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**Supplementary Information for:**

# *In vitro* synthesis and stabilization of amorphous calcium carbonate (ACC) nanoparticles within liposomes

**Chantel C. Tester, Ryan E. Brock, Ching-Hsuan Wu, Minna R. Krejci, Steven Weigand, and Derk Joester***

***Chemicals:*** Soy phosphatidylcholine, Soy phosphatidlyinositol (PC, Avanti Polar Lipids, Alabaster, AL). Calcium chloride dihydrate (BDH Chemicals, Westchester, PA). Ammonium carbonate, Sodium chloride, Dichloromethane, Chloroform (Mallinckrodt Baker, Phillipsburg, NJ). 40 nm calcite nanoparticles (NanoMaterials Technology Pte Ltd, Singapore). SephadexTM G-25, Nucleopore track-etched membranes (GE Healthcare Biosciences AB, Uppsala, Sweden). Unless otherwise noted, all solutions where prepared in ultra-pure water ($\rho$ = 18.2 MΩ·cm) prepared with a Barnstead NanoDiamond UF+UV purification unit.

***Liposome Preparation:*** Lipid films were prepared by rotary evaporation of a solution of PC (10 mg) in $CH_2Cl_2$ (10 mL) at 40˚C and 700 mbar. All subsequent steps were performed at room temperature. The lipid film was rehydrated with 10 mL of 1 M aqueous $CaCl_2$ for at least 30 minutes. The resulting vesicle suspension was extruded eleven times through a polycarbonate track-etched membrane (pore size 0.1, 0.2, 1.0 µm) using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Liposomes were subjected to size exclusion chromatography (Sephadex G-25, eluent: 1.5 M aqueous NaCl). Dynamic light scattering (Malvern Zetasizer nano) was used to confirm the size of the liposomes and to identify the fractions with the highest liposome concentration (viscosity 1.0383 cP, refractive index 1.345). Fractions were pooled, stored at 4 ºC, and used within 48 hours.

***$CaCO_3$ Precipitation:*** Precipitation was initiated by gently pipetting suspensions of $CaCl_2$-containing liposomes in 1.5 M aqueous NaCl with aqueous $(NH_4)_2CO_3$ (100 µL). Two concentrations of $(NH_4)_2CO_3$ were used: 1) 900 µL of liposome suspension with 100 µL of 1 M $(NH_4)_2CO_3$ and 2) 800 µL of liposome suspension with 200 µL 1.5 M $(NH_4)_2CO_3$, corresponding to 0.111 and 0.375 mol $(NH_4)_2CO_3$ per liter of liposome suspension, respectively. For comparison, bulk precipitation was carried out by mixing 1 M aqueous $(NH_4)_2CO_3$ (100 µL) with 1 M aqueous $CaCl_2$ (900 µL). Bulk precipitation reactions were analyzed within 15 min of the addition of ammonium carbonate. The precipitation resulted in rapid formation of ACC, vaterite, and calcite, with precipitates reaching µm sizes by the 15 min time point.

***Plunge Freezing*:** Copper grids coated with a lacy carbon support film (Ted Pella, Redding, CA) were rinsed with chloroform and plasma treated (E.A. Fischione Plasma Cleaner) for 12 s. Liposome suspensions were plunge frozen in liquid ethane using an automated vitrification robot (FEI Vitrobot™ Mark IV) at various times after the addition of ammonium carbonate solution. The liposome suspension (5 µL) was pipetted onto a grid within the vitrobot enclosure (25 ºC and 100% rel. humidity). Excess liquid was removed by automatic blotting (2 blots for 1 s each) before plunging into liquid ethane.

***Cryo-TEM:*** Imaging of liposomes less than 500 nm in diameter was performed at 120kV, on a field emission gun-equipped Jeol 1230 TEM employing a Gatan cryo-holder operating at approximately -180 °C. For optimum contrast of both inorganic precipitates and the phospholipid bilayer membrane, underfocusing was used for balancing mass-thickness and phase contrast.[1] Images were recorded using a Hamamatsu ORCA side mounted CCD camera (1024x1024 pixels). The size of the liposomes and encapsulated precipitates were measured using ImageJ software[2].

***Cryo-STEM/EDS:*** Liposomes extruded through a 1 µm track-etched membrane were analyzed using a Hitachi HD-2300 scanning transmission electron microscope (STEM), equipped with a field emission gun operating at 200 kV and a Gatan cryo-holder operating at approximately -170 °C. The size of the liposomes and encapsulated precipitates were measured using ImageJ software[2] (Figure 1). Energy-dispersive X-ray spectroscopy (EDS) spectra were measured using two Si(Li) EDS detectors (Thermo-Fischer). The high probe current required for EDS-analysis results in localized destruction of the sample by the electron beam.

