Calcium-responsive liposomes: Toward ion-mediated targeted drug delivery

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Abstract:

Liposomes are clinically approved supramolecular drug delivery platforms due to their ability to enhance the pharmacokinetic properties of encapsulated therapeutic agents. A key point for advancing liposomal drug delivery would be to control the timing and location of cargo release to maximize drug potency and minimize side effects. Toward this end, triggered release approaches have been developed that exploit either pathophysiological stimuli (passive release) including pH or external stimuli (active release) such as light. Here, we present a novel approach for triggering release of contents from liposomes driven by increased calcium at target sites, which plays an important role in biology related to certain diseases. In this chapter, we provide detailed experimental procedures for this project, including synthesis of calcium-responsive lipid switch **1**, evaluation of dye release properties and selectivity via fluorescence-based release assays as well as studies of morphology changes during release process by dynamic light scattering (DLS) and scanning transmission electron microscopy (STEM).

Keywords: liposomes, calcium, triggered release, drug delivery, fluorescent release assays, lipids.

1. Introduction

Liposomes have proven to be effective nanocarriers for drug delivery as they are able to encapsulate a wide range of cargo with different properties due to their unique bilayer structures.(Gregoriadis, 1973; Gregoriadis & Ryman, 1971) Liposomal complexes show significant advantages compared to free drugs including reduced drug toxicity(C.-C. Chang et al., 2008) and side effects(H.-I. Chang & Yeh, 2012) as well as improved therapeutic index.(Gabizon, 1992) To maximize drug efficacy and minimize off-target effects, efforts have been made to achieve enhanced control over the release of contents from liposomes.(Alaouie & Sofou, 2008; Arias, 2013; Preiss & Bothun, 2011) Strategies that have been reported for triggered release have primarily been divided into two categories, including passive release and active release.(Bibi, Lattmann, Mohammed, & Perrie, 2012) Passive release strategies utilize the differences in physiological conditions between healthy and diseased cells to drive release, including variations in pH,(Ellens, Bentz, & Szoka, 1984; Lee, Chen, Dettmer, O'Halloran, & Nguyen, 2007; Zhang et al., 2015) redox environment(Abbott, Jewell, Hays, Kondo, & Lynn, 2005; Ong, Yang, Cruciano, & McCarley, 2008; Park et al., 2010) and enzyme expression.(Elegbede et al., 2008; Guo, Wang, Wang, & Liu, 2012; Zhu, Kate, & Torchilin, 2012) For active release, external stimuli are exploited to trigger release, such as light,(Bayer, Alam, Mattern-Schain, & Best, 2014; Carter et al., 2014; Furuta et al., 1999; Z. Li, Wan, & Kutateladze, 2003) ultrasound(Ibsen et al., 2011; Liang et al., 2015; van Elk et al., 2014) and heat.(Chen et al., 2013; L. Li et al., 2010; Yatvin, Weinstein, Dennis, & Blumenthal, 1978)

Despite successful studies involving these stimuli-responsive liposomes in vitro, challenges remain to be solved to accommodate clinical applications. For passive release systems, a common issue is that pathophysiological conditions only exhibit minor variations from physiological conditions. For example, the extracellular pH of cancer cells is only slightly more acidic compared to normal cells (6.5-6.9 compared to 7.2-7.4), (Gillies, Liu, & Bhujwalla, 1994; Van Sluis et al., 1999) which makes it difficult to develop delivery systems possess selective response profiles based on such a small pH difference. On the other hand, actively triggered systems often face drawbacks associated with the delivery of external stimuli. In particular, UV light-induced release systems suffer from poor tissue penetration abilities and damage to healthy tissue.(Matsumura & Ananthaswamy, 2004) As a result, although certain liposome formulations have been approved for clinical use by FDA,(Davidson et al., 1994; Muggia et al., 1996) no stimuli-responsive liposomes have yet made it to the clinic.

Herein, we describe detailed procedures for an alternative strategy for liposomal

delivery instead triggered by the overabundance of chemical/metabolite profiles by exploiting molecular recognition events. Specifically, our initial system is designed to respond to elevated calcium concentrations.(Lou, Carr, Watson, Mattern-Schain, & Best, 2018; Lou, Zhang, & Best, 2019) Calcium plays an important role in biological processes including regulation of cell death,(Orrenius, Gogvadze, & Zhivotovsky, 2015) involvement in immune responses,(Gallo, Canté-Barrett, & Crabtree, 2006) and control over intra- or extra cellular signaling pathways.(Peacock, 2010) It has been shown that overabundance of calcium is a characteristic of diseases including Malaria,(Gazarini, Thomas, Pozzan, & Garcia, 2003) Alzheimer's disease,(Supnet & Bezprozvanny, 2010) and Gaucher's disease.(Vitner, Platt, & Futerman, 2010)

To achieve our goal, we designed and synthesized calcium-responsive lipid **1**, shown in Scheme 1B, by modifying a well-known calcium sensor developed by Tsien and co-workers.(Grynkiewicz, Poenie, & Tsien, 1985) To convert this sensor into a lipid that would be incorporated into liposomal membranes, hydrophobic lipid tails were installed. After chelating calcium, lipid **1** is expected to undergo a conformational change that can disrupt the membrane integrity and ultimately cause the encapsulated cargo release (Scheme 1A). Specifically, this lipid is designed to switch from a cylindrical bilayer-forming structure in free form to a cone-shaped non-bilayer structure upon calcium binding. In this article, we detail stepwise experimental procedures for this work, including the synthesis of lipid **1**, liposome preparation, fluorescent dye release assays for evaluating release efficacy, and dynamic light scattering (DLS) as well as scanning transmission electron microscopy (STEM) studies for understanding morphology change during the release processes.

[Insert Scheme 1 here]

Scheme 1. Calcium-triggered liposomal release. A) Cartoon for liposome release driven by membrane perturbation upon calcium binding. B) Hypothetical conformational changes of lipid switch **1** upon calcium binding to form **1-Ca**.

