GIANT LIPID VESICLES ENCAPSULATING A SYNTHETIC CYTOPLASM AS A
MODEL SYSTEM TO INVESTIGATE CELLULAR FISSION AND DIFFERENTIATION

A Dissertation in
Chemistry
by
Meghan Koback

© 2012 Meghan Koback

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2012
The dissertation of Meghan Koback was reviewed and approved* by the following:

Christine D. Keating  
Associate Professor of Chemistry  
Dissertation Advisor  
Chair of Committee

Nicholas Winograd  
Evan Pugh Professor of Chemistry

Scott Showalter  
Assistant Professor of Chemistry

Peter J. Butler  
Associate Professor of Bioengineering

Barbara J. Garrison  
Shapiro Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

Biological cells exhibit chemical heterogeneity in both their cytoplasmic interiors and their plasma membranes. This heterogeneity has consequences for cell function, such as polarity and asymmetric division, yet studying the underlying mechanisms has proven difficult due to the complexity of even the simplest living cell. Models of biological cells provide a tool for testing hypotheses in cellular biology. For example, giant lipid vesicles (GVs) are cell-sized structures composed of a lipid bilayer membrane surrounding an aqueous core, that serve as model cells or parts of cells. By encapsulating an aqueous two-phase system (ATPS) it has been possible to generate microcompartments within GVs. This work is interesting in that aqueous phase separation is one proposed mechanism for dynamic microcompartmentalization in the cytoplasm of biological cells, and our model provides a means for examining the consequences of this type of heterogeneity in a simple system. Previous work has demonstrated osmotic deflation, temperature, and pH-driven dynamic microcompartmentation within the GV interior, as well as structural and cytoplasmic polarity. This thesis examines the consequences of this initial polarity, focusing on asymmetric fission and differentiation in this model cell. While the overall goal of this thesis is concerned with polar ATPS GVs, Chapter 5 answers fundamental questions about the non-ideal behavior of two-polymer solutions, which has direct consequences for our artificial cell work.

Chapter 1 provides a brief introduction to ATPS GVs as a simple model system for phase-separation driven microcompartmentation of the cytoplasm. Background information on microcompartmentation in biological cells and its consequences for cell
function is presented. Examples of experimental models of the cytoplasm are reported, and shortcomings of these models are discussed. An introduction to GVs as membrane models, and model cells is provided. The chapter concludes with a summary of previous work incorporating an ATPS cytoplasm mimic inside GV model cells. Chapter 2 describes ATPS GVs presenting micron-scale domains in their bilayer membranes. It describes the effects of PEGylated lipids and temperature on domain localization, as well as the interplay between the membrane and interior chemistry. Additionally, osmotic deflation induced budding of these ATPS GVs with heterogeneous membranes is reported, which resulted in vesicles that were asymmetric, or polar, in their structure as well as the membrane and interior compositions. Chapter 3 builds on this initial polarity and describes the consequences of osmotic deflation of these asymmetric GVs. Further increases in external osmolality drove fission of polar GVs, to produce two non-identical daughter vesicles that were different in their membrane and interior “cytoplasmic” compositions. Additionally, osmotic-stress induced aqueous phase separation and polarity in daughter vesicles is reported. In Chapter 4, the differential segregation of denatured proteins and preferential accumulation in one daughter vesicle, but not the other, upon asymmetric fission of an ATPS GV is presented. The asymmetric segregation of aggregates is believed to play a role in cellular aging, and the work described in Chapter 4 provides insight into possible mechanisms by which this biased aggregate accumulation may occur in living systems. Chapter 5 describes observed non-ideal properties of ATPS, and measured polymer-polymer interactions, in order to gain insight into the driving forces of ATPS phase separation- information that is fundamentally important for our
synthetic cell work. Overall, this thesis presents important findings on ATPS phase behavior, and demonstrates a model cell capable of polarity and asymmetric cell division. This model cell could potentially serve as a test bed for examining hypotheses in cell biology (e.g. the role of membrane composition in cellular polarity; malfunctions in asymmetric inheritance; spatial and organization cues in polarity induction, etc.).
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. viii

LIST OF ABBREVIATIONS ................................................................................................. xvi

Acknowledgements ................................................................................................................ xviii

Chapter 1  Introduction ........................................................................................................... 1

1.1 Motivation for a Cytoplasm Mimic ................................................................................. 2
1.2 Bulk Models of the Intracellular Environment .............................................................. 4
  1.2.1 Polymer Solutions ................................................................................................... 5
  1.2.2 Aqueous Two-Phase Systems as Models of Intracellular Organization ............... 6
1.3 Introduction to Giant Lipid Vesicles as Model Cells ...................................................... 9
  1.3.1 GVs as Membrane Models ..................................................................................... 12
1.4 Incorporation of Intracellular Model into GVs as Primitive Model Cells .................. 19
1.5 Summary and Objectives ............................................................................................... 25
1.6 References ..................................................................................................................... 29

Chapter 2  Positioning Lipid Membrane Domains in Giant Vesicles by
Micro-organization of Cytoplasm Mimic ............................................................................ 38

2.1 Introduction ................................................................................................................... 38
2.2 Experimental Materials and Methods ......................................................................... 40
  2.2.1 Chemicals and Materials ...................................................................................... 40
  2.2.2 Preparation of Giant Vesicles with co-existing fluid phases encapsulating
      an Aqueous Two-Phase System ............................................................................... 41
  2.2.3 Preparation of budding ATPS GV samples for microscopy .................................. 43
  2.2.4 Instrumentation and software ............................................................................... 43
2.3 Results and Discussion ................................................................................................. 44
2.4 Conclusion .................................................................................................................... 60
2.5 References .................................................................................................................... 61

Chapter 3  Complete Budding and Asymmetric Division of Primitive Model Cells to
Produce Daughter Vesicles with Different Interior and Membrane Compositions .......... 67

3.1 Introduction .................................................................................................................... 67
3.2 Experimental Materials and Methods ......................................................................... 71
  3.2.1 Materials ................................................................................................................. 71
  3.2.2 Preparation of Giant Vesicles with co-existing fluid phases encapsulating
      an Aqueous Two-Phase System ............................................................................. 72
  3.2.3 Preparation of ATPS/GV samples for confocal microscopy ................................... 73
  3.2.4 Quantification of protein partitioning in ATPS-containing vesicles ....................... 74
  3.2.5 Instrumentation and Software ............................................................................... 75
3.3 Results and Discussion ................................................................................................. 75
3.4 Conclusion .................................................................................................................... 98
3.5 References .................................................................................................................................................. 100

Chapter 4  Differential Segregation of Denatured Proteins and Preferential Accumulation in One Daughter Vesicle Upon Asymmetric Division of Model Cells ................................. 109

4.1 Introduction .............................................................................................................................................. 109
4.2 Experimental Materials and Methods ..................................................................................................... 112
  4.2.1 Chemicals and Materials .................................................................................................................... 112
  4.2.2 Preparation of Giant Vesicles encapsulating an Aqueous Two-Phase System ........................................ 113
  4.2.3 Preparation of ATPS/GV samples for confocal microscopy ............................................................... 113
  4.2.4 Quantification of protein partitioning in ATPS-containing vesicles ................................................. 114
  4.2.5 Instrumentation and Software ........................................................................................................... 114
4.3 Results and Discussion ............................................................................................................................. 115
4.4 Conclusions .............................................................................................................................................. 129
4.5 References .............................................................................................................................................. 130

Chapter 5  Characterization of Multicomponent Polymer Solutions by Vapor Pressure Osmometry .................. 137

5.1 Introduction .............................................................................................................................................. 137
5.2 Experimental Materials and Methods ..................................................................................................... 140
  5.2.1 Materials ........................................................................................................................................... 140
  5.2.2 Sample Preparation ............................................................................................................................ 140
  5.2.3 Cloud Point Titration .......................................................................................................................... 141
  5.2.4 Vapor Pressure Osmometry .............................................................................................................. 142
5.3 Results and Discussion ............................................................................................................................. 143
  5.3.1 ATPS Phase Diagrams ....................................................................................................................... 144
  5.3.2 Osmolality of PEG 35 kDa/dextran 10 kDa ....................................................................................... 146
  5.3.3 Osmolality of PEG 35 kDa/PAAm 10 kDa ......................................................................................... 153
  5.3.1 Quantifying polymer-polymer interactions ....................................................................................... 159
5.4 Conclusions .............................................................................................................................................. 163
5.5 References .............................................................................................................................................. 164

Chapter 6  Conclusions and Future Directions .............................................................................................. 169

6.1 Conclusions .............................................................................................................................................. 169
6.2 Future Directions .................................................................................................................................... 172
6.3 References .............................................................................................................................................. 176
LIST OF FIGURES

Figure 1-1: Experimentally determined phase diagram for a PEG 8 kDa/ Dextran 10 kDa ATPS determined at 5 and 37°C. Solutions exist as two phases above the binodal curve for a given temperature, and as one phase below the curve. Solutions comprised of polymer weight percents that lie between the 5 and 37°C binodals will mix when heated to 37°C and will phase separate when cooled to 5°C. Points were determined by cloud point titration, and lines are a polynomial fit included to guide the eye. Image reprinted with permission from Andes-Koback et al. J. Am. Chem. Soc. 2011, 133, 9545-9555. Copyright 2011. American Chemical Society. .......................... 7

Figure 1-2: Structures of polyethylene glycol (PEG) and dextran monomers....................... 9

Figure 1-3: Structures of lipids found in biological and GV membranes. Image adapted and reproduced with permission from Eemen et al. Biotechnol. Agron. Soc. Environ. 2010, 14, 719-736. ........................................................................................................................................................................... 10

Figure 1-4: Structure of a Giant Vesicle having one lipid bilayer (unilamellar). Reprinted with permission from Menger and Angelova, Acc. Chem. Res. 1998, 31, 789-797. Copyright 1998 American Chemical Society. ................................................................. 11

Figure 1-5: Schematic demonstrating a possible mechanism for the spontaneous vesiculation to form a giant multilamellar vesicle upon the hydration of a dry phospholipid film. Solute-containing aqueous solution is added to the dry phospholipid film hydrating the outer monolayer. The bumps form on the surface because the surface area of the polar lipid heads increases with increasing hydration. Solution intercalates between these bumps as well as through the bilayers, resulting in encapsulation of the solute-containing solution, and eventually detachment from the surface to form sealed bilayer vesicles. Image reproduced with permission from Lasic et al. Biochem. J. 1988, 256, 1-11. ............................................................................................................. 11

Figure 1-6: Structures of saturated and unsaturated phospholipids. Top: Structure of the saturated phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). Bottom: Structure of the unsaturated phospholipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). .......................................................................................................................................................... 15

Figure 1-7: Schematic illustrating the different phase states of lipids in aqueous medium. Image adapted and reproduced with permission from Eemen et al. Biotechnol. Agron. Soc. Environ. 2010, 14, 719-736........................................................................................................................................................................... 15

Figure 1-8: Ternary phase diagram and optical micrographs of liquid-liquid phase coexistence for the lipid composition DOPC, DPPC, and cholesterol in the bilayer of GVs. (Top) Phase Diagram for DOPC, DPPC, and cholesterol at 25°C. (Bottom) Fluorescence micrographs of micron-scale domains in GVs. From left to right: 2:1
DOPC/DPPC + 30% cholesterol; 1:1 DOPC/DPPC + 30% cholesterol; and 1:2 DOPC/DPPC + 30% cholesterol. Scale bar is 20 µm. Image adapted and reproduced with permission from Veatch et al. Biophys. J. 2004, 86, 2910-2922.