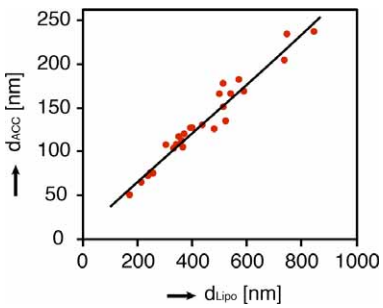


**Figure 1:** Plot of ACC nanoparticle diameter ($d_{ACC}$) versus that of the encapsulating liposome ($d_{Lipo}$). Particle size was measured from Cryo-STEM images of liposomes extruded through a 1 µm track-etched membrane at t = 20 h.

***Wide Angle X-ray Scattering:*** WAXS was conducted at beamline 5-ID-D at the Advanced Photon Source at Argonne National Laboratory. WAXS patterns were recorded using a Roper Scientific CCD camera system[3]. The sample to detector distance was 0.199 m, calibrated using lanthanum hexaboride powder. The wavelength of radiation was set to 0.95372 Å (13 keV), resulting in a $q$-range of 0.45-3.7 $Å^{-1}$. To minimize radiation damage, measurements were made while continuously flowing suspensions at a rate of 4 $\mu$L $s^{-1}$ through a quartz capillary (ID 0.15 cm, wall thickness 30 µm) flow-cell. The scattering from the empty capillary flow-cell, water, and 1 M $CaCl_2$ was also measured for background

subtraction. Data were acquired at different time points over the course of 11 h (Figure 2a) 1with an exposure time of 3 s. The resulting isotropic scattering patterns were azimuthally averaged using FIT2D[4], and corrected for varying incident beam intensity and sample transmission using a $CdWO_4$ crystal-coupled diode in the beam stop[3]. Suspensions of calcite nanoparticles (40 nm in diameter) at a range of concentrations were used to determine that the detection limit of the WAXS set up is between 0.1 and 1 $mg \cdot mL^{-1}$. Although we do not know the liposome volume fraction precisely, from cryo-TEM images we estimate the nanoparticle concentration to be well above this detection limit. When calculating the scattering intensity of liposome-encapsulated nanoparticles, the intensity of the liposome suspension at $t = 0$ was subtracted. Intensities were normalized to $\lambda$ = 1.54 Å.

***Temperature study:*** The influence of temperature on ACC stability was studied using the same WAXS set up but with 0.95372 Å (16 keV) radiation and a sample to detector distance of 287 mm ($q$ = 0.31-3.0 $Å^{-1}$). Liposome suspensions 10 h from ammonium carbonate addition were heated from 20˚C to 60˚C, at 10 degree increments (Figure 2b). At each temperature, the sample was allowed to equilibrate for 30 min before recording its WAXS pattern. Above 60ºC artifacts due to the evaporation of the sample become apparent.

***Simulated Crystal Structures and Diffraction:*** CrystalMaker (CrystalMaker software Ltd.) was used to calculate the diffraction pattern of calcite and vaterite [5].

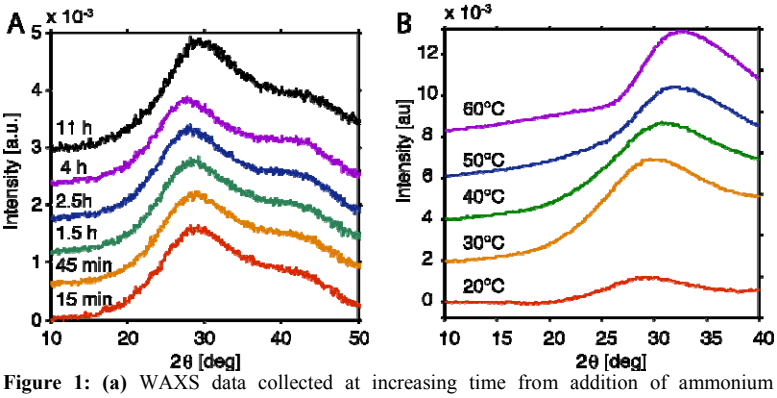


**Figure 1: (a)** WAXS data collected at increasing time from addition of ammonium carbonate to a suspension of 100 nm PC liposomes. **(b)** To establish temperature stability, WAXS was recorded from liposome suspensions (t = 10 h) heated at increasing temperature. Neither after 11 h at room temperature or after heating to 60ºC were Bragg peaks observed.

**References:**

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