2. Synthesis of calcium-responsive lipid **1**
   2.1 Equipment and materials
      2.1.1 Chemicals
         1. 4-Hydroxy-3-nitrobenzoic acid (CAS: 616-82-0, Fisher Scientific: A10562-14)
         2. 1-Dodecylamine (CAS: 124-22-1, Fisher Scientific: AAA1551522)
         3. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, CAS: 25952-53-8, AK Scientific: 965299)

4. Hydroxybenzotriazole (HOBt, CAS: 123333-53-9, Fisher Scientific: NC9296753)
5. N, N-Diisopropylethylamine (DIEA, CAS: 7087-68-5, Fisher Scientific: AC367841000)
6. 1,2-Dibromoethane (CAS: 106-93-4, Fisher Scientific: AAA127660B)
7. Potassium carbonate (K_2CO_3, CAS: 584-08-7, Fisher Scientific: AAA1662536)
8. Cesium carbonate (Cs_2CO_3, CAS: 534-17-8, Fisher Scientific: AC19204-0050)
9. 10% Palladium on carbon (CAS: 7440-05-3, 7440-44-0, highly flammable, Fisher Scientific: AC195030010)
10. Hydrogen gas (H_2, CAS: 1333-74-0, Airgas)
11. Argon (Ar, CAS: 7440-37-1, Airgas)
12. Ethyl bromoacetate (CAS: 105-36-2, AC15859-1000)
13. 1,8-Bis(dimethylamino)naphthalene (Proton sponge, CAS: 20734-58-1, Fisher Scientific: AC169860500)
14. Sodium iodide (NaI, CAS: 7681-82-5, Fisher Scientific: S324-100)
15. Hydrochloric acid (concentrated, HCl, highly corrosive, CAS: 7647-01-0, Fisher Scientific)
16. Potassium hydroxide (KOH, highly corrosive, CAS: 1310-58-3, Fisher Scientific: P251-500)
17. Sodium sulfate (Na_2SO_4, CAS: 7757-82-6, Fisher Scientific: s421-3)

2.1.2 Solvents

1. Dimethylformamide (DMF, CAS: 68-12-2)
2. Ethanol (EtOH, CAS: 64-17-5)
3. Ethyl acetate (EtOAc, CAS: 141-78-6)
4. Tetrahydrofuran (THF, CAS: 109-99-9)
5. Chloroform ($CHCl_3$, CAS: 67-66-3)
6. Dichloromethane (DCM, CH_2Cl_2, CAS: 75-09-2)
7. Acetone (CAS: 67-64-1)
8. Methanol (MeOH, CAS: 67-56-1)
9. MilliQ water purified from Millipore water system

2.1.3 Materials and equipment

1. Silica G TLC plate, with UV254, aluminum backed (Sorbtech: 1634126)
2. Silica gel, standard grade, 60 Å, 40-63 μm (Sorbtech: 30930M-25)
3. Neutral alumina, 32-63 μm (Sorbtech: 16050-5)
4. 50 mL, 100 mL, and 250 mL round bottom flasks; glass chromatography columns with reservoir; 125 mL, and 250 mL

separatory funnel; Büchner funnel with glass frits from Pyrex™, Synthware™ or Chemglass™.
5. Büchi R-124 rotary evaporator
6. Innovative Technologies (Newburyport, MA) Pure Solv MD-7 Solvent Purification System
7. Millipore water system (≥18 MΩ·cm triple water purification system)
8. Varian 500 MHz NMR spectrometer
9. JEOL DART-AccuTOF mass spectrometer
10. Q-Star XL quadrupole time-of-flight hybrid mass spectrometer (Applied Biosystems, Foster City, CA)

2.2 Procedure overview

The synthetic route to calcium-responsive lipid **1** is shown in Scheme 2. This began with reaction of 4-hydroxy-3-nitrobenzoic acid with dodecylamine under common amide bond coupling conditions to install the lipid tail of compound **3**. 1,2-Dibromoethane was then used to link two units of **3** together via the formation of the ethyl bridge of **4**. After that, catalytic hydrogenation was performed to reduce the nitro groups of **4** into the amine groups of **5a**, which were then alkylated by addition of ethyl bromoacetate to afford compound **5b**. The last step involved ester hydrolysis using potassium hydroxide followed by acidification to obtain lipid **1** in its protonated form. Detailed synthetic procedures are listed in the following sections as along with synthetic notes for each step.

[Insert Scheme 2 here]

Scheme 2. Synthesis of calcium-responsive lipid switch **1**. A dodecylamine group was coupled onto precursor **2** to produce the amide of **3**, followed by dimerization through reaction of the phenol group with dibromoethane to **4**, nitro reduction to the amines of **5a**, alkylation to introduce four ethylacetate groups to access **5b**, and finally ester hydrolysis to generate **1** in its protonated form.

2.2.1 Synthesis of compound **3**

1. Weigh compound **2** (1 g, 5.46 mmol, 1 eq.), dodecan-1-amine (1.52 g, 8.2 mmol, 1.5 eq.) and HOBt (1.256 g, 8.2 mmol, 1.5 eq.), and combine these in a 100 mL round bottom flask under a balloon of argon.
2. Dissolve the reaction mixture in 15 mL dry DMF and stir at 0 °C for 5 min.
3. Add EDCI (1.57 g, 8.2 mmol, 1.5 eq.) and DIEA (2.4 mL, 13.65 mmol, 2.5 eq.) into the round bottom flask.
4. Allow the reaction mixture to warm up to room temperature and further stir for 5 hours.

5. Quench the reaction by adding 3 mL of water and then pour the mixture into 100 mL of a 1 M aqueous solution of HCl.
6. Pour the reaction mixture into a separatory funnel and add 25 mL of EtOAc. Remove the organic layer (top), and repeat the EtOAc extraction procedure two more times.
7. Combine the EtOAc extractions in a separatory funnel, add 30 mL water, and remove the water layer (bottom). Repeat this wash step four more times with water and one time with brine (saturated aqueous solution of sodium chloride).
8. Transfer the EtOAc solution to an Erlenmeyer flask, add a few scoops of sodium sulfate (Na_2SO_4) to dry the solution, and filter through gravity filtration using a funnel fitted with a cotton ball.
9. Remove the solvent using a rotary evaporator.
10. Recrystallize product by dissolving the crude product in a minimal amount of heated solvent, 95% ethanol, followed by cooling and filtration to afford compound **3** as a pale yellow solid (1.54 g, 4.39 mmol, 80% yield).

