of vesicles ranging from 51–396 nm is possible if adequate sample preparation following this procedure is performed. The steps include fixation, drying by increasing ethanol concentrations, and chemical dehydration to avoid the formation of aggregates and ruptured vesicles caused by the vacuum and electron beam. On the other hand, **Figure 2C,D** shows liposome vesicles dried under room temperature and not subjected to any treatments described here, which means that they were prepared inadequately. As a result of the inadequate procedure, giant vesicles are formed, even after extrusion through a 0.2 µm pore size membrane. Ruptured vesicles are also observed in both panels as a result of vacuum and electron beam damage.

Nanoliposome vesicles have been explored as an encapsulation and delivery system for hydrophobic molecules, including resveratrol (3,5,4’-trihydroxystilbene), a bioactive compound against colorectal cancer cells. The encapsulation procedure can overcome the poor solubility of lipophilic compounds in addition to providing biocompatibility,

biodegradability, non-immunogenicity, and non-toxicity characteristics inherent to liposome nanocapsules[25]. Protocol adaptations must be taken into consideration depending on the administration route and purpose, such as the development of new liposome formulations for oral administration.

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**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1. Wang, X., Wang, Y., Chen, Z. G., Shin, D. M. Advances of cancer therapy by nanotechnology. *Cancer Research and Treatment: Official Journal of Korean Cancer Association.* **41** (1), 1 (2009).

2. Keller, B. C. Liposomes in nutrition. *Trends in Food Science & Technology.* **12** (1), 25-31 (2001).

3. Frézard, F., Schettini, D. A., Rocha, O. G., Demicheli, C. Lipossomas: propriedades físico-químicas e farmacológicas, aplicações na quimioterapia à base de antimônio. *Quimica Nova.* **28** (3), 511-518 (2005).

4. Ferreira, D. d. S., Lopes, S. C. d. A., Franco, M. S., Oliveira, M. C. pH-sensitive liposomes for drug delivery in cancer treatment. *Therapeutic Delivery.* **4** (9), 1099-1123 (2013).

5. Papachristos, A., Pippa, N., Ioannidis, K., Sivolapenko, G., Demetzos, C. Liposomal forms of anticancer agents beyond anthracyclines: present and future perspectives. *Journal of Liposome Research.* **25** (2), 166-173 (2015).

6. Akbarzadeh, A. et al. Liposome: classification, preparation, and applications. *Nanoscale Research Letters.* **8** (1), 102 (2013).

7. Bangham, A., Standish, M. M., Watkins, J. C. Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology.* **13** (1), 238-252 (1965).

