BIO-INSPIRED LIPOSOME-STABILIZED MULTI-PHASE ALL-AQUEOUS EMULSIONS FOR DIRECTED MINERAL SYNTHESIS

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by
Andrew T. Rowland

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The dissertation of Andrew T. Rowland was reviewed and approved* by the following:

Chris Keating  
Professor of Chemistry  
Dissertation Adviser  
Chair of Committee

Raymond Schaak  
DuPont Professor of Materials Chemistry

Scott A. Showalter  
Professor of Chemistry  
Professor of Biochemistry and Molecular Biology  
Graduate Program Chair

Peter Butler  
Associate Dean for Education and Graduate Professional Programs

Phillip C. Bevilacqua  
Distinguished Professor of Chemistry and Biochemistry and Molecular Biology  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

This thesis will describe the sequential development of a novel platform for bio-inspired materials synthesis. Each chapter builds upon the previous conceptually, starting with liposome-stabilized all-aqueous emulsions and gradually working toward an artificial mineralization vesicle comprised of multiple mineralizing phase separations.

Chapter 1 discusses the appeal of biominerals and the motivation to develop synthetic bio-inspired materials with similar properties. Biominerals contain mechanical and optical properties which exceed that of their geological equivalents. Biomineral synthesis routes utilized will be explained, namely mineralization vesicles and amorphous precursors, followed by a critical analysis of the synthetic methods inspired by those routes. Most synthetic methodologies only capture a few of the key concepts observed in Biology, which limits the intricacy of their product potential. This project addresses the need for more comprehensive and complex bioinspired methods in the form of multi-phase liquid emulsion microreactors.

Chapter 2 details the furthered development of liposome-stabilized aqueous two-phase system (ATPS) microreactors. The prolificacy of the system was expanded to encapsulate phases with different chemistries, including a salt-rich phase. This increases the potential of the system to include types of reactions not previously considered. The robustness of the system was also demonstrated by investigating the effects of liposome homogenization treatment. Liposome extrusion was shown to be not entirely necessary for the stabilization of different ATPS emulsions, as the interface permittivity was not significantly altered. Encapsulated CaCO₃ synthesis was demonstrated using both extruded and unextruded liposomes.

Chapter 3 describes the creation of artificial mineralization vesicles (AMVs) from large unilamellar vesicle (LUV)-stabilized PEG/dextran ATPS emulsions. Many of the basic concepts
that permeate all levels of AMV complexity were established, including intermediate chelation and enzyme partitioning. A monomeric chelator with calcium binding affinity higher than that of the LUVs but lower than that of carbonate was utilized to stabilize the system. The mineralization reaction was localized based on partitioning, as the enzyme that drives the reaction, urease, partitions almost exclusively to the PEG-rich phase. In order to demonstrate the importance of enzyme partitioning, carbonate salt was added directly to an AMV emulsion to induce decentralized mineralization.

Chapter 4 introduces another liquid-liquid phase separation to the artificial mineralization vesicle in the form of a mineralizing coacervate comprised of calcium and polyaspartic acid (PAA). The mineralization reaction was further localized to this coacervate, which converted to a CaCO₃-polymer composite material. The high concentration of PAA in the mineral stabilized the otherwise thermodynamically unfavorable amorphous calcium carbonate (ACC) morphology. Control over the relative size distributions of the coacervate phases and subsequent minerals was demonstrated. The unique core-shell morphologies of the minerals were illustrated through spectroscopic and electron imaging techniques. Various control materials were analyzed in order to highlight the importance of the individual AMV components to the mineral composition. Lastly, the breadth of potential material was expanded to include calcium phosphates as well.

Chapter 5 explores the coacervate and mineral forming capabilities of different polycarboxylates, both individually and simultaneously. Polyaspartic acid (PAA) coacervates resulted in smooth ACC spheres whereas polyglutamic acid (PGA) resulted in rougher sphere comprised of calcite and vaterite. Using PAA and PGA at the same time created ACC sphere with very porous interiors. The pores were attributed to the PGA regions that appear during the
mineralization reaction. In addition, fluorescent microscopy analysis of the minerals revealed distinct PAA and PGA-rich regions with the minerals. The PGA regions corresponded to the pores, the density of which was controlled by the relative polymer concentrations.

Lastly, Chapter 6 discusses the project in the context of other bio-inspired materials research. The gradual development of the mineralizing all-aqueous emulsion has resulted in a platform for bio-inspired materials synthesis capable of exerting unprecedented control over local organic content. Potential future directions for the project based on promising preliminary experiments will also be discussed.
# TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................. ix

LIST OF TABLES .................................................................................................................. xxii

LIST OF ABBREVIATIONS .................................................................................................. xxiv

ACKNOWLEDGEMENTS ...................................................................................................... xxvi

Chapter 1  Introduction ...................................................................................................... 1
  1.1 Sub-cellular compartmentalization ........................................................................... 1
    1.1.1 Membrane-bound organelles ........................................................................... 2
    1.2 Membrane-less organelles ................................................................................... 2
  1.2 Aqueous-aqueous phase separation ....................................................................... 2
    1.2.1 Segregative phase separation ......................................................................... 3
    1.2.2 Associative phase separation ......................................................................... 3
  1.3 Biomineralization .................................................................................................... 4
    1.3.1 Biogenic calcium carbonate ............................................................................ 4
  1.4 Biomineralization methods ..................................................................................... 6
    1.4.1 Biogenic mineralization vesicles ................................................................... 7
    1.4.2 Biogenic amorphous mineral precursors ....................................................... 7
  1.5 Artificial mineralization ......................................................................................... 10
    1.5.1 Synthetic mineralization vesicles .................................................................. 11
    1.5.2 Artificial liquid mineral precursors ............................................................... 12
  1.6 Summary .................................................................................................................. 16
  1.7 References ............................................................................................................... 20

Chapter 2  Stabilizing All-Aqueous Emulsions of Different Compositions Using
Liposomes Prepared via Gentle Hydration ......................................................................... 30
  2.1 Abstract .................................................................................................................... 30
  2.2 Introduction .............................................................................................................. 30
  2.3 Results ...................................................................................................................... 33
    2.3.1 Formation of stabilized all-aqueous emulsions .............................................. 33
    2.3.2 Influence of extrusion on emulsion size distribution ...................................... 35
    2.3.3 Examination of emulsion short-term stability .............................................. 36
    2.3.4 Vesicle distribution ....................................................................................... 37
    2.3.5 Permeability of the liposome-lined interface ............................................... 38
    2.3.6 Encapsulating calcium carbonate synthesis ............................................... 41
  2.4 Discussions .............................................................................................................. 42
  2.5 Conclusions ............................................................................................................. 44
  2.6 Methods .................................................................................................................. 45
  2.7 Supporting information ........................................................................................... 50
  2.8 References .............................................................................................................. 52
Chapter 6  Conclusions and Future Directions ........................................................................179

6.1 Conclusions .....................................................................................................................179
6.2 Future directions ..............................................................................................................180
  6.2.1 PAA:PEG:PAA block copolymers .............................................................................181
  6.2.2 Substituting the AMV interior phase .........................................................................182
  6.2.3 Coacervates with non-spherical shapes .....................................................................183
  6.2.4 Wetting glass surfaces with coacervates .................................................................184
6.3 References .......................................................................................................................187
LIST OF FIGURES

Figure 1-1: Schematic representations of the two forms of aqueous-aqueous phase separation: (a) segregative and (b) associative. (a) Segregative phase separation results in two polymer-rich phases, or a polymer-rich phase and a salt-rich phase. (b) Associative phase separation results in a dense, polymer-rich phase and a dilute phase. ..............................................................................................................................................3

Figure 1-2: Examples of functional biogenic calcium carbonates. (a) Scanning electron micrograph (SEM) of fractured nacre, comprised of alternating layers of CaCO$_3$ and organic material. Arrows notate organics. Reprinted from [29] with permission from Elsevier. (b) SEM sea urchin spine with organic material removed. Reprinted from [27] with permission from Annual Reviews. (c) Optical microscopy image of chiton surface with notated eyes. Black arrow notates eye with CaCO$_3$ lenses intact, white arrow notates arrow without. (d) Optical microscopy image of cross-section of chiton CaCO$_3$ lens. Reprinted from [35] with permission from Elsevier.................................5

Figure 1-3: (a) Ca$^{2+}$ and (b) Mg$^{2+}$ secondary ion mass spectrometry images of polished carinar process plates sections of a sea urchin tooth. (C) and (Pl) notate the epitaxial columns and plates respectively. Reprinted from [37] with permission from Elsevier.6

Figure 1-4: Cryo-SEM micrographs of cryo-sectioned neonatal calvaria. (a) Mineralizing bone (b) in proximity to mineral-containing vesicles (arrowheads). (b) Higher magnification view of intracellular mineral-containing vesicles. Inset: higher magnification view of mineral globules. Reprinted from [45] with permission from Elsevier..............................................................7

Figure 1-5: (Top) Microscopy images of silica minerals produced by various species of diatoms. The colors arise from diffraction effects, and do no reflect any pigmentation or staining of the silica. (Bottom) Simulated structures of the corresponding diatoms. Black corresponds to organic-rich regions, white to aqueous phase where the silica condenses. Note these computations correspond to a single time point of the assembly. (a) Arachnoidiscus ehrenbergii; (b) Surinella linearis v. helvetica; (c) Actinocyclus confluens. Scale bars = 10 μm. Reprinted images with permission from [53]. Copyright 2008 American Chemical Society.........................9

Figure 1-6: Giant lipid vesicles as models for biomimetic mineralization. (a) Giant vesicle hydrated with aqueous Ca$^{2+}$ solution prior to carbonate addition and (b) two hours after the initiation of carbonate diffusion. (c,d) SEM’s of amorphous calcium carbonate extracted from vesicles. Reproduced from [59] with permission from The Royal Society of Chemistry..........................................................11

Figure 1-7: SEM micrographs of CaCO$_3$ microspheres prepared from polyacrylic acid and calcium coacervates. Coacervates were mineralized using the ammonium carbonate diffusion method after (a) 3 minutes, (b) 30 minutes, (c) 1 hour, (d) 5 hours, (e) 18 hours, and (f) 24 hours of coacervate complexation time. Reprinted images with permission from [71]. Copyright 2007 American Chemical Society........13
Figure 1-8: Schematic representation on the differences between (a) coacervate-mediated biomineralization and (b) the polymer-induced liquid precursor (PILP) process. The important distinction is when liquid-liquid phase separation first occurs during the mineralization reaction. Phase separation occurs prior to the mineralization reaction in coacervate-mediated process, and during the reaction in the PILP process. ..............................................................14

Figure 1-9: Various synthetic biominerals produced via the polymer-induced liquid precursor process. (a-c) SEM micrographs of CaCO$_3$ structures prepared by injecting mineralizing solutions into different molds, then producing single crystal calcite crystals via the carbonate diffusion method. (a) Calcite nanowires extracted from a polycarbonate membrane. Reprinted with permission from [77]. Copyright 2011 Wiley. (b) Calcite scaffold extracted from a three-dimensional suspension of polystyrene spheres. Reprinted with permission from [78]. Copyright 2008 Wiley. (c) Patterned thin-film of calcite assembled atop hexagonally closed packed polystyrene spheres. Reprinted with permission from [79]. Copyright 2010 American Chemical Society. (d-e) TEM micrographs of fragments from a PILP-mineralized collagen-like scaffold. (d) Platelets of calcium phosphate broken off the larger scaffold structure. Arrows note platelets viewed from the side, which have a dark outline. (e) Fragment of mineralized collagen from the larger scaffold structure. Calcium phosphate platelets viewed from the side have a noticeable dark outline. Reprinted from [80] with permission from Elsevier...........................................15

Figure 1-10: Development of stabilized aqueous two phase system emulsions using liposomes prepared via gentle hydration. ..............................................................17

Figure 1-11: creation of an artificial mineralization vesicle from a liposome stabilized ATPS emulsion. Reprinted image with permission from [81]. Copyright 2015 American Chemical Society. ..................................................................................18

Figure 1-12: Incorporation of a mineralizing coacervate phase into the artificial mineralization vesicle. ..................................................................................18

Figure 1-13: Sequential phase separation events within the AMV using multiple polycarboxylates. ..................................................................................19

Figure 2-1: Schematic representation of the preparation of liposome-stabilized emulsions. ..................................................................................34

Figure 2-2: confocal microscopy with fluorescent overlay images of various vesicle-stabilized aqueous two-phase separation (ATPS) emulsions. Unextruded vesicles were able to effectively stabilize droplets of one phase within a continuous PEG-rich phase as well as extruded vesicles with identical lipid composition. (a,b) PEG/dextran emulsions; (c,d) PEG/Ficoll emulsions; (e,f) PEG/sulfate emulsions. (a,c,e) Extruded vesicles at the interface. (b,d,f) Unextruded vesicles at the interface. Rhodamine-tagged vesicles have been false colored red. Scale bar applies to all images. ..............................................................................35
Figure 2-3: Size histograms of droplets in extruded and unextruded vesicle-stabilized ATPS emulsions. In general, vesicle extrusion results in a decrease in average droplet diameter and distribution spread. (a) Measurements of vesicle (extruded and unextruded)-stabilized dextran-rich droplets; (b) measurements of vesicle-stabilized Ficoll-rich droplets; (c) measurements of vesicle-stabilized sulfate-rich droplets. Red bars indicate emulsions with extruded vesicles, blue indicate unextruded.

Figure 2-4: Confocal microscopy with fluorescent overlay images of various vesicle-stabilized ATPS emulsions, 24 hours after initial sample preparation. Vesicle extrusion does not appear to have a significant effect on the long-term stability of the emulsions. (a,b) PEG/dextran emulsions; (c,d) PEG/Ficoll emulsions; (e,f) PEG/sulfate emulsions. (a,c,e) Extruded vesicles at the interface. (b,d,f) Unextruded vesicles at the interface. Rhodamine-tagged vesicles have been false colored red. Scale bar in (a) applies to all parts except for (e,f).

Figure 2-5: (a) Experimental scheme used to monitor U15 diffusion through the liposome stabilized ATPS emulsion. Two droplets were placed adjacent on a silanized glass slide: one containing a liposome stabilized ATPS emulsion, the other labeled U15 in PEG-rich phase. Contact between the two and subsequent U15 diffusion was initiated through the addition of a coverslip. (b) Fluorescent confocal microscopy images depicting diffusion of labeled-RNA (U15) through vesicle-stabilized PEG/dextran ATPS emulsions. Inset refers to time after diffusion initiation. Rhodamine-tagged vesicles have been false-colored red, Alexa 647-tagged U15 blue.

Figure 2-6: (a) depiction of fluorescence recovery after photobleaching experiment on the fluorescent confocal microscope. Liposome-(extruded or unextruded) stabilized PEG/Dx ATPS emulsions were prepared with added fluorescent U15. A single dextran droplet was selected in the prepared. The droplet and immediate surrounding PEG-rich was bleached with maximum laser power. The average U15 fluorescence intensity was measured within a region of interest (ROI) than remained within the droplet for the entire experiment. (b) fluorescence recovery after photobleaching (FRAP) curves for U15 diffusion in vesicle stabilized PEG/dextran ATPS emulsions. Curves depict the fluorescent bleaching and recovery of Alexa-647 tagged U15 in the dextran-rich phase. The entire dextran-rich droplet was bleached and allowed to recover from the PEG-rich phase. Regardless of whether extruded or unextruded vesicles were used to stabilize the emulsion, the dextran-rich phase fully recovered in ~0.7 seconds.

Figure 2-S1: confocal microscopy with fluorescent overlay images of various vesicle-stabilized ATPS emulsions, 72 hours after initial sample preparation. (a,b) PEG/dextran emulsions; (c,d) PEG/Ficoll emulsions; (e,f) PEG/sulfate emulsions. (a,c,e) Extruded vesicles at the interface.

Figure 2-S2: fluorescence recovery after photobleaching (FRAP) curves for vesicle diffusion in vesicle stabilized PEG/dextran ATPS emulsions. Curves depict the fluorescent bleaching and recovery of Rhodamine-tagged (a) extruded and (b)
unextruded vesicles at the PEG/Dx interface. A fraction of the interface was bleached, and allowed to recover. The Rh fluorescence never fully recovers, indicating the vesicles do not freely diffuse from the interface.

Figure 2-S3: Relative Alexa-647 fluorescence in liposome-stabilized (a,d) PEG/Dx, (b,e) PEG/Ficoll, and (c,f) PEG/sulfate ATPS prepared with (a-c) extruded or (d-f) unextruded liposomes during U15 diffusion. Experimental setup depicted in Figure 2-4a was used for all samples. Time refers to time after addition of the cover slip and fusion of droplets. In all cases, U15 was able to diffuse through liposome-lined ATPS interface.

Figure 3-1: Illustration of enzymatic production of CaCO$_3$(s) mineral within artificial mineralization vesicles.

Figure 3-2: Ca(II) destabilizes bioreactor droplet structures by aggregating LUVs. (a) Illustration of the experiment, in which the effect of adding Ca$^{2+}$ to a sample of LUV-stabilized droplets is evaluated. At 5 mM Ca$^{2+}$ and above, the interfacial layer of LUVs is disrupted. (b) Confocal fluorescence microscopy images of LUV-coated ATPS emulsion droplets with different amounts of added Ca$^{2+}$ as indicated in each panel. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran for identification of Dx-rich droplets; red fluorescence represents Rhodamine-labeled lipid. Scale bar = 10 µm.

Figure 3-3. Ca$^{2+}$ chelators protect against vesicle aggregation. (a) Chemical structures of the four chelators tested. Malic acid (MA); Ethylenediamine disuccinic acid (EDDS); Glutamic acid diacetic acid (or N,N-bis(carboxymethyl)-L-glutamate) (GLDA); Ethylenediamine tetraacetic acid (EDTA). (b) Confocal microscopy images of LUV-ATPS emulsion droplets with 30 mM Ca$^{2+}$ and various chelators also at 30 mM. Chelators are noted on each panel. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran, indicating Dx-rich droplets and red fluorescence represents Rhodamine-labeled lipid. Scale bar = 10 µm.

Figure 3-4. Characterization of mineral formed in artificial mineralizing vesicles. (a, b) XRD analysis of CaCO$_3$ precipitates formed with (a) and without (b) the EDDS chelator. Traces are labeled as artificial mineralizing vesicles (AMVs, which contained the ATPS, a stabilizing layer of LUVs surrounding the dextran-rich phase droplets, and the chelator EDDS), and controls lacking the LUVs and/or EDDS as noted. Calcite and vaterite standard peaks are included for identification of peaks. (c) ATR-FTIR spectra of CaCO$_3$ precipitated in AMVs compared with controls that lack LUVs or EDDS; also shown for comparison is a calcite reference. Spectra have been normalized to the intensity at $\nu_3$ and offset for clarity. (d) SEM for mineral formed in AMVs with and without the EDDS chelator.

Figure 3-5. Darkfield optical microscope images showing enzyme-catalyzed calcium carbonate formation inside artificial mineralizing vesicles. (a) Reaction with 100
mM urea. (left) before and (right) 1 h after urea addition. Scale = 20 μm and applies to both images. (b) Reaction with 500 mM urea leads to more rapid mineralization. (left) 7 minutes after, and (right) 24 minutes after urea addition. Scale = 20 μm and applies to both images. Conditions: 30 mM Ca$^{2+}$, 30 mM EDDS, 30.77 units/mL urease.

**Figure 3-6.** Brightfield (DIC) and lipid fluorescence overlay illustrating that enzyme-catalyzed mineral formation is uniform across the population of artificial mineralizing vesicles. Samples were imaged 2 h after urea addition. Lipid fluorescence and transmitted light (DIC) channels have been overlaid to show location of mineral. (A) 25 mM EDDS; (B) 22.5 mM EDDS; scale = 10 μm applies to both A and B. (C) Lower-magnification view of 22.5 mM EDDS sample; scale = 25 μm. Conditions: 100 mM urea, 30 mM Ca$^{2+}$; 12.18 units/mL urease.

**Figure 3-7.** Emulsions were destabilized at long reaction times but mineral was retained in dextran-rich phase. (left) transmitted light (DIC) and (right) confocal fluorescence microscopy images of AMV sample 24 h after initiation of mineralization, with no enzyme inhibitor added. Conditions: 30 mM Ca$^{2+}$, 300 μg/mL urease, 200 mM urea, and 30 mM EDDS. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran, indicating Dx-rich phase and red fluorescence represents Rhodamine-labeled LUVs. Scale bar = 50 μm.

**Figure 3-8.** Non-enzymatic mineralization. LUV-stabilized PEG/dextran ATPS droplets with EDDS-chelated calcium, 13 minutes after direct addition of carbonate to the solution as Na$_2$CO$_3$. Rhodamine-labeled lipids are false-colored red. Conditions: 30 mM Ca$^{2+}$; 25 mM EDDS; 40 mM Na$_2$CO$_3$.

**Figure 3-S1.** Confocal microscopy images of LUV-ATPS emulsion droplets with 30 mM Ca$^{2+}$ and various chelator species also at 30 mM. Species are: A) Malic acid; B) EDDS; C) GLDA; D) EDTA at a [Ca$^{2+}$]/[Chelator] ratio as indicated in the inset. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran, indicating Dx-rich droplets and red fluorescence represents Rhodamine-labeled LUVs. Scale bars = 10 μm.

**Figure 3-S2.** Transmitted light (differential interference contrast) images for the EDDS-containing sample shown in Figure 2b of the main text. Droplets for which the equator was in-focus appear sharper, and were used in rough estimation of average droplet size as reported in text. Scale bars = 10 μm.

**Figure 3-S3.** ATR-FTIR spectrum for calcium citrate. Dotted line indicates peak at ~1570 cm$^{-1}$ described in the Methods.

**Figure 3-S4.** ATR-FTIR spectrum for CaCO$_3$ formed by enzymatic activity of urease in an ATPS, in the presence and absence of EDDS and LUVs. Dotted vertical line indicates $v_4$ peak, which at ~743 cm$^{-1}$ in the no EDDS, no LUVs sample is indicative of vaterite. Spectra for samples with EDDS and LUVs are included for comparison and also appear in Figure 3c.
Figure 3-S5. SEM images of CaCO$_3$ precipitates obtained from urease-mediated ATPS precipitation reactions identical to that in Figure 3, in the A) presence and B) absence of 20% v/v LUVs. Wide- and narrow-view images are displayed to give a better perspective of the distribution of precipitates within a particular sample. All scale bars are 5 microns.

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Figure 3-S6. Reaction progress as indicated by changes in pH upon urea hydrolysis. Dx-SNARF, which partitions into the dextran-rich phase, was used to monitor local pH changes. (A) Dx-rich droplets displaying pH changes. Images for the acid and base forms of the dye have been overlaid and false-colored for clarity (green = basic, red = acidic form; yellow indicates near-neutral pH in the overlaid images). Scale bars = 10 μm. (B) Trend of pH changes inside of Dx-rich droplets stabilized by LUVs, surrounded by PEG-rich continuous phase.

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Figure 3-S7. Darkfield optical microscopy for CaCO$_3$ deposition in artificial mineralizing vesicles (AMVs), from the same set of experiments as Figure 4a but showing a wider field of view. (left panels, both a and b) before mineralization, (right panels, both a and b) after mineralization has occurred. Mineralization was induced by addition of 100 mM urea to the solution. Scale = 20 μm and applies to all images. Conditions: 30 mM Ca$^{2+}$, 30 mM EDDS, 30.77 units/mL urease.

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Figure 3-S8. Comparison of transmission and ATR FTIR spectra for a CaCO$_3$ sample. Spectra have been normalized to the intensity at ν$_3$ for ease of comparison.

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Figure 4-1. Pre-organization of reaction microenvironment by liquid-liquid phase coexistence. (a,b) Concentrations of PAA and Ca$^{2+}$ determine structure and stability of aqueous/aqueous emulsion droplets. (a) Effect of adding PAA to lipid vesicle-coated PEG/dextran all-aqueous emulsion droplets in the absence of Ca$^{2+}$. Laser confocal microscopy fluorescence images as a function of added PAA 2-11 kDa (in mg/mL, image insets). (b) Illustration interpreting data in panel a as loss of interfacial liposomes and destabilization of all-aqueous emulsion upon the addition of PAA. (c) Effect of adding PAA to lipid vesicle-coated PEG/dextran all-aqueous emulsion droplets in the presence of 50 mM Ca$^{2+}$. Laser confocal microscopy fluorescence images as a function of added PAA (in mg/mL, image insets). Images are overlaid and false-colored for clarity. Red fluorescence represents Rhodamine-labeled liposomes; green fluorescence represents Alexa488-labeled PAA; blue fluorescence represents Alexa647-labeled dextran. Scale bars = 20 μm. (d) Illustration interpreting data in panel c as formation of Ca$^{2+}$/PAA-rich coacervate upon addition of PAA to Ca$^{2+}$–destabilized all-aqueous emulsion droplets.

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Figure 4-2: Calcium carbonate formation in coacervate-containing artificial mineralizing vesicles (AMVs). (a) Illustration depicting the formation of CaCO$_3$ inside AMVs. Prior to urea addition, three aqueous phases are present: PEG-rich phase, dextran-rich phase, and PAA/Ca$^{2+}$ coacervate. Lipid vesicles are absorbed at the PEG-rich/dextran-rich aqueous/aqueous interface, and urease partitions to the dextran-rich phase. Upon the addition of urea, carbonate binds to Ca$^{2+}$, displacing the PAA inside the coacervate, initiating mineralization. (b, c) Fluorescent confocal and transmitted
(DIC) stills of mineralization occurring inside AMVs. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca^{2+}; 100 mM urea, and (b) 10.5 mg/mL PAA 2-11 kDa; 10.5 units/mL urease, or (c) 14.75 mg/mL PAA 2-11 kDa; 12.45 units/mL urease. Inset times refer to time after urea addition. Arrows note the formed minerals, and fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). (d, e) Relative PAA fluorescence in each phase from data corresponding to (b) and (c), respectively, over the course of the reaction. Legend in (d) also applies to (e).

Figure 4-3: Particles produced in coacervate-containing AMVs are spherical, with size distributions tunable by changing initial coacervate sizes. Scanning electron micrographs of CaCO_{3} particles extracted from coacervate-containing AMVs at low (a) and high (b) magnification. (c, d) Volume distributions for initial coacervates (c) and resulting mineral microspheres (d) as a function of PAA concentration. Increased PAA systems (blue) contained 15 mg/mL PAA 2-11 kDa, and standard composition systems (red) contained 10.5 mg/mL PAA 2-11 kDa. Coacervate and particle volumes were calculated from diameters measured using confocal microscope (transmitted and PAA fluorescence channels) and SEM images, respectively. Coacervates measuring smaller than 1 micron in diameter could not be effectively resolved and were not included in this analysis. All emulsions used had the following composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca^{2+}; 12.45 units/mL urease; 100 mM urea.

Figure 4-4: Mineral microspheres contain PAA co-localized with carbonate. (a,b) Optical microscopy of mineral microspheres formed as in Figures 2 and 3 (standard conditions); (a) confocal fluorescence channel for Alexa 488-tagged PAA (green), and (b) transmitted (DIC) channel for the same particles. (c-i) MicroRaman characterization: (c) Raman spectra for mineral microspheres (top line) and for PAA alone (bottom line). Indicated peaks were used to create Raman maps (d, e) (d) Brightfield image of particles and Raman maps of peak intensity for the carbonate (red) and amide (stretches). Maps were acquired in the x,y-direction. (e) Brightfield image of particle used and Raman maps of peak intensity for the carbonate (red) and amide stretches (blue). Maps were acquired in the x,z-direction. Green line on the brightfield notates cross-section of particle where analysis was performed. Emulsions used to create the minerals had the following composition: 1:49 dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca^{2+}; 10.5 mg/mL PAA 2-11 kDa; 12.45 units/mL urease; 100 mM urea. Scale bar = 5 μm unless otherwise noted.

Figure 4-5: Structural characterization of mineral microspheres. (a) Scanning electron micrograph (SEM) of a particle that cracked open during washing. (b) Optical microscopy image of microtomed thin section of microspheres embedded in epoxy mounted atop silicon. (c) SEMs of mineral microspheres before (i., ii.) and after (iii., iv.) heat treatment: (i.) Non-heated particle and (ii.) higher magnification view of non-heated particle; (iii.) Heated particle and (iv.) higher magnification view of heated particle. (d) Scanning transmission electron micrograph (i.) and selected area electron diffraction patterns (or SAED) (ii.) of thin section of a non-heated particle.
the diffuse rings confirm the amorphous nature of the particle. Bright-field transmission electron micrograph (iii.) and selected area electron diffraction pattern (iv.) of thin section of a heated particle. The most prevalent diffraction ring corresponds to a d-spacing of 3.04 Å, characteristic of the {104} calcite plane. (e) XRD spectra of fresh particles, long-term aged particles in air, short-term aged particles in buffer, and heated particles. Calcite standard peaks are included for reference. The absence of peaks in all unheated sample XRD spectra is consistent with ACC. The heated particle XRD diffraction pattern showed strong peaks corresponding to calcite. (f) ATR-FTIR spectra of fresh particles, long-term aged particles in air, short-term aged particles in buffer, heated particles, and notated $\nu_1$ – $\nu_4$ carbonate ion vibrational bands.

**Figure 4-6.** Coacervate-containing AMVs can be adapted for production of other materials. (a) Calcium phosphate synthesis was performed by using a phosphate producing enzyme, alkaline phosphatase (ALP) in place of urease. The substrate is rac-glycerol 1-phosphate (RG1P), whose reaction with ALP releases phosphate anion. (b) ATR-FTIR spectrum of calcium phosphate particles made in coacervate-containing AMVs. Notated peaks refer to the $\nu_3$ and $\nu_4$ phosphate stretching modes; the broad appearance of these peaks suggest the material is amorphous calcium phosphate. Other prominent peaks in the spectrum are due to PAA incorporated in the particles. (c) SEM of particles. (d, e) Energy-dispersive X-ray spectroscopy (EDS) maps of (d) calcium and (e) phosphorous, acquired at the same location as the SEM image.

**Figure 4-S1.** Droplet size (left axis) and PAA partitioning (right axis) in AMVs as a function of total PAA concentration (bottom axis) in the absence of Ca$^{2+}$. Measurements were performed using the raw data associated with Figure 1a. Diameters implicitly assume that confocal sections capture droplet equators and consequently may systematically underestimate diameters. $K$ was calculated based on fluorescence intensity ratios. Error bars indicate the standard deviation of the mean of 32 measurements.

**Figure 4-S2.** PAA partitioning in AMVs as a function of total PAA concentration in the presence of 50 mM Ca$^{2+}$. Measurements were performed using the raw data associated with Figure 1b. $K$ was calculated based on fluorescence intensity ratios. Droplet size was not calculated due to nonspherical shapes for intermediate [PAA]. Error bars indicate the standard deviation of the mean of ~30 measurements.

**Figure 4-S3.** Laser confocal microscopy fluorescence images of coacervate-containing AMVs with 10.5 mg/mL PAA 2-11 kDa and 50 mM Ca$^{2+}$ with increasing NaCl (in mM, given in upper left of each image). Images have been overlaid and false-colored for clarity. Red channel represents Rhodamine-labeled lipid vesicles; green channel represents Alexa 488-labeled PAA; blue channel represents Alexa 647-labeled dextran. Scale bars = 10 μm.

**Figure 4-S4.** Droplet size (left axis) and PAA partitioning (right axis) in coacervate-containing AMVs as a function of total NaCl concentration (bottom axis) in the
presence of 50 mM Ca\(^{2+}\). Diameters implicitly assume that confocal sections capture droplet equators and consequently may systematically underestimate diameters. K was calculated based on the fluorescence intensity ratios. Measurements were performed using the raw data associated with Figure 4-S3. Error bars indicate the standard deviation of the mean of ~30 measurements.

**Figure 4-S5.** Changes in the amount of lipid vesicles (LUVs, for large unilamellar vesicles) present as interface stabilizers result in changes the coacervate and subsequent mineral volume distributions. Volume distributions of coacervates and minerals made inside AMVs with (a, b) increased LUV concentration (1.875 mg/mL) (c, d), standard LUV concentration (1.5 mg/mL) (e, f), and reduced LUV concentration (1.125 mg/mL). Regardless of LUV concentration, all emulsions had the same basic composition: 1:49 dextran:PEG volume ratio; 50 mM Ca\(^{2+}\); 10.5 mg/mL PAA 2-11 kDa; 12.45 units/mL urease; 100 mM urea. Note: differences in these size distributions from those in Figure 4-3 are due to reproducible differences in mixing technique (Figure 4-S6).