2.2.2 Synthesis of compound **4**

1. Weigh compound **3** (1.0381 g, 2.96 mmol, 2 eq.), Cs_2CO_3 (1.265 g, 3.88 mmol, 1.3 eq.) and K_2CO_3 (0.691 g, 5 mmol, 1.7 eq.), and combine these in a 100 mL round bottom flask, which then sealed and placed under a balloon of nitrogen.
2. Dissolve this mixture in 15 mL DMF followed by addition of 1,2-dibromoethane (0.1276 mL, 1.48 mmol, 1 eq.).
3. Heat the reaction at 120 °C by fitting the flask with a water-cooled condenser while allowing to stir overnight.
4. Remove the heat and allow the reaction to cool back down to room temperature and dilute the mixture with 150 mL water to form a yellow suspension.
5. Pour the suspension into a 250 mL separatory funnel and add 30 mL chloroform. Remove the bottom organic layer and repeat this extraction process two more times.
6. Combine the chloroform extractions back into the separatory funnel, add 50 mL water, and remove the aqueous layer (top). Repeat this wash step two more times with water and once with brine.
7. Collect the organic extraction in an Erlenmeyer flask. Add a few scoops of Na_2SO_4 and filter it off using a glass funnel fitted with a cotton ball. Remove the solvent using a rotary evaporator.
8. Perform column chromatography using a silica gel column via gradient elution from 50% EtOAc-hexane to 10% MeOH-DCM to obtain

compound **4** as a pale yellow solid. (0.9189 g, 1.264 mmol, 85% yield). TLC Rf=0.5 (5% MeOH-DCM as eluant).

2.2.3 Synthesis of compound **5a**

1. Weigh compound **4** (0.2431 g, 0.334 mmol, 1 eq.) in a 100 mL round bottom flask, and then seal under a balloon of argon.
2. Carefully transfer 10% Pd/C (0.0486 g, 0.2 eq.) into the reaction flask.
3. Add 20 mL EtOAc.
4. Replace the argon atmosphere with a hydrogen balloon.
5. Stir the reaction overnight at room temperature.
6. Once complete, carefully remove the Pd/C via vacuum filtration through glass fritted Büchner funnel coated with celite.
7. Remove the solvent using a rotary evaporator.
8. Purify the crude using a column packed with neutral alumina. Utilize gradient elution from chloroform to 1% MeOH-chloroform to obtain **5a** as a yellow solid. (130 mg, 0.195 mmol, 58% yield). TLC Rf=0.4 (eluant: 7.5% MeOH-chloroform).

2.2.4 Synthesis of compound **5b**

1. Weigh **5a** (40 mg, 0.06 mmol, 1 eq.), sodium iodide (47.7 mg, 0.318 mmol, 5.3 eq) and proton sponge (68.13 mg, 0.318 mmol, 5.3 eq.) in a small 1-dram vial, and seal this under argon.
2. Dissolve this mixture in 1 mL dry DMF.
3. Add ethyl bromoacetate (53 µL, 0.318 mmol, 5.3 eq.) and stir the reaction at 80 °C by using a fitted, water-cooled condenser for 24 hours.
4. After completion, remove the DMF solvent using a rotary evaporator.
5. Purify the crude using a column packed with silica gel via gradient elution from chloroform to 6% methanol-chloroform to afford **5b** as a brown solid. (35 mg, 0.035 mmol, 58% yield). TLC Rf=0.3 (10% methanol-chloroform as eluant).

2.2.5 Synthesis of compound **1**

1. Dissolve **5b** in a 1-dram vial in 800 µL THF and 200 µL methanol.
2. Add 150 µL of a 1 M aqueous solution of KOH and stir the reaction at room temperature overnight.
3. Remove the organic solvent using a rotary evaporator.
4. Re-dissolve the crude in 5 mL MilliQ water.
5. Add an aqueous solution of 4 M HCl dropwise. A precipitate will form once the pH reaches 1.
6. Obtain compound **1** via vacuum filtration using a Büchner funnel as a pale brown solid. (13 mg, 0.014 mmol, 73% yield).

2.3 Synthetic notes

2.3.1 General notes

1. ^{1}H and ^{13}C NMR spectra were taken using a Varian 500 MHz NMR spectrometer and analyzed with MestReNove software.
2. HRMS-DART analysis in positive mode works well for small molecules with molecular masses around or less than 1000. For detecting larger molecules or when negative mode is needed, ESI-MS is preferred.
3. Detailed structure analysis information can be found in (Lou et al., 2018)
4. When listed as such, dry solvents were all obtained using dry solvent delivery system.

2.3.2 Synthesis of compound **3**

1. Dry DMF is needed to ensure a relatively high reaction yield.
2. Layers may be hard to separate during extractions due to the poor solubility of lipid compounds. Addition of a small amount of methanol or brine can be considered to aid the separation.
3. DMF has a high boiling point and is therefore hard to remove using a rotary evaporator. Taking advantage of its solubility in water, washing the combined organic layer five times (or even more) is recommended.
4. An appropriate amount of ethanol is necessary for recrystallization to obtain pure product with high yield. To do so, first dissolve the crude in a tiny amount of hot ethanol in an Erlenmeyer flask. Keep adding hot ethanol slowly until all the crude has dissolved. Addition of one or two extra mLs of hot solvent is recommended at this point. Then, the flask is allowed to cool down to room temperature slowly, ideally placed in an insulated location, to ensure the crystallization process happens slowly and undisturbed.

2.3.3 Synthesis of compound **4**

1. Either K_2CO_3, Cs_2CO_3 or a combination of both can be used as the base. However, it is preferred to dry them in the oven prior to their use.
2. Anhydrous DMF is needed for this reaction.
3. Careful control of the reaction temperature is needed, as the boiling point of 1,2-dibromoethane is 128 °C. Overheating of the reaction may result in evaporation of the starting material.
4. It is recommended to track the reaction by TLC and adjust reaction time accordingly to ensure the complete conversion of the starting material and relatively high yield.

2.3.4 Synthesis of compound **5a**

1. Hydrogenation reactions represent a fire hazard since flammable Pd/C catalyst and hydrogen gas are involved. Extreme care must be taken when running this reaction.
2. Pd/C is highly flammable in air. It can also ignite organic solvent and hydrogen gas. Therefore, one should remove all of the organic solvents that are not necessary during the reaction and work up process. Pd/C is more dangerous post-reaction due to the presence of the hydrogen gas. Solvents with less of a fire hazard are preferred during workup including methanol or ethanol, while ether or THF should be avoided.
3. Double layer balloons for treatment with hydrogen are desired to prevent hydrogen gas from escaping from the balloon as much as possible.
4. Replacing the Ar atmosphere with H_2 is needed during reaction setup for safety purposes. However, considering argon has a much higher density comparing to hydrogen, it is recommended that hydrogen gas is introduced via a long needle to make sure all the argon can be removed.
5. Long needles used to introduce H_2 should be inserted as close to the reaction interface as possible to ensure significant contact with the reaction mixture. Also, needles can be clogged during the reaction, and thus is recommended to check them regularly.
6. It is recommended to track the reaction progress via TLC. To do so, hydrogen gas is replaced with a nitrogen atmosphere every time prior to removing an aliquot for TLC. After removal of the TLC aliquot, N_2 is then be replaced with H_2 again.
7. The addition of protic solvent can facilitate the reaction rate. If the reaction tends to be too slow, addition of methanol or ethanol can be considered.
8. Amine compounds can interact and stick on column if silica gel is used. To avoid that, alumina is preferred as the stationary phase for column chromatography. Also, slowly increasing the polarity of the elution system is essential when running the alumina column.
9. Mixed solvents of chloroform and methanol are needed to dissolve **5a** for transfer or for analysis such as by NMR.