8. Szoka, F., Papahadjopoulos, D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proceedings of the National Academy of Sciences of the United States of America.* **75** (9), 4194-4198 (1978).
9. Batzri, S., Korn, E. D. Single bilayer liposomes prepared without sonication. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **298** (4), 1015-1019 (1973).
10. Zumbuehl, O., Weder, H. G. Liposomes of controllable size in the range of 40 to 180 nm by defined dialysis of lipid/detergent mixed micelles. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **640** (1), 252-262 (1981).
11. Szebeni, J. et al. Oxidation and denaturation of hemoglobin encapsulated in liposomes. *Biochimica et Biophysica Acta (BBA) - General Subjects.* **798** (1), 60-67 (1984).
12. Coelho, J. F. et al. Drug delivery systems: Advanced technologies potentially applicable in personalized treatments. *EPMA Journal.* **1** (1), 164-209 (2010).
13. Pereira, P. R. et al. Purification and characterization of the lectin from taro (Colocasia esculenta) and its effect on mouse splenocyte proliferation in vitro and in vivo. *The Protein Journal.* **33** (1), 92-99 (2014).
14. Pereira, P. R. et al. Structural analysis and binding properties of isoforms of tarin, the GNA-related lectin from Colocasia esculenta. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics.* **1854** (1), 20-30 (2015).
15. Pereira, P. R. et al. High-resolution crystal structures of Colocasia esculenta tarin lectin. *Glycobiology.* **27** (1), 50-56 (2016).
16. Correa, A., Vericimo, M. A., Dashevskiy, A., Pereira, P. R., Paschoalin, V. M. F. Liposomal Taro Lectin Nanocapsules Control Human Glioblastoma and Mammary Adenocarcinoma Cell Proliferation. *Molecules.* **24** (3), 471 (2019).
17. Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analytical Biochemistry.* **83** (2), 346-356 (1977).
18. Merida, L. A. et al. Tarin stimulates granulocyte growth in bone marrow cell cultures and minimizes immunosuppression by cyclo-phosphamide in mice. *PLoS ONE.* **13** (11), e0206240 (2018).
19. Murtey, M. D., Ramasamy, P. Sample Preparations for Scanning Electron Microscopy – Life Sciences. In *Modern Electron Microscopy in Physical and Life Sciences*. Edited by Janecek, M., Kral, R., InTechOpen. (2016).
20. Andrade, C. A., Correia, M. T., Coelho, L. C., Nascimento, S. C., Santos-Magalhães, N. S. Antitumor activity of Cratylia mollis lectin encapsulated into liposomes. *International Journal of Pharmaceutics.* **278** (2), 435-445 (2004).
21. dos Santos Ferreira, D. et al. Development of a bone-targeted pH-sensitive liposomal formulation containing doxorubicin: physicochemical characterization, cytotoxicity, and biodistribution evaluation in a mouse model of bone metastasis. *International Journal of Nanomedicine.* **11**, 3737 (2016).
22. Pereira, P. R., Corrêa, A. C. N. T. F., Vericimo, M. A., Paschoalin, V. M. F. Tarin, a Potential Immunomodulator and COX-Inhibitor Lectin Found in Taro (Colocasia esculenta). *Comprehensive Reviews in Food Science and Food Safety.* **17** (4), 878-891 (2018).
23. Olson, F., Hunt, C., Szoka, F., Vail, W., Papahadjopoulos, D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **557** (1), 9-23 (1979).
24. Mui, B., Chow, L., Hope, M. J. Extrusion technique to generate liposomes of defined

size. *Methods in Enzymology*. **367**, 3 (2003).
25. Soo, E. et al. Enhancing delivery and cytotoxicity of resveratrol through a dual nanoencapsulation approach. *Journal of Colloid and Interface Science.* **462**, 368-374 (2016).