**Figure 4-S6.** Small changes in reaction protocol can influence particle size distributions. Histograms of volumes of organic-rich CaCO\(_3\) particles made using the same coacervate-containing AMV composition but with differing mixing techniques. (a) Particles made in a system which dextran-rich phase was introduced to the AMV synthesis before coacervates were. Prior to mineralization, the AMVs were vortex mixed for either 10 seconds (blue) or 3 minutes (red). (b) Particles made in a system which coacervates were introduced to before dextran-rich phase was. Prior to mineralization, the AMVs were vortex mixed for either 10 seconds (blue) or 3 minutes (red). (c) Histogram highlighting the greatest difference in size distribution due to mixing technique.

**Figure 4-S7.** Fluorescent PAA is found associated with solid mineral particles. (a) Alexa 488 fluorescent and (b) transmitted light (DIC) channels of minerals made inside coacervate-containing AMVs, doped with Alexa 488-tagged PAA. (c) Alexa 488 fluorescent and (d) DIC channels of minerals made inside coacervate-containing AMVs, NOT doped with Alexa 488-tagged PAA (i.e., only unlabeled PAA was present).

**Figure 4-S8.** (a) TGA-MS graph for microparticles made inside coacervate-containing AMVs with standard composition. (Left axis) Total relative weight of sample as a function of time (temperature). (Right axis) Mass spectroscopy signals of thermal decomposition by-products, namely water (18 amu) and carbon dioxide (44 amu). (b) Temperature profile of the heating rates used for each sample.

**Figure 4-S9.** Effect of liposomes and PAA on appearance of mineral particles. Scanning electron micrographs show CaCO\(_3\) minerals formed in solutions that contained a 1:49 dextran:PEG ATPS: (a,b) coacervate-containing AMVs without lipid vesicles (LUVs) at the interface (50 mM Ca\(^{2+}\); 10.5 mg/mL PAA), (c,d) AMVs without PAA present (20% by volume LUVs; 50 mM Ca\(^{2+}\)), (e,f) AMVs with neither LUVs nor PAA present (50 mM Ca\(^{2+}\)).
Figure 4-S10. Effect of PEG and dextran polymers on appearance of mineral particles. Scanning electron micrographs show CaCO$_3$ minerals formed in solutions that contained 50 mM Ca$^{2+}$ and 10.5 mg/mL PAA, with different polymer compositions. (a,b) dextran: PEG emulsions with (1:49 dextran: PEG volume ratio), (c,d) PEG-rich phase, and (e,f) buffer solution without PEG or dextran polymers. Note that while the Ca$^{2+}$ and PAA concentrations are constant across these samples, only the first two conditions led to Ca/PAA-rich coacervate formation; the buffer sample did not have coacervates present prior to mineralization. ..........................................................132

Figure 4-S11. Effect of Ca$^{2+}$ and PAA concentrations on appearance of mineral particles formed in the absence of PEG and dextran polymers. Scanning electron micrographs show particles prepared in (a,b) buffer solution not initially containing coacervates (50 mM Ca$^{2+}$; 10.5 mg/mL PAA), (c,d) buffer solution that containing a small volume of coacervates (50 mM Ca$^{2+}$; 0.525 mg/mL PAA), and (e,f) buffer solution containing a large volume of coacervates (1M Ca$^{2+}$; 10.5 mg/mL). .........................133

Figure 4-S12. Powder XRD of minerals made inside coacervate-containing AMV systems that were missing various components. AMVs compositions were identical those noted in Figure 4-S9. Calcite and vaterite standard peaks are included for reference. ..........................................................133

Figure 4-S13. SEMs of minerals after (a, b) short-term exposure to aqueous buffer solution and (c, d) long-term exposure to atmosphere. Buffer soaked particles were placed in 10 mM Tris buffer (pH = 8.5) at room temperature for 3 days. Atmosphere treated particles were dry and sat at room temperature for ~1 year. .........................134

Figure 4-S14. Powder XRD of calcium phosphate minerals made inside AMVs containing ALP and RG1P. .................................................................................................................................134

Figure 5-1: Fluorescent confocal images of AMVs containing 50 mM Ca$^{2+}$ chelated by different concentrations of various lengths of monodisperse polyaspartic acid (PAA). PAA was either (a, b, c) 10, (d, e, f) 50 or (g, h, i) 100 monomers in length. Coacervate formation was dependent on PAA length and concentration, and can only be observed in panels d, g, h, and i. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel. ..............................................................145

Figure 5-2: (a-d) Fluorescent confocal images of AMVs with coacervates comprised of different concentrations of monodisperse and bidisperse polyaspartic acid (PAA) and (e-h) low magnification and (i-l) high magnification scanning electron micrographs of particles synthesized inside AMVs with identical compositions. Arrows in (c) notate coacervates. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel. ......................................................................................148

Figure 5-3: Fluorescent confocal images of LUV-stabilized dextran-rich droplets in a continuous PEG-rich phase containing free Ca$^{2+}$ chelated by different concentrations of various lengths of monodisperse polyglutamic acid (PGA). PGA was either (a, b, c, d) 20 or (e, f, g, h) 100 monomers in length. Coacervate formation was extremely
dependent on PGA length and concentration, and can only be observed in panels e, f, and g. Arrows in g notate coacervates. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.

Figure 5-4: (a-d) Fluorescent confocal images of AMVs with coacervates comprised of different concentrations of monodisperse and bidisperse polyaspartic acid (PAA) and (e-h) low magnification and (i-l) high magnification scanning electron micrographs of particles synthesized inside AMVs with identical compositions. No coacervates are present inside panel a. Arrows in b, c, and d notate coacervates. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.

Figure 5-5: (a) Fluorescent confocal and transmitted (DIC) stills of mineralization occurring inside AMVs with 5 mg/mL PAA 100mer and 4 mg/mL PGA 100mer. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca$^{2+}$; 5 mg/mL PAA 100mer; 4 mg/mL PGA 100mer; 12.45 units/mL urease; 100 mM urea. Inset times refer to time after urea addition. Fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). Scale bar = 25 μm. (b) Relative PAA fluorescence in each phase corresponding to a over the course of the reaction. (c) Relative PGA fluorescence in each phase corresponding to a over the course of the reaction. (d) Illustration depicting the formation of CaCO$_3$ inside artificial mineralization vesicles (AMVs) containing both PAA and PGA. Prior to urea addition, three aqueous phases are present: PEG-rich phase, dextran-rich phase and PAA/PGA/Ca$^{2+}$ coacervate. Upon the addition of urea, carbonate displaces the PGA inside the coacervate. This results in an increase of PGA in the dextran-rich phase and the formation of PGA droplets inside the coacervate. Arrow notates a PGA-rich region.

Figure 5-6: Fluorescent confocal and transmitted (DIC) stills of mineralization occurring inside AMVs with 4 mg/mL PAA 100mer and 5 mg/mL PGA 100mer (a) before and (b) 20 minutes after the addition of urea. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca$^{2+}$; 4 mg/mL PAA 100mer; 5 mg/mL PGA 100mer; 12.45 units/mL urease; 100 mM urea. Fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). Scale bars = 50 μm.

Figure 5-7: SEM’s of minerals made inside AMVs containing different concentrations of 100mer PAA and 100mer PGA: (a-c) 5 mg/mL 100mer PAA, 4 mg/mL 100mer PGA; (d-f) 7.5 mg/mL 100mer PAA, 2.5 mg/mL 100mer PGA; (g-i) 2.5 mg/mL 100mer PAA, 7.5 mg/mL 100mer PGA.

Figure 5-8: MicroRaman characterization of CaCO$_3$ microspheres made inside AMVs containing both 100mer PAA and 100mer PGA. (a) Raman spectra for mineral microspheres (top line) and for PAA alone (bottom line). Indicated peaks were used to create Raman maps. (b-d) Brightfield image of particle and Raman maps of peak intensity for the carbonate (red) and amide (blue) stretches. Maps were acquired in the x,y-direction. Emulsions had the following compositions: 1:49 dex:PEG volume...
ratio; 20% by volume LUVs; 50 mM Ca\(^{2+}\); 12.45 units/mL urease; 100 mM urea; (b) 5 mg/mL 100mer PAA; 4 mg/mL 100mer PGA; (c) 7.5 mg/mL 100mer PAA; 2.5 mg/mL 100mer PGA; (d) 2.5 mg/mL 100mer PAA; 7.5 mg/mL 100mer PGA.

**Figure 5-9:** Optical microscopy of mineral microspheres formed as in Figure 6 (varying concentrations of PAA and PGA) (a, c, e) confocal fluorescence channel for Alexa 488-tagged PAA (green) and Alexa-647-tagged PGA (blue) and (b, d, f) transmitted DIC channel for the same particles. Insets in c and d show higher magnification view of the framed particle.

**Figure 5-10:** Conversion from the aspartate α configuration to the β configuration.

**Figure 5-S1:** (a) Temperature profile of the heating rates used for each TGA-MS experiment. (b) TGA-MS graph of CaCO\(_3\)-PAA particles made inside artificial mineralization vesicles with 10 mg/mL 50mer PAA and 5 mg/mL 100mer PAA. (Left axis) Total relative weight of sample as a function of time (temperature). (Right axis) Mass spectroscopy signals of thermal decomposition by-products, namely water (18 amu) and carbon dioxide (44 amu).

**Figure 5-S2:** Raman spectrum of a CaCO\(_3\) particle prepared inside an AMV containing only 50mer PAA (identical composition to Figure 2i). The position of the symmetric carbonate stretch (\(v_1\)) is indicative of ACC. All particles prepared using PAA all had the same spectrum.

**Figure 5-S3:** XRD of a CaCO\(_3\) particle prepared inside an AMV containing only 100mer PGA (identical composition to Figure 4l). Standard peaks for calcite and vaterite have been included for ease of identification. All particles prepared using PGA all had the same spectrum.

**Figure 5-S4:** XRD of a CaCO\(_3\) particle prepared inside an AMV containing 5 mg/mL 100mer PAA and 4 mg/mL 100mer PGA (identical composition to Figure 6a). Standard peaks for calcite and vaterite have been included for ease of identification. All particles prepared using both PAA and PGA all had the same spectrum.

**Figure 5-S5:** Fluorescent confocal and transmitted (DIC) stills of mineralization occurring inside AMVs with (a) 7.5 mg/mL PAA 100mer and 2.5 mg/mL PGA 100mer and (b) 2.5 mg/mL 100mer PAA and 7.5 mg/mL 100mer PGA. Based on the appearance of the final mineral products in Figures 5-7 and 5-9, we hypothesize the reactions proceed in the same manner as seen in Figure 5-5a. However, the limited depth of field in the x,y,z-directions obscures areas of the samples we believe to be more indicative. Emulsion composition: 1:49 D\(_x\):PEG volume ratio; 20% by volume LUVs; 50 mM Ca\(^{2+}\); 12.45 units/mL urease; 100 mM urea. Inset times refer to time after urea addition. Fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). Scale bar = 25 μm.

**Figure 6-1:** Fluorescent confocal image of AMVs prepared with PAA:PEG:PAA block copolymer. The copolymer was composed of 112mer PEG with 10mer PAA.
attached on both ends. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca^{2+}; 5 mg/mL PAA:PEG:PAA. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.

**Figure 6-2:** Fluorescent confocal and brightfield images of LUV-stabilized Ficoll-rich droplets in a continuous PEG-rich phase with PAA present (a) before and (b) after the addition of Ca^{2+}. PAA/Ca^{2+} coacervates form at the PEG/Ficoll interface upon the addition of Ca^{2+}. Emulsion composition: 1:24 Ficoll:PEG volume ratio; 20% by volume LUVs; 10 mg/mL PAA 2-11 kDa; (a) 0 mM Ca^{2+} or (b) 50 mM Ca^{2+}. PEG:Ficoll ATPS was initially 15%/15% PEG/Ficoll by weight total. Rhodamine-tagged LUVs have been false colored red and Alexa 488-tagged PAA has been false-colored green.

**Figure 6-3:** Fluorescent confocal and brightfield images of AMVs containing non-spherical coacervates comprised of (a) PAA or (b) PGA. Total PEG and dextran concentration has been increased by 50% over the standard composition. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca^{2+}; (a) 16 mg/mL 100mer PAA or (b) 10 mg/mL 100mer PGA. Rhodamine-tagged LUVs have been false colored red, Alexa 488-tagged PAA has been false-colored green, and Alexa-647 tagged PGA has been false-colored blue.

**Figure 6-4:** Creating non-spherical minerals from coacervates wetting glass surfaces. (a) Schematic representation of how glass coating affects coacervate wetting and the shape of the subsequent mineral. (b) Fluorescent confocal Z-stack of Ca^{2+}/PAA coacervates wetting a non-treated glass slide and (c) minerals made from those coacervates. (d) Fluorescent confocal Z-stack of Ca^{2+}/PAA coacervates not wetting a glass slide functionalized with PEG-silane and (e) minerals made from those coacervates. Solutions composition: 1 M Ca^{2+}; 7.5 mg/mL PAA 2-11 kDa. Alexa 488-tagged PAA has been false-colored green.
**LIST OF TABLES**

**Table 2-1:** Extruded and unextruded vesicle partitioning in the different ATPS tested. Vesicle partitioning was determined based on the relative fluorescent intensity of the Rhodamine label in the two phases. Uncertainty is the standard deviation of the average of ~400 measurements............................................................38

**Table 2-2:** U15 partitioning in different ATPS emulsions with extruded vesicles, unextruded vesicles, or no vesicles present. U15 partitioning was based on the relative fluorescent intensity of Alexa-647 in the two phases. Uncertainty is the standard deviation of the average of ~400 measurements............................................................38

**Table 3-1.** Partitioning of chelator species and chelated calcium; calculated values of chelator-calcium stability constants and free [Ca$^{2+}$] at the beginning of enzymatic reaction. $^a$Data calculated for 30 mM [Ca$^{2+}$] total, 30 mM chelator species, 100 mM ionic strength, pH 9.0. $^b$CaCO$_3$ K$_{sp}$ for ACC and calcite remain constant at $-\log(K_{sp}) = 6.40$ and 8.47, respectively, at 25 °C. $^c$Lipid $\beta'$ ranges from of 0.1-2.0. $^d$free [Ca$^{2+}$] for each chelator species was estimated by assuming a 1:1 ratio of Ca$^{2+}$: chelator binding, and calculating the effective stability constants, $\beta'$ as described in Supporting Information. ..................................................................................64

**Table 4-1:** Partitioning of Ca$^{2+}$, urease, and PAA in a PEG/dextran ATPS (V$_{Dex}$ : V$_{PEG}$ = 1 : 49), with and without 50 mM Ca$^{2+}$ and/or 10.5 mg/mL PAA 2-11 kDa, and 10.5 units/mL urease. $^a$Urease not determined in this ATPS because CaCO$_3$ could not be precipitated without Ca$^{2+}$; therefore, urease was not necessary here. $^b$Values normalized to 1 for the phase with lowest concentration, for ease of comparing fold-enrichment in other phases. ..................................................................................105

**Table 4-S1.** Concentrations of Ca$^{2+}$, urease, and polyaspartic acid (PAA) in a PEG/dextran/coacervate three-phase system (V$_{Dex}$ : V$_{PEG}$ = 1 : 49), with 50 mM Ca$^{2+}$, 10.5 mg/mL PAA 2-11 kDa, and 10.5 units/mL urease. $^a$Based on relative fluorescence intensity. ..................................................................................125

**Table 4-S2.** Mass compositions of particles made under different conditions, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 °C. The remaining mass was used to determine the organic: CaCO$_3$ mass ratio. The weight loss between 200 and 500 °C (second thermal decomposition step) was all attributed to organic material. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step. ..................................................................................126

**Table 5-1:** Concentration of Ca$^{2+}$ in PEG/dextran/coacervate A3PS with different weight ratios of bidisperse PAA. All separations had the same basic composition: V$_{Dex}$:V$_{PEG}$ = 1 : 9; 50 mM Ca$^{2+}$; 15 mg/mL total PAA. Error is the standard deviation of the average of 3 measurements..................................................................................146
Table 5-2: Mass compositions of CaCO₃-PAA particles made using mono- and bidisperse PAA, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 degrees Celsius, the relative amount of organic carbon (second thermal decomposition step) was calculated from the loss of percent mass between 200 and 500 degrees Celsius, and the relative amount of inorganic carbon (third thermal decomposition step) was calculated from the loss of percent mass between 500 and 800 degrees Celsius. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step .................................................................147

Table 5-3: Mass compositions of CaCO₃-PAA particles made using mono- and bidisperse PGA, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 degrees Celsius, the relative amount of organic carbon (second thermal decomposition step) was calculated from the loss of percent mass between 200 and 500 degrees Celsius, and the relative amount of inorganic carbon (third thermal decomposition step) was calculated from the loss of percent mass between 500 and 800 degrees Celsius. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step .................................................................151

Table 5-4: Mass compositions of CaCO₃-PAA particles made using both PAA and PGA, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 degrees Celsius, the relative amount of organic carbon (second thermal decomposition step) was calculated from the loss of percent mass between 200 and 500 degrees Celsius, and the relative amount of inorganic carbon (third thermal decomposition step) was calculated from the loss of percent mass between 500 and 800 degrees Celsius. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step .................................................................156
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta'$</td>
<td>effective stability constant</td>
</tr>
<tr>
<td>A3PS</td>
<td>aqueous three-phase system</td>
</tr>
<tr>
<td>AAS</td>
<td>atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ACC</td>
<td>amorphous calcium carbonate</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AMV</td>
<td>artificial mineralizing/mineralization vesicle</td>
</tr>
<tr>
<td>ATPS</td>
<td>aqueous two-phase system</td>
</tr>
<tr>
<td>ATR</td>
<td>attenuated total reflectance</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DOPE-PEG2k</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine poly(ethylene glycol) 2000 Da</td>
</tr>
<tr>
<td>DOPE-Rh</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)</td>
</tr>
<tr>
<td>Dx</td>
<td>dextran</td>
</tr>
<tr>
<td>EDDS</td>
<td>ethylenediamine disuccinic acid</td>
</tr>
<tr>
<td>EDS</td>
<td>energy-dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>Egg-PC</td>
<td>Egg-phosphatidylcholine</td>
</tr>
<tr>
<td>Egg-PG</td>
<td>Egg-phosphatidylglycerol</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GLDA</td>
<td>glutamic acid diacetic acid</td>
</tr>
<tr>
<td>K</td>
<td>partitioning coefficient</td>
</tr>
<tr>
<td>$K_c$</td>
<td>stress intensity factor</td>
</tr>
<tr>
<td>LLPS</td>
<td>liquid-liquid phase separation</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>MA</td>
<td>malic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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</tbody>
</table>
PAA  poly(aspartic acid)
PEG  poly(ethylene glycol)
PGA  poly(glutamic acid)
PILP  polymer-induced liquid precursor
PPDA  p-Phenylenediamine
RG1P  rac-glycerol 1-phosphate sodium salt
RNA  ribonucleic acid
ROI  region of interest
SAED  selected area electron diffraction
SEM  scanning electron microscope
SNARF  seminaphtharhodafluor
STEM  scanning transmission electron microscope
TEM  transmission electron microscope
TGA  thermogravimetric analysis
U15  RNA oligonucleotide, 15mer of U
XRD  X-ray diffraction
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Chapter 1

Introduction

Overview

The synthesis routes of biominerals offer a blueprint for the development of new synthetic materials with mechanical and optical properties that exceed what is currently available. This thesis will explain the development of a bio-inspired microreactor that serves as a platform for the synthesis of artificial biomaterials. In this introduction, I will detail the concepts necessary for a comprehensive understanding of bio-inspired mineralization microreactors. The basics of sub-cellular organization and liquid-liquid phase separation will be briefly discussed. Next, the properties and functions of biominerals will be explained to provide motivation for the development of bio-inspired materials. I will then discuss how Biology utilizes sub-cellular compartmentalization to direct biomineral assembly, providing the basis for bio-inspired mineralization systems. Examples of these bio-inspired systems will be discussed, including their respective advantages and shortcomings. Finally, I will explain how this project aims to address the questions left unaddressed by those prior systems.

1.1 Sub-cellular compartmentalization

The cell is colloquially referred to as the basic building block of Biology. However, the cell itself is divided into specialized compartments, each with a specific function. These sub-compartmental spaces serve as specialized reaction environments with regulated conditions, such as local ion concentrations. Compartmentalization within cells can be broken down into two broader categories: membrane-bound and membrane-less organelles.
1.1.1 Membrane-bound organelles

Several of these compartments are individually contained within lipid bilayers, a thin membrane formed from two layers of phospholipids. Cells themselves, as well as specific organelles such as mitochondria and vesicles, are surrounded by a proteolipid membrane. Vesicles themselves serve numerous functions, from reaction sites to intercellular transport of reactants and products. The lipid membrane serves as a semi-permeable barrier between the vesicle interior and exterior respectively.

1.1.2 Membrane-less organelles

A less known, but nonetheless vital, intracellular encapsulation method is the membraneless organelle. As the name applies, these compartments lack a lipid membrane and include assemblies such as the ribosome and carboxysome in bacteria. These protein condensates perform specific functions dictated by the encapsulated enzymes. Very recently, a new class of membraneless organelles has been identified as the product of intercellular liquid-liquid phase separation (LLPS). LLPS refers to the demixing of a single liquid phase into two separate, liquid phases: a dilute phase (the cytoplasm) and a condensate phase (the organelle). These ‘liquid organelles’ demonstrate fluid-like behaviors, such as the ability to merge, split, and flow with applied force, and include structures such as the cytoplasmic P granules and the nucleolus.

1.2 Aqueous-aqueous phase separation

The complex nature of biological systems has resulted in a need for simpler model systems. In regards to liquid organelles, this need has been met by aqueous-aqueous phase separations. Although aqueous-aqueous phase separations has been utilized in the field of Chemistry for years, the recent discovery of LLPS in Biology has given them additional
functionality as model systems for cells and membraneless organelles. Broadly speaking: phase separation can be categorized into two forms: segregative and associative separation (Figure 1-1).\textsuperscript{12}

\textbf{Figure 1-1:} Schematic representations of the two forms of aqueous-aqueous phase separation: (a) segregative and (b) associative. (a) Segregative phase separation results in two polymer-rich phases, or a polymer-rich phase and a salt-rich phase. (b) Associative phase separation results in a dense, polymer-rich phase and a dilute phase.

\subsection*{1.2.1 Segregative phase separation}

Segregative phase separation occurs due to the non-specific interactions between two polymers, or one polymer and a salt.\textsuperscript{13-14} This results in two aqueous phases, each rich with one of the species, hence the name aqueous two phase separation (ATPS). Traditionally, ATPS is a purification and separations method in Chemistry.\textsuperscript{15-16} In more recent years, ATPS’s have been utilized as models for the cytoplasm\textsuperscript{17} due to the high concentration of macromolecules present\textsuperscript{18} as well as cellular models in the form of stabilized ATPS emulsions.\textsuperscript{19-21}

\subsection*{1.2.2 Associative phase separation}

Associative phase separation results from associative interactions between two species. This results in a dense polymer-rich phase, known as a coacervate, and a dilute, “polymer-poor” phase.\textsuperscript{22} When the nature of these interactions is based on ion pairing, the process is known as complex coacervation.\textsuperscript{23} Coacervates have been used to model membraneless organelles due to
the similar nature of the interactions involved. Biologically derived molecules such as RNA are often used in these coacervate models.24-25

1.3 Biomineralization

Many biological processes utilize both membrane-bound and membrane-less organelles. My favorite example, and one of the most observable, is biomineralization. Biominerals are technically defined as biologically hard tissue, and includes structures such as bones, teeth, and exoskeletons.26 Another definition of a biomineral is an inorganic structure within organic components. The prior examples all contain vasculature or protein additives within their scaffolds. The presence of these organic additives alters and enhances the physical properties of the inorganic mineral beyond its native state.27 This introduction will largely be focusing on examples of the most common prolific biomineral: calcium carbonate.

1.3.1 Biogenic calcium carbonate

Calcium carbonate (CaCO₃) is one of the most abundant compounds in the Earth’s crust (about 4%)28 and can be found in countless biominerals. Various aquatic organisms enhance the mechanical properties of CaCO₃ in the form of biominerals to create effective support material. The sea shell mineral nacre, for instance, is comprised of alternating layers of CaCO₃ and organic material (Figure 1-2a).29 The sandwich-like structure allows force to be diffused across the layers, resulting in a sheer strength several orders of magnitude greater than that of CaCO₃ alone.30-31 Another interesting example comes from the sea urchin, an organism covered in calcite spines. Mineral spines are grown into elaborate, scaffold-like single crystals with incredible flexibility (Figure 1-2b).27,32 Beyond structural support, Biology also utilizes CaCO₃
birefringence to create specialized optical lenses.\textsuperscript{33-34} The refractive index of the mineral changes depending on the propagation direction of light, resulting in double diffraction. The sea mollusk chiton utilizes this phenomenon to create CaCO\textsubscript{3} lenses that can focus both above and below the surface of the ocean simultaneously (Figure 1-2c,d).\textsuperscript{35}

![Figure 1-2](image)

**Figure 1-2:** Examples of functional biogenic calcium carbonates. (a) Scanning electron micrograph (SEM) of fractured nacre, comprised of alternating layers of CaCO\textsubscript{3} and organic material. Arrows note organic. Reprinted from [29] with permission from Elsevier. (b) SEM sea urchin spine with organic material removed. Reprinted from [27] with permission from Annual Reviews. (c) Optical microscopy image of chiton surface with notated eyes. Black arrow notates eye with CaCO\textsubscript{3} lenses intact, white arrow notates arrow without. (d) Optical microscopy image of cross-section of chiton CaCO\textsubscript{3} lens. Reprinted from [35] with permission from Elsevier.

The exquisite structures that provide biominerals their enhanced properties can be attributed to their ability to direct the organic content on the sub-micron scale. For instance, the sea urchin tooth is largely made of calcite, yet is capable of biting through rocks of the same mineral. The fracture toughness of the tooth ($K_c = 0.97$ MPa\textperiodcentered m\textsuperscript{1/2}) exceeds that of pure calcite ($K_c = 0.12$-$0.54$ MPa\textperiodcentered m\textsuperscript{1/2}).\textsuperscript{36} The toughness of the tooth can be partially attributed to its scaffold-like microstructure (Figure 1-3).\textsuperscript{37} The tooth is comprised of an interconnected series of plates and columns (annotated as ‘C’ and ‘Pl’ respectively in Figure 1-3) comprised of calcite and the
relatively harder magnesium calcite, respectively. This structure directs the propagation of cracks, preserving the structural integrity of the tooth and increasing the fracture toughness.\textsuperscript{38} Proteins associated with the magnesium calcite columns contain a relatively higher concentration of aspartic acid residues compared to other regions.\textsuperscript{37} This implies that the local protein content directs the mineral structures. Similar phenomena can be observed in other sea creatures such as the ascidian, a filter feeder organism with a CaCO\textsubscript{3} shell. Within the shell, aspartic acid and glutamic acid-rich proteins correspond to regions of amorphous calcium carbonate and calcite respectively.\textsuperscript{39}

![Figure 1-3: (a) Ca\textsuperscript{2+} and (b) Mg\textsuperscript{2+} secondary ion mass spectrometry images of polished carinar process plates sections of a sea urchin tooth. (C) and (Pl) note the epitaxial columns and plates respectively. Reprinted from [37] with permission from Elsevier.](image)

1.4 Biomineralization methods

The unique optical and mechanical properties of biominerals are appealing in the design of synthetic materials. Thus, the synthesis mechanisms behind these bio-materials are also worth studying. Biological mineralization utilizes both encapsulation methods discussed prior: membrane-bound and membrane-less organelles.
1.4.1 Biogenic mineralization vesicles

The membrane-bound organelles come in the form of mineralization vesicles. These vesicles are lipid membrane-bound compartments that aid in the early stages of biominerals formation.\textsuperscript{40-41} The vesicles concentrate the precursors necessary for mineral formation for the sake of transport or for direct synthesis. The precursor material within the vesicles is carefully regulated via chelation in order to prevent catastrophe within the cell.\textsuperscript{42-43} Free Ca\textsuperscript{2+} ions, for instance, are used as signaling agents, and can even trigger premature cellular necrosis.\textsuperscript{44} Typically, mineralization vesicles are found in multi-cellular organisms and have been directly observed \textit{ex vivo} (Figure 1-4).\textsuperscript{45}

![Image](image_url)

\textbf{Figure 1-4}: Cryo-SEM micrographs of cryo-sectioned neonatal calvaria. (a) Mineralizing bone (b) in proximity to mineral-containing vesicles (arrowheads). (b) Higher magnification view of intracellular mineral-containing vesicles. Inset: higher magnification view of mineral globules. Reprinted from [45] with permission from Elsevier.

1.4.2 Biogenic amorphous mineral precursors

The other encapsulation method, aqueous-aqueous phase separation, comes in the form of an amorphous liquid precursor. Although never observed directly, there is mounting evidence of liquid phase precursors to many biominerals.\textsuperscript{46-47} Theoretically, the amorphous state of this phase allows both the micro and macro-structure of the mineral to be directed prior to
crystallization. The bulk of this evidence comes from \textit{ex vivo} experiments, in which synthetic polymers or polypeptides that are analogous to proteins associated with the early stages of biomineral formation are subjected to a mineralizing environment.\textsuperscript{48-49} For instance, the matrix protein Pif80 is thought to be instrumental in the formation of nacre. When added to a calcium salt solution, recombinant Pif80 units undergoes liquid-liquid phase separation in the form of a complex coacervate.\textsuperscript{50} I hypothesize that these precursor phases are highly analogous to the membraneless organelles observed in the cell.

The concept of liquid precursors provides a possible mechanism for the creation of intricate biomineral shapes. In principle, an amorphous precursor can be more easily shaped into the necessary shape than the final material. The mineral then retains the shape upon crystallization.\textsuperscript{46} Computational chemists have gone one step further and theorized the possibility of multiple phase separation events.\textsuperscript{51-52} In theory, hierarchical minerals are created step-wise through sequential phase separations, each one building upon the previous. Figure 1-5 depicts various species of diatoms, single-cell algae which create silica shells, and computational models of the proposed mineralization mechanisms used to create the shells.\textsuperscript{53} According to the model, the mineralization starts from a central point and builds radially. Each step in the process is comprised of a liquid phase separation followed by silica condensation. The theories presented here are not exclusive to diatoms or silica, however, and can be applied to other organisms as well. Other biominerals have hierarchical structural motifs that can be potentially explained by similar phase separation phenomena.\textsuperscript{39, 54-55}
It should be noted that mineralization vesicles and amorphous mineral precursors are not necessarily mutually exclusive and it is entirely possible that cells utilize both simultaneously. In fact, in the mineralization vesicles in Figure 1-4, amorphous mineral precursors can be observed. The exact state of matter of this precursor material is debatable, though that is not the goal of this thesis. Instead, I will be focusing on the synthetic analogues inspired by these systems.
1.5 Artificial biomineralization

Many materials researchers have emulated mineralization vesicles capable of producing synthetic biominerals. Rather than attempt perfect biological equivalents, the goal of these systems is to create materials by mimicking specific aspects of biological systems.