2.3.5 Synthesis of compound **5b**

1. The use of aqueous solvents during the workup should be avoided as the product may partition into the aqueous layer.
2. After removal of DMF using a rotary evaporator, the vial should be dried under high vacuum for long enough to make sure the DMF is

completely removed, which can be determined by NMR.

2.3.6 Synthesis of compound **1**

1. As the product of this reaction is purified via precipitation, any scale smaller than what we reported here is not recommended in case the precipitation is not sufficient for product collection.
2. Vacuum filtration may not be ideal for small scale reactions. Thus, centrifugation may serve as an alternative method. In that case, the supernatant should be discarded, and the residual crude has to be washed several times with water.
3. Once synthesized, compound **1** should be stored in a -20 °C freezer to minimize compound decomposition.

3. Liposome preparation (three types of samples, with encapsulated Nile red, sulforhodamine B and without dye)

Once synthetic compound **1** is in hand, this compound can be incorporated into liposomes at different percentages to be analyzed by dye release assays and microscopy studies. Preparation of separate samples of liposomes used in this chapter encapsulating different fluorescent dyes will be presented in this section.

3.1 Equipment and materials

1. L-α-phosphatidylcholine (Egg, Chicken, PC, Avanti Lipid: 840051)
2. Nile red (NR, CAS: 7385-67-3, Fisher Scientific: AC415710010)
3. Sulforhodamine B sodium salt (SRB, CAS: 3520-42-1, Fisher Scientific: AAA1476906)
4. MilliQ water purified from Millipore water system
5. Water bath
6. Dry ice
7. Isopropanol (CAS: 67-63-0)
8. Sephadex G-50 (Fisher Scientific: 45-000-018)
9. OHRUS analytical-grade balance
10. Fisherbrand™ Elite™ adjustable volume pipettes
11. Millipore water system (≥18 MΩ·cm triple water purification system)
12. Avestin LiposoFast Basic extruder with 200 nm polycarbonate membrane (Avestin, Ottawa, Canada)

3.2 Procedure

1. Prepare stock solutions of appropriate lipids and dyes as follows. Dissolve 4.41 mg Nile red in 2.77 mL chloroform to prepare a 5 mM stock solution. Dissolve 25 mg PC in 1.014 mL chloroform to prepare a 32 mM PC stock solution. For

lipid **1**, a 5 mM stock solution is made by dissolving 2.6 mg **1** in 580 µL of a 50% methanol-chloroform mixture. Dissolve 0.2323 g SRB in 20 mL MilliQ water to obtain a 20 mM Sulforhodamine B solution.

2. Pipette the proper amount of each stock solution into a clean 1-dram vial to match with the desired molar ratio in the liposomes that are being prepared. As an example, to make 250 µL of a 2 mM solution of Nile red-encapsulated liposomes containing 10% of **1** doped into PC, 13.3 µL of PC stock (85%), 10 µL of lipid **1** stock (10%) and 5 µL of Nile red stock (5%) are added into a vial. Nile red is calculated to be 5% of the total lipid content. For SRB-encapsulated liposomes and liposomes without dye, only PC (control liposomes) and PC/lipid **1** should be pipetted into the vial at this point.
3. Evaporate the organic solvent under nitrogen stream to afford a lipid film.
4. Leave the lipid film under high vacuum for at least one hour to remove any residual solvent.
5. Hydrate the lipid film with MilliQ water at 50 °C for three sets of 10 min with vigorous vortexing after each set. For SRB-encapsulated liposomes, 20 mM SRB stock solution should instead be used for the hydration. For example, if liposomes are prepared at the same scale mentioned in point 2 above, 250 µL of MilliQ water or SRB stock solution should be added.
6. Subject the resulting solution to ten freeze-thaw cycles by switching between a dry ice/acetone bath and a 50 °C water bath.
7. Extrude the liposome solution using an Avestin LiposoFast extruder equipped with 200 nm polycarbonate membrane for 15 passes.
8. For SRB-encapsulated liposomes, a size exclusive column packed with Sephadex G-50 is needed to remove unencapsulated dye. To do so, first pre-equilibrate Sephadex G-50 gel in MilliQ water for at least one hour. Pack a micro-column with the gel and load the liposome solutions onto the column. Run the column under normal pressure and take fractions every ~1 mL. The first pink fraction is typically the one with purified liposomes.

3.3 Notes

1. Lipid **1** can only dissolve in a mixture of methanol and chloroform due to its amphiphilic properties as a result of the hydrophobic tail region and hydrophilic head group.
2. The lipid stock solutions should be stored in -20 °C freezer after preparation.
3. Tiny amounts of compounds (< 5 mg) should be weighed using an analytical balance, preferably with five decimal places to ensure accuracy.
4. Normal pipette tips are not ideal for organic solvents. Solvent safe pipette tips should be used. If only normal tips are available, special care should be taken to make sure the proper volume is measured.

5. When forming the lipid film, a nitrogen flow is enough to remove small volumes of organic solvent. If more than 1 mL solvent is used, evaporation under rotary evaporator is instead recommended.
6. During freeze-thaw cycle, the surface of the vial should be wiped clean before subjecting it to hot water bath in case any dry ice resides on the surface.
7. For extrusion, more passes will result in more uniform size particles. An odd number of passes should be performed to ensure the final liposome solution ends up in a different syringe from where they start. To prevent dead volume and minimize bubbling during extrusion, pre-wetting the assembled extruder with proper buffer or MilliQ water can be considered.
8. Polycarbonate membranes should be checked after extrusion. If ripping is observed, the liposomes have to be re-extruded.
9. Sephadex G-50 used for column chromatography should be pre equilibrated with MilliQ or buffer for at least one hour prior to use. Other than a micro column, glass pipette can also be used. During the column, no pressure should be applied, and gravity elution is sufficient. When collecting fractions, the first fraction showing pink color is usually the liposome solution, which can be further checked by adding Triton X-100 under a UV lamp, after which an increase in fluorescent intensity should be observed denoting the release of encapsulated dye.
10. Prepared liposomes should be stored at 4 °C and should be used up within 2 days, after which decomposition becomes problematic.