Figure 1



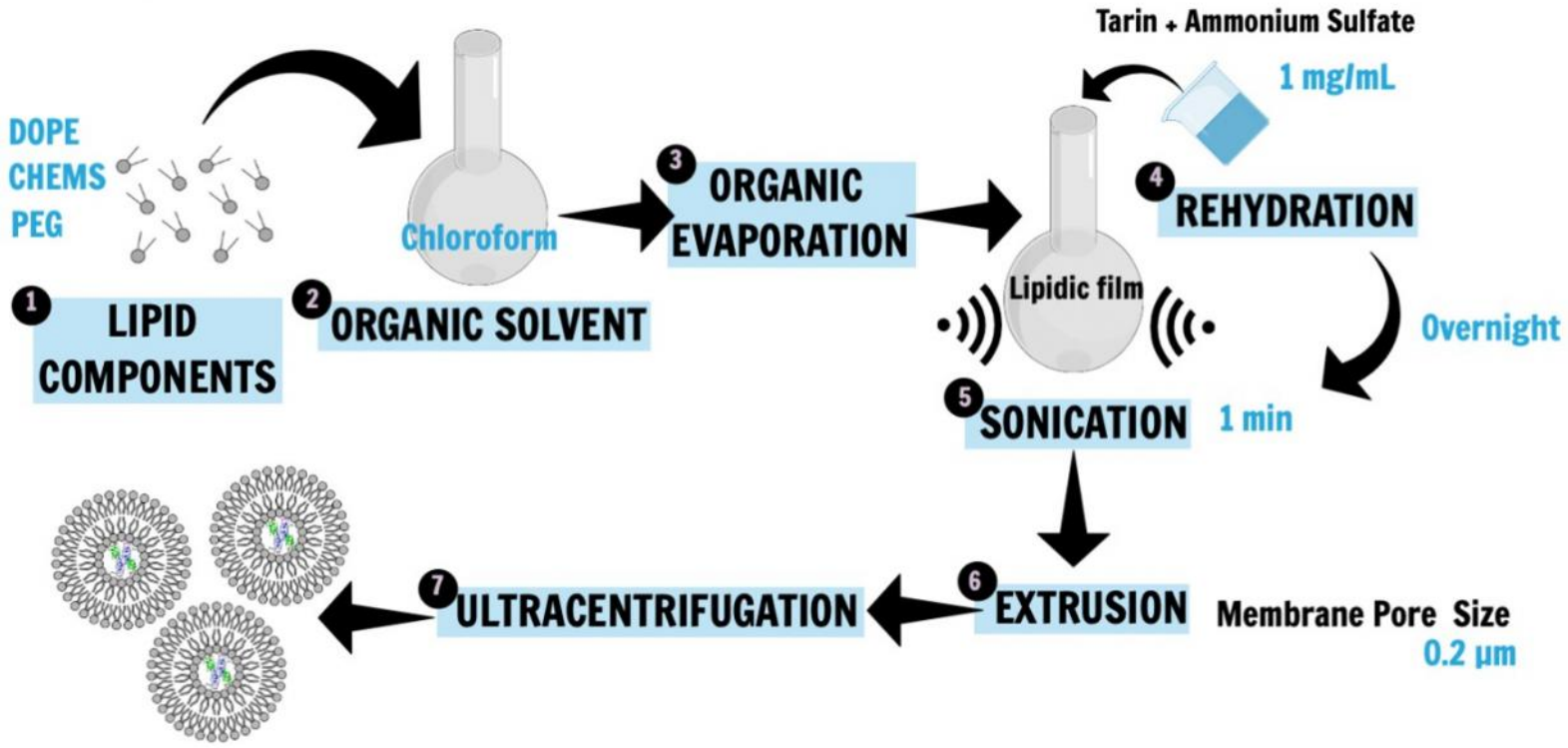

Figure 2



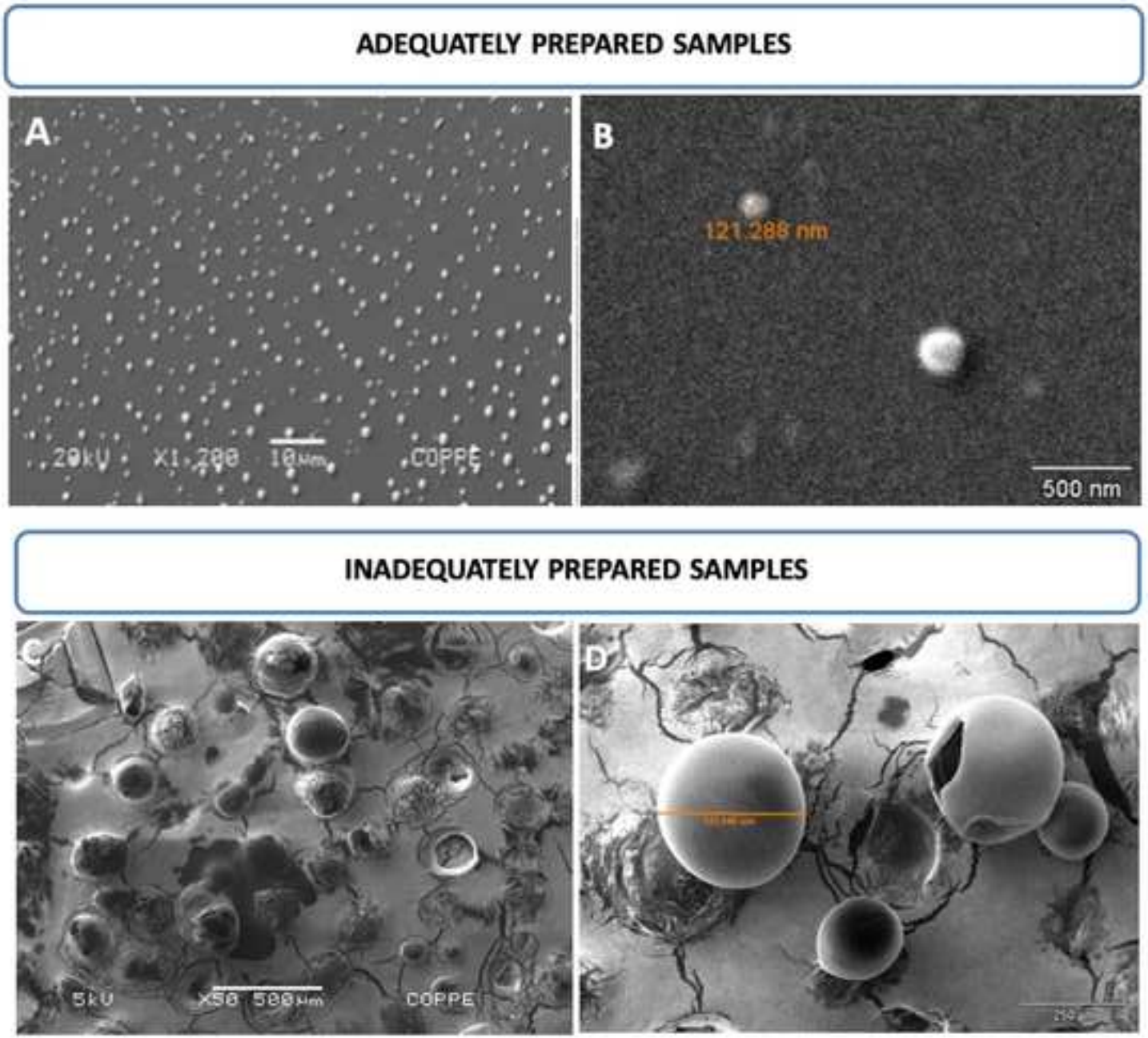

Table 1

| Liposome Components | Weight (g) | Concentration (mM) | Final volume |
|---|---|---|---|
| DOPE | 0.0420 | 5.7 | 10 mL |
| MPEG 2000-DSPE | 0.1059 | 3.8 | |
| CHEMS | 0.0024 | 0.5 | |

DOPE - 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine); MPEG 2000-DSPE - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol)-2000] (ammonium salt); CHEMS – cholesterylhemisuccinate.

Table 2



| Membrane pore size (µm) | Size distribution (nm) | Average size (nm) | Polydispersity index (PdI) | Peak (nm) | Entrapment Efficiency |
|---|---|---|---|---|---|
| 0.2 | 51 - 396 | 155 | 0. 168 | 94 ± 39 | 0.83 |

Size and polydispersity index were evaluated by dynamic light scattering, while encapsulation efficiency was determined according to Peterson[17].

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials R1.xls

| Name of Material/ Equipment | Company |
|---|---|
| Ammonium Sulfate | Sigma-Aldrich Co |
| Analitycal Ballance Mettler H10Tw | Mettler Inc. |
| Beckman DU-640 Spectrophotometer | Beckman Coulter |
| Bovine serum albumin (BSA) | Sigma-Aldrich Co |
| BUCHI Rotavapor R-300 Rotary Evaporator with Controller and V-300 Pump | Thermo Fischer Scientific |
| CHEMS (cholesterylhemisuccinate) | Sigma-Aldrich Co |