1.5.1 Synthetic mineralization vesicles

Synthetic mineralization vesicles imitate specific features of mineralization vesicles, namely a specialized mineralizing environment encapsulated by a semi-permeable membrane.\textsuperscript{56-58} In doing so, they can study select aspects of the mineralization vesicle. CaCO\textsubscript{3} is often used as the model mineral, due to its abundance in biominerals, and this introduction will speak to that specific mineral. For instance, Joester et al. developed a mineralization liposome using the agarose gel gentle hydration method (Figure 1-6).\textsuperscript{59} A giant vesicle was hydrated with free Ca\textsuperscript{2+}-rich aqueous solution. Excess Ca\textsuperscript{2+} outside the vesicle was removed using a purification method that does not disrupt the vesicle. The second precursor, carbonate, was added to the system using the ammonium carbonate ((NH\textsubscript{2})\textsubscript{2}CO\textsubscript{3}) diffusion method. Briefly, (NH\textsubscript{4})\textsubscript{2}CO\textsubscript{3} sublimes to NH\textsubscript{3} and CO\textsubscript{2} gases and dissolves to release free CO\textsubscript{3}\textsuperscript{2-}, which then binds to the Ca\textsuperscript{2+} to create the mineral.\textsuperscript{60} This diffusion method is not exclusive to this system or mineralization vesicles in general, and is frequently used as a slow, controllable mineralization technique. The encapsulated method was based on the locally concentrated Ca\textsuperscript{2+} inside the vesicle, contained by the membrane.
Other approaches do not necessarily utilize lipid membranes. For instance, polymersomes have also been used as synthetic mineralization vesicles.\textsuperscript{61} Regardless of the membrane composition, however, the basic approach remains consistent across most examples: aqueous precursor solution is encapsulated within a membrane, and an additional precursor diffuses through the membrane. Although effective, the preparation methods introduce a number of drawbacks to the scalability. For instance, most giant vesicles are incredibly heterogeneous in regards to membrane count and size distribution due to the nature of the preparation methods.\textsuperscript{62} The crop of viable vesicles for mineralization is greatly limited by this heterogeneity. Methods capable of asserting greater control over the dispersity, such as microfluidics, are restricted by their throughput.\textsuperscript{63-64} Furthermore, synthetic mineral vesicles are typically limited in the mineral structures they can produce. These analogues are designed to only capture one aspect of biogenic mineralization, and therefore do not provide much control over the mineral synthesis.
1.5.2 Artificial liquid mineral precursors

The other synthetic bio-mineralization approach, the amorphous liquid precursor, does not have a direct Biological analogue. Rather, systems have been developed from collective observations of mineral precursors with the goal of creating minerals with exceptional properties. One method has utilized complex coacervates as immediate precursors to minerals. A positively charged metal ion, such as free Ca\(^{2+}\), binds to a negatively charged polymer to create a separate liquid phase. Mineralization is induced through the addition of a counter-ion that binds to the calcium and displaces the chelating polymer. A substantial amount of the chelating polymer is incorporated into the final mineral structure. The presence of this polymer influences the mineral crystal structure, stabilizing otherwise thermodynamically unstable polymorphs. The mineral macrostructure, the shape, is dictated by that of the coacervate. For instance, Chujo et al created amorphous calcium carbonate (ACC) microspheres from complex coacervates comprised of polyacrylic acid and calcium (Figure 1-7). The size of the microspheres was dictated by the size of the coacervates, which in turn was dictated by the complexation time between polyacrylic acid and calcium. The particles were determined to be ~30% by weight polyacrylic, according to thermogravimetric analysis. Unfortunately, the coacervate-mediated method is somewhat limited in terms of the possible mineral structure that can be made. Shapes beyond spheroids are difficult to achieve due to the liquid nature of complex coacervates; spheres are the most energetically favorable conformation for suspended liquids due to surface tension.
Figure 1-7: SEM micrographs of CaCO$_3$ microspheres prepared from polyacrylic acid and calcium coacervates. Coacervates were mineralized using the ammonium carbonate diffusion method after (a) 3 minutes, (b) 30 minutes, (c) 1 hour, (d) 5 hours, (e) 18 hours, and (f) 24 hours of coacervate complexation time. Reprinted images with permission from [71]. Copyright 2007 American Chemical Society.

The other, slightly different and more prominent method is the polymer-induced liquid precursor (PILP). Much like a mineralizing coacervate, both free Ca$^{2+}$ and a negatively charged polymer are present in solution at the start. The polymer concentration is sufficiently low, however, that the critical threshold needed for phase separation is not met (Figure 1-8). A liquid phase only forms upon the addition of carbonate. The exact makeup of this phase is debatable, though it is thought to contain both polymer and amorphous mineral. Over time, this phase transitions into a mineral with polymer included. The appeal of the PILP method is the ability to control the mineral macrostructure by confining the mineralizing liquid in the desired shape. Since the PILP process starts with a dilute phase, as opposed to a coacervate phase, the mineralizing solution can be more easily drawn into different containers prior to mineralization. The mineral adapts the shape of the container as the reaction proceeds, allowing for elaborate, single-crystal structures.
Figure 1-8: Schematic representation on the differences between (a) coacervate-mediated biomineralization and (b) the polymer-induced liquid precursor (PILP) process. The important distinction is when liquid-liquid phase separation first occurs during the mineralization reaction. Phase separation occurs prior to the mineralization reaction in coacervate-mediated process, and during the reaction in the PILP process.

For instance, calcite nanowires have been produced using a porous membrane in combination with the PILP method (Figure 1-9a). Briefly, a dilute solution of calcium and polyacrylic acid was drawn into a polycarbonate track-etch membrane via capillary action, then mineralized via the ammonium carbonate diffusion method. After crystallization, the membrane was removed to free the single-calcite nanowires. Similar methods have been utilized to produce single crystal calcite scaffolds (Figure 1-9b) and honeycomb-pattern thin films (Figure 1-9c). In all cases, the resulting mineral has a comparatively low organic content in contrast to mineral produced via the coacervate-mediated method. The honeycomb film, for instance, was approximately 0.9% by weight organic material. The PILP method has also been used to create materials besides CaCO₃. Gower et al. mineralized a collagen-like scaffold with calcium
phosphate using a solution of free calcium, phosphate, and polyaspartic acid (Figure 1-9d,e).\textsuperscript{80} X-ray diffraction analysis of the mineralized fibers revealed the presence of hydroxyapatite, the primary calcium phosphate crystal structure found in bone.

Figure 1-9: Various synthetic biominerals produced via the polymer-induced liquid precursor process. (a-c) SEM micrographs of CaCO\textsubscript{3} structures prepared by injecting mineralizing solutions into different molds, then producing single crystal calcite crystals via the carbonate diffusion method. (a) Calcite nanowires extracted from a polycarbonate membrane. Reprinted with permission from [77]. Copyright 2011 Wiley. (b) Calcite scaffold extracted from a three-dimensional suspension of polystyrene spheres. Reprinted with permission from [78]. Copyright 2008 Wiley. (c) Patterned thin-film of calcite assembled atop hexagonally closed packed polystyrene spheres. Reprinted with permission from [79]. Copyright 2010 American Chemical Society. (d-e) TEM micrographs of fragments from a PILP-mineralized collagen-like scaffold. (d) Platelets of calcium phosphate broken off the larger scaffold structure. Arrows notate platelets viewed from the side, which have a dark outline. (e) Fragment of mineralized collagen from the larger scaffold structure. Calcium phosphate platelets viewed from the side have a noticeable dark outline. Reprinted from [80] with permission from Elsevier.

While the PILP method has been used to create some rather intricate structures, the technique does have its limitations. For one, the degree of control over the mineralization process is intangible. With the exception of the physical container which encapsulates the mineralizing environment, control over the micro-structure is rather limited. The method
provides no controllable direction for the incorporation of organic material, a key component of biominerals. Organic inclusions direct the crystal structure and morphology of biominerals, and spatial control over both those factors is essential.

1.6 Summary

The synthetic methods discussed prior aim to emulate one or two aspects of biomineralization systems. This has enabled the productions of synthetic biominerals with exquisite structures and new potential new vectors for biomedical science.\textsuperscript{66, 80} The simplicity of these methods, though, limits their localized control over the mineralization process. Structural and chemical motifs like asymmetry and differing organic content are key components in biominerals.\textsuperscript{39, 54-55} Fine control over the local reaction rates and local precursor availability are essential to the creation of these motifs. The best way to do so is to combine various aspects of these synthetic systems to create and all new platform for bio-inspired materials synthesis. My goal is not to create an exact duplicate of a biological mineralizing system, but rather develop something heavily inspired by Biology in order to create materials with dynamic, non-equilibrium structures. This thesis will detail the experimental development of a system we have dubbed the artificial mineralization vesicle (AMV).

Chapter 2 will discuss the further development of large-unilamellar vesicle (LUV)-stabilized ATPS emulsions as microreactors (Figure 1-10). A previous publication on the subject focused exclusively on one particular ATPS composition. This chapter expands the potential of the system by using vesicles to stabilize ATPS of different compositions, including a salt-rich phase. The chapter also examines the robustness of the system by demonstrating the influence of vesicle treatment on emulsion stability and effectiveness. We find these stabilized
emulsions to be an appealing alternative to giant vesicle reactors because of the increased homogeneity of the former.

**Figure 1-10:** Development of stabilized aqueous two phase system emulsions using liposomes prepared via gentle hydration.

Chapter 3 will detail the development of an artificial mineralization vesicle from these LUV-stabilized aqueous two phase separation emulsions (Figure 1-11). The AMV overcomes many of the shortcomings of synthetic mineralization vesicles discussed prior by encapsulating the reaction on the basis of partitioning. The enzyme which drives the mineralization reactions, urease, partitions exclusively to the AMV interior phase, thus localizing CaCO₃ formation. The mineral precursor, calcium, is bound by a chelator in order to prevent aggregation with the negatively charged phospholipid head-groups. The AMV presents a facile, yet equally effective alternative to traditional synthetic mineralization vesicles.
Chapter 4 will describe the incorporation of a third phase into the AMV in the form of a mineralizing complex coacervate (Figure 1-12). Much like the examples discussed prior, the coacervate is comprised of positively-charged Ca$^{2+}$ bound to a negatively-charged polycarboxylates, in this case polyaspartic acid. The coacervates convert to CaCO$_3$ microspheres with a high degree of polymeric incorporation. Unlike prior examples, though, the coacervate exists as part of a larger system that facilitates the mineralization reaction. This allows for the creation of dynamic, non-equilibrium mineral structures not previously possible with comparable methods. The chapter also briefly explores the synthesis of another biomineral, calcium phosphate, within AMVs.
Chapter 5, the final experimental chapter, will examine the influence of polycarboxylate length and identity on the coacervates and subsequent minerals. Small changes to the chelating polymer result in larger changes that reverberate throughout the reaction. For instance, changing polyaspartic acid to polyglutamic acid creates minerals with vastly different crystal structures and amounts of polymer content. The chapter also explores utilizing multiple polycarboxylates to induce subsequent phase separation events throughout the mineralization reaction (Figure 1-13). Multiple phase separations allows for the creation of minerals with tiered structures and non-uniform organic gradients of multiple polymers. This represents an important step toward directing the local organic content with artificial biominerals.

**Figure 1-13:** Sequential phase separation events within the AMV using multiple polycarboxylates

Lastly, Chapter 6 will summarize general conclusions and explore future directions for the AMV project. Possible experiments that will explore new methods controlling subsequent phase separation events within the AMVs and material gradients within the minerals will be discussed. Experiments with mineralizing coacervates outside of AMVs will also be described.
1.7 References


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Chapter 2
Stabilizing All-Aqueous Emulsions of Different Compositions using Liposomes Prepared via Gentle Hydration

2.1 Abstract

Artificial micro-reactors serve as effective models of biological micro-encapsulation as well as vectors for new bio-technologies. We previously reported large-unilamellar vesicle (LUV)-stabilized aqueous two-phase systems (ATPS), comprised of polyethylene glycol (PEG) and dextran (Dx), as all-aqueous emulsion micro-reactors. LUVs are defined by a narrow range of diameters, achieved by forcible extrusion of gentle hydration products. Although extrusion is successful at narrowing the liposome size distribution and removing undesired lipid aggregates, this time-intensive process often results in sample loss. Prior experiments also focused one ATPS composition, which limits the potential of the platform. Herein we investigate vortex mixing as alternative liposome treatment method in regards to preparing our stabilized emulsions. Our data illustrate that extrusion is not necessary to create these functional microreactors, as emulsions stabilized with unextruded liposomes are just as effective. Furthermore, liposomes are used to stabilize ATPS of differing chemical compositions, including a salt-rich phase, expanding the potential reactions possible in this system.

2.2 Introduction

Artificial bioinspired micro-reactors have been utilized to speculate on biological compartmentalization on the sub-cellular level, as well as develop new biotechnological and medical technologies. At the most basic level, a micro-reactor consists of a semi-permeable...
barrier, or membrane, which encapsulates the reaction of interest. The membrane should house
the reaction while still allowing for reactants and products to diffuse in and out of the reactor. A
variety of compartmentalization methods have been employed as micro-reactors, including
liposomes,\textsuperscript{1-2} polymersomes,\textsuperscript{3} colloidosomes,\textsuperscript{4-5} and liquid-liquid emulsions, both oil/water\textsuperscript{6-7} and
aqueous/aqueous. In the emulsions, droplets of one phase suspended in another comprise the
reactor interior and exterior respectively.

Aqueous two-phase systems (ATPS) are a common form of aqueous/aqueous phase
separation, either through two polymers or one polymer and salt. Above critical concentrations,
the systems separates into two phases, each enriched in one of the components. Conventionally,
ATPS has used as extraction and purification method of molecules of interest.\textsuperscript{8} More recently,
though, they have become appreciated as biomimetic media. These macromolecular-rich phases
resemble crowded biological fluids such as the cytoplasm, which can be upwards of 30%
biomacromolecules by volume.\textsuperscript{9} Crowding has been shown to have a significant effect on
biomolecular interactions and reactions, and should be considered when designing bioinspired
environments.\textsuperscript{9-10} ATPS also provide coexisting compartments with differing chemical and
physical properties. Encapsulation of molecules within these system is driven by preferential
partitioning between the two phases.\textsuperscript{11-12}

Droplets of one aqueous phase can be dispersed in the second, continuous aqueous phase
to create distinct microenvironments. Due to the low surface energy of the interface, surface
stabilizers are required to inhibit coalescence of the dispersed phase. A variety of different
approaches have been developed, including protein nanoparticles,\textsuperscript{13-14} silica nanoparticles,\textsuperscript{15}
cellulose particles,\textsuperscript{16-17} polyelectrolyte assembles,\textsuperscript{18} and liposomes.\textsuperscript{19-20} We previously reported
stabilized polyethylene glycol (PEG)/dextran (Dx) ATPS using large unilamellar vesicles
(LUVs) (~130 nm in diameter) as the emulsifiers to create Pickering-like emulsions. Our LUV-stabilized all-aqueous emulsions have been utilized to encapsulate ribozyme catalysis as well as CaCO$_3$ mineralization. One of the most reliable methods of liposome formation is the gentle hydration method, in which a dried thin film of lipids is slowly hydrated with a solution of interest. The resulting product is a heterogeneous mixture of lipid aggregates, and single and multi-layered liposomes. The homogeneity of these mixtures can be improved through the extrusion, in which the solution is forcibly passed through a porous polycarbonate membrane. Different pore sizes are utilized to create vesicles of different diameters; for instance, large unilamellar vesicles fall within the size regime of 100 to 1000 microns in diameter. Although very effective at homogenizing gentle hydration liposomes, most extruders can only process less than a milliliter of solution at a time. This volume restraint greatly limits the amount of liposomes that can be treated at one time, as well as the scalability of the entire system. Other methods of liposome homogenization, such as vortex mixing or sonication, should be considered as alternatives to extrusion.

In this chapter, the influence of extrusion of liposomes prepared via gentle hydration on the efficacy of stabilized all-aqueous emulsions was investigated. Various stabilized ATPS emulsions were prepared using liposomes with identical lipid compositions that were either extruded through 200 nm pores or left unextruded, and characterized based on their short-term stability, permittivity of reactants, and ability to encapsulate reactions. This chapter also builds upon our prior findings by examining other ATPS systems besides PEG/Dx. The LUV-stabilization mechanism is not exclusive to PEG/Dx, and can be used with ATPS emulsions of different compositions.
2.3 Results

Various ATPS emulsions were prepared and stabilized using extruded or unextruded liposomes prepared via gentle hydration. The emulsions were characterized using fluorescent confocal microscopy. In the context of this chapter, the term “vesicle” refers to a “lipid vesicle” and is used interchangeably with the term “liposome”.

2.3.1 Formation of stabilized all aqueous emulsions

Liposomes were prepared in PEG-rich phase (or PEG solution) using the gentle hydration method. All vesicles, regardless or treatment method, had an equal mole fraction of zwitterionic phosphatidylcholine and anionic phosphatidylylglycerol headgroups, with 2.8 mol % 2 kDa PEGylated lipid. Vesicles were then either passed through an extruder lined with polycarbonate membranes with 200 nm pores or placed on the vortex mixer for ~10 seconds. Vesicles passed through the membrane will hereto be referred to as “extruded” and vesicles placed on the mixer as “unextruded”. The gentle hydration method naturally results in an extremely heterogeneous mixture of single and multi-layered liposomes as well as lipid aggregates. Dynamic light scattering (DLS) analysis of the gentle hydration raw product revealed an average particle diameter of 3200 ± 200 μm. The extrusion process forcibly narrowed the size distribution of the liposomes, reducing the average particle diameter to 154 ± 4 μm. In doing so, however, a significant volume of the liposome stock was lost. PEG-rich phase does not extrude as easily as a buffer solution, and inevitably leaks from the mini-extruder during the process.26 Although the mini-extruder is not the only way to homogenize a liposome stock, other methods have similar drawbacks.27-28 Vortex mixing achieves the same narrowing effect, albeit to a lesser extent: average particle diameter of 270 ± 20 μm. The vortex mixer should still be considered a post-hydration treatment, however, given how little time and effort is required.
In order to test the flexibility of liposomes as stabilizers, three different ATPS were studied, each composed of water, polyethylene glycol (PEG 8 kDa), and a second polymer or a salt. The three compositions tested were PEG and dextran (Dx, 10 kDa), PEG and Ficoll (70 kDa), and PEG and sodium sulfate (SO$_4^{2-}$). Individual phases were separated then recombined with liposomes, extruded or unextruded, for a relative phase volume of 1:9 Dx/Ficoll/SO$_4^{2-}$:PEG. The added volume of liposomes in PEG-rich phase was included in the ratio. Mechanical agitation of the systems resulted in droplets of the denser phase in a continuous PEG-rich phase.

**Figure 2-1**: Schematic representation of the preparation of liposome-stabilized emulsions
In all cases, regardless of liposome treatment or ATPS composition, the vesicles adsorbed to the interfaces between the two phases (Figure 2-2).

**Figure 2-2:** confocal microscopy with fluorescent overlay images of various vesicle-stabilized aqueous two-phase separation (ATPS) emulsions. Unextruded vesicles were able to effectively stabilize droplets of one phase within a continuous PEG-rich phase as well as extruded vesicles with identical lipid composition. (a,b) PEG/dextran emulsions; (c,d) PEG/Ficoll emulsions; (e,f) PEG/sulfate emulsions. (a,c,e) Extruded vesicles at the interface. (b,d,f) Unextruded vesicles at the interface. Rhodamine-tagged vesicles have been false colored red. Scale bar applies to all images.

### 2.3.2 Influence of extrusion on emulsion size distribution

The reduction of average liposome size via extrusion also influences to size distributions within the stabilized droplets within the ATPS emulsions. On average, extruded vesicles create smaller, more homogeneous stabilized droplets in the continuous PEG-rich phase than the unextruded vesicles (Figure 2-3). Extrusion of vesicles reduces the average vesicle-stabilized dextran-rich phase droplet diameter from $13 \pm 2$ to $7 \pm 1$ μm. Likewise, the average Ficoll-rich diameter reduced from $9 \pm 3$ μm to $5 \pm 1$ μm. The stabilized sulfate-rich droplets displayed no apparent size decrease when using extruded vs unextruded vesicles ($10 \pm 4$ vs $9 \pm 4$ μm).
Figure 2-3: Size histograms of droplets in extruded and unextruded vesicle-stabilized ATPS emulsions. In general, vesicle extrusion results in a decrease in average droplet diameter and distribution spread. (a) Measurements of vesicle (extruded and unextruded)-stabilized dextran-rich droplets; (b) measurements of vesicle-stabilized Ficoll-rich droplets; (c) measurements of vesicle-stabilized sulfate-rich droplets. Red bars indicate emulsions with extruded vesicles, blue indicate unextruded.

2.3.3 Examination of emulsion short-term stability

The short-term stability of the liposome containing emulsions was tested by allowing the samples to equilibrate overnight. Extruded and unextruded vesicle-stabilized all-aqueous emulsions were prepared as described above with the addition of ampicillin to prevent bacterial growth. Samples were then deposited on silanized glass slides with 160 μm silicone spacers and sealed with a cover slip. After initial analysis on the confocal microscope, the sealed samples were placed in a dark, humid environment at room temperature (30°C) for 24 hours. The emulsions were then imaged again without further treatment (Figure 2-4). A similar experiment was performed where samples were allowed to sit for 72 hours as well (Figure 2-S1). Overnight, the droplets of the suspended phase coalesced to some extent in all samples. After 72 hours, coalescence results in significantly larger but still intact stabilized droplets. The PEG/dextran samples showed the least amount of coalescence whereas the PEG/sulfate showed the greatest. These observations are in line with the sample appearance after their initial appearance; the fresh dextran/PEG emulsions appear the most stable, with fairly homogeneous
size distributions of dextran droplets. Inversely, the sulfate droplets appear the least stable for the opposite reasons. It stands to reason this instability would be exacerbated over the course of 24 hours.

**Figure 2-4**: Confocal microscopy with fluorescent overlay images of various vesicle-stabilized ATPS emulsions, 24 hours after initial sample preparation. Vesicle extrusion does not appear to have a significant effect on the long-term stability of the emulsions. (a,b) PEG/dextran emulsions; (c,d) PEG/Ficoll emulsions; (e,f) PEG/sulfate emulsions. (a,c,e) Extruded vesicles at the interface. (b,d,f) Unextruded vesicles at the interface. Rhodamine-tagged vesicles have been false colored red. Scale bar in (a) applies to all parts except for (e,f).

### 2.3.4 Vesicle Distribution

The partitioning of extruded and unextruded vesicles in the different ATPS was investigated by measuring the relative Rhodamine fluorescence in each phase (Table 2-1). In all cases, the vesicles show at least slight preference for the suspended phase in all cases. However, the vesicles show stronger partitioning in the PEG/Ficoll ATPS than the PEG/dextran, and moreso in the PEG/sulfate. The nature of the liposome assembles at the interface was investigated using the fluorescence recovery after photobleaching (FRAP) technique.
Fluorescent liposomes at the ATPS interface were bleached with a high power laser using confocal microscopy. The diffusivity of the liposomes at the interface was determined by how quickly the fluorescent signal recovered at the bleached site. PEG/Dx ATPS emulsions were prepared using either extruded or unextruded vesicles, and part of the liposome lined-interface was bleached (Figure 2-S2). Regardless of whether the vesicles were extruded or not, the projected fits for the FRAP data show that the bleached portion will never fully recover. The lack of recovery indicates the vesicles do not diffuse at the interface.

Table 2-1: Extruded and unextruded vesicle partitioning in the different ATPS tested. Vesicle partitioning was determined based on the relative fluorescent intensity of the Rhodamine label in the two phases.

<table>
<thead>
<tr>
<th>ATPS composition (w/w)</th>
<th>Extruded vesicles</th>
<th>Unextruded vesicles</th>
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<tbody>
<tr>
<td>10%/10% dextran/PEG</td>
<td>1.8 ± 0.4</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>15%/10% Ficoll/PEG</td>
<td>4.3 ± 0.7</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>8%/10% sulfate/PEG</td>
<td>7 ± 2</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

Uncertainty is the standard deviation of the average of ~400 measurements.

2.3.5 Permeability of the liposome-lined interface

The permeability of the liposome-interfaces was investigated by tracking RNA diffusion through the systems. Figure 2-5a depicts the reaction scheme used to qualitatively follow RNA diffusion. Externally added fluorescently-labeled U15 freely diffuses through both the PEG and dextran-rich phases (Figure 2-5b). Over time, the RNA partitions into the dextran-rich phase, which aligns with its partitioning behavior (Table 2-2). Similar diffusion behavior can also be observed in emulsions prepared with PEG/Ficoll and PEG/sulfate ATPS (Figure 2-S3). In all cases, whether the vesicles were extruded or not does not inhibit the diffusion of U15 into the
dispersed phase. Given the nature of local gradients in these experiments, however, relative rates of diffusion cannot be inferred.

FRAP was used to directly examine the diffusion of U15 into liposome-stabilized dextran droplets. After bleaching an entire droplet, fluorescent U15 freely diffused from the PEG-rich phase into the dextran-rich phase (Figure 2-6a). Figure 2-6b depicts normalized U15 fluorescence in the dextran-rich phase before and after bleaching in emulsions prepared with extruded and unextruded vesicles. In both cases, the dextran-rich phase fully recovered almost instantaneously. The halftime of recovery, \( \tau_{1/2} \), for the dextran-rich phase was 0.8 seconds for the emulsion with extruded vesicles, and 0.9 seconds for the emulsion with unextruded. This difference in recovery time is negligible, indicating extrusion has no influence on the permeability of the emulsion interface.

Table 2-2: U15 partitioning in different ATPS emulsions with extruded vesicles, unextruded vesicles, or no vesicles present. U15 partitioning was based on the relative fluorescent intensity of Alexa-647 in the two phases.

<table>
<thead>
<tr>
<th>ATPS composition (w/w)</th>
<th>Extruded vesicles at interface</th>
<th>Unextruded vesicles at interface</th>
<th>No vesicles at interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%/10% dextran/PEG</td>
<td>1.7 ± 0.2</td>
<td>1.73 ± 0.09</td>
<td>1.65 ± 0.09</td>
</tr>
<tr>
<td>15%/10% Ficoll/PEG</td>
<td>1.03 ± 0.05</td>
<td>1.07 ± 0.07</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td>8%/10% sulfate/PEG</td>
<td>0.51 ± 0.05</td>
<td>0.49 ± 0.08</td>
<td>0.45 ± 0.06</td>
</tr>
</tbody>
</table>

Uncertainty is the standard deviation of the average of ~400 measurements.
Figure 2-5: (a) Experimental scheme used to monitor U15 diffusion through the liposome stabilized ATPS emulsion. Two droplets were placed adjacent on a silanized glass slide: one containing a liposome stabilized ATPS emulsion, the other labeled U15 in PEG-rich phase. Contact between the two and subsequent U15 diffusion was initiated through the addition of a coverslip. (b) Fluorescent confocal microscopy images depicting diffusion of labeled-RNA (U15) through vesicle-stabilized PEG/dextran ATPS emulsions. Inset refers to time after diffusion initiation. Rhodamine-tagged vesicles have been false-colored red, Alexa 647-tagged U15 blue.
Figure 2-6: (a) depiction of fluorescence recovery after photobleaching experiment on the fluorescent confocal microscope. Liposome-(extruded or unextruded) stabilized PEG/Dx ATPS emulsions were prepared with added fluorescent U15. A single dextran droplet was selected in the prepared. The droplet and immediate surrounding PEG-rich was bleached with maximum laser power. The average U15 fluorescence intensity was measured within a region of interest (ROI) than remained within the droplet for the entire experiment. (b) fluorescence recovery after photobleaching (FRAP) curves for U15 diffusion in vesicle stabilized PEG/dextran ATPS emulsions. Curves depict the fluorescent bleaching and recovery of Alexa-647 tagged U15 in the dextran-rich phase. The entire dextran-rich droplet was bleached and allowed to recover from the PEG-rich phase. Regardless of whether extruded or unextruded vesicles were used to stabilize the emulsion, the dextran-rich phase fully recovered in ~0.7 seconds.

2.3.6 Encapsulating calcium carbonate synthesis

We previously reported LUV-stabilized PEG/Dx emulsions as artificial mineralization vesicles (AMVs).\textsuperscript{20} In order to test whether unextruded vesicles could still encapsulate mineralization, PEG/dextran-based AMVs were prepared using either extruded or unextruded vesicles. Figure 2-7 depicts AMVs before and after the addition of urea to the emulsion as imaged by darkfield microcopy. Prior to the addition of urea, only the diffraction of PEG/Dx
interface can be observed in the images. After the addition of urea (~40 minutes), bright spots begin to appear. These spots are from amorphous calcium carbonate, meaning the AMVs successfully encapsulated the mineralization reaction.

![Image of darkfield microscopy images](image_url)

**Figure 2-7:** Darkfield microscopy images of artificial mineralization vesicles (AMVs) stabilized with either (a, b) extruded or (c, d) unextruded liposomes. (a, c) Before the addition of urea to the emulsion (pre-mineralization). (b, d) ~40 after the addition urea to the emulsion (post-mineralization).

### 2.4 Discussion

A variety of different compositions were studied to expand the potential of liposome-stabilized emulsions as microreactors. A PEG/Dx ATPS was previously used to create LUV-stabilized emulsions, so it stands to reason that system had the best chance of forming with unextruded vesicles. Ficoll is the polymer most structurally similar to dextran, and a PEG/Ficoll emulsion would theoretically behave in the same way. PEG/SO$_4^{2-}$ was selected as an outlier, in
which the salt is chemically dissimilar from the original interior phase. In all cases, liposomes of identical compositions can be used to stabilize various ATPS, including a phase with nearly 1 M salt present.\textsuperscript{31} This presents new opportunities for possible reactions that are more prolific in a PEG/salt ATPS.\textsuperscript{32-33} The most obvious difference between the different ATPS compositions is the vesicle partitioning (Table 2-1). Vesicle solubility affects the interfacial dynamics, which in turn affects the stability of the emulsion. Fewer vesicles at the surfaces means less total surface area that can be stabilized, meaning more coalescence into larger droplets can occur. This could be part of the reason for the differences in average droplet size.

The effects of extrusion, or lack thereof, on the emulsion functionality were also investigated. In general, unextruded liposomes appear to be effective at stabilizing the emulsions. Even after 24 hours, the suspended droplets still remained largely intact. The permeability of the interface was not comprised by unextruded liposomes, and the emulsion could still encapsulate a mineralization reaction. The mineral does form in slightly different locations, though, depending on whether the liposomes were extruded or not. In the AMVs formulated with unextruded liposomes, the minerals appear to have formed at the interface. This indicates that the unextruded liposomes have a higher Ca\textsuperscript{2+} binding affinity than extruded. Beyond that, the emulsion still performed its intended function.

The most obvious influence extrusion appears to have is the size distribution of the stabilized droplets. Emulsions prepared with extruded liposomes were slightly smaller in general. As stated above, extrusion greatly reduces to average liposome diameter. Assuming the total lipid concentration remains constant after extrusion, this increases the total liposome surface area. A greater total liposome surface area theoretically means more of the ATPS interface can be covered and stabilized. If all phase volumes are kept constant, then a greater
area of interface will result in smaller droplets of the suspended phase. The size polydispersity of the unextruded vesicle-stabilized ATPS can be attributed to the size polydispersity of the vesicles themselves. Lack of vesicle size control will result in a variety of differently sized dispersed phase droplets, depending on which sized vesicles are present at the interface.

One interesting trend is the influence of extrusion on vesicle partitioning in the system; extrusion seemingly increases the partitioning into the dispersed phase. This trend in most pronounced in the PEG/sulfate system, where extrusion results in nearly a twofold increase in partitioning. The difference in partitioning can possibly be attributed to lipid aggregates that form during the gentle hydration process. Aggregates typically adsorb to the ATPS interface, as can be observed in Figure 2-2b, and are not included in the fluorescence measurements. Extrusion forcibly breaks apart these aggregates, freeing up more lipids and liposomes to partition into the suspended phase.

2.5 Conclusions

Extrusion was shown to be a non-essential step in the formation of liposome-stabilized all-aqueous emulsions. Although the stability is somewhat comprised, emulsions stabilized with unextruded liposomes can still function as effective micro-reactors. In addition, it was shown that liposomes could be used to stabilize ATPS’s beyond PEG/dextran, including a sulfate-rich phase. The next logical step is to explore possible reactions that are more optimal in a salt-rich environment.
2.6 Methods

Materials. Calcium chloride dihydrate (≥99.5 % purity), urea, poly(ethylene glycol) (PEG) 8 kDa, Ficoll® PM 70, urease from jack bean (Canavalia ensiformis) of activity 8.3 U/mg, and ethylenediamine-\(N,N'\)-disuccinic acid trisodium salt solution (EDDS)(~35% in \(H_2O\)) was purchased from Sigma-Aldrich, Co. (St. Louis, MO). Dextran (Dx) 10 kDa was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Egg-phosphatidylcholine (Egg-PC), egg-phosphatidylglycerol (Egg-PG), 1, 2-dioleoyl-\(sn\)-glycero-3-phosphoethanolamine-poly(ethylene glycol) 2 kDa (DOPE-PEG 2 kDa), and 1,2-dioleoyl-\(sn\)-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE) lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Alexa-647 tagged U15 was custom ordered from Integrated DNA Technologies, Inc. (Coralville, IA).