4. Nile red release assay

4.1 Equipment and materials

1. 2 mM Nile red encapsulated liposomes prepared from Section 3, containing only PC or PC as well as different percentages of lipid **1**.
2. Calcium chloride hexahydrate ($CaCl_2 \cdot 6H_2O$, CAS: 7774-34-7, Fisher Scientific: C78-500)
3. Starna 50 µL quartz fluorimeter cell (Fisher Scientific: NC9234251)
4. PerkinElmer LS55 fluorimeter
5. Software: OriginPro 2017

4.2 Procedure

1. Prepare a 0.1 M calcium chloride solution by dissolving calcium chloride in MilliQ water
2. Add 100 µL of a 2 mM solution of liposomes into a micro quartz cuvette.
3. Measure the initial fluorescence intensity of this sample. (Excitation wavelength = 552 nm, excitation slit = 10 nm, emission slit = 5 nm, emission scan range: 580 nm - 700 nm).
4. Perform a titration by adding 0.5 µL of the 0.1 M calcium chloride for each data

point into the cuvette and take fluorescent intensity readings every time after adding calcium. (Increment ≈ 0.5 mM)

5. To another 100 µL solution of the exact same batch of liposomes, add 0.5 µL MilliQ water each time to measure the fluorescent intensity decrease caused by dilution.
6. Select fluorescent intensities at 620 nm and subtract out the intensity decrease due to dilution.
7. Repeat the experiments at least three times with different batches of liposomes.
8. The results are reported as a percentage of initial fluorescence. Representative release curves are shown in Figure 1a.

4.3 Notes

1. When adding calcium chloride into the cuvette, bubbles may form in the liposome solution. If bubbles are observed, the pipette tip can be used to gently remove them.
2. Pink particles can be seen during the titration process due to the disruption of the membrane and leakage of the encapsulated cargo. This may affect the light pathlength and cause signal fluctuation, which can be averaged out by repeating the experiment multiple times.
3. Error bars on the graph denote standard errors.

[Insert Figure 1 here]

Figure 1. A) Dose-dependent release of NR from PC liposomes containing 0%, 3%, 5% or 10% lipid **1**. Increased release can be achieved with higher percentages of lipid **1**. Insert: cartoon depiction for hydrophobic dye release assay and representative image before and after titration. B) Sulforhodamine B release study of liposomes containing lipid **1**. Liposomes containing 10% of lipid **1** yielded a greater fluorescence increase compared to PC liposomes. Insert: cartoon depiction for hydrophilic dye release assay.

5. SRB release assay

5.1 Equipment and materials

1. SRB encapsulated liposomes prepared according to section 3, containing only PC or PC spiked with 10% of lipid **1**.
2. Calcium chloride stock solution from section 4.
3. Triton X-100 (CAS: 9002-93-1)
4. Starna 50 µL quartz fluorimeter cell (Fisher Scientific: NC9234251)
5. PerkinElmer LS55 fluorimeter
6. Software: OriginPro 2017

5.2 Procedure

1. Prepare 10% Triton X-100 solution by dissolving 1 mL Triton into 10 mL MilliQ

water.

2. Add 100 µL of the appropriate liposome solution into a quartz cuvette and take the initial fluorescent reading. (Excitation wavelength = 550 nm, excitation slit = 7.5 nm, emission slit = 5.0 nm, emission scan range: 560 nm - 660 nm).
3. Add 1.0 µL of the calcium chloride solution into the cuvette each time and measure the fluorescent intensity every time after adding calcium. (Increment ≈ 1.0 mM)
4. After adding 20 mM calcium, add 2 µL 10% Triton X-100 to lyse the liposomes, and take a final fluorescence reading to approximate complete release.
5. Repeat the experiment at least three times with different batches of liposomes.
6. When processing the data, select fluorescent intensities at 585 nm and convert each point to a percentage of the fluorescence after triton X-100 addition to approximate the percentage of total dye released. A sample curve is shown in Figure 1b.

5.3 Notes

1. For the SRB release assay, the averaging of three scans for each fluorescent reading is recommended to minimize the noise.
2. Similar to the Nile red release assay, bubbles may be observed after adding a small volume of calcium stock solution into the cuvette. If so, gentle removal of the bubbles with pipette tips is suggested.
3. Error bars on graph indicate standard errors from at least three replicate experiments.

6. Nile red release with other metal ions

6.1 Equipment and materials

1. Zinc chloride ($ZnCl_2$, CAS: 7646-85-7)
2. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, CAS: 7786-30-3, 7791-18-6, Fisher Scientific: BP214-500)
3. Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$, CAS: 7791-20-0)
4. Cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$, CAS: 7791-13-1)
5. Iron(III) chloride ($FeCl_3$, CAS: 7705-08-0, Fisher Scientific: AA1235709)
6. Sodium chloride (NaCl, CAS: 7647-14-5, Fisher Scientific: S271-3)
7. Potassium chloride (KCl, CAS: 7447-40-7, Fisher Scientific: P217-500)
8. Copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$, CAS: 10125-13-0)
9. Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$, CAS: 13446-34-9)
10. 2 mM liposomes encapsulated with Nile red prepared according to section 3, containing only PC or PC as well as 10% lipid **1**.
11. Calcium chloride stock solution from section 4.
12. Starna 50 µL quartz fluorimeter cell (Fisher Scientific: NC9234251)
13. PerkinElmer LS55 fluorimeter

14. Software: OriginPro 2017

6.2 Procedure

1. Prepare a 0.1 M solution of the appropriate metal ion stock solution in MilliQ water
2. Add 50 µL of the appropriate liposome sample into a quartz cuvette and take the initial fluorescent reading. (Excitation wavelength = 552 nm, excitation slit = 10 nm, emission slit = 5 nm, emission scan range: 580 nm - 700 nm).
3. Add 5 µL of a 0.1 M metal ion stock solution into the cuvette and incubate for 40 min before measuring the fluorescence intensities. (Final concentration for the metal ion is approximately 10 mM).
4. Add 5 µL of MilliQ water into the exact same batch of liposomes to measure the intensity decrease caused by dilution.
5. Select intensities at 620 nm and subtract the intensity decrease from dilution. Report the results as a percentage of initial fluorescent intensity.
6. Repeat the whole experiment at least three times with different batches of liposomes. A representative bar graph is shown in Figure 2.