Figure 1-9: Shape changes of GV containing co-existing micron-scale liquid domains. (A) GV bends at the L_o/L_d interface to form a pear shape. (B) Addition of heat and sucrose drives GV fission, where the division plane is at the L_o/L_d phase boundary. Liquid disordered (L_d) phase is shown in blue (perylene) and the liquid ordered phase is (L_o) is shown in red (rhodamine). Scale bar is 5 µm. Reprinted by permission from Macmillan Publishers Ltd: Nature, from Baumgart et al. Nature, 2003, 425, 821-824. Copyright 2003.

Figure 1-10: Control of PNIPAAM compartment size in GVs by heating between 27 and 41°C. Image from Markström et al. Soft Matter 2007, 3, 587-595. Reproduced by permission of The Royal Society of Chemistry.

Figure 1-11: Gentle hydration method of ATPS-encapsulation within GVs. Lipids are dried to a thin lipid film and then hydrated with a polymer solution that has been heated above the phase transition temperature. The solution is then cooled down, and phase separation occurs in the bulk as well as inside the vesicle. Vesicles collect at the PEG/dextran ATPS interface and can be removed and transferred to a microscope slide for viewing. Inset shows the ATPS encapsulated within a GV, with the PEG-rich aqueous polymer phase surrounding the dextran-rich phase. Image taken with permission from Long et al. Proc. Natl. Acad. Sci. USA 2005, 102, 5920-5925. Copyright 2005.

Figure 1-12: Reversibility of protein microcompartmentation within an ATPS GV. Alexa Fluor 488-labeled soybean agglutinin (SBA) partitions to the dextran-rich phase at 5°C (top), is homogenously distributed at 21°C (middle), and again to the dextran-rich phase when the solution is cooled back down to 5°C. From left to right the images indicate transmitted light (DIC), membrane fluorescence (red indicates rhodamine-tagged lipid), and Alexa Flour 488 protein fluorescence. In DIC, the dextran-rich phase appears thicker than the PEG-rich phase due to the optical activity and refractive index differences of the polymer. Image taken with permission from Long et al. Proc. Natl. Acad. Sci. USA 2005, 102, 5920-5925. Copyright 2005.

Figure 1-13: Protein relocalization from the PEG-rich compartment to the dextran-rich compartment of an ATPS with a change in pH from 4.1 to 6.5. Sequential confocal images of fluorescently labeled human serum albumin (HSA) in a two-compartment PEG/dextran GV formed at pH 4.1 and changed to pH 6.5. Green indicates Alexa Fluor 488 protein fluorescence and red indicates rhodamine labeled membrane (DOPE-rhodamine). Scale bar is 5 µm. Reprinted with permission from Dominak et al. Langmuir 2010, 26, 5697-5705. Copyright 2010 American Chemical Society.

Figure 1-14: Dynamic budding of an ATPS GV. Transmitted DIC images show from left to right (A) budded ATPS GV before dilution with di water, (B) Budding induced by adding hypertonic sucrose solution. Temperature is 3°C. Scale bar is 10 µm. Image

Figure 2-1: ATPS-containing GV before (top) and after (bottom) budding was induced by osmotic stress. Fluorescence images have been false colored: blue indicates Alexa647, red indicates rhodamine, green indicates CF. Images: transmitted light DIC (left), Alexa 647-labeled PEG 5 kDa fluorescence (center), overlay of rhodamine-DOPE and CF-PEG-DSPE fluorescence images (right). T = 4 °C. Scale bar is 10 µm.

Figure 2-2: Confocal optical microscopy images of interior aqueous polymer and external lipid asymmetry in budded, ATPS-containing giant vesicles. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled dextran 10 kDa (A) or Alexa-labeled PEG 5 kDa (B, C). Images have been false colored: blue indicates Alexa647, red indicates rhodamine, green indicates CF. Panels: transmitted light DIC (left), Alexa 647-labeled polymer fluorescence, (center), overlay of rhodamine-DOPE and CF-PEG-DSPE images (right). T = 4 °C in (A) and (B), and T = 6 °C in (C). All scale bars are 10 µm.

Figure 2-3: Optical microscope images for ATPS/GV where Ld lipid domain harbors the PEG2000 functionalization. GVs were prepared with 1:1 DOPC: DPPC + 30 % cholesterol, and 2.2% DOPE-PEG2000, and imaged at 7 °C. The Ld phase of the vesicles was stained with 0.1% DOPE-rhodamine and is false colored red in the fluorescent images; the Lo phase was not labeled and appears dark. We also added 0.05% FITC-labeled dextran 500 kDa (green) to facilitate identification of the dextran-rich aqueous phase. The DOPE-PEG2000 is targeted to the Ld phase (red); the Ld phase coated the non-labeled PEG enriched bud and the Lo phase the dextran bud. Scale bar is 10 µm.

Figure 2-4: Effect of temperature on membrane domain localization in ATPS-containing GV. Different regions of the sample are shown initially at 4 °C (left), after heating to 35 °C (center), and subsequent cooling to 4 °C (right). Top panel is transmitted light, DIC; bottom panel is overlay of rhodamine-DOPE and CF-PEG-DSPE fluorescence. Fluorescence images have been false colored: red indicates rhodamine, and green indicates CF. Scale bar is 50 µm.

Figure 2-5: Effect of temperature on membrane domain localization in a single ATPS-containing GV. Fluorescence images have been false colored: green (left panels) indicates CF-PEG-DSPE, red (center panels) indicates rhodamine-DOPE, and blue (right panels) indicates Alexa647 PEG 5 kDa. Scale bar is 10 µm.

Figure 3-1: A portion of the phase diagram for PEG 8 kDa/dextran 10 kDa ATPS. The concentration used to prepare ATPS-containing GVs in this work was 7 wt % PEG 8 kDa and 10 wt% dextran 10 kDa, which lies just below the coexistence curve at 37 °C, such that the solution exists as a single phase at this temperature. At 5 °C, this composition is above the coexistence curve and exists as two distinct aqueous phases. Points were determined by cloud point titration, and lines are a polynomial fit included to guide the eye.
Figure 3-2: An ATPS-containing GV before (left panels) and after (right panels) budding was induced by exposure to a hypertonic sucrose solution. Fluorescence images have been false colored: blue indicates Alexa647, red indicates rhodamine, and green indicates carboxyfluorescein (CF). Images: transmitted light DIC (top), overlay of rhodamine-DOPE and CF-PEG-DSPE fluorescence images (center), and Alexa647-labeled PEG 5 kDa fluorescence (bottom). T= 4°C. Scale bar is 10 μm. 

Figure 3-3: Fission of an ATPS-containing GV in response to osmotic stress. Osmolality increases from left to right. Confocal fluorescence images have been false-colored: red indicates lipid fluorescence (DOPE-rhodamine) and blue indicates Alexa 647-conjugated dextran 10 kDa. The Alexa647 signal decreased over time due to photobleaching; the blue channel has been adjusted to make the partitioning of Alexa 647-conjugated dextran 10 kDa for each timepoint more apparent. T = 5°C. Scale bar is 10 μm. 

Figure 3-4: Schematic showing asymmetric fission of model cells. 

Figure 3-5: ATPS/GV before (top) and after (bottom) the addition of a membrane-bound protein. Alexa488-streptavidin was added to the external solution, upon which it bound to the biotinylated headgroups (DSPE-PEG 2000-biotin) in the L_α phase. Confocal images are as follows: DIC (top), DOPE-rhodamine (red, middle) and Alexa488-streptavidin (green, middle left) and Alexa647 fluorescence (bottom). T= 5°C. Scale bar = 10 μm. 

Figure 3-6: Effect of osmotic stress on two ATPS-containing GVs (A and B) in which lipid membrane phase coexistence was present. The membrane composition for both vesicles was 1:1 DOPC/DPPC + 30% cholesterol, with 2.4% DPPE-PEG-2K, 0.09% DSPE-PEG-2K-biotin, and 0.4% DOPE-rhodamine. Osmolality increases from left to right. Confocal fluorescence images have been overlaid and false-colored: red is DOPE-rhodamine, indicating the L_d membrane domain, and green is streptavidin-Alexa488, bound to DSPE-PEG-2K-biotin, which is partitioned into the L_α membrane domain. Blue indicates lectin SBA-Alexa 647. Arrows on the far right indicate the location of lipid nanotubes between the daughter vesicles. T = 5°C. Scale bars are 10 μm. 

Figure 3-7: Response of an ATPS-containing GV to osmotic stress. Here, the PEG-rich and dextran-rich daughter vesicles remained connected by a lipid nanotube. Osmolality increases from left to right. Fluorescence images have been false colored. Blue indicates Alexa647, green indicates carboxyfluorescein (CF), and red indicates Cy3 fluorescence. Images: overlay of DOPE-CF and streptavidin-Cy3 (bound to lipid DSPE-PEG-2000-biotin) fluorescence (top), and lectin SBA-Alexa647 fluorescence (bottom). For clarity, the color channels are displayed as upper and lower panels for the same timepoint. T = 5°C. Scale bar is 10 μm. 