Formation of aqueous two phase separations (ATPS). A 10 g stock ATPS was made by dissolving 1 g PEG 8 kDa and 1 g Dx 10 kDa in 8 g 10 mM pH 7.4 Tris buffer, for a final formulation of 10%/10% w/w PEG 8 kDa/Dx 10 kDa. Identical preparation methods were used to make 10 g stock ATPS with final formulations of 10%/15% w/w PEG 8 kDa/Ficoll 70 kDa and 10%/8% w/w PEG 8 kDa/SO_4^{2-}. The mixtures were stirred in 15 mL centrifuge tubes for 1 hour to dissolve the polymers. The PEG/Dx and PEG/Ficoll ATPS were allowed to sit at 5°C and phase separate overnight, while the PEG/SO_4^{2-} ATPS sat at 25°C. The individual phases were then carefully removed via pipet and stored in separate tubes at 5°C. All phases were disposed of one week after separation.
**Formation of liposomes.** All vesicles were initially prepared using the gentle hydration method. Phospholipids dissolved in chloroform were pooled in the bottom of borosilicate glass tubes with the following composition: 48.5 mol% Egg PC; 48.5 mol% Egg PG; 2.8 mol% DOPE-PEG-2kDa; 0.1 mol% Rh-DOPE. Lipids were dried under a gentle stream of argon (~5 psi) then under vacuum for 1 hour. Films were then hydrated with PEG-rich from a separated 10%/10% w/w PEG/dextran ATPS, PEG-rich phase from a separated 10%/15% PEG/Ficoll ATPS, or a 25% w/w PEG solution for a lipid concentration of 7.5 mg/mL. After incubating the thin films at 40°C for 72 hours, liposomes received one of two treatment methods, hereto referred to as extruded and unextruded. Extruded vesicles were passed through an Avanti Polar Lipids Mini Extruder lined with 100 nm membranes 11 times total. Unextruded vesicles were placed on a vortex mixer at maximum velocity for ~10 seconds and used without further treatment. Both extruded and unextruded vesicles were stored at 5°C prior to use and disposed of after one week.

**Dynamic light scattering.** 1 mL samples were prepared by diluting 30 μL of vesicle stock solution hydrated with PEG-rich phase from PEG/Dx ATPS, extruded or unextruded in 970 μL of isotonic fructose solution (183 mmol/kg). Average vesicle diameter was measured using dynamic light scattering analysis on the Malvern Zetasizer. 1 mL of undiluted gentle hydration raw product, hydrated with PEG-rich from PEG/Dx ATPS, was also analyzed.

**Droplet size analysis and fluorescence partitioning.** 20 μL emulsions were prepared by mixing 2 μL 7.5 mg/mL liposome stock solution, 16 μL separated PEG-rich phase, and 2 μL separated Dx-rich phase, for a final composition of 10% by volume vesicles and 1:9 Dx:PEG volume ratio. Samples used in time studies were also loaded with 50 μg/mL ampicillin to inhibit
any bacterial growth. Samples were mixed on the vortex mixer for ~3 minutes immediately prior to analysis. Identical volumes were used to create the stabilized PEG/Ficoll and PEG/SO$_4^{2-}$ ATPS emulsions. Both extruded and unextruded liposomes were used at equal concentrations. For partitioning and size analysis, 20 μL of sample was deposited on a silanized glass slide with silanized coverslip, separated by a 160 μm silicone spacer. Samples analyzed again after 24 hours were left on their slides and placed adjacent to 50 mL beaker of water under a large petri dish to prevent drying out. All samples were imaged on a Leica (Wetzlar, Germany) TCS SP5 PL confocal microscope using a 63x 1.4 NA APO objective or a 20x 0.7 NA APO objective.

**U15 diffusion.** 200 μL emulsions were prepared by mixing 5 μL 7.5 mg/mL liposome stock solution, 189 μL separated PEG-rich phase, and 6 μL separated Dx-rich phase, for a final composition of 2.5% by volume vesicles and 1:97 Dx:PEG volume ratio. Identical volumes were used to create the stabilized PEG/Ficoll and PEG/SO$_4^{2-}$ ATPS emulsions. Both extruded and unextruded liposomes were used at equal concentrations. 20 μL of the emulsion was deposited adjacent to 20 μL PEG-rich phase loaded with 0.5 μM Alexa 647-tagged U15 on a silanized glass slide with 160 μm silicone spacer. Contact between the two droplets was initiated by the addition of a silanized cover-slip. Fluorescent images were acquired over the course of U15 diffusion on the confocal microscope. For each experiment, two regions of interest (ROI’s) within the emulsion were selected: one inside an arbitrarily selected droplet of dispersed phase, one in the adjacent continuous phase. The ROIs would move in each image in order to account for sample drift while still allowing for the most accurate comparison between the two phases. Average fluorescent intensity in both ROIs over the course of diffusion was measured using ImageJ software.
Fluorescence recovery after photobleaching. 20 μL emulsions were prepared by mixing 2 μL 7.5 mg/mL liposome stock solution, 17 μL separated PEG-rich phase, and 1 μL separated Dx-rich phase, for a final composition of 10% by volume vesicles and 1:19 Dx:PEG volume ratio. Samples were also equilibrated with 0.5 μM Alexa Fluor 647-labeled poly U15. FRAP studies were performed with an excitation of 633 nm for the labeled poly U15. After selecting a stabilized dextran-rich phase droplet for analysis, a 10-frame prebleach sequence followed a 5-frame bleach at 100% 458, 476, 488, 514, 543, and 633 nm laser power for labeled poly U15. The entire droplet, as well as some of the surrounding continuous PEG-rich phase, was bleached with a square region of interest (ROI). Recovery was measured by tracking the fluorescent intensity during the postbleach sequence, using a circular ROI, 2 micron in diameter, confined to the droplet interior during the pre- and postbleach sequences. Different sized ROI’s were used for bleaching and measurements to account for sample drift throughout the experiment. For all FRAP experiments, the fluorescent noise was measured using 2 micron diameter circle as a background ROI with all lasers turned off and respective photomultiplier tubes (PMTs) turned on. Recovery data was normalized as described by Phiar et al. through the double normalization method.²⁹

Imaging CaCO₃ synthesis on the darkfield microscope. An adapted version of the previously reported artificial mineralization vesicle method was used.²⁹ Relative liposome volumes were changed to create better imaging conditions. A 100 μL ATPS with 10 vol. % LUVs or 10 vol. % unextruded vesicles of V₅DX : V₅PEG 1 : 49 was formulated, with 12.45 units/mL urease, 25 mM Ca²⁺ and 30 mM EDDS, and emulsified using a vortex mixer immediately prior to analysis. To
maintain the ATPS volume ratio, all stock solutions were prepared in PEG-rich phase. 50 μL of the emulsion was deposited on a silanized glass slide with no cover slip. The sample was then transferred to the microscope sample stage. To initiate the reaction, 5 M urea in PEG-rich phase was added to the emulsion for a final concentration of 100 mM urea, and a silanized glass coverslip was placed on top. Darkfield images were acquired periodically over the course of the reaction using an Olympus BX51 with darkfield illumination and a 100x objective.
2.7 Supporting information

Figure 2-S1: confocal microscopy with fluorescent overlay images of various vesicle-stabilized ATPS emulsions, 72 hours after initial sample preparation. (a,b) PEG/dextran emulsions; (c,d) PEG/Ficoll emulsions; (e,f) PEG/sulfate emulsions. (a,c,e) Extruded vesicles at the interface. (b,d,f) Unextruded vesicles at the interface. Rhodamine-tagged vesicles have been false-colored red.

Figure 2-S2: fluorescence recovery after photobleaching (FRAP) curves for vesicle diffusion in vesicle stabilized PEG/dextran ATPS emulsions. Curves depict the fluorescent bleaching and recovery of Rhodamine-tagged (a) extruded and (b) unextruded vesicles at the PEG/Dx interface. A fraction of the interface was bleached, and allowed to recover. The Rh fluorescence never fully recovers, indicating the vesicles do not freely diffuse from the interface.
Figure 2-S3: Relative Alexa-647 fluorescence in liposome-stabilized (a,d) PEG/Dx, (b,e) PEG/Ficoll, and (c,f) PEG/sulfate ATPS prepared with (a-c) extruded or (d-f) unextruded liposomes during U15 diffusion. Experimental setup depicted in Figure 2-4a was used for all samples. Time refers to time after addition of the cover slip and fusion of droplets. In all cases, U15 was able to diffuse through liposome-lined ATPS interface.
2.8 References


Chapter 3
Aqueous Emulsion Droplets Stabilized by Lipid Vesicles as Microcompartments for Biomimetic Mineralization


David Cacace conceived, designed and performed the initial experiments, analyzed the data, and wrote the initial draft of the manuscript. The author of this dissertation reproduced the initial work, performed later experiments, and assisted in the preparation of the final draft. Joshua Stapleton acquired and assisted in the analysis of the IR data. Daniel Dewey assisted in adapting the system for mineralization. Christine Keating conceived the experiments, and wrote the final draft of manuscript.

3.1 Abstract

Mineral deposition within living cells relies on control over the distribution and availability of precursors as well as the location and rates of nucleation and growth. This control is provided in large part by biomolecular chelators, which bind precursors and regulate their availability, and compartmentalization within specialized mineralizing vesicles. Biomimetic mineralization in self-assembled lipid vesicles is an attractive means of studying the mineralization process, but has proven challenging due to vesicle heterogeneity in lamellarity, contents and size across a population, difficulties encapsulating high and uniform precursor concentrations, and the need to transport reagents across an intact lipid bilayer membrane. Here we report the use of liposome-stabilized all-aqueous emulsion droplets as simple artificial mineralizing vesicles (AMVs). These biomimetic microreactors allow entry of precursors while retaining a protein catalyst by equilibrium partitioning between internal and external polymer-rich phases. Small molecule chelators with intermediate binding affinity were employed to control Ca\(^{2+}\) availability during CaCO\(_3\) mineralization, providing protection against liposome aggregation while allowing CaCO\(_3\)
formation. Mineral deposition was limited to the AMV interior, due to localized production of CO$_3^{2-}$ by compartmentalized urease. Particle formation was uniform across the entire population of AMVs, with multiple submicrometer amorphous CaCO$_3$ particles produced in each one. The all-aqueous emulsion-based approach to biomimetic giant mineral deposition vesicles introduced here should be adaptable for enzyme-catalyzed synthesis of a wide variety of materials, by varying the metal ion, enzyme, and/or chelator.

3.2 Introduction

Understanding and mimicking the materials synthesis capabilities of living organisms is of fundamental interest and could lead to new materials with desirable properties for mechanical, medical, or energy applications.$^{1-2}$ In vivo, specialized lipid vesicles play important roles in generating “privileged environments” for mineralization.$^{3-5}$ Their roles can include precursor accumulation, transport and/or provision of sites for controlled nucleation and growth. Organic components present in these vesicles interact with, and in some cases become part of, the resulting minerals.$^6$ Vesicles are important for both intracellular and extracellular biomineral deposition; in the latter, they are exported to extracellular matrix. Enzyme activity is known to play important roles in precursor availability in vivo, for example matrix vesicle phosphatases help regulate inorganic phosphate in the extracellular matrix during bone growth.$^{3}$ In vitro reconstitution of mineralizing vesicles is attractive as a means to learn how biology uses active organic matter to control the location, structure and properties of inorganic materials.

The simplest model for mineralizing vesicles is a lipid bilayer vesicle in which one precursor (e.g., Ca$^{2+}$) has been preloaded, and free precursor has been removed from the external solution; internal mineral deposition then occurs upon transport of a second precursor (e.g. CO$_3^{2-}$)
across the lipid bilayer membrane. Several minerals including CaCO$_3$, iron oxides, and calcium phosphates have been formed within lipid vesicles by this general approach. Not all precursors can readily cross the lipid bilayer. Ball and coworkers overcame this by encapsulating alkaline phosphatase to locally generate inorganic phosphate inside liposomes for calcium phosphate deposition. In vitro model studies of mineralization inside lipid vesicles have often employed submicrometer diameter vesicles, which are relevant for many intracellular mineralization events and are easier to prepare with reasonable size homogeneity than larger diameter vesicles. Recent interest in biomimetic mineralization in giant vesicles (>1 micron in diameter) is driven by the ability to image these structures in real-time by optical microscopy, and to incorporate fluorescently-tagged molecules, which could help elucidate new information about biomineralization. Additionally, these larger structures are of interest as microreactors.

It is now well appreciated that biological fluids such as cytoplasm are crowded, compartmentalized, and not equilibrated. The cell is rich in macromolecules and other solutes, which result in both physical (excluded volume) and chemical (attraction, repulsion) effects. In contrast, in vitro mineralization reactions, even those inside liposomes, are most often performed in much simpler media that lack macromolecular crowding agents. Additionally, mineralization precursors may be disruptive to normal cell processes and hence released only locally in vivo. For example, living cells are very sensitive to fluctuations in intracellular metal ion concentrations. Ca$^{2+}$ is important in cell signaling and its concentration is tightly regulated; apoptosis cascades can occur if Ca$^{2+}$ homeostasis is not maintained. Intracellular metal ions are generally bound by proteins and other biological chelators that serve to buffer their free ion concentrations and control availability. The importance of crowding, compartmentalization, controlled precursor availability and local precursor production in vivo
suggest that capturing these aspects of the biological milieu in our simplified biomimetic systems should be a priority.

Here, we produced CaCO$_3$ within artificial mineralizing vesicles (AMVs) by local enzymatic production of carbonate ions. CaCO$_3$ was chosen because it is the most prevalent biogenic mineral and its biological and biomimetic mineralization has been extensively studied.\textsuperscript{5-6,28} Our AMVs are enzyme-loaded, liposome-stabilized all-aqueous emulsion droplets (Figure 3-1). These structures are formed when ~100 nm diameter lipid vesicles (large unilamellar vesicles, or LUVs) self-assemble at the aqueous/aqueous interface of a PEG/dextran biphasic system\textsuperscript{29-32}, stabilizing enzyme-loaded droplets of dextran-rich phase in a continuous medium of PEG-rich phase.\textsuperscript{33} Small molecule chelators were included to control Ca$^{2+}$ availability by preventing Ca$^{2+}$ binding to lipid headgroups while still permitting CaCO$_3$ precipitation.

AMVs differ in several important ways from the traditional lipid bilayer vesicles that have been used previously as models for mineralizing vesicles. (1) The interior compartment is macromolecularly crowded due to the presence of ca. 30 wt% dextran, mimicking the excluded volume of intracellular environments.\textsuperscript{31,33-37} (2) Droplet contents are determined by partitioning between the interior and exterior phases and consequently are the same across the population. We take advantage of partitioning to load the AMVs with a biocatalyst, urease, which locally produces a mineralization precursor, CO$_3^{2-}$. Consequently, unlike for traditional lipid vesicles,\textsuperscript{7,38-39} we get excellent encapsulation efficiency of the biocatalyst and no separation step is needed to remove unencapsulated enzyme or mineral precursors from the continuous phase. Additionally, unlike new microfluidic-based giant vesicle protocols that enable efficient encapsulation,\textsuperscript{40} AMV preparation requires no specialized equipment and produces an entire population of structures on the ~ ten-micron scale simultaneously. (3) Because the AMVs are
superstructures of liposomes rather than individual liposomes, their delimiting membrane allows
diffusive transport for entry of precursors (e.g., Ca\(^{2+}\)) and does not require membrane
transporters or pore-forming molecules.\(^{33}\) The combination of lipid-stabilized, enzyme-loaded
all-aqueous emulsion droplets with intermediate chelation provides excellent control over the
mineralization process, enabling high-yield production of relatively monodisperse AMV
populations.

Figure 3-1: Illustration of enzymatic production of CaCO\(_3\)(s) mineral within artificial mineralization
vesicles

3.3 Results and Discussion

3.3.1 Composition of artificial mineralizing vesicles

Large unilamellar lipid vesicles (LUVs, \(~110\) nm diameter) were added to a biphasic
aqueous solution with mixing to form an all-aqueous emulsion. The LUVs used here had equal
mole fraction of zwitterionic phosphatidylcholine and anionic phosphatidylglycerol headgroups,
with 2.8 mole % 2 kDa PEGylated lipid. We used an aqueous two-phase system (ATPS)
composed of water, polyethylene glycol (PEG, 8 kDa) and dextran (Dx, 10 kDa), with a dextran-rich to PEG-rich phase volume ratio \( (V_{\text{Dx}} : V_{\text{PEG}}) \) of 1:49. We previously reported on this mineralization system in an unstabilized PEG/dextran ATPS with less extreme volume ratios (1:1 to 1:9), where we compared reaction rates in the different phases to buffer alone and found both that the reaction was nearly quantitatively contained to the dextran-rich phase and that the resulting \( \text{CaCO}_3 \) material was not greatly impacted by the presence of the PEG and dextran polymers.\(^{34}\) The PEG and dextran polymers used to generate the ATPS are nonionic and do not bind \( \text{Ca}^{2+} \) nor generate a polymer-induced liquid precursor (PILP).\(^{5,34}\) Here, droplet stabilization leads to a suspension of reactive dextran-rich droplets that will serve as our AMVs. Mechanical agitation of the ATPS in the presence of LUVs led to formation of vesicle-stabilized Dx-rich droplets suspended in a PEG-rich continuous phase, with droplet size determined by the amount of LUVs available to stabilize the interface.\(^{33}\) In contrast to other particulate stabilizers capable of forming Dx-in-PEG ATPS emulsions (e.g., latex beads, aggregated proteins),\(^{41,42}\) the lipid vesicle layer at the aqueous/aqueous interface provides chemistry similar to a biomembrane, with lipid headgroups facing the aqueous interior. Solutes can diffuse across the droplet interface between the individual LUVs, and are not required to cross a lipid bilayer.\(^{33}\)

The enzyme urease was compartmentalized into the droplet interiors by equilibrium partitioning between the PEG-rich and Dx-rich aqueous phases to form AMVs. Urease produces \( \text{CO}_3^{2-} \) when it hydrolyzes urea, such that \( \text{CaCO}_3 \) formation can be initiated by urea addition to a \( \text{Ca}^{2+} \)-containing sample.\(^{34,43-44}\) Measured urease concentrations were much higher in the Dx-rich phase of the 1:49 \( V_{\text{Dx}} : V_{\text{PEG}} \) system (10.9 ± 0.4 and 0.08 ± 0.008 mg/mL in the Dx-rich interior and PEG-rich external phases, respectively). The difference between this result and previous experiments at lower \( V_{\text{Dx}} : V_{\text{PEG}} \) which found weaker urease partitioning,\(^{34}\) can be understood as
a consequence of the extreme volume ratio. Local protein concentration increases as Dx-rich phase volume fraction decreases.\textsuperscript{29, 36, 45} At high concentration, protein multimerization and even formation of new, protein-rich phases can occur, further increasing partitioning.\textsuperscript{17, 46} This high enzyme loading would be difficult to achieve in traditional giant vesicle preparations, e.g. those based on gentle hydration or electroformation.\textsuperscript{38-39, 47-48}

![Figure 3-2: Ca(II) destabilizes bioreactor droplet structures by aggregating LUVs. (a) Illustration of the experiment, in which the effect of adding Ca\textsuperscript{2+} to a sample of LUV-stabilized droplets is evaluated. At 5 mM Ca\textsuperscript{2+} and above, the interfacial layer of LUVs is disrupted. (b) Confocal fluorescence microscopy images of LUV-coated ATPS emulsion droplets with different amounts of added Ca\textsuperscript{2+} as indicated in each panel. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran for identification of Dx-rich droplets; red fluorescence represents Rhodamine-labeled lipid included as a label for LUVs. Scale bar = 10 \( \mu \text{m} \).](image)

### 3.3.2 Intermediate chelation of Ca\textsuperscript{2+}

In the absence of added Ca\textsuperscript{2+} chelators, LUV aggregation and disruption of bioreactor structures occurred at Ca\textsuperscript{2+} concentrations above 5 mM (Figure 3-2).\textsuperscript{49-52} In biological
mineralizing systems, metal ions are generally bound by ligands that help control their local concentrations and deposition. We therefore approached the problem of Ca\(^{2+}\)-induced LUV aggregation by adding Ca\(^{2+}\) chelators. A range of small molecule chelators was evaluated to identify conditions where LUV-coated droplets were stable and CaCO\(_3\) precipitation remained possible. These included malic acid (MA), ethylenediamine disuccinic acid (EDDS), glutamic acid diacetic acid (GLDA) and ethylenediamine tetraacetic acid (EDTA); structures are shown in Figure 3-3a. Partitioning data for these chelators is shown in Table 3-1. In the absence of chelators, Ca\(^{2+}\) distribution throughout the ATPS is nearly uniform, with partitioning coefficient, K = 0.926 ± 0.034. The four chelators are structurally similar and all show a slight partitioning preference for the dextran-rich phase. Ca\(^{2+}\) partitioning is impacted by the presence of chelators, leading to a roughly two-fold enrichment in Ca\(^{2+}\) in the dextran-rich phase when EDDS, GLDA, or EDTA is present. MA, which both partitions less strongly in the absence of Ca\(^{2+}\) and binds a smaller fraction of the available Ca\(^{2+}\), also led to a slight, albeit lesser, enrichment of Ca\(^{2+}\) in the dextran-rich phase (Table 3-1). The extent of bound and free [Ca\(^{2+}\)] for each chelator species was estimated by assuming a 1:1 ratio of Ca\(^{2+}\): chelator binding, and calculating the effective stability constants (β’, Table 3-1; see Methods for details). An acceptable chelator in the context of our experiments should keep free [Ca\(^{2+}\)] below 5 mM, preventing LUV aggregation, while still allowing precipitation of CaCO\(_3\).
Table 3-1. Partitioning of chelator species and chelated calcium; calculated values of chelator-calcium stability constants and free [Ca^{2+}] at the beginning of enzymatic reaction. \(^{a,b,c}\)

<table>
<thead>
<tr>
<th>Chelator</th>
<th>(K_{\text{Chelator}})</th>
<th>(K_{\text{Ca}})</th>
<th>(\beta')</th>
<th>([\text{Ca}^{2+}])(_{\text{Free}}) (mM)(^d)</th>
<th>Did CaCO(_3) Precipitate?</th>
<th>Did LUVs Aggregate?</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Chelator</td>
<td>-</td>
<td>0.926 ± 0.034</td>
<td>0</td>
<td>30</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MA</td>
<td>0.724 ± 0.030</td>
<td>0.681 ± 0.010</td>
<td>1.80</td>
<td>15.2</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>EDDS</td>
<td>0.590 ± 0.016</td>
<td>0.522 ± 0.014</td>
<td>3.85</td>
<td>1.98</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>GLDA</td>
<td>0.584 ± 0.006</td>
<td>0.504 ± 0.011</td>
<td>5.35</td>
<td>0.36</td>
<td>Yes, low yield</td>
<td>No</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.641 ± 0.008</td>
<td>0.540 ± 0.008</td>
<td>9.42</td>
<td>0.003</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

\(^a\)Data calculated for 30 mM [Ca\(^{2+}\)] total, 30 mM chelator species, 100 mM ionic strength, pH 9.0. \(^b\)CaCO\(_3\) \(K_{sp}\) for ACC and calcite remain constant at \(-\log(K_{sp}) = 6.40\) and 8.47, respectively, at 25 °C. \(^c\)Lipid \(\beta'\) ranges from of 0.1-2.0. \(^d\)free [Ca\(^{2+}\)] for each chelator species was estimated by assuming a 1:1 ratio of Ca\(^{2+}\): chelator binding, and calculating the effective stability constants, \(\beta'\) as described in Supporting Information.

Based on their binding affinities for Ca\(^{2+}\), GLDA, EDTA and EDDS were expected to prevent LUV aggregation by Ca\(^{2+}\). We tested this prediction by imaging LUV-stabilized droplet samples that contained both 30 mM of Ca\(^{2+}\) and 30 mM of one of the chelators. Figure 3-3b summarizes the results, which confirmed that MA was not sufficient to stabilize LUVs against aggregation in the presence of Ca\(^{2+}\), but stable emulsions could be formed with any of the other three chelators. We observed decreasing droplet size with chelators of increasing Ca\(^{2+}\) affinity, indicating EDTA > GLDA > EDDS in protection against emulsion destabilization. Nonetheless, no LUV aggregation was apparent in these samples and LUV-stabilized dextran-rich droplets persisted. Additional confocal images displaying the stability of LUVs as a function of chelator concentration are shown in Figure 3-S1.
Figure 3-3. Ca$^{2+}$ chelators protect against vesicle aggregation. (a) Chemical structures of the four chelators tested. Malic acid (MA); Ethylenediamine disuccinic acid (EDDS); Glutamic acid diacetic acid (or N,N-bis(carboxymethyl)-L-glutamate) (GLDA); Ethylenediamine tetraacetic acid (EDTA). (b) Confocal microscopy images of LUV-ATPS emulsion droplets with 30 mM Ca$^{2+}$ and various chelators also at 30 mM. Chelators are noted on each panel. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran, indicating Dx-rich droplets and red fluorescence represents Rhodamine-labeled lipid. Scale bar = 10 µm.

3.3.3 Analysis of CaCO$_3$ precipitates

Urea was added to initiate CO$_3^{2-}$ production and CaCO$_3$ precipitation. As summarized in Table 3-1, for 30 mM of both chelator and Ca$^{2+}$, all samples except for those with EDTA (the chelator with highest Ca$^{2+}$ binding affinity) produced at least some mineral in the bulk suspension. Very little precipitate formed in the presence of GLDA, which also has a relatively high Ca$^{2+}$ affinity. Our observations are reasonable based on a comparison of the chelator binding affinities and the solubility product for CaCO$_3$ precipitate (Table 1). Although our
system is complex in composition and we did not attempt to correct for every factor’s impact on published thermodynamic constants (solution matrix effects, changes in CO$_3^{2-}$, ionic strength, and pH during the reaction, etc), the calculated values in Table 1 nonetheless provided useful estimates to guide chelator selection.$^{58}$ Because EDDS enabled both emulsion stabilization and good CaCO$_3$ yields, we selected EDDS for use in the artificial mineralizing vesicles described throughout the remainder of the manuscript. We note that the EDDS-containing droplet samples, while not as uniform in size as those with the stronger Ca$^{2+}$ chelator GLDA, were nonetheless reasonably homogeneous in size (diameter of $6.8 \pm 1.7$ microns, see Figure 3-S2). In the following discussion, AMVs refers to the fully-formed artificial mineralizing vesicles as illustrated in Figure 3-1: the ATPS (with urease-loaded dextran-rich droplets surrounded by PEG-rich phase), the layer of LUVs that stabilizes the droplets against coalescence, and the EDDS chelator which buffers [Ca$^{2+}$].

CaCO$_3$ can occur as several crystalline polymorphs (calcite, vaterite and aragonite) or as amorphous material.$^{59-62}$ Mineralization often progresses from an initial amorphous calcium carbonate (ACC) to vaterite and finally to the more stable calcite structure.$^{63}$ We had previously observed a mixture of vaterite and calcite polymorphs for urease-catalyzed CaCO$_3$ formed in the presence of PEG and dextran polymers but no chelators.$^{34}$ To determine the impact, if any, of the chelators, LUVs, and reaction encapsulation in these samples, precipitates were collected and analyzed by powder x-ray diffraction (XRD), scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR). Mineral was collected for analysis by centrifugation and washing to remove other reaction components (including AMVs, which dissociate upon dilution of the ATPS to a single phase).$^{33}$
XRD data for material formed in EDDS-chelated AMV samples showed very small peaks associated with calcite and a broad background consistent with ACC (Figure 3-4a). A control experiment that contained EDDS but lacked LUVs was similar but lacked the calcite peaks. CaCO$_3$ formed without EDDS was more crystalline; the XRD diffraction pattern had both strong sharp peaks corresponding to calcite and somewhat broader peaks associated with vaterite (Figure 3-4b). The relative amounts of these crystalline polymorphs differed depending on whether LUVs were present, with calcite much more prevalent in the LUV-containing sample. The lipid headgroups appear to act as structure-directing additives, facilitating formation of calcite.
Figure 3-4. Characterization of mineral formed in artificial mineralizing vesicles. (a, b) XRD analysis of CaCO$_3$ precipitates formed with (a) and without (b) the EDDS chelator. Traces are labeled as artificial mineralizing vesicles (AMVs, which contained the ATPS, a stabilizing layer of LUVs surrounding the dextran-rich phase droplets, and the chelator EDDS), and controls lacking the LUVs and/or EDDS as noted. Calcite and vaterite standard peaks are included for identification of peaks. (c) ATR-FTIR spectra of CaCO$_3$ precipitated in AMVs compared with controls that lack LUVs or EDDS; also shown for comparison is a calcite reference. Spectra have been normalized to the intensity at $v_3$ and offset for clarity. (d) SEM for mineral formed in AMVs with and without the EDDS chelator.

The presence of only small peaks, and a broad background in the XRD diffraction pattern for CaCO$_3$ formed in the AMVs (Figure 3-4a) suggested the possibility that this material was
amorphous (ACC); we used infrared spectroscopy to further investigate this possibility. Carbonate ions have four characteristic vibrational modes that are frequently used to discriminate between the various forms of calcium carbonate: symmetric stretching ($\nu_1$), out-of-plane bending ($\nu_2$), asymmetric stretching ($\nu_3$), and the in-plane bending ($\nu_4$).\textsuperscript{65-66} Attenuated total reflection (ATR) FTIR spectra for CaCO$_3$ formed in AMVs with and without EDDS chelator are shown in Figure 3-4c. A calcite reference sample is included for comparison. The vibrational spectrum for material formed in AMVs with EDDS is consistent with ACC. The presence of ACC is suggested by the appearance of a broad $\nu_1$ peak (~1050 cm$^{-1}$); this mode is infrared inactive in calcite, and relatively narrow in aragonite and vaterite. Splitting in the $\nu_3$ mode, a marked increase in the $\nu_2/\nu_4$ ratio (~6.8 in AMVs as compared with 2.3 for calcite), and the presence of physisorbed water (broad band at ~3300 cm$^{-1}$ and smaller peak ~1640 cm$^{-1}$) are strong indicators of ACC.\textsuperscript{66-69} Infrared spectra for material produced in the presence of EDDS chelator were similar whether or not LUVs were present. Small peaks in the 2800 – 3000 cm$^{-1}$ region (C-H stretching modes) and a shoulder ~ 1570 cm$^{-1}$ (carboxylate asymmetric stretching mode) may indicate bound EDDS in these samples (Figure 3-S3). Chelator binding is a documented means of stabilizing ACC against conversion to crystalline polymorphs.\textsuperscript{70-73} CaCO$_3$ formed in AMVs without EDDS appeared very similar to calcite based upon the peak shape and position of the $\nu_2$ (870 cm$^{-1}$), $\nu_3$ (1390 cm$^{-1}$), and $\nu_4$ (711 cm$^{-1}$) modes, and a $\nu_2/\nu_4$ ratio of 2.5.\textsuperscript{66-69,74} In samples that lacked both EDDS and LUVs, vaterite was observed in the vibrational spectrum ($\nu_4$ mode ~743 cm$^{-1}$), consistent with the XRD data in Figure 3-4b (Figure 3-S4).\textsuperscript{66}

SEM images of these samples, shown in Figure 3-4d, are consistent with the XRD and FTIR data. Material formed in AMVs with EDDS is quite uniform and made up of submicrometer sized particles. Material formed without chelator has much larger particle sizes
and a mixture of roughly cubic and spherical microparticles. SEM for materials formed with and without LUVs in the presence of each of the three chelators and with no chelator are shown in Figure 3-S5. In general, the presence or absence of a chelator was a stronger determinant of mineral morphology than was the presence or absence of LUVs. GLDA-containing samples produced a low yield of calcium carbonate that was otherwise similar to that formed in the presence of EDDS. Mineral formed in the presence of malic acid had a mixture of larger particle sizes, more similar to those of the controls that lacked chelator and smaller particles more similar to those seen for EDDS- or GLDA- containing samples. Particle size was not determined by the size of the AMVs: the CaCO$_3$ structures formed in the presence of chelator (EDDS, MA or GLDA) were smaller than the AMVs (compare CaCO$_3$ SEMs in Figure 3-S3d, top panel and SF5 with AMV images Figure 3-S2).