6.3 Notes

1. As the minimum volume for operating a sub-micro cuvette is 50 µL, care must be taken when adding exactly 50 µL liposome solution to avoid incorrect fluorescence readings.
2. As is mentioned in sections 4.3 and 5.3, any bubbles observed should be gently removed using pipette tips.
3. The 40 min incubation time is used to ensure the same ending point as the Nile red release assay.

[Insert Figure 2 here]

Figure 2. Selectivity of triggered release with different common metal cations. K^+, Na^+, and Zn^{2+} did not cause release, while Ca^{2+} resulted in the greatest release. (Final cation concentration = 10 mM)

7. DLS analysis

7.1 Equipment and materials

1. Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW laser operating at λ =633 nm.
2. Nile red-encapsulated liposome samples before and after calcium treatment from section 4.
3. MilliQ water
4. Disposable plastic micro cuvette (For size measurement at a 173° scattering angle, a minimum of volume 40 µL, Malvern Panalytical Inc.)

7.2 Procedure

1. Dilute the samples before and after calcium addition 1:10 with MilliQ water. As an example, add 5 µL liposome solution into 45 µL MilliQ water.
2. Pipette 50 µL of the diluted sample into the micro cuvette.
3. Insert the cuvette into instrument and allow the sample to equilibrate for 30 seconds at 20 °C.
4. Measure the Z-average reading of each set of liposome samples.
5. Repeat the experiments at least three times with different batches of liposomes. A sample result is shown in Figure 3.

7.3 Notes

1. The micro cuvette is designed for at least 40 µL sample. As always, care has to be taken to make sure there are no bubbles present during measurement.
2. For MilliQ water used in DLS experiments, it is preferred to filter this through filter prior to use to prevent the influence from any residual particle or dust left in MilliQ that can interfere with the results.

[Insert Figure 3 here]

Figure 3. DLS studies before and after triggering release driven by Ca^{2+}. Dramatic changes in particle size were observed only when lipid **1** is present.

8. STEM imaging

8.1 Equipment and materials

1. Zeiss Auriga 40 microscope operating in scanning transmission (STEM) mode with a beam energy of 30 keV
2. Two batches of 2 mM liposomes composed of entirely PC or 10% lipid **1** and PC without dye encapsulation prepared according to the procedure listed in section 3.2.
3. A 0.5% (w/v) solution of phosphotungstic acid

8.2 Procedure

1. Treat two liposome samples with 20 mM calcium to prepare for three different liposome samples ready for imaging, including PC liposomes containing 10% lipid **1** before (only treated with water) and after (treated with calcium) triggering release and PC liposomes after treating with the same amount of calcium.
2. Immobilize a drop (5-10 µL) of each solution onto a carbon film supported by a 200-mesh copper grid.
4. Stain with 0.5% (w/v) solution of a phosphotungstic acid solution.
3. Store the sample in a desiccator overnight prior to examination.

4. Take images and process the images with ImageJ.
5. Repeat the experiment three times with different batches of liposomes. Sample images are shown in Figure 4.

8.3 Notes

1. For electron microscopy studies, no dyes should be encapsulated within the membrane.
2. Dilution of the liposome samples may be desired to reduce sample aggregation.
3. Beam energy has to be carefully determined to minimize the damage to the liposome samples.

[Insert Figure 4 here]

Figure 4. STEM images of liposomes: A) containing 10% of **1** before calcium addition, B) containing 0% of **1** after 20 mm calcium addition, and C) containing 10% of **1** after 20 mm calcium addition. The scale bar in each image denotes 200 nm.

9. Summary and conclusions

   The calcium-responsive lipid switch **1** provides a novel approach to triggering release utilizing the differences in the abundance of this critical cation between healthy and diseased cells. Detailed fluorescent dye release assays were performed to evaluate the release efficacy of the system, as well as morphology analysis via DLS and STEM to understand the mechanism of release based on changes in lipid self-assembly properties. Similar to this strategy, other aberrantly expressed biomolecules can also act as exciting targets for liposomal delivery systems using different lipid switches.