Figure 3-8: Confocal fluorescence images collected during asymmetric division of ATPS-containing GV presenting micron-scale lipid domains (lipid composition was 1:1 DOPC/DPPC + 30% cholesterol, with 2.2% DPPE-PEG-2K, 0.08% DSPE-PEG2K-carboxyfluorescein, and 0.08% DOPE-rhodamine). Osmolality increases
from left to right (130 ± 1.5 mmol/kg to 238 ± 5.5 mmol/kg). Fluorescence images have been false colored: red indicates DOPE-rhodamine in the $L_d$ membrane domain, and green indicates DSPE-PEG 2000-carboxyfluorescein, in the $L_o$ membrane domain, and blue indicates Alexa 647-lectin SBA. The Alexa647 signal decreased over time due to photobleaching; the blue channel has been adjusted to make the partitioning of SBA apparent for each timepoint. $T = 5^\circ$C. Scale bar is 10 μm.

**Figure 3-9:** Division of ATPS-containing GVs with excess area of either $L_d$ or $L_o$ membrane domain. Membrane compositions were: 1:1 DOPC/DPPC + 30% cholesterol (A), 1:2 DOPC/DPPC + 30% cholesterol (B). Osmolality increases from left to right. Fluorescence images have been overlaid and false-colored. Blue indicates lectin SBA-Alexa 647, red indicates $L_d$ domain lipid (DOPE-rhodamine), and green indicates $L_o$ domain streptavidin-Alexa488 (bound to lipid DSPE-PEG – 2K-biotin). $T = 5^\circ$C. Scale bar is 10 μm.

**Figure 3-10:** Membrane inheritance in daughter vesicle upon complete budding (top) and fission (bottom) of ATPS-containing vesicles for which the membrane domain areas and aqueous domain volumes are mismatched. Osmolality increases from left to right. Fluorescence images have been false-colored: blue indicates Alexa647, red indicates rhodamine, and green indicates Alexa488. Images: overlay of DOPE-rhodamine and streptavidin-Alexa488 (bound to lipid DSPE-PEG-2000-biotin) fluorescence (top), and lectin SBA-Alexa647 fluorescence (bottom). For clarity, the color channels are displayed as upper and lower panels for the same timepoint. $T = 5^\circ$C. Scale bar is 10 μm.

**Figure 3-11:** Second-generation aqueous phase separation and budding in a daughter vesicle. Membrane composition was 1:2 DOPC/DPPC + 30% cholesterol. Osmolality increases from left to right. Panels top to bottom are transmitted light (DIC), membrane fluorescence, and interior protein fluorescence. Confocal fluorescence images have been overlaid and false-colored. Red indicates $L_d$ domain lipid (DOPE-rhodamine), and green indicates $L_o$ domain lipid (streptavidin-Alexa 488, bound to DSPE-PEG 2000-biotin), and blue indicates the lectin, SBA-Alexa 647, which is partitioned into the dextran-rich interior aqueous phase. $T = 5^\circ$C. Scale bar is 10 μm.

**Figure 3-12:** Aqueous phase separation in each of resulting vesicles after complete budding to form two daughter vesicles connected by a lipid nanotube. Membrane composition was 1:1 DOPC/DPPC + 30% cholesterol. Osmolality increases from left to right. Top row is transmitted light (DIC). Fluorescence images have been overlaid and false-colored. Green indicates $L_d$ domain lipid (DOPE-CF), red indicates $L_o$ domain lipid (streptavidin-Cy3, bound to lipid DSPE-PEG 2000-biotin), and blue indicates lectin SBA-Alexa 647 (note that the red and green dyes are reversed as compared with previous figures). Arrows highlight the location of newly-formed aqueous phases within each of the daughter vesicles. $T = 5^\circ$C for the first three panels and 32 °C for the last three panels. Scale bar is 10 μm.
Figure 4-1: pH-dependent partitioning of HSA. Fluorescently labeled HSA and PEG 5 kDa in PEG 8 kDa/dextran 500 kDa ATPS/GVs formed in neutral pH (A) and pH 4.1 (B). Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right). Scale bar is for both images and is 10 µm.

Figure 4-2: Effects of increasing osmolality on denatured protein in an ATPS GV. Panels A-C represent increasing external osmolality. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right).

Figure 4-3: Effects of increased sucrose on a denatured protein in polar mother vesicle. Osmolality increases from A-D. GV composed of egg PC/DOPG with 1.8 mol % PEGylated lipid encapsulating PEG 8 kDa/dextran 500 kDa formed at pH 4.1. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right). Scale bar = 10 µm, T= 25°C.

Figure 4-4: Inheritance of denatured protein by PEG-rich daughter vesicle upon asymmetric division. The external osmolality is increased from Panels A-F. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right). Scale bar = 10 µm, temperature = 25°C.

Figure 4-5: HSA aggregates at Bulk ATPS interface. The ATPS composition used here is: 11.7% PEG 8 kDa, 12.0 % dextran 500 kDa in 1mM MES buffer at 25°C. From left to right: transmitted DIC, polymer fluorescence (middle), protein fluorescence (far right). Blue represents Alexa 647-PEG 5 kDa partitioned to the PEG-rich phase, and Alexa 488-HSA is shown in green. Scale bar = 10 µm.

Figure 4-6: Fibrinogen aggregates at Bulk ATPS interface. The ATPS composition used here is: 11.7% PEG 8 kDa, 12.0 % dextran 500 kDa in 1mM MES buffer at 25°C. From left to right: transmitted DIC, polymer fluorescence (middle), protein fluorescence (far right). Blue represents Alexa 647-fibrinogen, and FITC-dextran 10 kDa, partitioned to the dextran-rich phase, is shown in green. Scale bar = 10 µm.

Figure 4-7: Asymmetric inheritance of degraded protein. Egg PC/DOPG vesicles with 3.9 wt% PEG 8 kDa/ 4 wt% dextran 500 kDa formed in 1mM MES, pH 4.1 buffer. Panels A-C represent increasing osmolality. From left to right: lipid membrane fluorescence, polymer fluorescence (middle), protein fluorescence (far right). Red
represents rhodamine, Fitc-dextran 10 kDa partitioned to the dextran-rich phase is shown in green, and blue represents Alexa 647-fibrinogen. Scale bar = 10 µm. T=25°C.  

Figure 4-8: Asymmetric inheritance of degraded protein and second budding of ATPG GV. Egg PC/DOPG vesicles with 3.9 wt% PEG 8 kDa/4 wt% dextran 500 kDa formed in 1mM MES, pH 4.1 buffer. Panels A-E represent increasing osmolality and heat. From left to right: lipid membrane fluorescence, polymer fluorescence (middle), protein fluorescence (far right). Red represents rhodamine, Fitc-dextran 10 kDa partitioned to the dextran-rich phase is shown in green, and blue represents Alexa 647-fibrinogen. Scale bar = 10 µm.  

Figure 5-1: The chemical structures of Polyethylene glycol (PEG), dextran, and polyacrylamide (PAAm). Dextran as shown here is in its simplest form.  

Figure 5-2: Phase diagram for PEG 35 kDa/dextran 10 kDa ATPS. Experimentally determined binodals at 5(☐), 25 (●) and 37°C(○). Points were determined by cloud point titration, and lines are a polynomial fit included to guide the eye.  

Figure 5-3: Phase diagram for PEG 35 kDa/PAAm 10 kDa ATPS. Experimentally generated binodals at 25°C (●) and 37°C (○). Points were determined by cloud point titration, and lines are included to guide the eye.  

Figure 5-4: Vapor Pressure Osmometry data for PEG 35 kDa two-component [PEG + water] solutions at 25°C (○) and 37°C (●). Osmolality is plotted vs. PEG 35 kDa molality and each point is the average of triplicate readings on identical samples.  

Figure 5-5: Vapor Pressure Osmometry data for PEG 35 kDa/dextran 10 kDa at 25°C (A) and 37°C (B). Osmalalities of 3-component [PEG + dextran + water] solutions as a function of PEG molality, where dextran was held constant and the molality of PEG was varied (●). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [dextran + water] osmalalities.  

Figure 5-6: Vapor Pressure Osmometry data for PEG 35 kDa/dextran 10 kDa at 25°C on four different days. Osmolalities of 3-component [PEG + dextran + water] solutions as a function of PEG molality, where dextran was held constant and the molality of PEG was varied (●). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [dextran + water] osmalalities.  

Figure 5-7: Vapor Pressure Osmometry data for PAAm 10 kDa two-component [PAAm + water] solutions at 25°C (○) and 37°C (●). Osmolality is plotted vs. PAAm 10 kDa molality and each point is the average of triplicate readings on identical samples.  

Figure 4-8: Vapor Pressure Osmometry data for PAAm10 kDa/ PEG 35 kDa at 25 °C (A) and 37°C (B) Osmalalities of 3-component PAAm/PEG solutions as a function of
PAAm molality, where PEG was held constant and the molality of PAAm was varied (○). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [PAAm + water] osmolalities.

Figure 5-9: Vapor Pressure Osmometry data for PAAm10 kDa/PEG 35 kDa at 25 °C (A, C, E) and 37 °C (B, D, E) on three different days. Osmalalities of 3-component PAAm/PEG solutions as a function of PAAm molality, where PEG was held constant and the molality of PAAm was varied (○). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [PAAm + water] osmolalities.