3.3.4 Observation of CaCO$_3$ formation in artificial mineralizing vesicles

We focused on EDDS-stabilized AMVs for optical microscopy characterization during mineralization because only these samples had both stable AMVs and an ability to form appreciable quantities of CaCO$_3$ under our reaction conditions. Urease activity was verified in Ca$^{2+}$-free AMVs by following the increase in pH upon reaction with urea (Figure 3-S6). We then performed urease-catalyzed mineralization while observing populations of single AMVs by optical microscopy. Figure 3-5 shows the appearance of mineral particles inside AMVs by darkfield optical microscopy. Prior to urea addition, the AMVs are visible due to scattering from the LUV-coated aqueous/aqueous interface. After urea addition bright spots appear within each AMV, indicating the formation of CaCO$_3$. The lipid vesicle layer surrounding the AMVs remains present and surrounds the newly formed mineral, which appears as multiple small
particles per AMV as mineral deposition continues. This can be viewed in overlaid fluorescence and transmitted light images shown in Figure 3-6. The amount of material formed depends on the ratio of chelator to Ca\(^{2+}\), with faster reaction times and more CaCO\(_3\) observed at lower concentrations of EDDS (compare Figure 3-6a and b). Because the polymer and enzyme contents of AMVs result from equilibrium partitioning into the Dx-rich phase of the ATPS, they are the same across the population of AMVs. Consequently, mineral formation is quite uniform across the population, with similar mineral loading in each structure (see Figures 3-6c and 3-S7, which show wider fields of view). This is an important distinction from traditional giant lipid vesicles as biomimetic mineralization reactors, which can be quite heterogeneous in interior contents from vesicle to vesicle, and as such may exhibit nonuniform mineral formation across a population.\(^7, 39, 48\) For this reason, giant vesicle-based mineralization studies often focus on individual vesicles. Here it is possible to image populations of multiple, very similar single AMVs simultaneously. We note that AMV sizes, although varying some from experiment-to-experiment based on LUV availability to stabilize the interface, are also relatively uniform within a population. In our previous work on (non-mineralizing) all-aqueous emulsions we saw a strong dependence of droplet size on LUV concentration, with higher LUV concentrations able to stabilize more surface area. We have not yet explored intentionally varying the droplet size for the mineralization experiments,\(^33\) which differ by the presence of Ca\(^{2+}\), EDDS, and urease, as well as reaction products such as NH\(_3\) that together result in a slight destabilization of the emulsion as compared to systems that lack these added components, but presumably the same mechanism would still provide some degree of size control. The pre-mineralization samples shown in Figure 3-S7 darkfield images, which are more readily quantified than the confocal fluorescence of Figure 3-6c because the entire structures are in the focal plane, had mean
diameters of 4.2 and 5.5 μm, with standard deviations 33 and 34% of those values, respectively. Post-mineralization samples were slightly larger, with mean diameters of 6.5 and 7.3 μm, and standard deviations 25 and 22% of those values. Increased AMV size during mineralization suggests that the amount of interface that could be stabilized by the available LUVs was reduced by the reaction, for example by reducing repulsions between LUVs.33

Figure 3-5. Darkfield optical microscope images showing enzyme-catalyzed calcium carbonate formation inside artificial mineralizing vesicles. (a) Reaction with 100 mM urea. (left) before and (right) 1 h after urea addition. Scale = 20 μm and applies to both images. (b) Reaction with 500 mM urea leads to more rapid mineralization. (left) 7 minutes after, and (right) 24 minutes after urea addition. Scale = 20 μm and applies to both images. Conditions: 30 mM Ca\(^{2+}\), 30 mM EDDS, 30.77 units/mL urease.
Figure 3-6. Brightfield (DIC) and lipid fluorescence overlay illustrating that enzyme-catalyzed mineral formation is uniform across the population of artificial mineralizing vesicles. Samples were imaged 2 h after urea addition. Lipid fluorescence and transmitted light (DIC) channels have been overlaid to show location of mineral. (A) 25 mM EDDS; (B) 22.5 mM EDDS; scale = 10 μm applies to both A and B. (C) Lower-magnification view of 22.5 mM EDDS sample; scale = 25 μm. Conditions: 100 mM urea, 30 mM Ca\(^{2+}\); 12.18 units/mL urease.

Despite some increase in mean diameter, AMVs were stable during the enzymatic reaction for up to several hours, long enough to observe localized CaCO\(_3\) precipitation. At longer times they began to coalesce, forming large, often irregular droplets and in some cases bicontinuous emulsions. Decreased droplet stability at long times could be due to the increased pH and ionic strength due to ammonia and carbonate production continuing after available Ca\(^{2+}\) has been depleted. It was possible to stop the reaction at desired timepoints by adding a urease inhibitor, PDDA, which helped to prevent droplet destabilization. Even in inhibitor-free samples, morphological changes at long reaction times did not result in a loss of compartmentalization.

Figure 3-7 shows structures observed after 24 hr with no added inhibitor/stabilizer; the CaCO\(_3\) remains in the dextran-rich phase despite the more complex shapes adopted by this phase as it is
destabilized. Similar bicontinuous microstructures can be intentionally produced in stabilized PEG/dextran ATPS.\textsuperscript{75} In future it may be possible to take advantage of these morphological transformations during mineral deposition to produce different CaCO\textsubscript{3} morphologies. Some biominerals, such as the sea urchin skeletal plate have open network morphologies that have been successfully mimicked by templating in bicontinuous emulsions.\textsuperscript{76-78} At the low mineral loadings studied here, however, morphological changes in the mineralizing compartment do not greatly impact the morphology of the resulting material. Rather, we observe aggregates of material that most likely formed in separate droplets but have come together upon droplet coalescence due to LUV destabilization (Figure 3-7). Based on the optical contrast and morphology, and consistent with the normal progression of CaCO\textsubscript{3} polymorphs, it appears likely that these CaCO\textsubscript{3} minerals are in the process of conversion from ACC to vaterite and calcite.

![Figure 3-7](image)

Figure 3-7. Emulsions were destabilized at long reaction times but mineral was retained in dextran-rich phase. (left) transmitted light (DIC) and (right) confocal fluorescence microscopy images of AMV sample 24 hr after initiation of mineralization, with no enzyme inhibitor added. Conditions: 30 mM Ca\textsuperscript{2+}, 300 µg/mL urease, 200 mM urea, and 30 mM EDDS. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran, indicating Dx-rich phase and red fluorescence represents Rhodamine-labeled LUVs. Scale bar = 50 µm.

An interesting consequence of the distinct thermodynamic phases inside vs. outside these bioreactors is that even in the absence of localized CO\textsubscript{3}\textsuperscript{2-} production, mineral formation still predominantly occurs inside these structures. Figure 3-8 shows mineralization in structures identical to the AMVs but lacking urease. Here, CO\textsubscript{3}\textsuperscript{2-} was instead added to solution as Na\textsubscript{2}CO\textsubscript{3}. Although the mineral formed has larger particle size than the enzyme-catalyzed mineral formed
in Figures 3-4 and 3-5, and some of it is found outside the droplets in the continuous PEG-rich phase, unlike the enzyme-catalyzed material, it nonetheless is predominantly found inside the LUV-stabilized droplets. One explanation for this observation is the two-fold higher [Ca\(^{2+}\)] inside due to partitioning of the Ca-EDDS complex (Table 3-1). Beyond this, the chemistry of CaCO\(_3\) formation is surprisingly complex and can occur through nonclassical pathways that involve formation of stable prenucleation clusters and liquid-liquid phase separation of these clusters.\(^{79-81}\) Based on the size-dependent partitioning of other charged solutes and the relative surface energies of other dense liquid phases in PEG/dextran ATPS,\(^{29}\) it is likely that these clusters would partition into the dextran-rich phase more strongly than Ca\(^{2+}\) or CO\(_3^{2-}\) alone, and that any new precursor-dense liquid would also energetically prefer the dextran-rich phase. We therefore speculate that nonclassical CaCO\(_3\) pathways may enhance localization of the mineral within the dextran-rich droplets. In the fully-constructed AMVs that also contain urease, local production of CO\(_3^{2-}\) by the compartmentalized enzyme clearly provides additional control over mineralization location (compare Figures 3-5 or 3-6 with Figure 3-8).

**Figure 3-8.** Non-enzymatic mineralization. LUV-stabilized PEG/dextran ATPS droplets with EDDS-chelated calcium, 13 minutes after direct addition of carbonate to the solution as Na\(_2\)CO\(_3\). Rhodamine-labeled lipids are false-colored red. Conditions: 30 mM Ca\(^{2+}\); 25 mM EDDS; 40 mM Na\(_2\)CO\(_3\).
3.4 Conclusions

*In vivo,* free Ca\(^{2+}\) concentrations are tightly regulated. During CaCO\(_3\) biomineralization, Ca\(^{2+}\) is bound by chelating carboxylated polysaccharides, which assist in calcium compartmentalization within lipid-bound mineralizing vesicles.\(^3\)-\(^4\), \(^24\)-\(^27\) Our bioinspired AMVs capture several key features of their biological counterparts, including control over Ca\(^{2+}\) availability by chelation, compartmentalization of reagents and reactions, macromolecular crowding, and mineral formation within lipid headgroup-delimited compartments. We note that the membrane curvature for the vesicles in our system is much higher than for a continuous lipid bilayer of similar size to our AMVs, such as a giant vesicle, and of opposite curvature from mineralization inside an individual LUV (i.e. convex vs. concave). Unlike biological mineralizing vesicles or giant lipid vesicles, these AMVs are simple to prepare with uniform composition and high macromolecule encapsulation yields, do not require post-synthesis removal of unencapsulated reagents (e.g., by dialysis or chromatography), and can be produced in relatively large quantities. Individual AMVs are large enough to image using optical microscopy and since solutes can diffuse between the LUVs at their boundary without direct transport across a lipid bilayer,\(^33\) entry/egress is simplified. As such, they are attractive test beds for evaluating the effect of biological peptides, carbohydrates, or lipids thought to serve as nucleation sites and/or mineralization-directing organic additives.

We anticipate that this approach to AMVs should be transferrable to other mineralization processes (e.g., calcium phosphates, iron oxides, etc) that can benefit from localized enzyme activity to produce a precursor and/or alter the local environment by, for example, changing the pH. Chelator selection may need to be optimized for different metal ions and mineral compositions to maintain effective intermediate affinity. Most water-soluble proteins will
partition to some extent into the dextran-rich phase of a PEG/dextran ATPS, allowing their accumulation inside the AMVs. Extensive research on protein partitioning in PEG/dextran biphasic systems, including approaches to affinity partitioning, has been reported due to the use of these systems in bioseparations.

3.5 Methods

Materials. Calcium chloride dihydrate (≥99.5 % purity), urea, ethylenediaminedisuccinic acid (EDDS), ethylenediaminetetraacetic acid (EDTA), malic acid (MA), anhydrous copper(II) chloride, poly(ethylene glycol) (PEG) 8 kDa, dextran (Dx) 10 kDa, urease from jack bean (Canavalia ensiformis) 483 kDa of activity 35 U/mg, urease from jack bean (Canavalia ensiformis) 544 kDa of activity 1,538.5 U/mg and ovalbumin 40 kDa were purchased from Sigma Chemical Co. (St. Louis, MO). N,N-bis(carboxymethyl)-L-Glutamate (GLDA) was purchased from Tokyo Chemical Co. (Tokyo, Japan). Amino-dextran 10 kDa was purchased from Molecular Probes, Inc. (Eugene, OR). Egg-phosphatidylcholine (PC), egg-phosphatidylglycerol (PG), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol) 2 kDa (DOPE-PEG 2 kDa), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-Rh) lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Instrumentation and Microscopy. UV/Visible measurements were taken with a Hewlett-Packard 8453 diode array spectrometer (Palo Alto, CA) with Aglient ChemStation software. Fluorescence was measured using a Fluorolog Horiba Jobin Yvon fluorimeter (Edison, NJ) and Fluorolog software. Atomic absorption of calcium was measured using Shimadzu Ca/Mg
hollow cathode lamp operating at 422.7 nm, wavelength, 5 nm slit width, 7 mA input current, 15 and 50 PSI acetylene and air respective input pressures; samples were analyzed using WizAARD software. Fluorescence microscopy images were taken with a Leica TCS SP5 PL confocal microscope (Wetzlar, Germany) using a 63x 1.4 NA APO objective with DIC optics in tandem with sequential laser excitation of 544 and 633 nm and emission bands of 560-600 and 650-700 nm for Rhodamine and Alexa 647-labeled Dextran 10 kDa, respectively, on a 63x oil immersion objective; images were analyzed using LAS-AF software. X-ray diffraction patterns were collected on a Si zero-background sample holder using PANAnalytical X Pert Pro MPD Theta-Theta Diffractometer (Almelo, The Netherlands) across a 20-70° 2θ range at 45 kV acceleration, 40 mA Cu Kα radiation using a 0.5° divergence fixed-slit width and 10 mm beam mask in tandem with a PIXcel detector over an analysis time of 15 minutes; the resulting patterns were analyzed using Jade+ 9 software from MDI (Livermore, CA). SEM images were acquired with a Hitachi S-3500N, operated at high vacuum mode at a 5-7 mm working distance, and a 5 keV accelerating voltage; a ~10 Å gold coating was applied to samples using an in-house metal evaporator prior to imaging. Darkfield images in Figure 4a were acquired on an Olympus BX61 microscope (Melville, NY) using a 40x 0.75 NA air objective with darkfield optics with an Olympus DP71 camera and CellSens Dimension v1.7 software. Darkfield images in Figure 4b were acquired on a Nikon TE-200 inverted microscope using a 60 x 0.5 NA oil objective with a darkfield oil condenser with a Photometrics CoolSNAP HQ camera and ImagePro 7 software. Because dextran-rich AMVs are more dense than the continuous PEG-rich phase, they tend to sediment to onto the coverslip, hence collection of images is facilitated by using the inverted microscope geometry, particularly for longer times after sample preparation, when more droplet setting has occurred.
**Formation of bulk ATPS, ATPS LUV emulsions.** A 20 g stock ATPS of 10%/10% w/w PEG 8 kDa/Dx 10 kDa was made in 10 mM pH 7.4 Tris buffer, referred to as the “ATPS stock”. The phases were separated by centrifugation and recombined to make ATPS of desired $V_{Dx} : V_{PEG}$ ratios and total volumes as detailed previously. Note that the PEG-rich phase this ATPS working solution is composed of both PEG-rich phase from the ATPS stock as well as LUVs hydrated in PEG-rich phase (see below); these are combined together to make the total PEG-rich phase volume ($V_{PEG}$).

LUVs were prepared by extrusion of multilamellar vesicles formed by gentle hydration of lipid films. Specifically, initial lipid mixtures of 48.56, 48.51, 2.83, and 0.10 mol % of Egg-PC, Egg-PG, DOPE-PEG 2 kDa, and Rh-DOPC were pooled in a silanized glass tube with an additional wash of ~49 µL chloroform, for a final volume of ~150 µL; this mixture was stirred rapidly by hand while Ar was blown into the tube slowly (~1 PSI), which dried the lipid mixture into a film on bottom of the glass. The vial was placed within a vacuum desiccator and vacuum was pulled via pump for at least two hours to remove any chloroform remaining in the lipid film. For hydration, 500 µL of PEG-rich phase from the ATPS stock was added to the glass vial, capped tightly with parafilm, and placed in a 37 °C incubator for at least 48 hours. The resulting heterogeneous vesicle mixture was allowed to cool to room temperature and then extruded through a 200 nm pore 11 times using an Avanti mini-extruder, creating ~200 nm LUVs and removing any excess non-LUV lipid material. The LUVs were stored in a 2 mL silanized glass vial under Ar gas at room temperature until use, and used within a week. This PEG-rich LUV stock was then added to an ATPS working solution at 20% v/v of LUVs, making sure that the additive total of PEG-rich phase and LUVs equal the final desired PEG-rich phase volume ($V_{PEG}$); this ATPS was then mixed.
vigorously via pipette to create an ATPS LUV emulsion. This emulsion was observed to be stable for between a few hours to up to a day, depending on amount of LUVs added.

**Selection and Addition of Chelators and Ca$^{2+}$ to ATPS LUV emulsions; localized CaCO$_3$ precipitation.** We determined the appropriate Ca$^{2+}$ intermediate chelator species to use in our system using the mathematical guidelines outlined by Bers and colleagues,$^{58}$ which describes the relationships between the multiple binding sites of a ligand and a metal, the pH, and ionic strength. A chelator here is described as "intermediate" if it could bind Ca$^{2+}$ well enough to prevent Ca-LUV interactions, but not enough for CaCO$_3$ precipitation to occur. By knowing the optimal chelator-Ca$^{2+}$ stability constants reported in literature for each of our chelators used,$^{55,56,82,83}$ the starting pH, the ending pH, and the solubility product of CaCO$_3$,$^{84}$ we were able to use Bers's equations to identify the range of chelator species to use based on their binding strength to Ca$^{2+}$.

To determine the effect of Ca$^{2+}$ and chelators upon the stability ATPS LUV emulsions, a 200 µL ATPS with 20 vol. % LUVs of V$_{Dx}$ : V$_{PEG}$ 1 : 49 was first made up and emulsified via pipette. To this emulsion, in order, urease, a particular chelator (if applicable), fluorescent dextran, and Ca$^{2+}$ were added to their final concentrations, making sure to mix the emulsion via pipette after each addition. Urease was solvated as a 40 mg/mL stock in buffer, and Ca$^{2+}$ as a 3 M stock in PEG-rich phase. The chelators were also introduced in PEG-rich phase, with the exception of EDDS, which could only be purchased in aqueous solution. A 50 µL sample of the suspended emulsion was dispersed as a single droplet on a silanized glass cover slip with a 150 µL silicone spacer and placed on the microscope stage. After waiting a few minutes to allow some of the Dx-rich droplets to settle, an area of interest was determined visually and DIC/fluorescence images were taken. To initiate CaCO$_3$ precipitation in the case of darkfield images, urea from a 5 M in
PEG-rich phase stock was added to the top of the emulsion and very gently stirred to a final concentration of 200 mM urea. The resulting precipitation reaction was monitored in real-time over a 30-minute time period.

**Partitioning Determination.** A 10 mL ATPS of $V_{PEG} : V_{Dx}$ of 3 : 1 was formulated with no LUVs, and an equivalent concentration of Ca$^{2+}$ and chelator species to the above experiments were added. The ATPS was mixed thoroughly, left in a refrigerator at 5 °C overnight, and allowed to warm to room temperature before analysis, where aliquots of each polymer-rich phase were taken and diluted appropriately into DI H$_2$O. Calcium concentrations were determined by atomic absorption spectrophotometry, chelator concentrations by visible absorbance spectroscopy in the presence of 30 mM Cu$^{2+}$ for EDDS, GLDA, and EDTA and 50 mM Cu$^{2+}$ for MA ($\lambda_{\text{max}} = 760, 660, 725, 825$ nm for Cu$^{2+}$ complexes of MA, EDDS, GLDA and EDTA, respectively), and urease concentration by tryptophan fluorescence ($\lambda_{\text{Ex}} = 280$ nm, $\lambda_{\text{Em}} = 332$ nm). At least 6 calibration standards were utilized for each method, resulting in $R^2$ values of at least > 0.99. Urease could not be directly quantified in the PEG-rich phase, where its concentration was low. Therefore, urease concentration in the PEG-rich phase was calculated by using the total number moles of urease added, the volumes of each polymer-rich phase, and the determined urease concentration in the Dx-rich phase.

**Infrared Spectroscopy.** Infrared spectra were collected using a Bruker Vertex V70 spectrometer (Bruker Optics, Billerica, MA) using a Harrick MVP Pro Star attenuated total reflection (ATR) accessory with a diamond crystal, which was operated under N$_2$-rich conditions to remove atmospheric moisture. All spectra were acquired between 4000-400 cm$^{-1}$ at 4 cm$^{-1}$ resolution by
averaging 100 scans and using an DLaTGS detector, which was recorded using Opus software; intensities are reported here as $-\log(R/R_o)$, where $R$ is the reflectance of the sample, and $R_o$ is the reflectance of the reference (clean diamond crystal), taken before each sample. We chose to employ ATR-FTIR to minimize sample perturbation and eliminate any effects that might arise via the use of hygroscopic salts (see comparison of transmission and ATR FTIR in Figure 3-S8).

Analysis of CaCO$_3$ products using XRD, SEM and ATR-FTIR. A 1 mL ATPS with or without 20 % v/v LUVs and $V_{Dx} : V_{PEG} = 1 : 49$ was formulated, emulsified via pipette, and the appropriate amount of urease, Ca$^{2+}$, and chelator species (typically 300 µg/mL, 30 mM and 30 mM, respectively) were added as described above. Control samples with no chelator with and without LUVs were also formulated. Urea from a 5 M PEG-rich phase stock solution was added to each emulsion for a final concentration of 200 mM, and the emulsion was vortexed via pipette to initiate the precipitation reaction. After one hour, the emulsion was centrifuged at 12600 $\times$ g to separate the precipitant, and the remaining liquids were removed via pipette. The CaCO$_3$ pellet was resuspended with 1 mL of 10 mM Tris base (pH = 9.5), followed by two additional centrifuge and washing cycles. After the final centrifuge step, the buffer was removed via pipette and the centrifuge tube placed within crushed dry ice until analysis to prevent any unwanted polymorph or morphological changes by CaCO$_3$. Immediately prior to analysis, a minimum amount of 95% EtOH (typically ~30 µL) was used to resuspend the precipitate, which was used on an appropriate sample stage for XRD, SEM, or ATR-FTIR.

ATR-FTIR analysis of CaCO$_3$ standard mixed with matrix materials. CaCO$_3$ powder (0.01 g, Sigma-Aldrich) was placed within a centrifuge tube, along with one of the following: 10 mM
Tris base (pH = 9.5), 10% w/w PEG 8 kDa, 10% w/w Dx 10 kDa, 10 mg/mL urease, 20% v/v LUVs, or 30 mM of MA, EDDS or GLDA; 10 mM Tris base was used rather than the typical buffer (pH 9.5 instead of 7.4) to prevent CaCO$_3$ dissolution. The centrifuge tubes were vortexed gently for an hour and then washed as per the procedure above, and the final powder within the tubes placed on dry ice. ATR-FTIR analysis was performed by handling the samples as described above.

**Comparison of Transmission and Attenuated Total Reflection (ATR)FTIR.** The preponderance of calcium carbonate FTIR literature has been collected in transmission mode. This approach entails diluting a small amount of analyte in an infrared transparent salt (KBr, KCl, NaCl) and then applying pressure using a press and die to form a monolithic free standing pellet. Given the relative ease with which ACC can undergo phase transformation and the hygroscopic nature of the common diluents, one must always consider how transmission preparation may alter the sample. As such, we chose to employ ATR-FTIR for these analyses to minimize sample perturbation and eliminate any effects that might arise via the use of hygroscopic salts. However, the use of ATR is not without limitations. One issue is that the position and shape of peaks within a spectrum acquired via ATR cannot be directly compared to those acquired via transmission. Particularly in the case inorganic compounds like calcium carbonate the position shift for strong peaks can be substantial, on the order of 30 cm$^{-1}$ (see $\nu_3$ in Figure 3-S8). Less intense peaks like the $\nu_4$ may show a $\sim 1$ cm$^{-1}$ shift in ATR compared to transmission (Figure 3-S8). In short, ATR is a viable approach for analyzing these materials but one must use care in how transmission spectra are used to guide interpretation.

The spectrum of calcium citrate (Figure 3-S3) supports the assignment of the $\sim 1570$ cm$^{-1}$ peak to a calcium carboxylate species in samples prepared using EDDS. Peaks analogous to
those in EDDS, but significantly weaker, may indicate similar coordination when malic acid is used as the chelator. These peaks are absent when samples are prepared without the use of a chelator. However, the splitting and broadening of the ν₃ mode that has been observed for ACC cannot be entirely ruled out as the source of peaks in the 1550-1650 cm⁻¹ region. ⁶⁶
3.6 Supporting information

**Figure 3-S1.** Confocal microscopy images of LUV-ATPS emulsion droplets with 30 mM Ca\(^{2+}\) and various chelator species also at 30 mM. Species are: A) Malic acid; B) EDDS; C) GLDA; D) EDTA at a [Ca\(^{2+}\)]/[Chelator] ratio as indicated in the inset. Images have been overlaid and false-colored for clarity. Blue fluorescence represents Alexa 647-labeled dextran, indicating Dx-rich droplets and red fluorescence represents Rhodamine-labeled LUVs. Scale bars = 10 µm.
**Figure 3-S2.** Transmitted light (differential interference contrast) images for the EDDS-containing sample shown in Figure 2b of the main text. Droplets for which the equator was in-focus appear sharper, and were used in rough estimation of average droplet size as reported in text. Scale bars = 10 µm.

**Figure 3-S3.** ATR-FTIR spectrum for calcium citrate. Dotted line indicates peak at ~1570 cm\(^{-1}\) described in the Methods.
Figure 3-S4. ATR-FTIR spectrum for CaCO$_3$ formed by enzymatic activity of urease in an ATPS, in the presence and absence of EDDS and LUVs. Dotted vertical line indicates $v_4$ peak, which at ~743 cm$^{-1}$ in the no EDDS, no LUVs sample is indicative of vaterite. Spectra for samples with EDDS and LUVs are included for comparison and also appear in Figure 3c.

Figure 3-S5. SEM images of CaCO$_3$ precipitates obtained from urease-mediated ATPS precipitation reactions identical to that in Figure 3, in the A) presence and B) absence of 20% v/v LUVs. Wide- and narrow-view images are displayed to give a better perspective of the distribution of precipitates within a particular sample. All scale bars are 5 microns.
**Figure 3-S6.** Reaction progress as indicated by changes in pH upon urea hydrolysis. Dx-SNARF, which partitions into the dextran-rich phase, was used to monitor local pH changes. (A) Dx-rich droplets displaying pH changes. Images for the acid and base forms of the dye have been overlaid and false-colored for clarity (green = basic, red = acidic form; yellow indicates near-neutral pH in the overlaid images). Scale bars = 10 μm. (B) Trend of pH changes inside of Dx-rich droplets stabilized by LUVs, surrounded by PEG-rich continuous phase.

**Figure 3-S7.** Darkfield optical microscopy for CaCO$_3$ deposition in artificial mineralizing vesicles (AMVs), from the same set of experiments as Figure 4a but showing a wider field of view. (left panels, both a and b) before mineralization, (right panels, both a and b) after mineralization has occurred. Mineralization was induced by addition of 100 mM urea to the solution. Scale = 20 μm and applies to all images. Conditions: 30 mM Ca$^{2+}$, 30 mM EDDS, 30.77 units/mL urease.
Figure 3-S8. Comparison of transmission and ATR-FTIR spectra for a CaCO$_3$ sample. Spectra have been normalized to the intensity at $\nu_3$ for ease of comparison.
3.7 References


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Chapter 4
Bioinspired Mineralizing Microenvironments Generated by Liquid-Liquid Phase Coexistence


The author of this dissertation and David Cacace contributed equally. The author of this dissertation conceived, designed and performed the experiments, analyzed the data, and assisted in the preparation of the final manuscript draft. David Cacace conceived, designed and performed initial experiments, data analyzed the data pertinent to Figure 4-1 and Table 4-1. Nuerxida Pulati designed and performed the experiments and data analysis pertinent to Figure 4-5. Morgan Gulley assisted in size analysis experiments. Christine Keating conceived the experiments, and wrote the final manuscript draft.

4.1 Abstract

Living organisms direct the location, structure, and properties of biominerals by tightly controlling reactant concentration profiles, biopolymer identity and availability, and other aspects of reaction microenvironments. Such control at the microscale is difficult to exert in synthetic systems, especially when producing sufficient material for post-synthesis characterization. Inspired by the scalability of emulsions and the effectiveness of liquid-liquid phase separation in organizing subcellular biochemistry, we introduce mineralizing microreactors based on vesicle-coated multiphase droplets of a semi-stabilized, all-aqueous Pickering emulsion. Each droplet contains both a Ca\(^{2+}\)/polyaspartate-rich coacervate phase and second, adjacent phase hosting a carbonate-producing enzyme. Precipitation begins at the interface and results in coacervate-templated particle formation. The resulting stable, polycarboxylate-rich amorphous calcium carbonate microspheres have smooth surfaces and dense shell/porous core morphology. Pre-organization of mineralizing media by phase
coexistence is a powerful way to shape reaction microenvironments that should be broadly generalizable to other materials; adaptation for calcium phosphate is demonstrated.

4.2 Introduction

Mineralization by living organisms occurs in specialized microenvironments that provide exquisite reaction control, including precursor concentration profiles, the distribution and identity of structure-directing biomolecules, and the local physical environment (pH, surface functional groups, macromolecular surfaces).\textsuperscript{1-2} The resulting biominerals are tailor-made for their functions in the organism, often containing organic components that contribute to their morphological, optical and/or mechanical properties.\textsuperscript{3} Such control over materials synthesis, at ambient temperature and pressure in an aqueous reaction environment, goes beyond what is generally possible through nonbiological methods. Consequently, researchers have sought to produce artificial versions of biomineralizing microenvironments, including mineral deposition vesicle mimics.\textsuperscript{4-9} Tradeoffs between designing microreactors that maximize control over the reaction environment and those amenable to scaleup to produce larger quantities of material can pose challenges. For example, microfluidic strategies offer exquisite control,\textsuperscript{10-11} but low throughput, while reactions performed in free solution offer scaleup at the expense of control. We overcome these challenges by adopting another biological compartmentalization strategy: phase separation. Macromolecule-rich intracellular liquid condensates are a new paradigm for understanding how cells compartmentalize molecules and control local microenvironments. These structures, which include such well-known membraneless organelles as the nucleolus,\textsuperscript{12} p-granules,\textsuperscript{13} and stress granules, are now thought to form by associative phase separation, or coacervation, of proteins and nucleic acids.\textsuperscript{14-15} Coacervates have been implicated in the
formation of protein-based hard materials in squid beak, but have not generally been considered sites of biogenic mineralization. However, low-complexity intrinsically disordered proteins are implicated in biogenic mineralization. Biomimetic mineralization and drug encapsulation strategies have appeared based on including chelating polymers including simple polypeptides such as polyaspartic acid (PAA) in reactions that form, for example, calcium carbonates. Liquid droplets termed polymer-induced liquid precursors (PILPs) can form in these solutions during the mineralization process, for example as carbonate ions are generated to react with Ca$^{2+}$ present in the solution. Amorphous and/or liquid precursors enable remarkable shape control and have been used to produce single crystal calcite in complex forms such nanorods and inverse opals. We reasoned that multiple stable, organic-rich aqueous phases could be used to pre-organize reaction microenvironments for materials synthesis, providing control over local availability of inorganic and organic reaction participants.

Here, we introduce coacervate-containing artificial mineralizing vesicles (AMVs) that combine inspiration from both liquid condensates and mineralizing vesicles, and demonstrate their use for production of hybrid organic/inorganic microspheres. Lipid vesicle-stabilized all-aqueous emulsion droplets serve as structured microreactors whose adjacent phase compartments dictate by equilibrium partitioning the initial local concentrations of a metal ion (Ca$^{2+}$), a polymeric chelator (polyaspartic acid, PAA), an enzyme (urease, which produces CO$_3^{2-}$), and two macromolecular crowders (polyethylene glycol and dextran). This structured reaction microenvironment continues to shape concentration profiles as its composition changes during production of calcium carbonate. Dilution to disrupt the emulsion releases spherical microparticles with a porous core/dense shell morphology that incorporate ~30 wt/wt% organics. They remain stable as amorphous calcium carbonate (ACC) for at least 1 year at room
temperature, or can be converted to porous polycrystalline calcite spheres by heating. Many aspects of this bioinspired mineralization platform are tunable, for example particle size can be varied by changing the initial coacervate size and composition. Adaptation for production of other materials is possible by changing the enzyme/substrate pair to produce other precursors.