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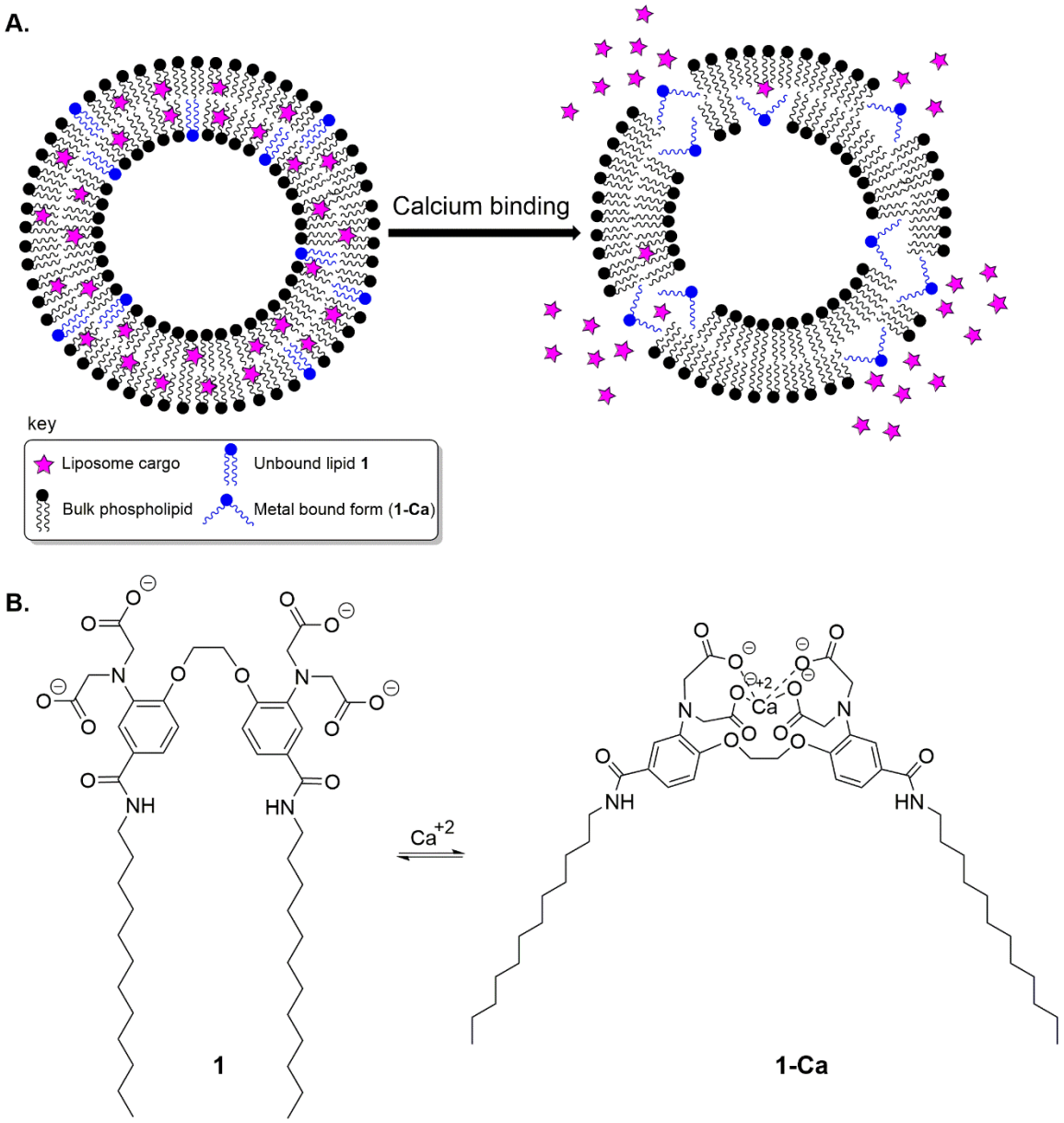
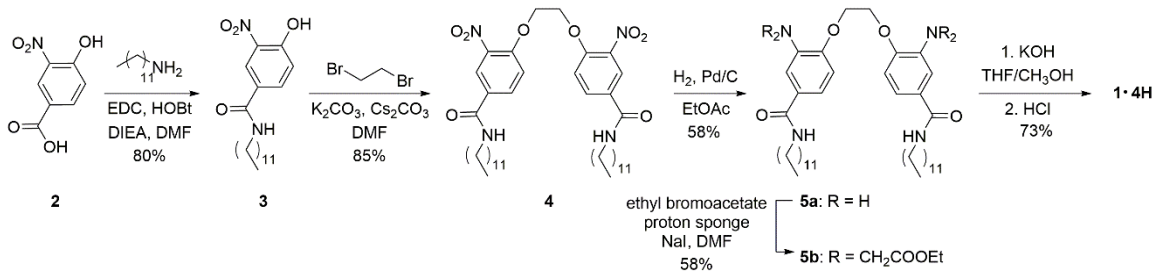
Figures:



Scheme 1. Calcium-triggered liposomal release. A) Cartoon for liposome release driven by membrane perturbation upon calcium binding. B) Hypothetical conformational changes of lipid switch **1** upon calcium binding to form **1-Ca**.



Scheme 2. Synthesis of calcium-responsive lipid switch **1**. A dodecylamine group was coupled onto precursor **2** to produce the amide of **3**, followed by dimerization through reaction of the phenol group with dibromoethane to **4**, nitro reduction to the amines of **5a**, alkylation to introduce four ethylacetate groups to access **5b**, and finally ester hydrolysis to generate **1** in its protonated form.

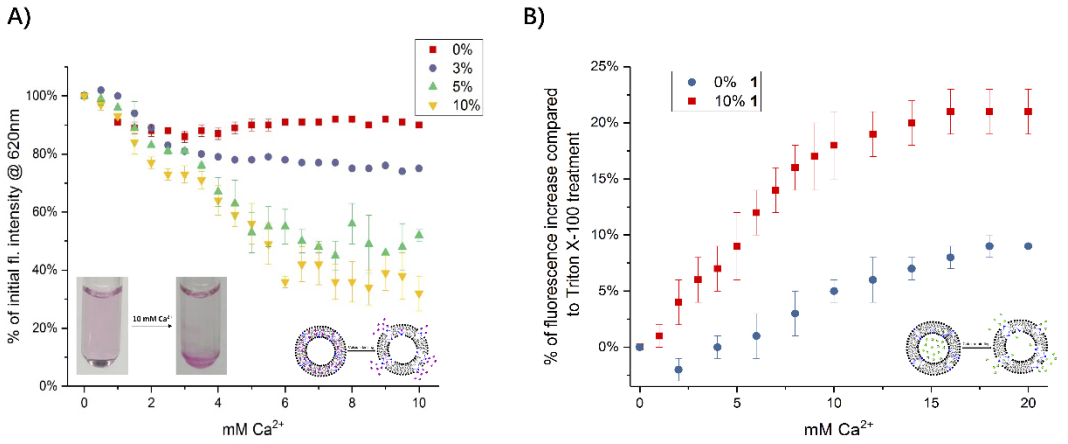


Figure 1. A) Dose-dependent release of NR from PC liposomes containing 0%, 3%, 5% or 10% lipid **1**. Increased release can be achieved with higher percentages of lipid **1**. Insert: cartoon depiction for hydrophobic dye release assay and representative image before and after titration. B) Sulforhodamine B release study of liposomes containing lipid **1**. Liposomes containing 10% of lipid **1** yielded a greater fluorescence increase compared to PC liposomes. Insert: cartoon depiction for hydrophilic dye release assay.