Figure 5-10: Excess osmolality Δ Osm from VPO studies of PEG 35 kDa-dextran 10 kDa at 25 °C (A) and 37°C (B). Δ Osm vs. m₂m₃, the product of molal concentrations of dextran 10 kDa and PEG 35 kDa; the slope is μ₂₃/RT. PEG 35 kDa was the variable solute.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF488</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td>AF647</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>ATPS</td>
<td>aqueous two-phase system</td>
</tr>
<tr>
<td>CF</td>
<td>carboxyfluorescein</td>
</tr>
<tr>
<td>$C_d$</td>
<td>concentration of a solute in dextran-rich aqueous phase</td>
</tr>
<tr>
<td>$C_p$</td>
<td>concentration of a solute in PEG-rich aqueous phase</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DI H$_2$O</td>
<td>18.2 MΩ-cm Nanopure water</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-$sn$-glycerol-3-phosphocoline</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyl-$sn$-glycerol-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DOPG</td>
<td>1,2-dioleoyl-$sn$-glycerol-3-[phospho-$rac$-(1-glycerol)]</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-$sn$-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>DSPE</td>
<td>1,2-distearoyl-$sn$-glycerol-3-phosphoethanolamine</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GV</td>
<td>giant vesicle</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>$K$</td>
<td>partition coefficient</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>$L_d$</td>
<td>liquid disordered</td>
</tr>
<tr>
<td>$L_o$</td>
<td>liquid ordered</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>R</td>
<td>intensity ratio</td>
</tr>
<tr>
<td>SBA</td>
<td>soybean agglutinin</td>
</tr>
<tr>
<td>$T_{misc}$</td>
<td>miscibility temperature</td>
</tr>
<tr>
<td>VPO</td>
<td>vapor pressure osmometry</td>
</tr>
<tr>
<td>wt %</td>
<td>weight percent</td>
</tr>
</tbody>
</table>
Acknowledgements

First, I would like to thank my advisor Dr. Christine D. Keating for her constant support and guidance over the past six years. Under her guidance I learned how to become a better scientist and independent thinker. Chris helped me to hone my writing and communication skills, through editing my written work and presenting opportunities to practice conveying my research to others. She always set the bar high-and for that I am truly grateful. It was because of her constant encouragement and high standards that I learned what I am truly capable of. She helped me to become the scientist and person that I am today.

I thank my committee members Dr. Andy Ewing, Dr. Nick Winograd, Dr. Scott Showalter, and Dr. Peter Butler for their support and helpful discussions along the way.

I must thank the ladies -Nicole, Elaine, Suzanne, and Missy- at the Microscopy and Cytometry Facility for all of their help.

I also must acknowledge my fellow labmates. I thank Dr. Lisa Dominak, Dr. Jackie Keighron, and Dr. Ann-Sofie Cans for help with initial training and discussions regarding the artificial cells project. You ladies made work an enjoyable experience and I will never forget some of the great heart-to-hearts we had while in our 514 office. A special thanks to Dan Dewey for his constant support and help, especially at the end
when I needed him to take on the responsibility of managing the confocal and for always being there to troubleshoot. Ben Smith, Bill Aumiller, Dr. Stacey Dean, and Dr. Sarah Brunker- you mean more to me than you will ever know. I would like to thank you for being a shoulder for me to lean on when things were tough, a voice of reason when I lost my way, and for being great listeners when I needed to talk. Not only were you there for me when I needed you the most, but you also made graduate school a fun place to be. I will never forget all of the wonderful times we shared together. Kristi Liddell, Brad Davis, David Kirby and David Cacace- thank you for being such amazing labmates.

Ben, when I first met you I could have never imagined the friendship we would have. You are a true friend- one that I will always cherish. I cannot thank you enough for all that you have done for me. I hope that our friendship continues long after graduate school.

Bill, you have been an awesome friend to me. I will never forget all of the fun times we have shared- too many to name. Just the thought of them makes me laugh. Thank you for the person that you are and the friendship that we have.

I couldn’t have made it through graduate school without the support of my friends and family. I thank Maureen Desmond for being the best friend a girl could ask for. Perhaps you, more than anyone, really understood how I felt during this process. Dana- you have always known that I could do this and were proud of me each and every step of
the way. Thank you for being such a true, wonderful friend. Lisa- you came into my life at a time when I needed a friend. You are such an incredible person. I cherish our friendship and am excited for the memories yet to come.

My parents, Jerry and Peggy, my sisters, Leigh Anne, Jenny, and Jill, and my grandmother, “Nan”, never doubted that I could do this. Your encouraging words and love helped me get through this program. At the end, I kept repeating a phrase that my dad said to me, “You will do this, you will do this”. Thank you for never doubting my ability. Thank you for being proud of me…every step of the way.

My second parents, Kitty and Paul, also must be thanked for their support throughout my graduate career. Your love for me has always been evident. I am proud to be a part of your family. Thank you for all of your love and support.

And last but not least, to my family, Jason and Jackson. This is for you. Jackson, my son, you are my world. Some people said that having a child in graduate school would only make things that much more challenging. I think the opposite is true. You made every day a great day. After a stressful day at work, I came home to you. Your smile, your hugs and kisses, and hearing you say “I love you” made everything better. You are the best child a mother could ask for. I love you. Jason, from the first day I arrived at Penn State you have been my number one cheerleader. You encouraged me when I had to take my entrance exams all the way up until the end when I was writing my
thesis and finishing experiments. In the beginning, it was you who would bring me McDonald’s mandarin chicken salads to South Frear at 8 o’clock at night because I couldn’t leave the hot room. It was you who would listen to me practice my presentations over and over again when preparing for my oral comprehensive exams. You understood when I had to work long nights on the confocal or would stay up all night reading and writing. And at the end, it was you who took on the majority of the “mom” and “dad” duties, as well as household chores so that I could spend extra time working on my dissertation. You always knew that I could do this. Because of you, I was able to. I am forever grateful. And I am excited about the next chapter of our lives together. I love you.
Chapter 1

Introduction

Portions of this material were reproduced in part from Dominak, L. M.; Keighron, J.; Andes-Koback, M.; Keating, C.D. “Towards Synthesis of an Artificial Cell.” For submission to International Review of Cell and Molecular Biology (IRCMB).

Biological cytoplasm is not a dilute homogenous soup, but rather a crowded milieu characterized by complex spatial architecture. The placement of cytoplasmic constituents in their correct locations is critical for proper cell function, such as growth and division, but the generation and maintenance of this sub-cellular organization is not well understood. One hypothesis is phase separation of the cytoplasm, which would allow for localized regions, or compartments, in the absence of an intervening membrane. Examination of this hypothesis in biological cells has been precluded by the complexity of even single celled organisms. Models of biological cytoplasm, encapsulated within an artificial cell, provide a simple means for examining phase-separation driven microcompartmentation and its consequences for cell structure and function. This thesis describes the application of model cells encapsulating a cytoplasm mimic to investigate the consequences of cell polarity, namely fission and differentiation, in this simple system. Additionally, this thesis examines fundamental properties of our cytoplasm mimic. A brief overview of the motivation for this thesis work, relevant background, and experimental results are presented in the following paragraphs.
1.1 Motivation for a Cytoplasm Mimic

The cytoplasm is a fundamental, yet critically important component of all living cells. It contains the cytosol, organelles, and other biomolecules, with the exception of the cell nucleus, and is the site at which many important biochemical processes and cellular activities occur.\(^1\) This intracellular environment is crowded\(^2\), containing high total concentrations of macromolecules (~200-300mg/mL proteins and nucleic acids)\(^3\), and occupies a considerable fraction of the cell volume (~30 weight %)\(^4\). The cytoplasm is also characterized by complex spatial organization.\(^1,5,6\) The most obvious forms are those provided by membrane-bound organelles, such as the mitochondria or Golgi apparatus, which sequester biomolecules inside of, and control the flow of molecules in and out of, distinct subcellular compartments.\(^1\) Other types of organization, in which molecules are spatially localized in the absence of a delineating membrane, often termed microcompartmentation, have also been identified.\(^6\) This is an important characteristic of the cytoplasm, as it is this feature- the maintenance of molecular constituents in their proper locations- that is crucial for the facilitation and regulation of intracellular reactions and proper cell function.

Microcompartmentation has been identified in many cell types and in both prokaryotic and eukaryotic cells. In the bacteria *Escherichia coli* and *Bacillus subtilis*, DNA polymerase and accessory proteins are not free floating in the cytoplasm, but are fixed to the cellular envelope.\(^5\) Likewise, in the bacterium *Caulobacter crescentus* several proteins including kinases and chemotaxis receptors are organized to specific locations prior to cell division.\(^5\) Non-membrane bound compartments that sequester proteins and
metabolic pathways have also been identified in bacteria. In eukaryotic cells, the nucleus contains substructures not bound by a membrane (For example, the nucleolus, the Cajal body), and the cytoskeleton and organelles themselves are non-randomly distributed. Heterogeneity in the form of spatial gradients of ions and small molecules is also common. For example, in muscle cells the concentration of calcium ions is higher in the nuclear and subsarcolemmal regions than in other parts of the cytoplasm. Additionally, compartmentation of sequential enzymes, in which multiple enzymes come together to form a complex in order to maximize enzyme efficiency, have been reported for the Krebs cycle, glycolysis, and nucleic acid production.

Intracellular microcompartmentation has consequences for cell function. For example, mitochondria localize near sources of ATP utilization to overcome diffusion barriers. Enzymes of the de novo purine biosynthesis pathway co-localize when the dispersion media is depleted of purines to allow for substrate channeling and regulation of metabolic flux. In C. crescentus, differential phosphorylation across the pre-divisional cell is important for asymmetric inheritance of cellular processes, such as DNA replication, and generation of different cell types (stalk or swarmer daughter cells). Furthermore, compartmentation and subsequent asymmetric inheritance of denatured proteins are potential senescence factors involved in cellular aging, and malfunctions in asymmetric division has been implicated in cancer.

Compartmentation is important for cellular organization and proper cell function, yet in many cases the origins and maintenance are not well understood. In addition to organelles, molecular gradients, and metabolic assemblies, other forms of intracellular organization exist. For example, phase separation of the cytoplasm has been reported in
the human eye lens. Although this is an example of micrcompartmentation, phase separation is caused by intracellular inhomogeneities due to unfavorable protein interactions that occur with natural aging. An example in which phase separation-driven compartmentation facilitates proper cell function was recently found in the cytoplasm of the nematode worm *Caenorhabditis elegans*. During development, germ granules in *C. elegans* single-cell embryos, known as P granules, localize to the anterior position upon polarization. Brangwynne et al. demonstrated that P granules exhibit liquid-like behaviors including fusion and wetting, and localize by a condensation/dissolution mechanism. This phase transition is interesting in that it may explain some of the observations in biological cytoplasm. Indeed, it is consistent with a long believed hypothesis by several researchers that the cytoplasm may undergo phase separation creating distinct phases, or compartments. This is physically reasonable since bulk solutions of macromolecules are known to phase separate at weight % much less than those found in biological cytoplasm. However, extensive evidence for or against this hypothesis is lacking due to the complexity of even the simplest living cells. Non-living models of biological cytoplasm that mimic basic properties provide a means for examining the physical basis and consequences of intracellular organization.