| Chloroform | Sigma-Aldrich Co |
| Copper (II) Sulfate (Pentahydrate) | Sigma-Aldrich Co |
| Coverslips (13mm diameter) | Thermo Scientific Nunc |
| DOPE(1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine) | Lipoid GMBH |
| Ethanol Absolute | Sigma-Aldrich Co |
| Folin -Ciocalteu phenol reagent | Sigma-Aldrich Co |
| Glutaraldehyde | Sigma-Aldrich Co |
| HEPES | Sigma-Aldrich Co |
| Hexamethyldisilazane (HMDS) | Sigma-Aldrich Co |
| JEOL JSM-6460 LV Sacnning Electron Microscope | JEOL LTD |
| Mini Extruder 7 | Avanti Polar Lipids |
| MPEG 2000-DSPE 1,2-distearoyl-sn-glycero-3- phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) | Lipoid GMBH |
| Optima L-90k Ultracentrifuge | Beckman Coulter |
| Phosphate Buffer | Sigma-Aldrich Co |
| Poli-L-lysine | Sigma-Aldrich Co |
| Potassium L-tartrate monobasic | Sigma-Aldrich Co |
| Sodium Carbonate | Sigma-Aldrich Co |
| Sodium chloride | Sigma-Aldrich Co |
| Sodium Deoxycholate (DOC) | Sigma-Aldrich Co |
| Sodium Dodecyl Sulfate | Sigma-Aldrich Co |
| Sodium Hydroxide | Sigma-Aldrich Co |
| Sodium phosphate dibasic anhydrous | Sigma-Aldrich Co |
| TESCAN VEGA 3 Scanning Electron Microscope | Tescan |
| Trichloroacetic Acid (TCA) | Sigma-Aldrich Co |
| Zetasizer Nano ZSP | Malvern Panalytical LTD |
| Ultrasonic cleaning bath model 2510 | Branson |