4.3 Results

4.3.1 Formation of Ca\(^{2+}\)/polypeptide-rich coacervates within artificial mineralizing vesicles

Coacervate-containing AMVs were prepared by adding Ca\(^{2+}\) and PAA (2-11 kDa) to vesicle-stabilized all-aqueous emulsions of polyethylene glycol (PEG, 8 kDa)/dextran (10 kDa) biphasic systems.\(^{22}\) PEGylated, negatively-charged lipid vesicles ~150 nm in diameter were used.\(^{22}\) Emulsion stability relies on electrostatic repulsion between the negatively-charged lipid vesicles, which can be disrupted by increased solution ionic strength or by Ca\(^{2+}\) binding to lipid headgroups.\(^{22,23}\) We therefore began by identifying conditions under which PAA and Ca\(^{2+}\) could be added to form a coacervate phase with minimal emulsion destabilization. Figure 4-1a,b shows the effect of adding PAA to this emulsion in the absence of Ca\(^{2+}\). Mean droplet size increased from 1.7 ± 0.2 without PAA to ~12 µm at 10.5 mg/mL PAA (Figure 4-S1). Increased droplet size suggests coalescence due to reduced interface stabilization by adsorbed liposomes.\(^{22}\) Indeed, as PAA concentration was increased, liposome fluorescence at the aqueous/aqueous interface decreased and liposome aggregates appeared in the continuous phase. By 10.5 mg/mL PAA, no interfacial liposome layer was visible (Figure 4-1a). Increased ionic strength is expected to increase liposome packing at the interface,\(^{22}\) and does not account for these observations. Liposome redistribution can, however, be understood in terms of electrostatic repulsion between the negatively-charged lipid vesicles and negatively-charged PAA. PAA partitioning between the
Dx-rich and PEG-rich phases remained fairly constant at $K = 0.3 – 0.5$ over the range of PAA concentrations used (Figure 4-S1); this corresponds to a two-to-threefold higher PAA concentration in the Dx-rich droplets as compared to the PEG-rich continuous phase. Hence, as more PAA was added, the droplets became increasingly negatively-charged, and interfacial liposome assembly was prevented.\textsuperscript{25,26,27,28}

Figure 4-1. Pre-organization of reaction microenvironment by liquid-liquid phase coexistence. (a,b) Concentrations of PAA and Ca\textsuperscript{2+} determine structure and stability of aqueous/aqueous emulsion droplets. (a) Effect of adding PAA to lipid vesicle-coated PEG/dextran all-aqueous emulsion droplets in the absence of Ca\textsuperscript{2+}. Laser confocal microscopy fluorescence images as a function of added PAA 2-11 kDa (in mg/mL, image insets). (b) Illustration interpreting data in panel a as loss of interfacial liposomes and destabilization of all-aqueous emulsion upon the addition of PAA. (c) Effect of adding PAA to lipid vesicle-coated PEG/dextran all-aqueous emulsion droplets in the presence of 50 mM Ca\textsuperscript{2+}. Laser confocal microscopy fluorescence images as a function of added PAA (in mg/mL, image insets). Images are overlaid and false-colored for clarity. Red fluorescence represents Rhodamine-labeled liposomes; green fluorescence represents Alexa488-labeled PAA; blue fluorescence represents Alexa647-labeled dextran. Scale bars = 20 µm. (d) Illustration interpreting data in panel c as formation of Ca\textsuperscript{2+}/PAA-rich coacervate upon addition of PAA to Ca\textsuperscript{2+}–destabilized all-aqueous emulsion droplets.
Figure 4-1c,d shows the effect of adding increasing amounts of PAA to the emulsion in the presence of 50 mM Ca\(^{2+}\). Without PAA, Ca\(^{2+}\) interacts with phospholipid headgroups and causes liposome aggregation, destabilizing the emulsion. This can be regulated through Ca\(^{2+}\) chelation.\(^{23}\) Here, PAA serves as the Ca\(^{2+}\) chelator. At PAA concentrations as low as 2.5 mg/mL, a third phase was observed (green droplets in Figure 4-1c). This new Ca\(^{2+}\)/PAA-rich coacervate phase is wet by the dextran-rich phase droplets (blue in Figure 4-1c). As PAA concentration was increased, vesicle aggregates were less pronounced and an interfacial vesicle layer around the dextran-rich droplets became clear. Reduced intensity in the green channel indicates that PAA partitioning into the Ca\(^{2+}\)/PAA-rich coacervate decreases with increasing [PAA] (Figure 4-S2). By 10.5 mg/mL PAA, droplets that contained both the Dx-rich phase and the Ca\(^{2+}\)/PAA-rich coacervate phase were surrounded by uniform vesicle coronas. When the PAA concentration was further increased (>10.5 mg/mL), the Ca\(^{2+}\)/PAA-rich phase decreased in size and by 17.5 mg/mL PAA disappeared entirely, consistent with formation of soluble Ca\(^{2+}\)/PAA complexes.\(^{29,30,31,32}\) Compared to the initial emulsion without PAA or Ca\(^{2+}\), these droplets are much larger (compare Figure 4-1a, first panel with Figure 4-1c, last panel). Cation/anion ratio-dependent stability is common for coacervates formed by ion pairing; such coacervates are also sensitive to ionic strength.\(^{33-34}\) When NaCl was added to the coacervate-containing AMVs, the coacervates dissolved into the Dx-rich phase as the NaCl concentration increased, becoming notably smaller in volume at 100 mM NaCl and disappearing entirely by 150 mM NaCl (Figures 4-S3, 4-S4). We selected 50 mM Ca\(^{2+}\) and 10.5 mg/mL PAA, with no added NaCl, as our standard conditions for coacervate-containing AMVs.
4.3.2 Distribution of Ca$^{2+}$, urease, and PAA

Partitioning for Ca$^{2+}$, urease and PAA in PEG/dextran biphasic and PEG/dextran/coacervate triphasic systems was determined using atomic absorption spectroscopy and fluorescence (Table 4-1, Table 4-S1). Ca$^{2+}$ concentrations were nearly the same in both phases of PEG/dextran biphasic systems, while urease and PAA preferred the dextran-rich phase to varying degrees. In triphasic systems that correspond to coacervate-containing AMVS, Ca$^{2+}$ was strongly localized to the coacervate phase, reaching a local concentration of $1.34 \pm 0.05$ M, while the PEG-rich and Dx-rich phases had much lower concentrations of ~30 and 130 mM Ca$^{2+}$, respectively. Urease partitioned mainly to the Dx-rich phase ($4.35 \pm 0.085$ mg/mL), with PEG-rich and coacervate phase urease concentrations 20-fold lower. Hence, the two components necessary for CaCO$_3$ precipitation, Ca$^{2+}$ and CO$_3^{2-}$ (the latter formed by urease), are physically located within two separate compartments, coacervate and Dx, respectively. The polymeric Ca$^{2+}$ chelator PAA was also strongly localized in the coacervate phase (Table 4-1, Table 4-S1).

Table 4-1: Partitioning of Ca$^{2+}$, urease, and PAA in a PEG/dextran ATPS ($V_{\text{Dex}} : V_{\text{PEG}} = 1 : 49$), with and without 50 mM Ca$^{2+}$ and/or 10.5 mg/mL PAA 2-11 kDa, and 10.5 units/mL urease.

<table>
<thead>
<tr>
<th>Added to standard PEG/Dex ATPS:</th>
<th>Ca$^{2+}$</th>
<th>Urease</th>
<th>PAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>with Ca$^{2+}$</td>
<td>0.99 ± 0.08</td>
<td>0.0075 ± 0.0008</td>
<td>N/A</td>
</tr>
<tr>
<td>with PAA</td>
<td>N/A</td>
<td>N/A $^a$</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>C$<em>{PEG}$: C$</em>{Dex}$: C$_{PAA}$$^b$</td>
<td>1 : 4.2 : 43</td>
<td>1 : 20.1 : 1.15</td>
</tr>
</tbody>
</table>

$^a$ Urease not determined in this ATPS because CaCO$_3$ could not be precipitated without Ca$^{2+}$; therefore, urease was not necessary here. $^b$ Values normalized to 1 for the phase with lowest concentration, for ease of comparing fold-enrichment in other phases.
4.3.3 CaCO₃ formation

Urea was added to initiate local enzymatic production of carbonate by urease in the dextran-rich phase of coacervate-containing AMVs, as illustrated in Figure 4-2a. Because the interfacial vesicles do not block entry to the droplet interiors, urea can be added to the PEG-rich continuous phase. Reactions were followed by confocal fluorescence imaging on an inverted optical microscope. Since samples contain many droplets above the viewing plane, the largest and densest droplets, which contain the largest coacervates, are preferentially imaged. As shown in Figure 4-2b, by ~8 minutes after urea addition, the PAA-rich coacervate droplets, which were initially spherical and surrounded by dextran-rich phase on all sides, began to deform and interact with the AMV’s liposome shell; this process continued as the reaction progressed. Such changes in shape and wetting behavior indicate changes in the composition of the phases. At about 11 minutes, precipitated calcium carbonate is seen in the transmitted light images (lower panel), and the PAA-rich coacervate phase has begun to dissolve, releasing PAA into the Dx-rich phase (Figure 4-2b, d). This can be understood as a consequence of CaCO₃ formation, which removes Ca²⁺ previously bound by PAA; without the bound Ca²⁺, PAA can no longer form a coacervate. Although large droplets dominate these images, several smaller coacervate-containing AMVs can also be seen. In these smaller (≤10 µm diameter) droplets, regions of brightest PAA fluorescence remained spherical throughout the reaction, corresponding to what appear to be denser spheres of CaCO₃ in the transmitted light images. We hypothesized that these smaller droplets, which behaved differently during the reaction than their larger neighbors, might be more representative of our sample. We repeated this experiment at 14.75 mg/mL PAA, which maximizes emulsion stability at the expense of coacervate volume and PAA partitioning, (see Figure 4-1c). Figure 4-2c,e show images for the reaction time course of this sample, which
progresses similarly as for Figure 4-2b except that PAA-rich spheres persist upon coacervate dissolution despite the large size of the coacervate-containing AMVs.

**Figure 4-2:** Calcium carbonate formation in coacervate-containing artificial mineralizing vesicles (AMVs). (a) Illustration depicting the formation of CaCO$_3$ inside AMVs. Prior to urea addition, three aqueous phases are present: PEG-rich phase, dextran-rich phase, and PAA/Ca$^{2+}$ coacervate. Lipid vesicles are absorbed at the PEG-rich/dextran-rich aqueous/aqueous interface, and urease partitions to the dextran-rich phase. Upon the addition of urea, carbonate binds to Ca$^{2+}$, displacing the PAA inside the coacervate, initiating mineralization. (b, c) Fluorescent confocal and transmitted (DIC) stills of mineralization occurring inside AMVs. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca$^{2+}$; 100 mM urea, and (b) 10.5 mg/mL PAA 2-11 kDa; 10.5 units/mL urease, or (c) 14.75 mg/mL PAA 2-11 kDa; 12.45 units/mL urease. Inset times refer to time after urea addition. Arrows notate the formed minerals, and fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). (d, e) Relative PAA fluorescence in each phase from data corresponding to (b) and (c), respectively, over the course of the reaction. Legend in (d) also applies to (e).
4.3.4 Mineral characterization

Solids were collected from mineralization experiments by dilution with 10 mM Tris buffer (pH ~8) to convert the system to a single phase, releasing interfacial liposomes into solution and allowing collection of precipitates by centrifugation. Scanning electron microscope images show smooth microspheres (Figure 4-3a). Concave spots are observed on some particles (Figure 4-3b), which may reflect coacervate shape during the mineralization process and/or interactions between microspheres before they had dried completely. Figure 4-3c and 4-3d show the distribution of initial coacervate volumes and mineral particle volumes, respectively. Data for our standard composition, corresponding to Figure 4-2b, is shown in red. The distribution of coacervate volumes is quite broad, with a mean ~6 x 10^3 μm^3, while the resulting particles show a tighter distribution and an average ~1.5 x 10^3 μm^3. We hypothesize that the narrowing of size distribution from the coacervates to the mineral microspheres is a result of differences in the rate of CaCO_3 formation and coacervate solidification, which depends not only on coacervate volume but also on CO_3^{2−} flux from the urease-rich dextran phase into the Ca-rich coacervate phase. Smaller coacervate droplets, produced either by increasing the PAA concentration similarly to Figure 4-2c and Figure 4-1c or by adding more liposomes to decrease average AMV volume, resulted in smaller mineral microspheres (Figure 4-3, blue histograms, and Figure 4-S5, respectively). Thus, although coacervate size does not directly translate into mineral microsphere size, it can be used to control the distribution of mineral sizes.
Figure 4-3: Particles produced in coacervate-containing AMVs are spherical, with size distributions tunable by changing initial coacervate sizes. Scanning electron micrographs of CaCO$_3$ particles extracted from coacervate-containing AMVs at low (a) and high (b) magnification. (c, d) Volume distributions for initial coacervates (c) and resulting mineral microspheres (d) as a function of PAA concentration. Increased PAA systems (blue) contained 15 mg/mL PAA 2-11 kDa, and standard composition systems (red) contained 10.5 mg/mL PAA 2-11 kDa. Coacervate and particle volumes were calculated from diameters measured using confocal microscope (transmitted and PAA fluorescence channels) and SEM images, respectively. Coacervates measuring smaller than 1 micron in diameter could not be effectively resolved and were not included in this analysis. All emulsions used had the following composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca$^{2+}$; 12.45 units/mL urease; 100 mM urea.

Fluorescence imaging confirmed the presence of labeled PAA throughout the particles (Figures 4-4, 4-S7), while powder XRD showed no features, suggesting that these particles were amorphous (Figure 4-5e). Vibrational spectroscopy (ATR-FTIR and microRaman) supported
assignment of these particles as amorphous calcium carbonate (ACC),\textsuperscript{36-37} and further indicated the presence of polyaspartic acid (Figures 4-4, 4-5 and Supporting Information). MicroRaman optical sectioning (z-stack) of individual particles suggested a dense shell surrounding a less-dense core, with organic and inorganic components co-localized in both the shell and core regions (Figure 4-4d,e).

Figure 4-4: Mineral microspheres contain PAA co-localized with carbonate. (a,b) Optical microscopy of mineral microspheres formed as in Figures 2 and 3 (standard conditions); (a) confocal fluorescence channel for Alexa 488-tagged PAA (green), and (b) transmitted (DIC) channel for the same particles. (c-e) MicroRaman characterization: (c) Raman spectra for mineral microspheres (top line) and for PAA alone (bottom line). Indicated peaks were used to create Raman maps (d, e) (d) Brightfield image of particles and Raman maps of peak intensity for the carbonate (red) and amide stretch (blue). Maps were acquired in the x,y-direction. (e) Brightfield image of particle used and Raman maps of peak intensity for the carbonate (red) and amide stretches (blue). Maps were acquired in the x,z-direction. Green line on the brightfield notates cross-section of particle where analysis was performed. Emulsions used to create the minerals had the following composition: 1:49 dex:PEG volume ratio; 20% by volume LUVs; 50 mM Ca\textsuperscript{2+}; 10.5 mg/mL PAA 2-11 kDa; 12.45 units/mL urease; 100 mM urea. Scale bar = 5 μm unless otherwise noted.
Microsphere interiors were imaged by SEM of broken particles and by TEM of microtomed epoxy-embedded particles (Figure 4-5a,b); images reveal a porous internal structure and denser shell, in accordance with the microRaman data in Figure 4. Selected area electron diffraction shows no crystallinity (Figure 4-5d, top). Thermogravimetric analysis with mass spectrometry detection (TGA-MS) was used to determine the amount of water and organic material incorporated in these particles. We find 13.6% by mass water and 28.8% organics, with the rest inorganics (assumed to be CaCO₃, Figure 4-S8 and Table 4-S2). High loadings (~10 w/w%) have been reported for encapsulants such as lysozyme in CaCO₃-based nanoparticle drug delivery vehicles formed by co-precipitation or coacervation methods, generally in the presence of a polyanion such as heparin or polyacrylic acid.¹⁸, ³⁸ We investigated the role of each major component of our coacervate-containing AMV system by performing a series of control experiments lacking vesicles, PAA, the PEG/dextran ATPS, and/or dextran-rich phase (Figures 4-S9-S11). Omission of liposomes resulted in a heterogeneous particle population, with many hollow particles, consistent with surface wetting-related changes in droplet organization (Figure 4-S9). Particles formed in the absence of PAA contained a mixture of crystalline forms (calcite and vaterite, see Figure 4-S12). SEM images of these particles showed greater heterogeneity in size and shape than our standard conditions (Figure 4-S9). When both PAA and Ca²⁺ were present, coacervation was only observed for macromolecularly-crowded media (ATPS or PEG-rich phase alone), and did not occur at our standard Ca²⁺ and PAA concentrations in the absence of the neutral polymers. Although coacervates were not present, urease-catalyzed carbonate production nonetheless led to mineralization, producing smaller and less spherical particles (Figure 4-S10). It was possible to induce coacervation in the absence of the PEG and dextran polymers by increasing the Ca²⁺: PAA ratio either by increasing Ca²⁺ at constant PAA or by
decreasing PAA. Particles formed in these systems were <2 μm diameter, semi-spherical and clustered into aggregates (Figure 4-S11). TGA indicated similar water content for all of the PAA and Ca-containing compositions tested (13.6-16.1% by mass, Table 4-S2). The ratio of organics to inorganics (i.e., CaCO₃) ranged from a high of 0.5 for the coacervate-containing AMVs to 0.14 for 50 mM Ca²⁺ and 0.525 mg/mL PAA, which gave a low volume of coacervate. Interestingly, the sample that had no coacervates present prior to urea addition (50 mM Ca²⁺, 10.5 mg/mL PAA, in buffer rather than ATPS) led to nearly as high of organic content as the standard composition (0.43 vs. 0.5, Table 4-S2). We hypothesize that a PILP-type mechanism occurred in which the carbonate ions helped to drive formation of coacervates rich in Ca²⁺, PAA, and CO₃²⁻. Unlike the coacervate-containing AMVs, however, these conditions did not lead to uniform, individual smooth mineral microspheres (Figure 4-S11).
Figure 4-5: Structural characterization of mineral microspheres. (a) Scanning electron micrograph (SEM) of a particle that cracked open during washing. (b) Optical microscopy image of microtomed thin section of microspheres embedded in epoxy mounted atop silicon. (c) SEMs of mineral microspheres before (i., ii.) and after (iii., iv.) heat treatment: (i.) Non-heated particle and (ii.) higher magnification view of non-heated particle; (iii.) Heated particle and (iv.) higher magnification view of heated particle. (d) Scanning transmission electron micrograph (i.) and selected area electron diffraction patterns (or SAED) (ii.) of thin section of a non-heated particle: the diffuse rings confirm the amorphous nature of the particle. Bright-field transmission electron micrograph (iii.) and selected area electron diffraction pattern (iv.) of thin section of a heated particle. The most prevalent diffraction ring corresponds to a d-spacing of 3.04 Å, characteristic of the {104} calcite plane. (e) XRD spectra of fresh particles, long-term aged particles in air, short-term aged particles in buffer, and heated particles. Calcite standard peaks are included for reference. The absence of peaks in all unheated sample XRD spectra is consistent with ACC. The heated particle XRD diffraction pattern showed strong peaks corresponding to calcite. (f) ATR-FTIR spectra of fresh particles, long-term aged particles in air, short-term aged particles in buffer, heated particles, and notated v₁ – v₄ carbonate ion vibrational bands.
4.3.5 Stability and heat treatment of hybrid organic-inorganic microspheres

Removal of the organic inclusions by slow heating (0.1°C/min to 500 °C, held for 8 h) resulted in conversion to porous, polycrystalline calcite while retaining the overall spherical shape of the original organic-rich ACC particles (Figure 4-5). Thin sections of this heated material showed increased porosity throughout the core, and SAED indicated polycrystalline calcite (Figure 4-5d, right panels). XRD confirmed conversion to calcite. Infrared spectra also showed changes consistent with loss of ACC and gain of calcite. In contrast, dry unheated samples stored in air on the benchtop remain ACC indefinitely (>12 months so far, Figure 4-5e,f), and samples stored in water remained unchanged for at least 3 days (Figure 4-S13). ACC is normally expected to convert to more stable forms (i.e., calcite) over time particularly when heated or exposed to water (even as humid air), although freeze-drying or additives (Mg²⁺ or organics) can slow conversion.³⁹⁻⁴¹ We attribute the stability of ACC in our microparticles to their high organic content, which prevents restructuring. The ability to produce stable organic-rich ACC that can be stored under ambient conditions for long times and later converted to crystalline form is potentially interesting as a means of repairing synthetic or biological materials.⁴²

4.3.6 Calcium phosphate production in coacervate-containing AMVs

We replaced the urease/urea enzyme/substrate pair with alkaline phosphatase and rac-glycerol 1-phosphate to adapt the coacervate-containing AMVs for calcium phosphate production (Figure 4-6a). XRD of the resulting particulate material showed no features, and ATR-FTIR spectra were consistent with PAA-rich amorphous calcium phosphate (Figure 4-6b, 4-S14).⁴³ Figure 4-6 shows SEM images and EDS elemental mapping for calcium phosphate
particles produced by this process, which were smaller than the ACC particles but otherwise similar in appearance as smooth, round microspheres.

Figure 4-6. Coacervate-containing AMVs can be adapted for production of other materials. (a) Calcium phosphate synthesis was performed by using a phosphate producing enzyme, alkaline phosphatase (ALP) in place of urease. The substrate is rac-glycerol 1-phosphate (RG1P), whose reaction with ALP releases phosphate anion. (b) ATR-FTIR spectrum of calcium phosphate particles made in coacervate-containing AMVs. Notated peaks refer to the $\nu_3$ and $\nu_4$ phosphate stretching modes; the broad appearance of these peaks suggest the material is amorphous calcium phosphate. Other prominent peaks in the spectrum are due to PAA incorporated in the particles. (c) SEM of particles. (d, e) Energy-dispersive X-ray spectroscopy (EDS) maps of (d) calcium and (e) phosphorous, acquired at the same location as the SEM image.

4.4 Discussion

The approach used here is related to polymer-induced liquid precursor (PILP) processes introduced by Gower. PILP-based mineralization strategies have been applied to CaCO$_3$, 

115
calcium phosphates and amino acid crystals, constituting a new type of nonclassical crystallization pathway relevant for biomimetic mineralization. PILPs employ solution conditions where formation of liquid phases occurs only after the addition of CO$_3^{2-}$. Therefore, PILPs appear to be coacervate droplets that contain Ca$^{2+}$, PAA and CO$_3^{2-}$. In our work, the coacervate phase exists prior to the onset of CO$_3^{2-}$ production. Separating the coacervation and reaction steps by pre-forming Ca$^{2+}$–rich coacervate droplets offers greater control over reaction location and microenvironment structuring. For example, we took advantage of this to control coacervate volume and position relative to the enzyme-rich phase responsible for CO$_3^{2-}$ production. Complex emulsion types can also be produced by microfluidics, however the all-aqueous emulsion approach to generating coacervate-containing AMVs enables batch production of mineralization-competent droplets and facile release and collection of the particle products. This is important for producing sufficient sample for characterization (e.g., by XRD, TGA) and for any downstream applications that require scale-up.

Aqueous multiphase emulsion droplets represent a general approach for materials synthesis beyond the examples shown here. Globular proteins generally accumulate in the dextran-rich phase of PEG/dextran systems, which should enable swapping out the urease for another system-dependent enzyme to produce a desired precursor or change in conditions such as pH to drive mineral formation. Although the PEG/dextran system is the most common, phase separation can be achieved in a wide variety of aqueous polymer solutions. Similarly, coacervation to form droplets rich in mineral precursors is not restricted to polycarboxylates and Ca$^{2+}$, and can be envisioned based on chelation or ion pairing between many polymer/precursor pairs. Thus, the basic approach introduced here should be adaptable for enzymatic production of materials with other inorganic and organic components.
4.5 Methods

**Materials.** Calcium chloride dihydrate (≥99.5 % purity), urea, poly-(α,β)-DL-aspartic acid sodium salt (PAA) 2-11 kDa, poly(ethylene glycol) (PEG) 8 kDa, urease from jack bean (*Canavalia ensiformis*) of activity 8.3 U/mg, alkaline phosphatase of activity 20 U/μL, rac-Glycerol 1-phosphate sodium salt hydrate, and Amicon Ultra centrifugal filters were purchased from Sigma-Aldrich, Co. (St. Louis, MO). Dextran (Dex) 10 kDa was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Alexa Fluor™ 488 hydrazide, dextran conjugated Alexa Fluor™ 647 10 kDa, Premium Grade (1-ethyl-3-(3-dimethylaminopropyl)carboimide hydrochloride) (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), Sulfo-NHS-acetate, SNARF™-1 carboxylic acid acetate succinimidyl ester, and Zebra™ Spin Desalting Columns were purchased from Thermo Fisher Scientific, Co. (Waltham, MA). Egg-phosphatidylcholine (Egg-PC), egg-phosphatidylglycerol (Egg-PG), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol) 2 kDa (DOPE-PEG 2 kDa), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE) lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Poly(L-aspartic acid sodium salt) 14 kDa was purchased from Alamanda Polymers, Inc. (Huntsville, AL).

Fluorescently labeled PAA was prepared in-house by conjugating Alexa Fluor™ 488 hydrazide to carboxylic acid residues using an EDC linker. The labeling reaction was performed step-wise to ensure maximum labeling efficiency and purity. 12.3 mg of PAA 14 kDa was dissolved in 1 mL 0.1 PBS buffer. 25-molar equivalent sulfo-NHS-acetate was added to 100 μL of the PAA solution to cap the terminal amines. The solution was gently mixed at room temperature for 1 hour. Excess sulfo-NHS-acetate was removed using a Zebra™ Spin Desalting Column (7 kDa MWCO) that had been equilibrated with 0.1 M MES buffer, pH ~6. 0.1 molar equivalent EDC
and 0.25 molar equivalent sulfo-NHS acetate were added to the purified polymer to form the reactive intermediate. After 15 minutes, the excess EDC was quenched using 10 molar excess 2-mercaptoethanol. The polymer was purified again with a desalting spin column equilibrated with 0.1 PBS buffer. The purified polymer was added directly to ~1 mg of Alexa 488 hydrazide and mixed at room temperature for 2 hours. Excess label was removed with a desalting spin column, and the solution was concentrated using an Amicon Ultra centrifugal filter (MWCO 3 kDa). The labeled polymer was then stored at -22℃.

**Formation of PEG/Dex ATPS.** All ATPS stocks were prepared to 10 w/w% PEG 8 kDa and 10 w/w% Dex 10 kDa. The appropriate masses of both polymers were dissolved in 10 mM pH 7.4 Tris buffer. Buffer was manually prepared in house using a combination of Tris hydrochloride and Tris base. The two phases were then allowed to fully separate overnight at 5℃, then removed via pipet. The PEG-rich phase and Dex-rich phase from this system were used for all subsequent sample preparation.

**Formation of lipid vesicles.** Large unilamellar vesicles (LUVs) were prepared by extrusion of heterogeneous lipid assemblies formed by gentle hydration of lipid films in PEG-rich phase. Lipid composition was always 48.56, 48.51, 2.83, and 0.10 mol % of Egg-PC, Egg-PC, DOPE-PEG 2 kDa, and Rh-DOPE, respectively. Lipid stocks were pooled in a borosilicate tube with an additional ~50 μL chloroform, for a final volume of ~200 μL; this mixture was stirred rapidly by hand while Ar was blown into the tube slowly (~1 PSI), which dried the lipid mixture into a film on bottom of the glass. The vial was placed within a vacuum desiccator and vacuum was pulled via pump for at least two hours to remove any chloroform remaining in the lipid film. For
hydration, 500 µL of PEG-rich phase from the ATPS stock was added to the glass vial for a final lipid concentration of 7.5 mg/mL, capped tightly with parafilm, and placed in a 37 °C incubator for at least 48 hours. The heterogeneous mixture cooled to room temperature and then extruded through a 200 nm pore 11 times using an Avanti mini-extruder, creating ~150 nm LUVs and removing any excess non-LUV lipid material. The LUVs were stored in a HDPE vial at 5°C and used within a week.

**Effect of PAA on ATPS with and without Ca\(^{2+}\).** To determine the effect of PAA on ATPS LUV emulsions with and without 50 mM Ca\(^{2+}\), a number of 100 µL ATPS with 20 vol. % LUVs of \(V_{\text{Dex}} : V_{\text{PEG}}\) 1 : 49 was formulated and emulsified via pipette. The additive volume of the LUVs contributed to the final PEG-rich volume. 50 mM Ca\(^{2+}\) was added to some of these emulsions and remixed. To all the emulsions, varying concentrations of PAA, Alexa 647-tagged dextran and Alexa 488-tagged PAA (except for a control sample) were added and the samples remixed again. To prevent excess dilution of the ATPS, PAA and Ca\(^{2+}\) were made as 50 mg/mL and 3 M stocks in PEG-rich phase, respectively. A 50 µL sample of each emulsion was dispersed on an in-house silanized glass cover slip with a 150 µL silicone spacer. A silanized cover slip was placed on top, and the sample was placed on the microscope stage to image. After waiting a few minutes to allow some of the dextran-rich droplets to settle, an area of interest was determined visually, and DIC/fluorescence images were taken. All fluorescent and DIC images were acquired on a Leica (Wetzlar, Germany) TCS SP5 PL confocal microscope using a 63x 1.4 NA APO objective. Images were analyzed using a combination of Leica LAS-X software and ImageJ (National Institutes of Health).
Urease, PAA and Ca$^{2+}$ Partitioning. A 20 mL ATPS of $V_{\text{Dex}} : V_{\text{PEG}}$ of 1 : 49 was formulated with no LUVs, and an equivalent concentration of Ca$^{2+}$ and PAA to the above experiments was added. The samples were mixed thoroughly, left in a refrigerator at 5 °C overnight and warmed to room temperature before analysis, where aliquots of each polymer-rich phase were removed and diluted appropriately into DI H$_2$O. Atomic absorption of calcium was measured using a Shimadzu AA-7000 Atomic Absorption Spectrophotometer (Kyoto, Japan) with Ca/Mg hollow cathode lamp operating at 422.7 nm wavelength, 5 nm slit width, 7 mA input current, 15 and 50 PSI acetylene and air respective input pressures; samples were analyzed using WizAArd software. Fluorimetry was used to detect urease via tryptophan fluorescence ($\lambda_{\text{Ex}} = 280$ nm, $\lambda_{\text{Em}} = 332$ nm) as well as Alexa 488-labeled PAA partitioning ($\lambda_{\text{Ex}} = 494$ nm, $\lambda_{\text{Em}} = 516$ nm). Fluorescence was measured using a Fluorolog Horiba Jobin Yvon fluorimeter (Edison, NJ) and Fluorolog software, using 5 nm excitation and emission slit widths. For urease, background interference caused by PEG prevented determining the exact concentration of urease in the PEG-rich phase. Instead, the PEG-rich phase concentration was calculated by using the total number moles of urease added, the volumes of each polymer-rich phase, and the determined urease concentration in the Dex-rich phase. Each method was blanked against DI H$_2$O and at least 6 calibration standards were utilized for each method, resulting in $R^2$ values of at least > 0.99.

Monitoring mineralization in situ using confocal microscopy. A 100 μL ATPS with 20 vol. % LUVs of $V_{\text{Dex}} : V_{\text{PEG}}$ 1 : 49 was formulated, with the appropriate concentrations of urease, Ca$^{2+}$ and PAA, and emulsified using a vortex mixer immediately prior to analysis. PAA and dextran were equilibrated with Alexa 488-tagged PAA and Alexa 647-tagged dextran respectively. To maintain the ATPS volume ratio, all stock solutions were prepared in PEG-rich
phase. 50 μL of the emulsion was deposited on a silanized glass slide with a 150 μL spacer, and no cover slip. The sample was then transferred to the microscope sample stage. To initiate the reaction, 5 M urea in PEG-rich phase was added to the emulsion for a final concentration of 100 mM urea, and a silanized glass coverslip was placed on top.

Preparation of CaCO$_3$ particles for bulk and individual analysis. A 1 mL ATPS with 20 vol. % LUVs of V$_{\text{Dex}}$ : V$_{\text{PEG}}$ 1 : 49 was formulated, with 12.45 units/mL urease, 50 mM Ca$^{2+}$ and 10.5 mg/mL PAA, and emulsified using a vortex mixer. Urea from a 5 M stock solution in PEG-rich phase was added to the emulsion for a final concentration of 100 mM, and the emulsion was vortexed via pipette to initiate the reaction. After one hour, samples were centrifuged at 16100 × g to separate the precipitant, and the supernatant was removed via pipette. Pellets were resuspended with 1 mL of 10 mM Tris buffer (pH = 8.5), followed by three additional centrifuge and washing cycles. Mineral samples were then dried in a SpeedVac vacuum concentrator at room temperature for two hours. Dried samples were then stored under atmosphere at room temperature. Differences in emulsion or mineral composition are notated in individual figure captions.

Mixing effects on the size distributions of coacervates and minerals. AMVs can be made by first establishing a PEG/coacervate phase separation or a PEG/dextran phase separation. The former was achieved with the following order of addition: PAA stock solution, calcium stock solution, PEG-rich phase, urease stock solution, dextran-rich phase, LUV stock solution. The latter was achieved with the following order of addition: PAA stock solution, PEG-rich phase, urease stock solution, dextran-rich phase, calcium stock solution, LUV stock solution. All
experiments in this paper used emulsions in which the PEG/coacervate phase separation was established first with the exception of Figure 4-S6.

**Preparation of calcium phosphate particles for bulk and individual analysis.** The ATPS used for these emulsions and stock solutions was prepared using 10 mM buffer, pH=8.5. The increased pH better accommodates the enzyme used for phosphate mineralization. A 1 mL ATPS with 20 vol. % LUVs of V$_{\text{Dex}}$ : V$_{\text{PEG}}$ 1 : 49 was formulated, with 0.2 units/mL alkaline phosphatase, 50 mM Ca$^{2+}$ and 10.5 mg/mL PAA, and emulsified using a vortex mixer. *rac*-Glycerol 1-phosphate sodium salt hydrate (RG1P) from a 1 M stock solution in PEG-rich phase was added to the emulsion for a final concentration of 50 mM, and the emulsion was vortexed via pipette to initiate the reaction. After one hour, samples were centrifuged at 16100 $\times$ g to separate the precipitant, and the supernatant was removed via pipette. Pellets were resuspended with 1 mL of 10 mM Tris buffer (pH = 8.5), followed by three additional centrifuge and washing cycles. Mineral samples were then dried in a SpeedVac vacuum concentrator at room temperature for two hours. Dried samples were then stored under atmosphere at room temperature.