1.2 Bulk Models of the Intracellular Environment

Experiments performed in bulk solution provide a controlled environment for testing hypotheses regarding the physical basis of biological cytoplasm and for studying
biochemical processes that occur within the cellular interior. In designing a model of the intracellular environment, a simple starting point is the cytoplasmic medium. Polymer solutions containing one or more macromolecular species have been used to mimic the crowding and compartmentation phenomena exhibited by biological cytoplasm, as discussed below.

1.2.1 Polymer Solutions

The cytoplasm is a crowded environment that contains high concentrations of biomolecules, such as proteins and nucleic acids. This milieu is known as crowded because no single macromolecular species exists in high concentrations, but rather all of the biomolecules occupy a large fraction of the total cell volume. This occupied volume is not available to other molecules and results in non-specific steric repulsion that has kinetic and thermodynamic consequences. Despite this, biological reactions are often studied in dilute aqueous solutions where crowding is negligible. Polymers such as polyethylene glycol (PEG), dextran, and Ficoll, as well as the proteins BSA and hemoglobin have been used as crowding agents to mimic this aspect of the cytoplasm. Indeed, the effects of macromolecular crowding from the aforementioned (bio)polymers are profound. For example, macromolecular crowding has been shown to nonspecifically enhance compaction or protein folding, protect proteins from denaturation, and increase the rates of slow transition-state limited associations.
1.2.2 Aqueous Two-Phase Systems as Models of Intracellular Organization

When two or more incompatible polymers are mixed at appropriate concentrations in aqueous solution, phase separation occurs resulting in two aqueous phases, or microcompartments.\textsuperscript{21-23} Phase separation of dissimilar polymers in bulk solution, known as aqueous two-phase systems (ATPS), is a phenomenon that has been characterized for a myriad of biopolymer pairs.\textsuperscript{21-23} Immiscibility arises due to the high molecular weight of the polymers combined with interactions (van der Waals, hydrogen bonding, and ionic forces) between the polymer segments and can be influenced by temperature, inorganic salts, and pH.\textsuperscript{21-23}

Phase diagrams can be constructed to determine the conditions necessary and the polymer concentrations needed to achieve immiscibility (Figure 1-1). One way to generate a phase diagram is by performing a cloud point titration.\textsuperscript{23} In this method, one aqueous polymer solution is added drop wise to the other aqueous polymer solution until the solution turns opaque, an indication that the polymer solution is entering the two-phase region. Water is then added to the point at which the solution turns clear. The weights of the polymer solutions are recorded at each step, and the procedure is repeated. A graph of the polymer weight percent compositions is constructed, and the compositions at which the solution is one or two-phases is determined by a binodal curve. At polymer weight percents below the binodal the solution exists as a single phase, while above the line phase separation will occur.
Figure 1-1: Experimentally determined phase diagram for a PEG 8 kDa/ Dx 10 kDa ATPS determined at 5 and 37°C. Solutions exist as two phases above the binodal curve for a given temperature, and as one phase below the curve. Solutions comprised of polymer weight percents that lie between the 5 and 37°C binodals will mix when heated to 37°C and will phase separate when cooled to 5°C. Points were determined by cloud point titration, and lines are a polynomial fit included to guide the eye. Image reprinted with permission from Andes-Koback et al. J. Am. Chem. Soc. 2011, 133, 9545-9555. Copyright 2011. American Chemical Society.

ATPS have long been used for the separation of proteins, organelles, and intact cells.\textsuperscript{21-23} Biomolecules will partition between the two aqueous phases based on their size and chemistry, and partitioning can be improved by functionalizing the solute and/or polymer by, for example, streptavidin-biotin chemistry or by attaching biomolecules to scaffolds.\textsuperscript{24,25} Long et al. demonstrated that attaching protein A to gold nanoparticles substantially improved partitioning (2000:1) into the dextran-rich phase of a polyethylene glycol (PEG)/dextran ATPS as compared to free protein (which had a 5:1 dextran to PEG ratio).\textsuperscript{25} For most solutes partitioning is not easy to predict and must be done experimentally. Despite this, ATPS are preferred over organic separation media because
the high water content (~60-90%) provides a non-denaturing environment for biomaterials, and the polymers have a stabilizing effect on particle structure and biological activity.\textsuperscript{23}

The most well characterized ATPS is composed of the synthetic polymer polyethylene glycol (PEG) and the polysachharide dextran in water or buffer, the structures of which are shown in Figure 1-2.\textsuperscript{21-23} We use this ATPS and take advantage of its basic characteristics - a crowded, compartmentalized environment that is non-denaturing to biomolecules – to create a simple model of the cytoplasm. However, bulk solutions alone do not accurately mimic biological cytoplasm. In addition to crowding and compartmentation, biological cells are characterized by small volumes ($1 \times 10^{-17}$ L for small bacterial cells and as high as 0.5 µL for some of the largest oocytes)\textsuperscript{9} which restricts the number of total molecules it can contain and impacts intracellular reactions.\textsuperscript{26} Giant lipid vesicles (GVs) provide “cell-sized” volumes bound by a semi-permeable lipid bilayer membrane and have proven useful as models of cells or parts of cells. A brief introduction to GVs is discussed below, and is followed by a summary on work done encapsulating cytoplasm models within these cell-sized structures.
1.3 Introduction to Giant Lipid Vesicles as Model Cells

GVs are composed of bilayer membranes surrounding an aqueous core. The membrane is composed of double chain amphiphiles, most notably glycerophospholipids, but can also contain cholesterol, integral membrane proteins, and ion channels. Examples of common lipids found in biological and GV membranes are provided in Figure 1-3. GVs spontaneously form in aqueous solution to form micron-scale (1-100 µm) structures (Figure 1-4 and Figure 1-5). A myriad of molecules such as proteins, enzymes, and nucleic acids, as well as polymers, hydrogels, and small vesicles have been encapsulated in the GV interior during vesicle formation (e.g. gentle hydration, electroformation) or post formation by micro-injection techniques. GVs have been used as simple membrane models to understand their more complex biological counterparts, as cell-sized reaction vessels, and more recently as models of primitive cells. A brief introduction to GVs membrane models and as primitive model cells is presented below.
**Figure 1-3:** Structures of lipids found in biological and GV membranes. Image adapted and reproduced with permission from Eemen et al. *Biotechnol. Agron.Soc. Environ.* **2010**, *14*, 719-736.

Figure 1-5: Schematic demonstrating a possible mechanism for the spontaneous vesiculation to form a giant multilamellar vesicle upon the hydration of a dry phospholipid film. Solute-containing aqueous solution is added to the dry phospholipid film hydrating the outer monolayer. The bumps form on the surface because the surface area of the polar lipid heads increases with increasing hydration. Solution intercalates between these bumps as well as through the bilayers, resulting in encapsulation of the solute-containing solution, and eventually detachment from the surface to form sealed bilayer vesicles. Image reproduced with permission from from Lasic et al. Biochem. J. 1988, 256, 1-11.
1.3.1 GVs as Membrane Models

Insight into the membranes of biological cells has been provided from studies using giant vesicles as membrane models. The basic structure of a vesicle provides researchers with a tool to characterize and measure physical properties of membrane components in an isolated environment. The nature of vesicle formation allows for a controlled composition of lipids and the reconstitution of certain proteins into the final structure.\textsuperscript{27-33} These artificial cell models provide a simple tool for the study of vesicle membrane dynamics, as well as morphological changes with relevance to understanding the more complex membranes of biological cells.

\textit{Single component membranes}

GVs have been used as model biomembrane systems for elucidating physicochemical properties of biological membranes. Mechanical analysis of GV membranes has provided information about the elastic properties of membranes including bending rigidity, stretching elasticity, and spontaneous curvature.\textsuperscript{28,45} For example, incorporation of cholesterol into the bilayer has been shown to increase membrane bending stiffness,\textsuperscript{46} while the addition of PEGylated lipids increases membrane flexibility.\textsuperscript{47} GVs have also been used to study the effects of composition on membrane permeability. Lipid vesicles devoid of proteins or peptide channels can allow water and small molecules to pass through,\textsuperscript{43,44,48} and the reconstitution of ion channels and pumps allows for ions to cross the membrane barrier.\textsuperscript{33} GV reconstitution has also provided a tool to examine membrane-protein interactions\textsuperscript{49} and the role of integral proteins on
membrane processes, such as contraction and growth, and cell spreading. Additionally, GVs can undergo a variety of cytomimetic transformations such as fusion, fission, endocytosis, budding, and birthing, thus providing a tool for understanding the physical parameters governing membrane shape and morphological transitions.

Although a great deal of information has been gained from studies on single component membranes, biological cells exhibit heterogeneity in their plasma membrane, which has implications for signal transduction, membrane trafficking, and processes such as fission. An example of this is lipids that are capable of phase separating into co-existing micron-scale domains, a two-dimensional compartmentation that is analogous to the three-dimensional compartmentation described above. A brief introduction to work incorporating lipid domains in the bilayers of GV membranes follows.

**Membrane Heterogeneity**

Biological cell membranes are composed of hundreds of proteins and lipids, and exhibit lateral inhomogeneities believed to be important in cellular functions such as signal transduction pathways, cell adhesion and migration, and lipid and protein sorting. Although protein interactions have long been recognized as a driving force of lateral membrane heterogeneities, a recent question researchers have set out to answer is what role do lipids play in the lateral organization of biomembranes. Due to the structural variety of lipids, intramembrane interactions may drive phase separation into co-existing liquid domains (~100 nm in diameter) that are enriched in certain lipids and proteins. Visualization of such structures in biological membranes has been precluded by the
optical limits of detection, and other techniques such as detergent extraction are indirect.  