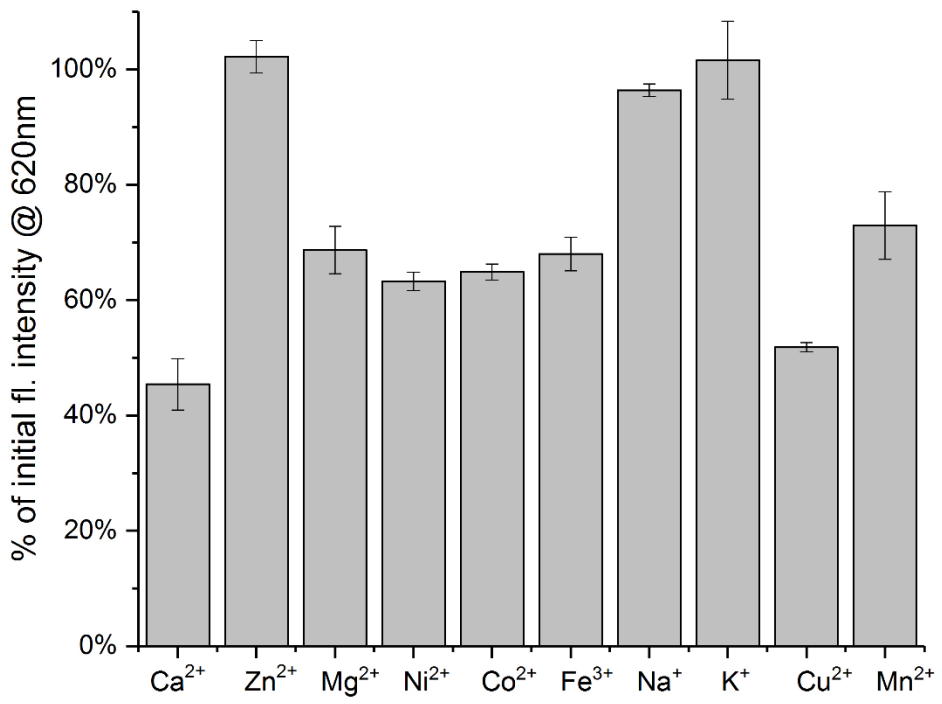


Figure 2. Selectivity of triggered release with different common metal cations. K^+, Na^+, and Zn^{2+} did not cause release, while Ca^{2+} resulted in the greatest release. (Final cation concentration = 10 mM)

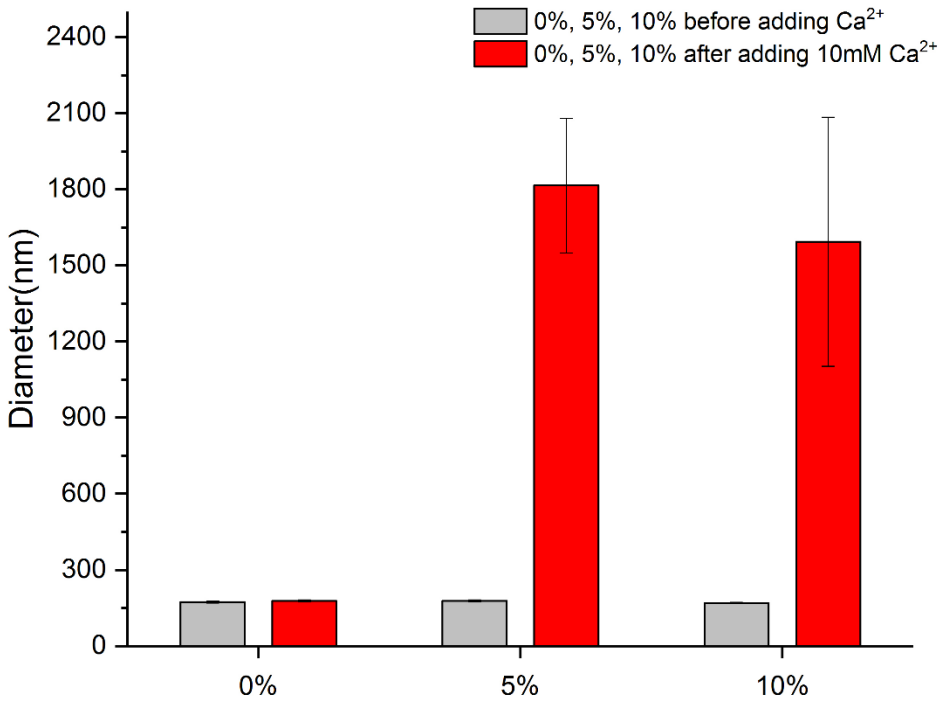


Figure 3. DLS studies before and after triggering release driven by Ca^{2+}. Dramatic changes in particle size were observed only when lipid **1** is present.

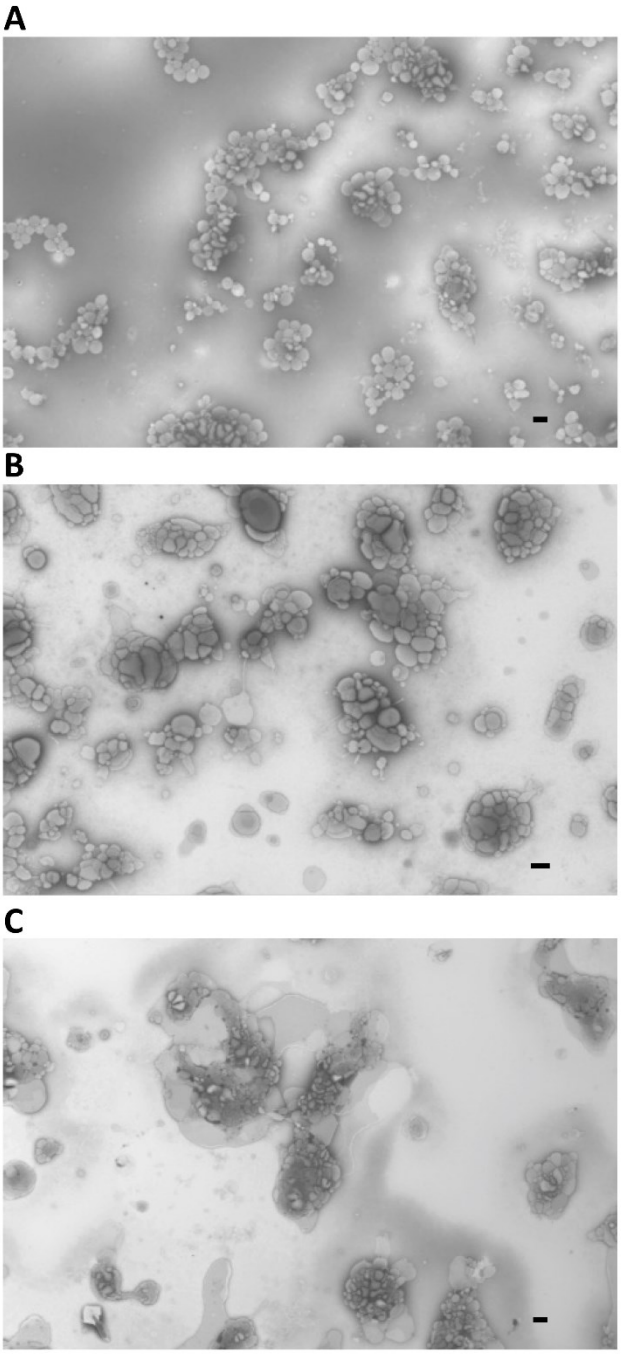


Figure 4. STEM images of liposomes: A) containing 10% of **1** before calcium addition, B) containing 0% of **1** after 20 mm calcium addition, and C) containing 10% of **1** after 20 mm calcium addition. The scale bar in each image denotes 200 nm.