**X-ray powder diffraction (XRD).** Particles were prepared as described above. XRD patterns were collected on a Si zero-background sample holder using Malvern Panalytical (Malvern, UK) XPert Pro MPD Theta-Theta Diffractometer across a 10- 70° 2θ range at 45 kV acceleration, 40 mA Cu Kα radiation using a 0.5° divergence fixed-slit width and 10 mm beam mask in tandem with a PIXcel detector over an analysis time of 15 minutes; the resulting patterns were analyzed.
using Jade software from MDI (Acceptable shift on the weekly instrument test is +/- 0.03 deg. for 2-theta).

**Vibrational spectroscopy.** Infrared spectra were collected using a Bruker (Billerica, MA) Vertex V70 spectrometer using a Harrick MVP Pro Star attenuated total reflection (ATR) accessory with a diamond crystal, which was operated under N₂-rich conditions to remove atmospheric moisture. All spectra were acquired between 4000-400 cm⁻¹ at 6 cm⁻¹ resolution by averaging 100 scans, which was recorded using OPUS software; intensities are reported here as – log(R/R₀), where R is the reflectance of the sample, and R₀ is the reflectance of the reference (clean diamond crystal), taken before each sample. Raman maps were acquired on a Horiba (Kyoto, Japan) LabRam with back illuminated detector (2048x512 pixel) and 300 g/mm grating with spectral resolution of ~4 cm⁻¹. Each individual spectrum had an acquisition time of 5 seconds. The x,y was acquired with a 532 nm laser while the x,z map was acquired with a 633 nm laser. Particles were dispersed on a non-silanized glass slide with no further treatment.

**Electron Microscopy.** Scanning electron microscopy (SEM) images were acquired with an FEI (Hillsboro, OR) Nova NanoSEM 630 SEM, operated at high vacuum mode at a 5 mm working distance, and a 5 keV accelerating voltage. ETD and TLD detectors were utilized for low and high magnification imaging, respectively. Elemental maps were acquired using energy-dispersive X-ray spectroscopy (EDS) on the same instrument, operated at a 15 keV accelerating voltage and utilizing an ETD detector. A ~10 Å iridium coating was applied to samples using sputter coater prior to imaging. Samples for transmission electron microscopy (TEM) were embedded in epoxy then sliced into thin sections using ultramicrotome (Leica EM UC6)
thickness of 70 ~ 120 nm. TEM images and electron diffraction patterns were acquired with an FEI Talos F200X transmission electron microscopy, using 200 kV electron beam. Low electron energies were used to acquire image due to sample sensitivity to electron beam.

**Thermogravimetry.** Minerals were analyzed with a TA Instruments (New Castle, DE) Discovery Series TGA Q5500 coupled with Discovery MS. ~5 mg of each mineral sample was loaded into a 100 μL high temperature platinum pan. The system was purged with nitrogen gas and heated at an uneven rate: 10 minutes at a 30°C isothermal, heated to 200°C at a rate of 10°C/min, 20 minutes at a 200°C isothermal, heated to 500°C at a rate of 10°C/min, 20 minutes at a 500°C isothermal, heated to 800°C at a rate of 10°C/min, and 20 minutes at an 800°C isothermal. A non-linear heating rate was used to decompose the sample as efficiently as possible. Gravimetric data was acquired and analyzed using TA instruments TRIOS software. Accompanying mass spectroscopy data was acquired alongside the gravimetric data using TA instruments Process Eye software.
### 4.6 Supporting information

**Table 4-S1.** Concentrations of Ca\(^{2+}\), urease, and polyaspartic acid (PAA) in a PEG/dextran/coacervate three-phase system (V\(_{\text{Dex}}\) : V\(_{\text{PEG}}\) = 1 : 49), with 50 mM Ca\(^{2+}\), 10.5 mg/mL PAA 2-11 kDa, and 10.5 units/mL urease.

<table>
<thead>
<tr>
<th></th>
<th>Ca(^{2+}) (mM)</th>
<th>Urease (μg/mL)</th>
<th>PAA (intensity units)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PEG-rich phase</strong></td>
<td>31 ± 1</td>
<td>217 ± 4</td>
<td>55 ± 2</td>
</tr>
<tr>
<td><strong>Dextran-rich phase</strong></td>
<td>130 ± 5</td>
<td>4360 ± 90</td>
<td>1080 ± 40</td>
</tr>
<tr>
<td><strong>Coacervate phase</strong></td>
<td>1340 ± 50</td>
<td>250 ± 10</td>
<td>4780 ± 30</td>
</tr>
</tbody>
</table>

\(^a\)Based on relative fluorescence intensity
Table 4-S2. Mass compositions of particles made under different conditions, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 °C. The remaining mass was used to determine the organic: CaCO₃ mass ratio. The weight loss between 200 and 500 °C (second thermal decomposition step) was all attributed to organic material. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step.

<table>
<thead>
<tr>
<th>Mineralizing environment</th>
<th>[Ca²⁺] (mM)</th>
<th>[PAA] (mg/mL)</th>
<th>Coacervates present?</th>
<th>Water content (% w/w)</th>
<th>Organic content (% w/w)</th>
<th>Organic: CaCO₃ weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard composition (PEG/dextran ATPS w/ PAA, LUVs)</td>
<td>50</td>
<td>10.5</td>
<td>yes</td>
<td>13.59</td>
<td>28.60</td>
<td>0.4964</td>
</tr>
<tr>
<td>PAA omitted</td>
<td>50</td>
<td>0</td>
<td>no</td>
<td>7.13</td>
<td>11.04</td>
<td>0.1349</td>
</tr>
<tr>
<td>LUVs &amp; PAA omitted</td>
<td>50</td>
<td>0</td>
<td>no</td>
<td>3.65</td>
<td>2.56</td>
<td>0.0488</td>
</tr>
<tr>
<td>PEG/dextran ATPS omitted</td>
<td>50</td>
<td>10.5</td>
<td>no</td>
<td>15.13</td>
<td>25.03</td>
<td>0.4183</td>
</tr>
<tr>
<td>PEG/dextran ATPS omitted &amp; extra Ca²⁺</td>
<td>1000</td>
<td>10.5</td>
<td>yes (high volume)</td>
<td>13.80</td>
<td>22.83</td>
<td>0.3603</td>
</tr>
<tr>
<td>PEG/dextran ATPS omitted &amp; reduced [PAA]</td>
<td>50</td>
<td>0.525</td>
<td>yes (low volume)</td>
<td>16.07</td>
<td>10.30</td>
<td>0.1399</td>
</tr>
</tbody>
</table>
**Figure 4-S1.** Droplet size (left axis) and PAA partitioning (right axis) in AMVs as a function of total PAA concentration (bottom axis) in the absence of Ca$^{2+}$. Measurements were performed using the raw data associated with Figure 1a. Diameters implicitly assume that confocal sections capture droplet equators and consequently may systematically underestimate diameters. K was calculated based on fluorescence intensity ratios. Error bars indicate the standard deviation of the mean of 32 measurements.

**Figure 4-S2.** PAA partitioning in AMVs as a function of total PAA concentration in the presence of 50 mM Ca$^{2+}$. Measurements were performed using the raw data associated with Figure 1b. K was calculated based on fluorescence intensity ratios. Droplet size was not calculated due to nonspherical shapes for intermediate [PAA]. Error bars indicate the standard deviation of the mean of ~30 measurements.
**Figure 4-S3.** Laser confocal microscopy fluorescence images of coacervate-containing AMVs with 10.5 mg/mL PAA 2-11 kDa and 50 mM Ca\(^{2+}\) with increasing NaCl (in mM, given in upper left of each image). Images have been overlaid and false-colored for clarity. Red channel represents Rhodamine-labeled lipid vesicles; green channel represents Alexa 488- labeled PAA; blue channel represents Alexa 647-labeled dextran. Scale bars = 10 μm.

**Figure 4-S4.** Droplet size (left axis) and PAA partitioning (right axis) in coacervate-containing AMVs as a function of total NaCl concentration (bottom axis) in the presence of 50 mM Ca\(^{2+}\). Diameters implicitly assume that confocal sections capture droplet equators and consequently may systematically underestimate diameters. K was calculated based on the fluorescence intensity ratios. Measurements were performed using the raw data associated with Figure 4-S3. Error bars indicate the standard deviation of the mean of ~30 measurements.
Figure 4-S5. Changes in the amount of lipid vesicles (LUVs, for large unilamellar vesicles) present as interface stabilizers result in changes the coacervate and subsequent mineral volume distributions. Volume distributions of coacervates and minerals made inside AMVs with (a, b) increased LUV concentration (1.875 mg/mL) (c, d), standard LUV concentration (1.5 mg/mL) (e, f), and reduced LUV concentration (1.125 mg/mL). Regardless of LUV concentration, all emulsions had the same basic composition: 1:49 dextran:PEG volume ratio; 50 mM Ca\(^{2+}\); 10.5 mg/mL PAA 2-11 kDa; 12.45 units/mL urease; 100 mM urea. Note: differences in these size distributions from those in Figure 4-3 are due to reproducible differences in mixing technique (Figure 4-S6).
Figure 4-S6. Small changes in reaction protocol can influence particle size distributions. Histograms of volumes of organic-rich CaCO$_3$ particles made using the same coacervate-containing AMV composition but with differing mixing techniques. (a) Particles made in a system which dextran-rich phase was introduced to the AMV synthesis before coacervates were. Prior to mineralization, the AMVs were vortex mixed for either 10 seconds (blue) or 3 minutes (red). (b) Particles made in a system which coacervates were introduced to before dextran-rich phase was. Prior to mineralization, the AMVs were vortex mixed for either 10 seconds (blue) or 3 minutes (red). (c) Histogram highlighting the greatest difference in size distribution due to mixing technique.
**Figure 4-S7.** Fluorescent PAA is found associated with solid mineral particles. (a) Alexa 488 fluorescent and (b) transmitted light (DIC) channels of minerals made inside coacervate-containing AMVs, doped with Alexa 488-tagged PAA. (c) Alexa 488 fluorescent and (d) DIC channels of minerals made inside coacervate-containing AMVs, NOT doped with Alexa 488-tagged PAA (i.e., only unlabeled PAA was present).

**Figure 4-S8.** (a) TGA-MS graph for microparticles made inside coacervate-containing AMVs with standard composition. (Left axis) Total relative weight of sample as a function of time (temperature). (Right axis) Mass spectroscopy signals of thermal decomposition by-products, namely water (18 amu) and carbon dioxide (44 amu). (b) Temperature profile of the heating rates used for each sample.
Figure 4-S9. Effect of liposomes and PAA on appearance of mineral particles. Scanning electron micrographs show CaCO$_3$ minerals formed in solutions that contained a 1:49 dextran:PEG ATPS: (a,b) coacervate-containing AMVs without lipid vesicles (LUVs) at the interface (50 mM Ca$^{2+}$; 10.5 mg/mL PAA), (c,d) AMVs without PAA present (20% by volume LUVs; 50 mM Ca$^{2+}$), (e,f) AMVs with neither LUVs nor PAA present (50 mM Ca$^{2+}$).

Figure 4-S10. Effect of PEG and dextran polymers on appearance of mineral particles. Scanning electron micrographs show CaCO$_3$ minerals formed in solutions that contained 50 mM Ca$^{2+}$ and 10.5 mg/mL PAA, with different polymer compositions. (a,b) dextran: PEG emulsions with (1:49 dextran: PEG volume ratio), (c,d) PEG-rich phase, and (e,f) buffer solution without PEG or dextran polymers. Note that while the Ca$^{2+}$ and PAA concentrations are constant across these samples, only the first two conditions led to Ca/PAA-rich coacervate formation; the buffer sample did not have coacervates present prior to mineralization.
Figure 4-S11. Effect of Ca\(^{2+}\) and PAA concentrations on appearance of mineral particles formed in the absence of PEG and dextran polymers. Scanning electron micrographs show particles prepared in (a,b) buffer solution not initially containing coacervates (50 mM Ca\(^{2+}\); 10.5 mg/mL PAA), (c,d) buffer solution that containing a small volume of coacervates (50 mM Ca\(^{2+}\); 0.525 mg/mL PAA), and (e,f) buffer solution containing a large volume of coacervates (1M Ca\(^{2+}\); 10.5 mg/mL).

Figure 4-S12. Powder XRD of minerals made inside coacervate-containing AMV systems that were missing various components. AMVs compositions were identical those noted in Figure 4-S9. Calcite and vaterite standard peaks are included for reference.
Figure 4-S13. SEMs of minerals after (a, b) short-term exposure to aqueous buffer solution and (c, d) long-term exposure to atmosphere. Buffer soaked particles were placed in 10 mM Tris buffer (pH = 8.5) at room temperature for 3 days. Atmosphere treated particles were dry and sat at room temperature for ~1 year.

Figure 4-S14. Powder XRD of calcium phosphate minerals made inside AMVs containing ALP and RG1P.
4.7 References


5.1 Abstract

Biominerals such as spines and teeth have mechanical strength that exceeds that of its inorganic mineral equivalents. The enhanced strength of biominerals can be attributed to their hierarchical structures which are dictated by differences in the local organic content. Controllable variations in local organic content within a mineral structure can be difficult to achieve synthetically. Herein we present a method that utilizes multiple liquid-liquid phase separation events within an artificial mineralization vesicle to achieve multiple organic gradients within calcium carbonate microspheres. This method utilizes two distinct coacervate phases, comprised of calcium and either polyaspartic or polyglutamic acids, as mineral precursors. The mineral and coacervate forming capabilities of different lengths of either polycarboxylate were examined alone, then combined. The minerals made using both polycarboxylates were formed through subsequent phase separation events, resulting in minerals with distinct polyaspartic and polyglutamic acid-rich regions. The ability to create controllable, differentiated organic gradients within the same mineral represents an important step toward achieving the same level of structural control as Biology.

5.2 Introduction

Biominerals are well-known for their mechanical toughness, which can be several orders of magnitude higher than that of the native minerals.\textsuperscript{1-5} For instance, the sea urchin tooth is
largely made of calcium carbonate (CaCO$_3$), yet is capable of biting through rocks of the same mineral.$^6$ The fracture toughness ($K_c$) of the sea urchin tooth is roughly twice that of pure calcite (0.97 MPa•m$^{1/2}$ vs 0.54 MPa•m$^{1/2}$).$^7$ One reason for this enhanced toughness is a scaffold-like microstructure; the sea urchin tooth is comprised of interconnected micron-scale calcite plates and columns (Figure 1-3).$^8$ This structure directs the propagation of cracks, preserving the structural integrity of the tooth and increasing the fracture toughness. Hierarchical structures that enhance mechanical such as these can be found in countless biominerals, including bone.$^9$-$^{10}$

Within these hierarchies, organisms exert exquisite control over the local organic content of the mineral. These organics act as local structure directing agents.$^{11}$-$^{13}$ Returning to the sea urchin tooth example, the alternating plates and columns of the scaffold are comprised of calcite and magnesium calcite, respectively.$^{14}$ The presence of magnesium in calcite increases both the elastic modulus and hardness, which is thought to contribute to sea urchin tooth toughness.$^{15}$ Proteins associated with the magnesium calcite columns contain a significantly higher concentration of aspartic acid residues compared to other regions.$^{14}$ This suggests that the local protein content directs the incorporation of magnesium, and by extension the local physical properties. A similar example can be observed in the ascidian, a filter feeder organism is comprised of layers of calcite and amorphous calcium carbonate (ACC).$^{16}$ Proteins associated with the ACC regions within ascidians contain a high degree of aspartic acid residues. Meanwhile, proteins associated with the calcite regions are rich with glutamic acid.$^{16}$ Other instances where the local protein content dictates the local crystal structure can be found in other CaCO$_3$-creating organisms as well.$^{17}$-$^{19}$ The reason for the presence of ACC is unknown, though it is hypothesized that the organism exploits the differences in mechanical strength to disperse impact energy.
The structural hierarchies observed in biominerals have inspired many synthetic analogues, with the eventual goal of developing materials with comparable mechanical properties.\textsuperscript{20-23} Although these analogues do succeed in illustrating some degree of structural hierarchy, they lack the same dynamic organic gradients observed in genuine biominerals. The current synthetic methods used in the development synthetic biominerals do not allow for the same degree of spatial control as seen in Biology. Herein, we detail a new platform for bio-inspired materials synthesis that utilizes liquid-liquid phase separation (LLPS) to template mineral synthesis. LLPS has only just recently been recognized as an important aspect of subcellular compartmentalization\textsuperscript{24-27} and as a potential first step in biomineral synthesis.\textsuperscript{28-32} We recently reported an artificial mineralization vesicle (AMV) that utilizes a complex coacervate as a mineral precursor.\textsuperscript{33} We now build upon our previous methods through the incorporation of a second phase separating event. Subsequent LLPS events are theorized to be instrumental in the synthesis of silica minerals in diatoms.\textsuperscript{34-37} Each phase separations produces another layer of mineral which builds upon the previous. We present a test of that theory within the confines of our AMV by utilizing multiple polycarboxylates. The individual coacervate and mineral forming capabilities of different lengths of polyaspartic acid (PAA) and polyglutamic acid (PGA) will be evaluated. Then, both polycarboxylates will be used in combination to create organic-mineral composites with an unprecedented degree of control over the organic inclusions.

5.3 Results

AMVs and CaCO\textsubscript{3} microspheres were prepared using varying concentrations and lengths of polyaspartic acid (PAA) and polyglutamic acid (PGA). AMVs were characterized using confocal fluorescence microscopy, and atomic absorption spectroscopy (AAS) while the
minerals were characterized using scanning electron microscopy (SEM), X-ray diffraction (XRD), microRaman spectroscopy, thermal gravimetric analysis (TGA), and confocal fluorescence microscopy. The individual and cooperative coacervate and mineral forming capabilities of the different polycarboxylates will be discussed.

5.3.1 Different lengths of polyaspartic acid

Three different lengths of disperse PAA were used: 10 monomers long (1,400 Da), 50 monomers long (7,000 Da), and 100 monomers long (14,000 Da). These lengths span the potential weight range of the polydisperse PAA used in Chapter 4 (2,000 to 11,000 Da). In order to test the potential coacervate forming capabilities of the different lengths of PAA, AMVs were prepared with different concentrations of PAA at constant [Ca$^{2+}$] of 50 mM. The resulting emulsions were imaged using fluorescent confocal microscopy (Figure 5-1). Regardless of polymer concentration, the 10mer PAA did not form any coacervates. 50mer formed coacervates only at the lowest tested concentration (10 mg/mL), which equals 71.4 mM of the PAA monomer. The 100mer formed coacervates, regardless of total polymer concentration. Furthermore, the coacervates formed by the 100mer appeared much larger than those formed by the 50mer. Generally, longer polymers have greater coacervate forming capability, which explains why only the 50mer and 100mer formed coacervates.$^{38-39}$ Based on these observations, we chose to focus on the 50mer and 100mer for additional study.
Figure 5-1: Fluorescent confocal images of AMVs containing 50 mM Ca\(^{2+}\) chelated by different concentrations of various lengths of monodisperse polyaspartic acid (PAA). PAA was either (a, b, c) 10, (d, e, f) 50 or (g, h, i) 100 monomers in length. Coacervate formation was dependent on PAA length and concentration, and can only be observed in panels d, g, h, and i. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.

Bulk aqueous three phase systems (A3PS) were prepared using PEG-rich phase, dextran-rich phase, free Ca\(^{2+}\), and different concentrations of 50mer and 100mer PAA. The total PAA concentration was kept constant, with different mass ratios of the 50mer and 100mer present.

The calcium concentration in each phase was measured using atomic absorption spectroscopy (AAS) (Table 5-1). In general, the calcium concentration in the coacervate phase decreases as the amount of 100mer PAA is increased. Inversely, the calcium concentration in coacervate phase increases alongside the 100mer PAA. These data agree with the prior optical microscopy characterization (Figure 5-1.) In this context, coacervate-forming capability correlates with how
well the polymer can coordinate to calcium ions. Thus, longer PAA is capable of binding more calcium into the coacervate phase as expected.38-39

Table 5-1: Concentration of Ca\(^{2+}\) in PEG/dextran/coacervate A3PS with different weight ratios of bidisperse PAA. All separations had the same basic composition: V\(_{\text{Dex}}\):V\(_{\text{PEG}}\) = 1 : 9; 50 mM Ca\(^{2+}\); 15 mg/mL total PAA

<table>
<thead>
<tr>
<th>[Ca(^{2+})] (mM)</th>
<th>50mer only</th>
<th>2:1 50mer:100mer</th>
<th>1:1 50mer:100mer</th>
<th>1:2 50mer:100mer</th>
<th>100mer only</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-rich phase</td>
<td>40 ± 1</td>
<td>33.5 ± 0.6</td>
<td>36 ± 2</td>
<td>32 ± 1</td>
<td>34.9 ± 0.6</td>
</tr>
<tr>
<td>Dextran-rich phase</td>
<td>87.8 ± 0.7</td>
<td>71.6 ± 0.5</td>
<td>58.3 ± 0.6</td>
<td>45.7 ± 0.5</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>Coacervate</td>
<td>600 ± 200</td>
<td>1240 ± 90</td>
<td>1200 ± 300</td>
<td>1040 ± 20</td>
<td>1450 ± 60</td>
</tr>
</tbody>
</table>

Error is the standard deviation of the average of 3 measurements.

The composition of the minerals was investigated using thermogravimetric analysis paired with mass spectroscopy (TGA-MS).40-41 Minerals were prepared inside AMVs with a constant concentration of total PAA, but with different mass ratios of the 50mer and the 100mer. The relative amounts of the different components of the mineral, the water, the organic material, and the inorganic carbonate, were determined based on the weight loss at each thermal decomposition step (Figure 5-S1). The relative amount of organic material present in the mineral increases as the amount of the 100mer present in the emulsion increases (Table 5-2). This could relate to the fact that coacervates containing more of the100mer PAA have higher [Ca\(^{2+}\)] (Table 5-1). The reason for the observed trend could also relate to the polymer behavior during the mineralization process. It’s worth noting all these minerals all have lower organic content than those prepared with polydisperse PAA (Table 4-S2). Both systems had different total amounts of PAA present at the start of the reaction, though, so a difference in organic content is expected.
Table 5-2: Mass compositions of CaCO₃-PAA particles made using mono- and bidisperse PAA, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 degrees Celsius, the relative amount of organic carbon (second thermal decomposition step) was calculated from the loss of percent mass between 200 and 500 degrees Celsius, and the relative amount of inorganic carbon (third thermal decomposition step) was calculated from the loss of percent mass between 500 and 800 degrees Celsius. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[50mer PAA] (mg/mL)</th>
<th>[100mer PAA] (mg/mL)</th>
<th>Water content (% w/w)</th>
<th>Organic content (% w/w)</th>
</tr>
</thead>
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<tr>
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<td>14.51</td>
<td>16.85</td>
</tr>
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<td>2:1 50mer:100mer</td>
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<td>5</td>
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<td>5</td>
<td>10</td>
<td>13.04</td>
<td>20.73</td>
</tr>
<tr>
<td>100mer only</td>
<td>0</td>
<td>15</td>
<td>11.53</td>
<td>20.60</td>
</tr>
</tbody>
</table>

AMVs were prepared using different concentrations of the two polymers (Figure 5-2). Unlike the AAS and TGA experiments, the concentrations featured are based on the optimized experimental conditions for stable AMV formation. The fairly homogeneous size distribution and lack of lipid aggregation indicate that the AMVs are fairly stable, regardless of which length is present. Minerals formed within these AMVs, though, were shown to be extremely dependent on the length of polymers present. For instance, minerals formed within coacervates containing only the 100mer did not have symmetrical sphere structures. Rather, the particles were a heterogeneous mix of spheres and oblique spheroids. Particles formed using only the 50mer PAA had similar warped structures, albeit to a lesser extent. When both lengths were present, though, the CaCO₃ microspheres appeared to be much more spherical and homogeneous. These particles were much more comparable to the ones prepared using the polydisperse PAA (Figure
4-3). Some of the larger, damaged particles also appear to have a core-shell structure, where a solid shell surrounds a porous interior (Figure 5-2l). The core-shell structure is another structural motif of the polydisperse PAA particles (Figure 4-5a). Regardless of which lengths were used, all the prepared minerals were confirmed to be amorphous calcium carbonate via Raman spectroscopy (Figure 5-S2). Presumably, the high amount of organic content present in the mineral stabilizes the ACC structure. We can assume the surface energies involved in the coacervate and mineral formation are being affected by the polymers present, and that having multiple lengths present results in a more uniform, spherical structures.

Figure 5-2: (a-d) Fluorescent confocal images of AMVs with coacervates comprised of different concentrations of monodisperse and bidisperse polyaspartic acid (PAA) and (e-h) low magnification and (i-l) high magnification scanning electron micrographs of particles synthesized inside AMVs with identical compositions. Arrows in (c) note coacervates. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.
5.3.2 Different lengths of polyglutamic acid

Two different lengths of monodisperse PGA were used inside the AMVs: 20 monomers (3,000 Da) and 100 monomers (15,000 Da). These lengths were selected based on their availability from the manufacturer (Alamanda Polymers). Similar to the experiments with PAA, AMVs were prepared with different concentrations of PGA against a steady concentration of calcium in order to test their coacervate forming capabilities. The resulting emulsions were imaged using fluorescent confocal microscopy (Figure 5-3). Similar to the 10mer PAA, the 20mer PGA did not form coacervates at any of the concentrations present. The 100mer PGA did form coacervates, but only below a critical concentration. Even then, the coacervates that do form appear very different from the ones made using PAA. For one, the phase contrast between the coacervate and the dextran-rich phase is significantly dimmer, to the point where it’s difficult to distinguish the phases (Figure 5-3g). In addition, the size of the PGA-based coacervates present is much smaller than the PAA-based ones observed. Lastly, the critical polymer concentration for the dissolution of coacervates is lower for the PGA than PAA, even with comparable lengths. Coacervates form with 15 mg/mL PAA present (107 mM of the monomer) but not with 12.5 mg/mL PGA (83 mM of the monomer). These observations indicate that PGA has a lower coacervate forming capability than PAA in the AMV systems.
Figure 5-3: Fluorescent confocal images of LUV-stabilized dextran-rich droplets in a continuous PEG-rich phase containing free Ca$^{2+}$ chelated by different concentrations of various lengths of monodisperse polyglutamic acid (PGA). PGA was either (a, b, c, d) 20 or (e, f, g, h) 100 monomers in length. Coacervate formation was extremely dependent on PGA length and concentration, and can only be observed in panels e, f, and g. Arrows in g note coacervates. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.

The influence of the PGA length on mineral composition was investigated using TGA-MS, similar to the experiments with PAA. Minerals were prepared inside AMVs with a constant concentration of total PGA, but with different mass ratios of the 20mer and the 100mer. (Table 5-3). Overall, the minerals made inside PGA-based coacervates contained significantly less organic content as compared to the PAA-based coacervates. For instance, the lowest percentage of organic observed for the PAA-based minerals was 16.76% (Table 5-2). The highest percentage achieved for the PGA-based minerals was 10.77%. This difference in organic content likely explains the difference in crystal structure. Unlike the PAA-based minerals, which were all amorphous, all the PGA-based minerals are a mixture of calcite and vaterite, as confirmed by XRD (Figure 5-S3).
Table 5-3: Mass compositions of CaCO$_3$-PAA particles made using mono- and bidisperse PGA, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 degrees Celsius, the relative amount of organic carbon (second thermal decomposition step) was calculated from the loss of percent mass between 200 and 500 degrees Celsius, and the relative amount of inorganic carbon (third thermal decomposition step) was calculated from the loss of percent mass between 500 and 800 degrees Celsius. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[20mer PGA] (mg/mL)</th>
<th>[100mer PGA] (mg/mL)</th>
<th>Water content (% w/w)</th>
<th>Organic content (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mer only</td>
<td>10</td>
<td>0</td>
<td>6.44</td>
<td>10.30</td>
</tr>
<tr>
<td>3:1 20mer:100mer</td>
<td>7.5</td>
<td>2.5</td>
<td>8.60</td>
<td>10.77</td>
</tr>
<tr>
<td>1:3 20mer:100mer</td>
<td>2.5</td>
<td>7.5</td>
<td>7.84</td>
<td>10.35</td>
</tr>
<tr>
<td>100mer only</td>
<td>0</td>
<td>10</td>
<td>5.27</td>
<td>6.60</td>
</tr>
</tbody>
</table>

Mineral structures prepared using both lengths of PGA showed major dependence on length (Figure 5-4). Despite not forming an initial coacervate, the 20mer PGA still formed spherical particles similar to the PAA-based particles. These particles, however, did not have the same core-shell structure as previously observed. Rather, the interiors of PGA-based particles have a starburst growth pattern that implies a different mechanism. Furthermore, large pores form on the surface of the mineral at higher concentrations of the 100mer PGA. We can’t confirm why or how these pores formed, though it may have to do with the sudden loss of water during mineral formation. The TGA data confirms that these PGA-based particles (Table 5-3) contain less water than the PAA-based particles.
Figure 5-4: (a-d) Fluorescent confocal images of AMVs with coacervates comprised of different concentrations of monodisperse and bidisperse polyaspartic acid (PAA) and (e-h) low magnification and (i-l) high magnification scanning electron micrographs of particles synthesized inside AMVs with identical compositions. No coacervates are present inside panel a. Arrows in b, c, and d notate coacervates. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.

5.3.3 Combining monodisperse PAA and PGA

AMVs were prepared using both 100mer PAA and 100mer PGA. These lengths were chosen based on their previously established coacervate-forming capabilities. Depending on the concentrations of the polycarboxylates present, either one or more coacervate-phases form inside the AMVs. Figure 5-5a depicts mineralization inside AMVs containing 5 mg/mL PAA and 4
mg/mL PGA, which results in only one coacervate phase. According to the relative fluorescence intensities in each phase, the coacervates are comprised of mostly PAA with some PGA present. The majority of the PGA is present in the dextran-rich phase. Upon the addition of urea, PGA partitions from the coacervate into the dextran-rich phase (Figure 5-5c). However, not all PGA is displaced to the dextran-rich phase. Smaller, PGA-rich regions form inside the coacervate soon after. We can assume that this phase separation occurs due to the local PGA reaching a critical concentration. These droplets persist throughout the entire reaction, and can be observed at the end. Whether these PGA-rich droplets are coacervates, though, is uncertain. As the PGA moves into the dextran-rich phase or condenses in the coacervate, the PAA is further concentrated into the coacervate (Figure 5-5b). This behavior contradicts prior behavior, in which the PAA would dissolve into the surrounding dextran-rich phase as the reaction proceeded (Figure 4-2d).
Figure 5-5: (a) Fluorescent confocal and transmitted (DIC) stills of mineralization occurring inside AMVs with 5 mg/mL PAA 100mer and 4 mg/mL PGA 100mer. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca\textsuperscript{2+}; 5 mg/mL PAA 100mer; 4 mg/mL PGA 100mer; 12.45 units/mL urease; 100 mM urea. Inset times refer to time after urea addition. Fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). Scale bar = 25 μm. (b) Relative PAA fluorescence in each phase corresponding to a over the course of the reaction. (c) Relative PGA fluorescence in each phase corresponding to a over the course of the reaction. (d) Illustration depicting the formation of CaCO\textsubscript{3} inside artificial mineralization vesicles (AMVs) containing both PAA and PGA. Prior to urea addition, three aqueous phases are present: PEG-rich phase, dextran-rich phase and PAA/PGA/Ca\textsuperscript{2+} coacervate. Upon the addition of urea, carbonate displaces the PGA inside the coacervate. This results in an increase of PGA in the dextran-rich phase and the formation of PGA droplets inside the coacervate. Arrow notates a PGA-rich region.
Multiple coacervate phases can form with the same AMV but only with specific concentrations of both polycarboxylates. Figure 5-6 depicts AMVs with 4 mg/mL PAA and 5 mg/mL PGA. Under these conditions, each AMV can form one coacervate rich in PAA, and one or more coacervates rich in PGA. These PGA coacervates can form around the PAA coacervate, within the PAA coacervate, or both. Upon the addition of urea, the reaction proceeds in the exact same manner as depicted in Figure 5-5a, and the end products look very similar. It seems that regardless of where the polycarboxylates start in the system, the final mineral product reaches an equilibrium.