To understand how lipid interactions give rise to membrane domains, a basic understanding of the properties that drive lipid phase separation is necessary. Lipids have a characteristic melting temperature ($T_m$), above which they are in a fluid liquid disordered ($L_d$) phase and below exist in a solid ($S_o$) phase. Lipids with saturated acyl chains tend to have high melting temperatures, while unsaturated lipids are characterized by a lower $T_m$. For example, the $T_m$ of saturated DPPC$^{61}$ is 41°C, while that of unsaturated DOPC$^{61}$ is -20°C; the structures of these lipids are shown in Figure 1-6. Binary lipid mixtures can undergo phase separation into a solid ($S_o$) phase and a liquid disordered ($L_d$) phase between the $T_m$ of the two lipid components.$^{62}$ In the presence of cholesterol, a new phase state known as liquid ordered ($L_o$) is formed (Figure 1-7). This phase is enriched in cholesterol, which associates with saturated phospholipid acyl chains, resulting in lipids that are highly ordered but have a translational diffusion between that of the $S_o$ and the $L_d$ phases. In lipid bilayers, ternary mixtures of a high melting temperature lipid, a low melting temperature lipid, and cholesterol can generate co-existing phase domains, $L_d$ and $L_o$, when the experimental temperature is between the $T_m$ of the saturated and unsaturated constituents.$^{62,63}$
Figure 1-6: Structures of saturated and unsaturated phospholipids. Top: Structure of the saturated phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). Bottom: Structure of the unsaturated phospholipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

Figure 1-7: Schematic illustrating the different phase states of lipids in aqueous medium. Image adapted and reproduced with permission from Eemen et al. Biotechnol. Agron.Soc. Environ. 2010, 14, 719-736.
Keller and co-workers used GVs as a tool to determine the basic conditions necessary for the formation of lipid domains, driven by $L_\alpha/L_d$ phase separation, in bilayer membranes. They examined various three-component mixtures consisting of a high melting temperature lipid, a low melting temperature lipid, and a sterol, and determined the compositions capable of and factors influencing (temperature, sterol structure) liquid-liquid immiscibility in these simple bilayer systems.$^{62,64}$ A phase diagram for DOPC, DPPC, and cholesterol at a constant temperature of 25°C is shown in Figure 1-8. As the composition of DOPC:DPPC:cholesterol is varied, the ratio of the $L_\alpha$ to $L_d$ phase is changed, which effects the relative sizes of the micron-scale domains. For example, a ratio of 2:1 DOPC/DPPC + 30% cholesterol results in larger $L_d$ domains (as shown in bright areas), while 1:2 DOPC/DPPC + 30% cholesterol produces larger $L_\alpha$ domains (dark areas).$^{64a}$
Figure 1-8: Ternary phase diagram and optical micrographs of liquid-liquid phase coexistence for the lipid composition DOPC, DPPC, and cholesterol in the bilayer of GVs. (Top) Phase Diagram for DOPC, DPPC, and cholesterol at 25°C. (Bottom) Fluorescence micrographs of micron-scale domains in GVs. From left to right: 2:1 DOPC/DPPC + 30% cholesterol; 1:1 DOPC/DPPC + 30% cholesterol; and 1:2 DOPC/DPPC + 30% cholesterol. Scale bar is 20 μm. Image adapted and reproduced with permission from Veatch et al. *Biophys. J.* 2004, 86, 2910-2922.

GV membranes have also been used to examine the associated properties of the $L_o$ and $L_d$ phases. The $L_o$ phase is slightly thicker than the $L_d$ phase due to the ordering of the acyl chains. This height mismatch results in a line tension, a one-dimensional equivalent of surface tension in bulk-phase separated systems, at the $L_o/L_d$ interface which controls the kinetics of phase separation, domain size, and influences vesicle shape. Baumgart et al. measured micron-scale phase domains in GVs by micropipette aspiration and determined that line tension is strongly composition dependent and decreases towards the mixing/demixing point. Keller et al. used optical microscopy to examine the effects of line tension on domain size. They found that small $L_o$ and $L_d$ domains grow into larger domains by coalescence and colliding with one
another- not through Ostwald ripening, a process where growth occurs at the expense of the smaller domains- supporting the idea that line tension is important in generating phases comparable to vesicle dimensions (10 µm). Additional studies revealed that vesicles bend to minimize the energy at the liquid-liquid interface, resulting in complex shapes including buds, pears, prolaters, and when subjected to temperature and osmotic stress can undergo vesicle fission (Figure 1-9).  

**Figure 1-9:** Shape changes of GV containing co-existing micron-scale liquid domains. (A) GV bends at the L_o/L_d interface to form a pear shape. (B) Addition of heat and sucrose drives GV fission, where the division plane is at the L_o/L_d phase boundary. Liquid disordered (L_d) phase is shown in blue (perylene) and the liquid ordered phase is (L_o) is shown in red (rhodamine). Scale bar is 5 µm. Reprinted by permission from Macmillan Publishers Ltd: Nature, from Baumgart et al. Nature, 2003, 425, 821-824. copyright 2003.

Even if the membranes of biological cells do not have micron scale lipid domains as observed in these simple model systems, the basic biophysics that govern phase
separation in GV membranes is applicable to living cells. For example, phase separation has been demonstrated in giant plasma membrane vesicles (GPMVs), which are derived from the plasma membranes of biological cells. GPMVs, which contain integral and peripheral membrane proteins and hundreds of different lipids, can separate into co-existing L_o and L_d domains below 25°C. This evidence is exciting in that it bridges the gap between model membranes and the biological systems they are meant to mimic.

1.4 Incorporation of Intracellular Model into GV as Primitive Model Cells

Although a myriad of biomolecules have been encapsulated within GV, with very few exceptions they have been homogenously distributed. Biological cytoplasm is marked by spatial architecture and heterogeneity. This has been achieved by encapsulation of polymers, such as the temperature responsive polymer poly(N-isopropylacrylamide) (PNIPAAm). PNIPAAm is characterized by a lower critical solution temperature (LCST), below which the polymer exists in the sol-state, and above which a coil-to-globule transition of individual polymer molecules takes place and a hydrogel is formed. When encapsulated in GV, the hydrogel acts as a compartment for the immobilization of nanoparticles. Jesorka et al. demonstrated that hydrogel formation was reversible and the size of the compartment could be controlled by slight variations in temperature. For example, further heating after hydrogel formation resulted in shrinkage causing the polymer density in the gel compartment to increase more than 20-fold, and the diameter of the compartment to decrease to ~1/3 the vesicle diameter (Figure 1-10). Additionally, this work demonstrated that multiple compartments could be
formed in a single GV by fusion of two or more vesicles that contained PNIPAAm in their vesicle interior. Although primitive, this work demonstrates a simple cytoplasm capable of dynamic and reversible microcompartmentation within a vesicle.

![Images of vesicles at different temperatures](image)

**Figure 1-10:** Control of PNIPAAm compartment size in GVs by heating between 27 and 41°C. Image from Markström et al. *Soft Matter* 2007, 3, 587-595. Reproduced by permission of The Royal Society of Chemistry.

Our group has encapsulated a PEG/dextran ATPS within GVs to mimic the crowding and microcompartmentation phenomena of biological cytoplasm. We form ATPS/GVs by the gentle hydration method. Briefly, a single-phase polymer solution is added to a lipid film and incubated above the polymer transition temperature, allowing for spontaneous vesiculation. This protocol yields a heterogeneous population of vesicle sizes, as well as both multi- (many bilayers) and unilamellar (single bilayer) vesicles. The solution is then cooled down below the polymer transition temperature, and phase separation occurs in both the bulk ATPS solution as well as the GV interior (**Figure 1-11**). Phase separation into PEG-rich and dextran-rich aqueous phases within the GV, or model cell, interior is interesting because it allows for distinct compartments in the absence of a delineating membrane.
Figure 1-11: Gentle hydration method of ATPS-encapsulation within GV's. Lipids are dried to a thin lipid film and then hydrated with a polymer solution that has been heated above the phase transition temperature. The solution is then cooled down, and phase separation occurs in the bulk as well as inside the vesicle. Vesicles collect at the PEG/dextran ATPS interface and can be removed and transferred to a microscope slide for viewing. Inset shows the ATPS encapsulated within a GV, with the PEG-rich aqueous polymer phase surrounding the dextran-rich phase. Image taken with permission from Long et al. Proc. Natl. Acad. Sci. USA 2005, 102, 5920-5925. Copyright 2005.

Biomolecules, such as proteins or DNA, can be localized to sub-regions of the vesicle interior by partitioning between the aqueous phases, and the degree of localization can be controlled by slight adjustments in temperature, osmotic pressure, and pH.\textsuperscript{21-23} For example, Long et al. found that the lectin protein Soybean agglutinin (SBA), a carbohydrate binding protein, favored the dextran-rich phase of a PEG 4.6 kDa/Dx 10
22 kDa ATPS at 5°C. When the solution was heated to 21°C, the ATPS was converted to a single phase resulting in a homogenous distribution of the protein, as shown in Figure 1-12.

*Figure 1-12: Reversibility of protein microcompartmentation within an ATPS GV. Alexa Fluor 488-labeled soybean agglutinin (SBA) partitions to the dextran-rich phase at 5°C (top), is homogenously distributed at 21°C (middle), and again to the dextran-rich phase when the solution is cooled back down to 5°C. From left to right the images indicate transmitted light (DIC), membrane fluorescence (red indicates rhodamine-tagged lipid), and Alexa Flour 488 protein fluorescence. In DIC, the dextran-rich phase appears thicker than the PEG-rich phase due to the optical activity and refractive index differences of the polymer. Image taken with permission from Long et al. Proc. Natl. Acad. Sci. USA 2005, 102, 5920-5925. Copyright 2005.

This process was reversible with temperature. When the solution was cooled back down to 5°C, the solution phase separated into distinct microcompartments, and the protein was once again localized to the dextran-rich phase. Additionally, dynamic protein localization has been achieved by changes in pH. Dominak et al. demonstrated that human serum albumin (HSA) concentrated in the dextran-rich phase of a PEG 8 kDa/Dx
500 kDa ATPS at pH 6.5, but at partially/fully denaturing pH (4.1 or 12) localized to the PEG-rich phase.\textsuperscript{43} Figure 1-13 shows relocalization of HSA in response to pH changes. Exposure of the protein to high and low pH resulted in denaturation and partitioning into the more hydrophobic PEG-rich phase. Relocation in response to external pH changes is a general phenomenon, and was observed for several proteins that varied in size and isoelectric point.