| Catalog Number | Comments/Description |
|---|---|
| A4418 | |
| 417870 | |
| 8043-30-1090 | |
| 5470 | |
| 05-001-022PM | |
| C6512 | |
| 48520-U | CAUTION |
| 209198 | |
| EW-01839-00 | |
| 565600.1 | |
| 32205 | |
| F9252 | |
| G5882 | |
| H3375 | |
| 440191 | CAUTION |
| 610000 | |
| 588200.1 | |
| PN LL-IM-12AB | |
| P3619 | |
| P8920 | |
| 243531 | |
| S7795 | |
| S7653 | |
| D6750 | |
| L3771 | |
| S8045 | |
| RES20908-A7 | |
| #657874 | |
| 91230 | |

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| | |
|---|---|
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| Department: | BIOCHEMISTRY |
| Institution: | UNIVERSIDADE FEDERAL DO RIO DE JANEIRO |
| Title: | PREPARATION AND CHARACTERIZATION OF NANOLIPOSOMES FOR THE ENTRAPMENT OF BIOACTIVE HYDROPHILIC GLOBULAR PROTEINS |
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**Manuscript JoVE59900**

We believe we have fully addressed reviewer concerns. The text was modified according to the editor suggestions, with all modifications highlighted in green. The narration text was highlighted in yellow. All video concerns were addressed. After all text modifications, language was revised by a specialized editing company in order to improve English grammar and syntax.

We hope that the manuscript and video in its revised form will be found suitable for publication as a research article in this reputable journal.

**CHANGES TO BE MADE BY THE AUTHOR(S) REGARDING THE MANUSCRIPT:**

**1. This is commercial. Please remove it.**
**Answer:** The commercial name was removed as requested (Line 96).

**2. Please specify the volume.**
**Answer:** The volume of the volumetric flask was specified (250mL) in line 101.

**3. What is this angle?**
**Answer:** The equipment arm should be inclined at 25° to maintain the volumetric flask in contact with water bath without affecting evaporation efficiency or damaging the sample. The standard position can vary according to equipment brand.
A note containing this explanation was included in lines 111-113.

**4. What solvent list?**
**Answer:** The solvent table that accompanies the equipment manual. This document lists the appropriate vacuum value according to the solvent. However, in this case, as the water bath temperature was set to 40 °C, the vacuum value was corrected to 207 mbar. For this reason, the authors judged appropriate to remove this information (line 121).
The boiling point was accidentally mistyped and was corrected in the revised manuscript (line 121) and in the video.

**5. In the condenser?**
**Answer:** The evaporated solvent collected in the condenser must be discarded into a special recipient, which will be collected by a specialized company responsible for the appropriate discard.
A sentence with this information was included in the Note of Lines 129-131.

**6. What volume is used? 10 mL?**
**Answer:** The final volume was added, as requested (Line 134).

**7. Please specify the size of the syringe.**
**Answer:** The size of the syringe was specified (1mL) and the sentence was modified (Line 158-159).