Figure 5-6: Fluorescent confocal and transmitted (DIC) stills of mineralization occurring inside AMVs with 4 mg/mL PAA 100mer and 5 mg/mL PGA 100mer (a) before and (b) 20 minutes after the addition of urea. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca\(^{2+}\); 4 mg/mL PAA 100mer; 5 mg/mL PGA 100mer; 12.45 units/mL urease; 100 mM urea. Fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). Scale bars = 50 μm.
As previously established, PAA-based coacervates result in more organic content in the mineral than PGA-based minerals. The data follow this trend somewhat, as the amount of organic present increases as the PAA:PGA weight ratio increases (Table 5-4). Overall, though, all the PAA/PGA based minerals prepared had higher organic content than any of the PAA-based minerals.

Table 5-4: Mass compositions of CaCO₃-PAA particles made using both PAA and PGA, acquired using thermal gravimetric analysis paired with mass spectroscopy (TGA-MS). The relative amount of water (first thermal decomposition step) was calculated from the loss of percent mass between 30 and 200 degrees Celsius, the relative amount of organic carbon (second thermal decomposition step) was calculated from the loss of percent mass between 200 and 500 degrees Celsius, and the relative amount of inorganic carbon (third thermal decomposition step) was calculated from the loss of percent mass between 500 and 800 degrees Celsius. The MS signals for water (18 amu) and carbon dioxide (44 amu) were used to determine the effective end point for each decomposition step.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[100mer PAA] (mg/mL)</th>
<th>[100mer PGA] (mg/mL)</th>
<th>Water content (% w/w)</th>
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<tr>
<td>1:3 PAA:PGA</td>
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<td>3:1 PAA:PGA</td>
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<td>15.07</td>
<td>27.34</td>
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</tbody>
</table>

The influence of these PGA-rich droplets can be observed in the CaCO₃ mineral interiors (Figure 5-7). The mineral interiors are very porous, despite having a relatively solid shell. The pores are roughly the same size as the PGA-rich regions that form soon after the addition of urea to the emulsion. We hypothesize that these structures are responsible for the mineral interior. As carbonate displaces the chelating polymer, the mineral forms around these PGA-rich droplets. No mineral forms inside these phases, which instead dissolve during the removal of the CaCO₃ spheres from the emulsion. The size of these pores changes depending on the initial
concentrations of the PAA and PGA present in the AMV (Figure 5-7). Given the dynamic phase
behavior involved during the mineralization, we cannot assert how controllable this change is.
At the moment, it appears that more similar concentrations of the two polycarboxylates result in
greater porosity.

![SEM images of minerals](image)

**Figure 5-7:** SEM’s of minerals made inside AMVs containing different concentrations of 100mer PAA and 100mer PGA: (a-c) 5 mg/mL 100mer PAA, 4 mg/mL 100mer PGA; (d-f) 7.5 mg/mL 100mer PAA, 2.5 mg/mL 100mer PGA; (g-i) 2.5 mg/mL 100mer PAA, 7.5 mg/mL 100mer PGA.

Individual calcium carbonate particles were analyzed using micro Raman spectroscopy.

Most, if not all, particles had the same basic spectrum (Figure 5-8a). The spectrum is mostly
comprised of peaks that can be attributed to PAA (or PGA). The most prominent CaCO₃ peak
Corresponds to the carbonate symmetric stretch, which can be observed around ~1077 cm⁻¹. This
particular frequency corresponds to the ACC structure, which agrees with the XRD analysis
(Figure 5-S4). Further, spatial characterization of the individual particles was performed to
acquire a better understanding of the mineral microstructure. Raman maps of individual particles, made using different PAA:PGA weight ratios, were acquired. The relative peak intensity of the carbonate symmetric stretch and the primary amide carbonyl stretch were used to map out the relative material density of the CaCO\textsubscript{3} and PAA/PGA, respectively (Figure 5-8b-d). The maps provide a limited perspective of the interiors of the particles. For instance, the particles that were shown to be mostly hollow prior (Figure 5-7c) appear hollow in the maps (Figure 5-8b). Likewise, particles that were mostly solid (Figure 5-7i) still appear mostly solid (Figure 5-8d). The other key observation is that the CaCO\textsubscript{3} and PAA/PGA appear rather closely associated to one another. Rather than create distinct regions rich in either CaCO\textsubscript{3} or polymer, the two materials are almost indistinguishable.

**Figure 5-8:** MicroRaman characterization of CaCO\textsubscript{3} microspheres made inside AMVs containing both 100mer PAA and 100mer PGA. (a) Raman spectra for mineral microspheres (top line) and for PAA alone (bottom line). Indicated peaks were used to create Raman maps. (b-d) Brightfield image of particle and Raman maps of peak intensity for the carbonate (red) and amide (blue) stretches. Maps were acquired in the x,y-direction. Emulsions had the following compositions: 1:49 dex:PEG volume ratio; 20% by volume LUVs; 50 mM Ca\textsuperscript{2+}; 12.45 units/mL urease; 100 mM urea; (b) 5 mg/mL 100mer PAA; 4 mg/mL 100mer PGA; (c) 7.5 mg/mL 100mer PAA; 2.5 mg/mL 100mer PGA; (d) 2.5 mg/mL 100mer PAA; 7.5 mg/mL 100mer PGA.

In order to see how the different polycarboxylates incorporate into the mineral, CaCO\textsubscript{3} spheres were prepared using trace amounts of fluorescently labeled PAA and PGA. The
minerals were then imaged using fluorescent confocal microscopy (Figure 5-9). Both labels are clearly present in the mineral, meaning both PAA and PGA are incorporated regardless of concentration. In addition, the two polymers appear in different regions of the same particle. The pores within the particles can be observed in the DIC channel through differences in refractive index, and the PGA channel overlaps with those pores. This supports the hypothesis that the hollow regions within the particles are due to the PGA regions within the mineralizing coacervate.

![Figure 5-9: Optical microscopy of mineral microspheres formed as in Figure 6 (varying concentrations of PAA and PGA) (a, c, e) confocal fluorescence channel for Alexa 488-tagged PAA (green) and Alexa-647-tagged PGA (blue) and (b, d, f) transmitted DIC channel for the same particles. Insets in c and d show higher magnification view of the framed particle.](image)
5.4 Discussion

Polyaspartic acid and polyglutamic acid appear to have different coacervate and mineral forming capabilities, despite their structural similarities. The PAA structure reported by the manufacturer, though, does not necessarily apply to the actual material. Individual monomers within a single PAA chain adopt one of two conformations: α and β. In the β isomer, one of the methyl groups from the side chain is incorporated into the carbon backbone. PAA can interchange between these two conformations during the chemical post-processing (Figure 5-10).\textsuperscript{46-47} The unpredictability of this isomerization is an important consideration when discussing the polymer structural influences on coacervate and mineral formation. Thus, this discussion will attempt to address the differences between PAA and PGA in broader terms.

![Figure 5-10: Conversion from the aspartate α configuration to the β configuration.](image)

The different phase forming behaviors of PAA and PGA could relate to their relative calcium binding affinities. PAA has been shown to be a stronger inhibitor of calcium mineral formation than PGA.\textsuperscript{48-49} PAA also adopts the stable β-sheet conformation in the presence of calcium, whereas PGA adopts a random coil.\textsuperscript{50} From these collective observations, we can assume conclude that PAA has a higher binding affinity than PGA. This differences can be exploited to create a dynamic mineralizing environment, where multiple phase separation events are used to template a non-equilibrium mineral structure. AMVs made using both PAA and PGA have one coacervate at the start of the reaction. Although both polycarboxylates are
present in the coacervate, the majority of the PGA is in the dextran-rich phase. Upon the addition of urea, the PGA begins to redistribute throughout the AMV. Some of the PGA partitions into the dextran-rich phase. In the prior AMV systems, we asserted that the carbonate displaces the chelating polymer in the mineralizing coacervate. Here, we can assume that PGA is displaced first due to binding affinity. The PGA also condenses into smaller droplets within the larger mineralizing coacervates (Figure 5-5d). The formation of these droplets likely occurs due to reaching a critical PGA concentration within the coacervate. Minerals extracted from these reactions are highly porous, with overlapping pores roughly the same size as the PGA droplets. We assert that the mineral forms around these PGA droplets as opposed to within, and they dissolve upon removal from the emulsion. If mineral does form inside these droplets, the mineral is non-continuous with the larger structure. The shows that subsequent phase separations can be utilized to direct the mineral formation.

Inversely, the PAA becomes more concentrated in the coacervate over time. This could be due to the high concentration of PGA present in the dextran-rich phase, and the charge repulsions between the two negatively-charged polymers. The exclusionary effects of the PGA results in a higher concentration of local PAA within the coacervate. This likely explains the increased amount of organic material present in the mineral as confirmed by TGA-MS (Table 5-4). Even if less total polymer is present at the start of the reaction, the interactions between multiple polycarboxylates influences the incorporation of the polymer throughout the reaction. Potentially, charge repulsions between the PAA in the coacervate and the PGA in the dextran-rich phase cause the former to become more concentrated over the reaction. Thus, the final mineral product has a higher concentration of polymer.
The different locations of the PAA and PGA throughout the reaction also influences their incorporation into the mineral. Fluorescent microscopy imaging of fluorescently-labeled minerals indicate the two polymers are in different locations throughout the structure. The PGA-rich regions correspond to the pores within the mineral, which aligns with the theory of secondary phase separation. Furthermore, the density of these PGA regions within the coacervate is directed by the initial concentrations of PAA and PGA. In addition to controlling to the porosity of the mineral, this also controls the gradients of the two polymers.

5.5 Conclusions

The coexistence of two polymer gradients within the same mineral is rather novel in the field of bioinspired mineralization. Individual CaCO$_3$ made in this method are entirely ACC, however, it’s not unreasonable to assume the local crystal structures could convert over time. PGA-based particles were either vaterite or calcite, so the PGA-rich regions may destabilize and become crystalline over time.$^{51}$ It may be possible to encourage this conversion through minor post synthesis treatment such as heating.$^{52}$ Biology utilizes differences in organic content to dictate local crystal structure, and in turn the local mechanical properties.$^{16, 19}$ We believe this work represents an important step toward achieving the same level of structural control as seen in biominerals.

5.6 Methods

Materials. Calcium chloride dihydrate ($\geq$99.5 % purity), urea, poly(ethylene glycol) (PEG) 8 kDa, urease from jack bean (Canavalia ensiformis) of activity 8.3 U/mg, and Amicon Ultra centrifugal filters were purchased from Sigma-Aldrich, Co. (St. Louis, MO). Dextran (Dex) 10 kDa was
purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Alexa Fluor™ 488 hydrazide, Alexa Fluor™ 647 hydrazide, Premium Grade (1-ethyl-3-(3-dimethylaminopropyl)carboimide hydrochloride) (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), and Zebra™ Spin Desalting Columns were purchased from Thermo Fisher Scientific, Co. (Waltham, MA). Egg-phosphatidylcholine (Egg-PC), egg-phosphatidylglycerol (Egg-PG), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol) 2 kDa (DOPE-PEG 2 kDa), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE) lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Poly-L-aspartic acid sodium salt (PAA) 1.4 kDa, PAA 6.8 kDa, PAA 14 kDa, poly-L-glutamic acid sodium salt (PGA) 3 kDa, and PGA 15 kDa were purchased from Alamanda Polymers, Inc. (Huntsville, AL). Fluorescently labeled PAA was prepared in-house by conjugating Alexa Fluor™ 488 hydrazide to carboxylic acid residues using an EDC linker. The labeling reaction was performed step-wise to ensure maximum labeling efficiency and purity. 12.3 mg of PAA 14 kDa was dissolved in 1 mL 0.1 PBS buffer. 25-molar equivalent sulfo-NHS-acetate was added to 100 μL of the PAA solution to cap the terminal amines. The solution was gently mixed at room temperature for 1 hour. Excess sulfo-NHS-acetate was removed using a Zebra™ Spin Desalting Column (7 kDa MWCO) that had been equilibrated with 0.1 M MES buffer, pH ~6. 0.1 molar equivalent EDC and 0.25 molar equivalent sulfo-NHS acetate were added to the purified polymer to form the reactive intermediate. After 15 minutes, the excess EDC was quenched using 10 molar excess 2-mercaptoethanol. The polymer was purified again with a desalting spin column equilibrated with 0.1 PBS buffer. The purified polymer was added directly to ~1 mg of Alexa 488 hydrazide and mixed at room temperature for 2 hours. Excess label was removed with a desalting spin column, and the solution was concentrated using an Amicon Ultra centrifugal filter (MWCO 3 kDa). The
labeled polymer was then stored at -22°C. An adapted method was used to prepare the fluorescently labeled PGA.

**Formation of PEG/Dex ATPS.** All ATPS stocks were prepared to 10 w/w% PEG 8 kDa and 10 w/w% Dex 10 kDa. The appropriate masses of both polymers were dissolved in 10 mM pH 7.4 Tris buffer. Buffer was manually prepared in house using a combination of Tris hydrochloride and Tris base. The two phases were then allowed to fully separate overnight at 5°C then removed via pipet. The PEG-rich phase and Dex-rich phase were used for all subsequent sample preparation.

**Formation of LUVs.** Large unilamellar vesicles (LUVs) were prepared by extrusion of heterogeneous lipid assemblies formed by gentle hydration of lipid films in PEG-rich phase. Lipid composition was always 48.56, 48.51, 2.83, and 0.10 mol % of Egg-PC, Egg-PC, DOPE-PEG 2 kDa, and Rh-DOPE, respectively. Lipid stocks were pooled in a borosilicate tube with an additional ~50 μL chloroform, for a final volume of ~200 μL; this mixture was stirred rapidly by hand while Ar was blown into the tube slowly (~1 PSI), which dried the lipid mixture into a film on bottom of the glass. The vial was placed within a vacuum desiccator and vacuum was pulled via pump for at least two hours to remove any chloroform remaining in the lipid film. For hydration, 500 μL of PEG-rich phase from the ATPS stock was added to the glass vial for a final lipid concentration of 7.5 mg/mL, capped tightly with parafilm, and placed in a 37 °C incubator for at least 48 hours. The heterogeneous mixture cooled to room temperature and then extruded through a 200 nm pore 11 times using an Avanti mini-extruder, creating ~150 nm LUVs and
removing any excess non-LUV lipid material. The LUVs were stored in a HDPE vial at 5°C and used within a week.

**Formation of AMVs.** A 100 μL ATPS with 20 vol. % LUVs of V\textsubscript{Dex} : V\textsubscript{PEG} 1 : 49 was formulated, with 50 mM Ca\textsuperscript{2+} and appropriate concentrations of PAA and PGA, and emulsified using a vortex mixer immediately prior to analysis. To maintain the ATPS volume ratio, all stock solutions were prepared in PEG-rich phase. 50 μL of the emulsion was deposited on a silanized glass slide with a 150 μL spacer, and a cover slip. All fluorescent and DIC images were acquired on a Leica (Wetzlar, Germany) TCS SP5 PL confocal microscope using a 63x 1.4 NA APO objective. Images were analyzed using a combination of Leica LAS-X software and ImageJ (National Institutes of Health).

**Preparation of CaCO\textsubscript{3} particles for bulk and individual analysis.** A 1 mL ATPS with 20 vol. % LUVs of V\textsubscript{Dex} : V\textsubscript{PEG} 1 : 49 was formulated, with 12.45 units/mL urease, 50 mM Ca\textsuperscript{2+} and appropriate concentrations of PAA and PGA, and emulsified using a vortex mixer. Urea from a 5 M stock solution in PEG-rich phase was added to the emulsion for a final concentration of 100 mM, and the emulsion was vortexed via pipette to initiate the reaction. After one hour, samples were centrifuged at 16100 \times g to separate the precipitant, and the supernatant was removed via pipette. Pellets were resuspended with 1 mL of 10 mM Tris buffer (pH = 8.5), followed by three additional centrifuge and washing cycles. Mineral samples were then dried in a SpeedVac vacuum concentrator at room temperature for two hours. Fully dried samples were then stored under atmosphere at room temperature. Differences in emulsion or mineral composition are noted in individual figure captions.
**Ca$^{2+}$ Partitioning.** A 1 mL ATPS of V$_{Dex}$ : V$_{PEG}$ of 1 : 9 was formulated with no LUVs, 50 mM Ca$^{2+}$, and 15 mg/mL total PAA for each sample. The ATPS was mixed thoroughly, left in a refrigerator at 5 °C overnight to separate and warmed to room temperature before analysis, where aliquots of each polymer-rich phase were removed in triplicate and diluted appropriately into DI H$_2$O. Atomic absorption of calcium was measured using a Shimadzu AA-7000 Atomic Absorption Spectrophotometer (Kyoto, Japan) with Ca/Mg hollow cathode lamp operating at 422.7 nm wavelength, 5 nm slit width, 7 mA input current, 15 and 50 PSI acetylene and air respective input pressures; samples were analyzed using WizAArd software.

**Monitoring mineralization in situ using confocal microscopy.** A 100 μL ATPS with 20 vol. % LUVs of V$_{Dex}$ : V$_{PEG}$ 1 : 49 was formulated, with 12.45 units/mL urease, 50 mM Ca$^{2+}$ and appropriate concentrations of PAA and PGA, and emulsified using a vortex mixer immediately prior to analysis. PAA and PGA were equilibrated with Alexa 488-tagged PAA and Alexa 647-tagged PGA respectively. To maintain the ATPS volume ratio, all stock solutions were prepared in PEG-rich phase. 50 μL of the emulsion was deposited on a silanized glass slide with a 150 μL spacer, and no cover slip. The sample was then transferred to the microscope sample stage. To initiate the reaction, 5 M urea in PEG-rich phase was added to the emulsion for a final concentration of 100 mM urea, and a silanized glass coverslip was placed on top. DIC/fluorescence images were acquired every 15 seconds over the course of the reaction to mitigate dye bleaching.

**X-ray powder diffraction (XRD).** Particles were prepared as described above. XRD patterns were collected on a Si zero-background sample holder using Malvern Panalytical (Malvern, UK)
XPert Pro MPD Theta-Theta Diffractometer across a 20-70° 2θ range at 45 kV acceleration, 40 mA Cu Kα radiation using a 0.5° divergence fixed-slid width and 10 mm beam mask in tandem with a PIxcel detector over an analysis time of 15 minutes; the resulting patterns were analyzed using Jade software from MDI (Acceptable shift on the weekly instrument test is +/- 0.03 deg. for 2-theta.).

**Vibrational spectroscopy.** Raman spectra and maps were acquired on a Horiba (Kyoto, Japan) LabRam with back illuminated detector (2048x512 pixels) a 600 g/mm grating (spectral resolution ~2 cm⁻¹), and a 532 laser operating at 45 mW. The laser was focused and the images were acquired using a 100x NA 0.9 WD 0.2 mm objective. Each individual spectrum had an acquisition time of 10 seconds. Particles were dispersed on a non-silanized glass slide with no further treatment.

**Electron Microscopy.** Scanning electron microscopy (SEM) images were acquired with an FEI (Hillsboro, OR) Nova NanoSEM 630 SEM, operated at high vacuum mode at a 5 mm working distance, and a 5 keV accelerating voltage. ETD and TLD detectors were utilized for low and high magnification imaging, respectively. A ~10 Å iridium coating was applied to samples using sputter coater prior to imaging.

**Thermogravimetry.** Minerals were analyzed with a TA Instruments (New Castle, DE) Discovery Series TGA Q5000 coupled with Discovery MS. ~5 mg of each mineral sample was loaded into a 100 μL high temperature platinum pan. The system was purged with nitrogen gas and heated at an uneven rate: 10 minutes at a 30°C isothermal, heated to 200°C at a rate of
10°C/min, 20 minutes at a 200°C isothermal, heated to 500°C at a rate of 10°C/min, 40 minutes at a 500°C isothermal, heated to 800°C at a rate of 10°C/min, and 20 minutes at an 800°C isothermal.

A non-linear heating rate was used to decompose the sample as efficiently as possible.

Gravimetric data was acquired and analyzed using TA instruments TRIOS software.

Accompanying mass spectroscopy data was acquired alongside the gravimetric data using TA instruments Process Eye software.
5.7 Supporting information

Figure 5-S1: (a) Temperature profile of the heating rates used for each TGA-MS experiment. (b) TGA-MS graph of CaCO$_3$-PAA particles made inside artificial mineralization vesicles with 10 mg/mL 50mer PAA and 5 mg/mL 100mer PAA. (Left axis) Total relative weight of sample as a function of time (temperature). (Right axis) Mass spectroscopy signals of thermal decomposition by-products, namely water (18 amu) and carbon dioxide (44 amu).

Figure 5-S2: Raman spectrum of a CaCO$_3$ particle prepared inside an AMV containing only 50mer PAA (identical composition to Figure 2i). The position of the symmetric carbonate stretch ($\nu_1$) is indicative of ACC. All particles prepared using PAA all had the same spectrum.
Figure 5-S3: XRD of a CaCO$_3$ particle prepared inside an AMV containing only 100mer PGA (identical composition to Figure 4l). Standard peaks for calcite and vaterite have been included for ease of identification. All particles prepared using PGA all had the same spectrum.

Figure 5-S4: XRD of a CaCO$_3$ particle prepared inside an AMV containing 5 mg/mL 100mer PAA and 4 mg/mL 100mer PGA (identical composition to Figure 6a). Standard peaks for calcite and vaterite have been included for ease of identification. All particles prepared using both PAA and PGA all had the same spectrum.
Figure 5-S5: Fluorescent confocal and transmitted (DIC) stills of mineralization occurring inside AMVs with (a) 7.5 mg/mL PAA 100mer and 2.5 mg/mL PGA 100mer and (b) 2.5 mg/mL 100mer PAA and 7.5 mg/mL 100mer PGA. Based on the appearance of the final mineral products in Figures 5-7 and 5-9, we hypothesize the reactions proceed in the same manner as seen in Figure 5-5a. However, the limited depth of field in the x,y,z-directions obscures areas of the samples we believe to be more indicative. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca$^{2+}$; 12.45 units/mL urease; 100 mM urea. Inset times refer to time after urea addition. Fluorescent channels have been false-colored and overlaid: Alexa 488-tagged PAA (green); Rhodamine-tagged liposomes (red), Alexa 647-tagged dextran (blue). Scale bar = 25 μm.
5.8 References


Chapter 6

Conclusions and Future Directions

6.1 Conclusions

Liposome-stabilized all-aqueous emulsions are appealing alternatives to giant vesicles in the development of bio-inspired micro-reactors. Stabilized emulsions are inherently more homogeneous than giant vesicles in both size and encapsulated reactants. In this thesis, it was demonstrated that the liposomes with minimal homogenization can function as effective stabilizers. Other emulsion stabilizers, such as polysaccharide protein particles, are more synthesis intensive than the gentle hydration method, which makes liposomes the more prolific option. In addition, it was shown that liposomes could be used to stabilize an aqueous phase with nearly 1 M salt present. Normally, this concentration of salt would be problematic to boundaries such as lipid membranes. This system could be used to potentially encapsulate reactions optimized for salt-rich environments.

Liposome-stabilized all-aqueous emulsions were used to develop an artificial mineralization vesicle (AMV) with enhanced control over the mineralization process. The use of a mineralizing coacervate phase in the context of a multi-phase system allows for asymmetric structures and organic gradients not achievable with comparable methods, such as polymer-induced liquid precursors (PILPs). Utilizing multiple polycarboxylates, namely polyaspartic acid (PAA) and polyglutamic acids (PGA), caused additional phase separation events to occur throughout the reaction. We hypothesized that differences in calcium binding affinity led to these separations, which resulted in CaCO₃ minerals with distinct regions rich in either PAA or PGA. Control over local polymer content during synthesis allows Biology to create multiple
polymorphs of the same mineral within a structure.\textsuperscript{7-8} The experiments reported here represent an important step toward a comparable of control in the development of bio-inspired synthetic materials.

Our use of multiple phase separations is comparable to theories on the synthesis of multi-layer silica diatom shells.\textsuperscript{9} Liquid phase separation is followed by silica condensation, and subsequent mineralization events build upon one another to build the shell. Our work with multiple polycarboxylates inside AMVs suggests that this type of conceptual approach can be applied to calcium-based minerals like those observed in ascidians.\textsuperscript{7} The Asp-rich and Glu-rich found in these minerals could potentially phase separate in the presence of calcium, similar to polycarboxylate-based coacervates. Over the course of the mineralization reaction, these phase separate into different regions based on their protein composition. The crystal structures of the result minerals are then dictated by the proteins present.

6.2 Future directions

Going forward, this project should continue to seek out new methods to exert greater control over the mineralization process. The mechanical and optical properties of biominerals are owed to the localized differences organic content, and achieving that same level of control in synthetics is the ultimate goal of bioinspired materials. In regards to the AMV platform, there’s a number of parameters that influence the mineralization reaction. The AMV platform is comprised of many components, including a PEG/dextran ATPS, liposomes, enzymes, salt, and chelating polymers. Changing any one of these parameters has a significant influence on the final mineral product in terms of structure and organic content. Chapter 4 explored this concept by removing individual components to create control materials, and Chapter 5 by changing the
chelating polymer. Exploring the influence of each parameter reveals potentially new methods of locally controlling the mineralization process in regards to shape, structure, or organic content. Preliminary experiments along these lines were experiments, but never elaborated upon. However, I believe these experiments demonstrate the potential of the AMV platform.

6.2.1 PAA:PEG:PAA block copolymers

Various PAA:PEG:PAA block copolymers are available for purchase from Alamanda Polymers. These block copolymers are long chains of PEG with PAA chains on either end. Preliminary experiments tested the coacervate forming capabilities of one of these copolymers by creating AMVs. The AMVs did contain coacervates within the dextran-rich phase (Figure 6-1). The resulting coacervates had a different appearance from those of pure PAA, implying different phase forming behavior. The charge density of the block copolymer is drastically different from that of PAA due to the addition of the neutrally charged PEG chain. This difference in charge density affects how the block copolymer is incorporated into the coacervate. If that’s the case, it may be possible to induce sequential phase separations events like those observed in Chapter 5 (Figure 5-5a). I believe it’s worth performing a similar set of experiments to examine the coacervate and mineral forming capabilities of these polymers. Using a block copolymer in combination with PAA and PGA could allow for more phase separation events in the same reactors, asserting even greater control over the mineral structure. The block copolymers are available in a variety of different PAA:PEG:PAA ratios that could all demonstrate different behaviors.
Figure 6-1: Fluorescent confocal image of AMVs prepared with PAA:PEG:PAA block copolymer. The copolymer was composed of 112mer PEG with 10mer PAA attached on both ends. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca$^{2+}$; 5 mg/mL PAA:PEG:PAA. Rhodamine-tagged LUVs have been false-colored red and overlaid with the brightfield channel.

6.2.2 Substituting the AMV interior phase

Chapter 2 demonstrated that liposomes within a certain size range could be used to stabilize Ficoll-rich and sulfate-rich droplets within a continuous PEG-rich phase. Coacervate incorporation within a stabilized PEG/Ficoll ATPS emulsion was attempted in order to determine whether Ficoll could be used as a substitute for dextran in the coacervate-containing AMV. When all components were mixed together simultaneously, the coacervates formed outside of the Ficoll droplets. The LUVs adsorbed to the PEG/coacervate interface instead of the PEG/Ficoll interface. If the emulsion was prepared without calcium, however, and calcium was added at a later step, coacervates would form at the stabilized PEG/Ficoll interface (Figure 6-2). The mineral forming capabilities of these interface coacervates was never explored, unfortunately. Theoretically, mineralizing this system could create a mineral shell around a microreactor interior. This structure would be analogous to mineral-creating single cell organisms such as coccolithophores.$^{12-13}$ Coccolithophores protects themselves with shells comprised of individual,
beautiful CaCO$_3$ plates. Non-equilibrium Ficoll AMVs provide a potential synthesis route for creating a comparable structure.

![Before Ca$^{2+}$ addition](image1.png) ![After Ca$^{2+}$ addition](image2.png)

**Figure 6-2:** Fluorescent confocal and brightfield images of LUV-stabilized Ficoll-rich droplets in a continuous PEG-rich phase with PAA present (a) before and (b) after the addition of Ca$^{2+}$. PAA/Ca$^{2+}$ coacervates form at the PEG/Ficoll interface upon the addition of Ca$^{2+}$. Emulsion composition: 1:24 Ficoll:PEG volume ratio; 20% by volume LUVs; 10 mg/mL PAA 2-11 kDa; (a) 0 mM Ca$^{2+}$ or (b) 50 mM Ca$^{2+}$. PEG:Ficoll ATPS was initially 15%/15% PEG/Ficoll by weight total. Rhodamine-tagged LUVs have been false colored red and Alexa 488-tagged PAA has been false-colored green.

### 6.2.3 Coacervates with non-spherical shapes

Coacervates typically adopt spherical shapes to minimize surface energy in solution.$^{14}$ Within the AMVs, it’s possible to alter the shape of the coacervate by altering the relative magnitude of interfacial tensions.$^{14}$ Preliminary experiments increased the concentration of PEG and dextran in the emulsion in order to change the wetting behavior of the coacervate. This created AMVs with hemispherical coacervates that contacted both the dextran and PEG-rich phases (Figure 6-3). Upon the addition of urea, the coacervates morphed back into spheres and the minerals maintained this spherical shape. The interiors of this minerals were never investigated, though,
and structural gradients may have formed as the result of the initial, non-spherical shapes. I believe these minerals are worth investigating, as they could present a facile method of creating asymmetry.

**Figure 6-3:** Fluorescent confocal and brightfield images of AMVs containing non-spherical coacervates comprised of (a) PAA or (b) PGA. Total PEG and dextran concentration has been increased by 50% over the standard composition. Emulsion composition: 1:49 Dx:PEG volume ratio; 20% by volume LUVs; 50 mM Ca\(^{2+}\); (a) 16 mg/mL 100mer PAA or (b) 10 mg/mL 100mer PGA. Rhodamine-tagged LUVs have been false colored red, Alexa 488-tagged PAA has been false-colored green, and Alexa-647 tagged PGA has been false-colored blue.

### 6.2.4 Wetting glass surfaces with coacervates

The other method explored to create non-spherical shapes was surface wetting. Unlike the prior example, this system does not utilize AMVs. Rather, coacervates attached a glass substrate will be mineralized in dilute solution. Coacervates are highly charged liquids and will wet a substrate depending on its surface charge (Figure 6-4a). Preliminary experiments showed that the wetting behavior of a coacervate could be altered through the surface chemistries of a
glass slide (Figure 6-4b,d). Furthermore, minerals do appear to retain these “edges” upon mineralization (Figure 6-4c). The primary problem with this project was particle retrieval; it was difficult to separate minerals that wet the glass surface vs those suspended in the dilute. In most samples, the latter was much more prominent. A microfluidic device could be utilized to separate settling coacervates from those in solution. In addition to asymmetric shapes, the minerals could also internal material gradients similar to those observed in Chapter 4 (Figure 4-5d). Adhesion to the glass surface will result in uneven rates of carbonate diffusion. The throughput of bulk solution experiments would allow for more prolific work.

**Figure 6-4:** Creating non-spherical minerals from coacervates wetting glass surfaces. (a) Schematic representation of how glass coating affects coacervate wetting and the shape of the subsequent mineral. (b) Fluorescent confocal Z-stack of Ca$^{2+}$/PAA coacervates wetting a non-treated glass slide and (c) minerals made from those coacervates. (d) Fluorescent confocal Z-stack of Ca$^{2+}$/PAA coacervates not wetting a glass slide functionalized with PEG-silane and (e) minerals made from those coacervates. Solutions composition: 1 M Ca$^{2+}$; 7.5 mg/mL PAA 2-11 kDa. Alexa 488-tagged PAA has been false-colored green.
These are just a few ideas based on some preliminary experiments I performed. Over the years, friends and colleagues have suggested dozens of ways (many of which I’ve forgotten) to tweak the individual parameters. I think the ideas I’ve presented here are all viable and should be considered starting points for future group members. However, I also believe this platform has the potential to flourish beyond my imagination, and I eagerly await the first AMV publication without my name attached.
6.3 References


VITA

Andrew T. Rowland

Education
2019
The Pennsylvania State University- University Park, PA
Ph.D. in Chemistry: Advisor Dr. Christine D. Keating

2013
Lafayette College- Easton, PA
B.S. in Chemistry

Selected Publications

2. Cabrera, K. D.; Rowland, A. T.; Szarko, J. M.; Diaconescu, P. L.; Nataro, C. Monodentate phosphine substitution in \([\text{Pd}(\kappa^3\text{-dppf})(\text{PR}_3)] \text{[BF}_4]_2 \) (dppf = 1,1’-bisdiphenylphosphino)ferrocene) compounds. *Dalton Transactions* 2017, 46, 5702-5710


Selected Presentations