\textbf{Figure 1-13:} Protein relocalization from the PEG-rich compartment to the dextran-rich compartment of an ATPS with a change in pH from 4.1 to 6.5. Sequential confocal images of fluorescently labeled human serum albumin (HSA) in a two-compartment PEG/dextran GV formed at pH 4.1 and changed to pH 6.5. Green indicates Alexa Fluor 488 protein fluorescence and red indicates rhodamine labeled membrane (DOPE-rhodamine). Scale bar is 5 \(\mu\)m. Reprinted with permission from Dominak \textit{et al.} \textit{Langmuir} 2010, 26, 5697-5705. Copyright 2010 American Chemical Society.

Exposure of ATPS GVs to osmotic stress results in a budded or polar geometry.\textsuperscript{44} When placed in a hypertonic solution, water is drawn out of the vesicle interior, causing osmotic deflation, which has many consequences. First, the reduction in volume allows for more membrane than is needed to coat the now smaller aqueous polymer phases.
Additionally, the PEG- and dextran-rich aqueous polymer solutions become concentrated, resulting in an increased interfacial tension between them. As a result, the vesicle undergoes a shape change from a spherical to a more energetically favorable budded geometry, where the bud contains one of the aqueous polymer phases and the body of the vesicle contains the other (Figure 1-14). Budding results in asymmetry in both the internal polymer phases and biomolecule composition, giving rise to polarity. Budding is observed with increasing frequency with an increase in external osmolality, an increase in temperature, and is affected by factors that influence the membrane elasticity. For example, incorporation of PEGylated lipids facilitated GV budding, while membranes containing high amounts (40 mol %) of cholesterol reduced the incidence of budding. Albeit simple, this work demonstrated that a random symmetry-breaking step could induce polarity in ATPS GV model cells providing an experimental system for investigating the effects of biochemical polarity.

Figure 1-14: Dynamic budding of an ATPS GV. Transmitted DIC images show from left to right (A) budded ATPS GV before dilution with di water, (B) Budding induced by adding hypertonic
1.5 Summary and Objectives

ATPS GVs provide an experimental model system for studying the physical and chemical basis of intracellular organization. Previous work in our laboratory has demonstrated that ATPS GVs mimic macromolecular crowding and dynamic microcompartmentation—critical aspects of biological cytoplasm. Exposure of these model cells to osmotic stress drives GV budding, resulting in polarity in both the structure and internal protein distribution. This thesis is concerned with building on this initial polarity and examining the consequences thereof, in this simple system. In addition, this thesis describes fundamental studies on two-polymer solutions, specifically the osmotic pressure, to gain an understanding of the interactions occurring in ATPS.

Chapter 2 describes localization of lipid membrane microdomains to specific “poles” of asymmetric GVs in response to local internal composition. Interior aqueous microdomains were generated in a simple model cytoplasm composed of a PEG/dextran ATPS encapsulated in the vesicles. The GV membrane composition used was a modification of a DOPC/DPPC/cholesterol mixture known to form micrometer-scale liquid ordered and liquid disordered domains; we added lipids with PEG 2000 Da-modified headgroups. Osmotically induced budding of the ATPS-containing GVs led to structures where the PEG-rich and dextran-rich interior aqueous phases were in contact with different regions of the vesicle membrane. Liquid ordered (L_o) membrane domains
rich in PEG-terminated lipids preferentially coated the PEG-rich aqueous phase vesicle “body”, while coexisting liquid disordered (Ld) membrane domains coated the dextran-rich aqueous phase “bud”. Membrane domain positioning resulted from interactions between lipid headgroups and the interior aqueous polymer solutions, e.g., PEGylated headgroups with PEG and dextran polymers. Heating resulted first in patchy membranes where Lo and Ld domains no longer showed any preference for coating the PEG-rich vs dextran-rich interior aqueous volumes, and eventually complete lipid mixing. Upon cooling lipid domains again coated their preferred interior aqueous microvolume. This work shows that nonspecific interactions between interior aqueous contents and the membrane that encapsulates them can drive local chemical heterogeneity, and offers a primitive experimental model for membrane and cytoplasmic polarity in biological cells.

In Chapter 3, I report complete budding and asymmetric fission of ATPS GV's possessing micron-scale membrane domains to produce daughter vesicles that are chemically distinct in both interior and membrane compositions. ATPS containing vesicles formed buds when sucrose was added externally, such that they became not only morphologically asymmetric but also asymmetric in both their interior and their membrane compositions. Further increases in osmolality drove formation of two chemically distinct daughter vesicles, which were in some cases connected by a lipid nanotube (complete budding), and in others were not (fission). In all cases, separation occurred at the aqueous-aqueous phase boundary, such that one daughter vesicle contained the PEG-rich aqueous phase and the other contained the dextran-rich aqueous phase. PEGylated lipids localized in the Lo domain resulted in this membrane domain preferentially coating the PEG-rich bud prior to division, and subsequently the PEG-rich
daughter vesicle. Varying the mole ratio of lipids resulted in excess surface area of $L_o$ or $L_d$ membrane domains such that, upon division, this excess portion was inherited by one of the daughter vesicles. In some cases, a second “generation” of aqueous phase separation and budding could be induced in these daughter vesicles. Asymmetric fission of a simple self-assembled model cell, with production of daughter vesicles that harbored different protein concentrations and lipid compositions, is an example of the seemingly complex behavior possible for simple molecular assemblies. These compartmentalized and asymmetrically dividing ATPS-containing GVs could serve as a test bed for investigating possible roles for spatial and organizational cues in asymmetric cell division and inheritance.

Chapter 4 examines the asymmetric distribution of denatured proteins, human serum albumin (HSA) and fibrinogen, in non-living artificial cells. The asymmetric distribution and inheritance of damaged proteins is required for establishing cellular age asymmetry in biological cells. During asymmetric division, aggregated and damaged protein accumulates in the mother but not the daughter cells, culminating in an “aged” mother cell and a “young” daughter cell. Age asymmetry has also been observed in biological cells that divide asymmetrically to produce two non-identical daughter cells. Examination of this age asymmetry has been difficult in vivo due to the complexity of biological cells. To encapsulate denatured proteins in GVs, we formed vesicles at pH 4.1, where the proteins were partially acid unfolded; denaturation resulted in preferential partitioning of the proteins to the more hydrophobic PEG-rich phase of the vesicle. Exposure of polarized and non-polarized vesicles (i.e., budded model cells with asymmetrically distributed “cytoplasmic” components) containing denatured HSA to
increased osmotic stress resulted in the formation of aggregates, which localized at the PEG/dextran phase boundary. In some instances, further exposure to osmotic stress and heat induced GV fission such that the PEG-rich daughter vesicle retained higher concentrations of the denatured protein (fibrinogen and HSA). Although this work is much simpler than the complex machinery required for age asymmetry in biological cells, sorting based on aggregate formation and non-specific interactions, as shown here, likely plays a role in polar segregation of damaged proteins in living systems.

The work discussed in chapter 5 reports osmolality data for aqueous solutions containing two polymers and evaluates the potential for vapor pressure osmometry as a technique to acquire information about the polymer-polymer interactions occurring in these systems. Understanding the osmotic pressure of these solutions is important for controlling vesicle behavior in response to osmotic stress. During vesicle formation, samples are cooled to allow for phase separation. ATPS/GVs collect at the bulk ATPS interface and are then transferred to a solution, which is often different from that in which the vesicles were formed. The bilayer membrane of our vesicles is water permeable, and thus it is important to match the osmotic pressure of the internal solution with that of the external solution in which the vesicles are dispersed, and to be able to control the osmotic pressure with simple solutions such as sucrose. In this work we chose to examine Polyethylene glycol (PEG)/ Polyacrylamide (PAAm) and PEG/dextran polymer pairs, as these ATPS show a temperature and salt dependence. Examination of solution osmolality as a function of temperature for these two-polymer systems demonstrated non-additive behavior as compared to data obtained for the single polymer solutions (e.g., [dextran + water], [PEG + water], [PAAm + water]). Other researchers have used osmolality data,
obtained by vapor pressure osmometry, to determine interaction potentials for a small solute and a (bio)polymer in aqueous solution. We examined if this is a viable method for our two-polymer systems. In this chapter, I report osmolality values as a function of temperature for the polymer pairs known to exhibit temperature-dependent phase behavior, and evaluate vapor pressure osmometry as a technique to characterize these interactions.

1.6 References


60. Munro, S. Lipid rafts: elusive or illusive? Cell 2003, 15, 377-388.

61. The abbreviations for the lipids stand for DOPC = 1,2-Dioleoyl-sn-glycero-3-phospho-choline, DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphocholine


Chapter 2

Positioning Lipid Membrane Domains in Giant Vesicles by Micro-organization of Cytoplasm Mimic


Preparation of ATPS GVs with co-existing micron-scale membrane domains and data analysis using confocal microscopy, along with the writing of the manuscript, was done in collaboration with Dr. Ann-Sofie Cans of the Keating group. Dr. Cans performed initial experiments analyzing micron-scale domains in ATPS GVs and investigated the localization of these domains in the presence of lipids with PEGylated headgroups. The author of this dissertation was responsible for preparation of ATPS GVs with co-existing domains, confocal microscopy experiments, and data analysis, specifically focusing on the effects of temperature on these model cells.