**8. What does this mean? Reunite?**
**Answer:** The sentence was rewritten to avoid misunderstandings (Line 164).

**9. Please be specific.**
**Answer:** A sentence was added (Line 180-181) to specify that the minimum volume is

determined according to the rotor used.

**10. Please use the SI unit (Pa) for pressure. Microns have different meanings.**
**Answer:** The corresponding value in Pascal was included, as requested (Lines 201 and 216).

**11. Clicking or pressing?**
**Answer:** The word clicking was replaced by pressing (Line 214).

**12. Is to be determined?**
**Answer:** The modification was included (line 236). In this study the lipid concentration was not determined, however the information is given in case the readers decide to do this.

**13. What volumes are needed?**
**Answer:** The sentence was rewritten to clarify the volumes required for the SDS and NaOH solutions (lines 249-250).

**14. What volumes are needed?**
**Answer:** A note was included to inform how to determine the volume required for reagents A and B (lines 256-259). The volumes required for DOC and TCA were included in line 250.

**15. How? By vortexing?**
**Answer:** "by vortexing" was added to the sentence (Line 276).

**16. How many test tubes?**
**Answer:** The sentence was rewritten to specify that the transference should be done to a new test tube (Lines 294-295).

**17. Do you mean the tubes in step 2.3.1 and 2.3.2?**
**Answer:** Yes. Tube specifications were added as required (Line 301).

**18. Do you mean the tubes in step 2.3.1 and 2.3.2?**
**Answer:** Yes. Tube specifications were added as required (Line 306).

**19. DLS equipment?**
**Answer:** The word DLS was added to specify the equipment (Line 347).

**20. From which step?**
**Answer:** The step corresponding to the liposomal preparation was added (Line 349).

**21. Can this be rephrased to: set the equipment parameters as follows:?**
**Answer:** The sentence was rephrased, as requested (Line 352).

**22. Press "Start"**
**Answer:** The word "click" was replaced by "press", as requested (Line 355).

**23. Please update step number.**
**Answer:** The step number was updated, as requested (Line 365).

**24. From which step?**
**Answer:** The corresponding step was added (Line 381).

**25. It seems that this reference is not listed in the references. Please included it; each**

**reference cited in text must appear in the reference list.**
**Answer:** The reference is already in the reference list but the year was accidentally mistyped and was corrected in the manuscript.

## CHANGES TO BE MADE BY THE AUTHOR(S) REGARDING THE VIDEO:

**1. 1:32: The video shows 270 mbar while the written manuscript (step 1.4.5) says 207 mbar. Please be consistent. Please also fix typo (minimum instead of minimum).**
**Answer:** The vacuum value and the word "minimum" were corrected in the video, as requested.

**2. 06:23-6:30: This detail is not included in the written manuscript. Please add a step in section 3 of the manuscript for stability determination.**
**Answer:** The corresponding sentence from the narration was added to the manuscript (Lines 338-339).

**3. Please show the title card at the end of the video.**
**Answer**: A title card was included at the end of the video, as requested.

**4. While the voiceover narration is playing, the music volume should be lowered by about 6 dB.**
**Answer:** The music volume was lowered, as requested.

**5. 1:00 - This shot of adding the components to a flask needs to be extended or cut out. It is currently not on screen long enough, and the viewer does not have enough time to process what they are seeing.**
**Answer:** The shot was extended, as suggested.

**6. 1:06, 1:23, 1:30, 1:51, 1:54, 2:09, 2:18, 2:19, 2:22, 2:27, 2:37, 3:49, 4:37, 5:42, 6:13, 6:18, 6:38, 7:22, 7:27, 8:16 - The edits here are jump cuts, which tend to have a jarring effect on the viewer. They should be smoothed out with crossfades instead.**
**Answer:** The edits were modified as suggested.

**7. 2:27 - The quick camera zoom seen here should be cut out.**
**Answer:** The quick camera zoom was cut out, as requested.

**8. 3:52 - This shot should be extended a bit. There is detail there for the viewer to see, but they are not allowed much time to do so.**
**Answer:** The shot was extended, as requested.