2.1 Introduction

Biological cells exhibit chemical heterogeneity in both their plasma membranes and their interiors.\textsuperscript{1-5} In addition to obvious forms of heterogeneity such as the presence of membrane-encapsulated organelles and cytoskeletal structures in eukaryotic cells, the biochemical composition of the cytoplasm itself is nonuniform.\textsuperscript{3,6} Although both cytoplasmic and membrane microcompartmentation are thought to be critical aspects of cellular structure, their origins and consequences are not yet well understood. Giant lipid vesicle (GV)\textsuperscript{7-9} membrane model systems have incorporated lateral heterogeneity in the form of coexisting microscale lipid domains.\textsuperscript{10} Relatively few cell models have yet been constructed that incorporate heterogeneous aqueous interiors; examples include GV containing hydrogels or small vesicles.\textsuperscript{11-16} We recently reported a cytoplasm mimic based on vesicle-encapsulated aqueous solutions of poly(ethyleneglycol) (PEG) and dextran, which can separate into two coexisting aqueous phases.\textsuperscript{15-17} Aqueous two-phase
systems (ATPS) can form when two polymers or a polymer and a salt are mixed in water at sufficiently high polymer concentrations.\textsuperscript{18-20} For example, in this work we encapsulate ATPS composed of \textasciitilde{}15 weight % total polymer (7-8\% PEG 8 kDa, and 7-10\% dextran 10 kDa). For comparison, the cytoplasm has between 17 and 35 weight \% macromolecules.\textsuperscript{21}

Bulk PEG/dextran ATPS have an upper, PEG-rich aqueous phase and a lower, dextran-rich aqueous phase. Here, and in previous work,\textsuperscript{15-17} we take advantage of slight changes in the phase diagram (i.e., in the polymer compositions required for phase separation) that occur as a function of temperature to encapsulate coexisting PEG-rich and dextran-rich microvolumes within giant lipid vesicles (GVs). For the lipid and polymer compositions used here, this results in formation of ATPS-containing GVs (ATPS/GVs) in which an interior dextran-rich aqueous phase is surrounded by an outer PEG-rich aqueous phase.\textsuperscript{16} Solutes, such as proteins, added to ATPS can preferentially accumulate into one of the phases; separations are possible for solutes with different partitioning behavior.\textsuperscript{15,16,18-20,22} We have used partitioning between the coexisting PEG-rich and dextran-rich microvolumes to control local protein concentrations within these model cells.\textsuperscript{15,16}

Exposure to hypertonic sucrose solutions results in osmotic shrinkage of the ATPS/GVs, which has several consequences. The reduction of volume generates excess membrane area, facilitating deformation to form nonspherical geometries. Concentration of the PEG and dextran polymers inside the vesicle improves solute partitioning between the aqueous phases and increases the interfacial tension at the aqueous-aqueous interface between the PEG-rich and dextran-rich microvolumes.\textsuperscript{15,16} This increased interfacial
tension, coupled with the excess membrane surface area, can result in expulsion of the interior dextran-rich aqueous phase from within the PEG-rich phase to form one or more “buds” on a PEG-rich GV “body”.\textsuperscript{16} Since the contents of the aqueous phases are chemically distinct, budding generates compositional asymmetry in the synthetic cytoplasm. When proteins such as soybean agglutinin were partitioned into the dextran-rich phase, this asymmetry led to differences in protein concentration in the buds as compared with the body of the vesicles, a primitive model of polarity.\textsuperscript{16}

Herein, we report localization of lipid membrane microdomains in response to the local composition of our ATPS cytoplasm mimic within the vesicles. We accomplished this by building on the chemical asymmetry of the aqueous interior of budded ATPS/GVs to control the location of lipid domains in the vesicle membrane, generating asymmetry in membrane chemistry. Lipid domains presenting PEGylated headgroups preferentially coated the PEG-rich aqueous microvolumes of the budded ATPS/GVs. The resulting model cells exhibit chemical polarity in both their exterior membrane and interior aqueous compositions. Such interplay between internal and external microcompartmentation seems not only possible, but likely, in biological cells.

2.2 Experimental Materials and Methods

2.2.1 Chemicals and Materials

All lipids, 1,2-dioleoyl-\textit{sn}-glycero-3-phosphocholine (DOPC), 1,2- Dipalmitoyl-\textit{sn}-Glycero-3-Phosphocholine (DPPC), cholesterol, 1,2-dioleoyl-\textit{sn}-glycero-3-
phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000 ] (DOPE-mPEG 2000), 1,2- Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethyleneglycol)-2000] (DPPE-mPEG 2000), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)2000-N'-carboxyfluorescein] (DSPE-mPEG 2000- FITC), and 1,2-dioleoyl-sn-glycero-3- phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-rhodamine) were purchased from Avanti Polar Lipids (Alabaster, AL). The polymers: PEG 5 kDa, PEG 8 kDa, dextran 10 kDa, and FITC-dextran 500 kDa, sodium phosphate buffer salts, sucrose, and sodium azide were purchased from Sigma-Aldrich. The Alexa Fluor 647–dextran 10 kDa and Alexa Fluor 647- conjugated carboxylic acid, succinimidylester were purchased from Invitrogen (Eugene, OR). Press-to-Seal silicone isolators from Invitrogen were used to enclose ATPS GV preparations on microscope slides for imaging. Water in the experiments was purified to a resistivity of $\leq 18.2$ M$\Omega$ with a Barnstead NANOPure Diamond system (Dubuque, IA).

2.2.2 Preparation of Giant Vesicles with co-existing fluid phases encapsulating an Aqueous Two-Phase System

The giant vesicles were prepared using a modification$^{15,16}$ of the gentle hydration technique$^{23}$ as previously described. We note that vesicles prepared in this way are heterogeneous, with multilamellar, oligolamellar, and unilamellar vesicles of various sizes all present in the sample. Since we are also incorporating the PEG and dextran polymers for ATPS formation, we additionally see GVs having single aqueous phases as well as the desired 2- aqueous phase/GVs. We have discussed the relative amounts of various structures formed previously,$^{15}$ here we focused on ATPS-containing GVs $>5$
microns in diameter that appeared to be unilamellar via fluorescence microscopy (i.e.,
they were not multilamellar; they may be uni- or oligolamellar).

Briefly, lipids in chloroform solution were added to a clean glass test tube in the
mole ratios of 34.7% DOPC, 34.7% DPPC, 30% cholesterol, 2.2% DPPE-PEG2000 or
DOPE-PEG2000, and 0.05% DOPE-rhodamine. In a majority of the experiments 0.05%
DSPE-PEG2000-FITC was also added to the lipid mixture. The lipids were dried under a
gentle stream of argon gas to form a thin coating of lipid film in the vial. Residuals of
chloroform were eliminated by vacuum desiccation for 1-2h. A solution of 1 mL of a
freshly made single phase ATPS solution heated to 37°C was added to the lipid films.
The samples were incubated at 37°C for 2-3 days allowing giant vesicles to form and
encapsulate the single-phase polymer solution. The ATPS solutions used in these
experiments were prepared by dissolving 7.5–8 wt. % of PEG 8 kDa and 8 wt. % of
dextran 10 kDa in 5 mM pH 7.0 sodium phosphate buffer with the addition of 1mM
sodium azide to prevent bacterial growth in the vesicle solution. Cloud point titrations
were performed at 5°C and 37°C to determine the composition at which the ATPS
existed as one phase at 37°C and two at 5°C. In some experiments, the dextran phase of
the ATPS was labeled by adding Alexa Fluor 647- dextran 10 kDa to a final
concentration of 77 μg/mL or by adding FITC-labeled dextran 500 kDa to a final
concentration of 400 μg/mL. In the experiments where the PEG phase in the ATPS was
labeled, Alexa Fluor 647 dextran 5 kDa was added to the ATPS to a final concentration
of 12-30 μg/mL.
2.2.3 Preparation of budding ATPS GV samples for microscopy

After the ATPS GV was formed the sample vials were cooled to 5°C, allowing the bulk and the vesicles with encapsulated polymer solutions to phase separate. The ATPS-GVs accumulated at the interface of the bulk separated polymer solutions from which a few microliters of vesicle solution were collected with a micropipette and added to a shallow well made of a silicon-to-seal isolator attached to the top of a microscope coverslip. In some experiments, vesicles were monitored at isotonic conditions. These vesicles were diluted by adding cold PEG-rich top phase. When preparing budding vesicles, the vesicles were induced to bud by subjecting them to a hypertonic solution as described previously; typically, 1 µL of ATPS/GV suspension was placed into 165 µL of 130 mM sucrose solution (osmolality of this ATPS in bulk is ~ 110 mmol/kg). For experiments where solution was added to the samples in real time at a constant temperature of 5°C, the coverslip sample vial was left as an open volume. For experiments where vesicles were studied as a function of temperature, the sample was heated from 5 °C to 49 °C and the sample well was covered with a second coverslip to avoid evaporation of solution during the experiment.

2.2.4 Instrumentation and software

ATPS GV images were acquired on a Nikon TE 200 inverted microscope (Nikon Plan Fluor 100X 1.4 NA objective) with a CoolSNAP HQ CCD camera (Photometrics, Tucson, AZ, 1392 x 1040 pixels) or with an Olympus IX-70 laser scanning confocal
inverted microscope (LSCM) (Nikon Plan Apo 60X 1.4 NA objective). Line scans of confocal images were used to quantify fluorescence intensities.

A temperature-controlled PE-100 microscope stage, a PE-94 control unit (both from Linkam, ± 0.1°C), and a circulating water bath from VWR (model 1160A) were used to control GV suspension temperature. A microprobe (model IT-21) from Harvard apparatus and a Physitemp BAT-12 readout unit (± 0.1°C) were used to directly measure GV suspension temperature. Photoshop v7. (Adobe Systems, San Jose, CA) was used to obtain and process microscope images.

2.3 Results and Discussion

We prepared ATPS-containing GVs by gentle hydration in warm polymer solutions as described previously, except that here we used lipid compositions capable of forming micron-scale fluid domains. Briefly, polymer concentrations for the ATPS to be encapsulated within the vesicles were determined by examination of the phase diagram for PEG 8 kDa/dextran 10 kDa in water at 4 and 37 °C. ATPS compositions were selected such that the solution would exist as a single phase at 37 °C and as two phases at 4 °C. GVs were prepared by gentle hydration of ternary lipid mixtures in these polymer compositions at 37 °C. The lipid composition used in this work (1:1 DOPC/DPPC + 30% cholesterol) was selected based on phase diagrams for ternary lipid mixtures reported by the Keller group. This lipid mixture displays phase coexistence for the liquid ordered (L_o) and liquid disordered (L_d) phases, which form micron-scale domains in GVs for a range of lipid compositions. Our earlier work
on ATPS/GV budding indicated that the membrane flexibility contributed by PEGylated headgroups facilitated formation of budded geometries.\textsuperscript{16} We therefore modified the 1:1 DOPC/DPPC + 30% cholesterol recipe by including lipids with PEGylated headgroups (2.2\% PEG-DPPE). We also added fluorescent lipids to track the location of the domains: 0.1\% rhodamine-DOPE, which is known to partition into the DOPC-rich L\textsubscript{d} domain,\textsuperscript{27} and 0.05\% carboxyfluorescein (CF) labeled PEG 2000-modified DSPE (0.05\% CF-PEG-DSPE), to report on the distribution of PEGylated lipids.

\textbf{Figure 2-1:} ATPS-containing GV before (top) and after (bottom) budding was induced by osmotic stress. Fluorescence images have been false colored: blue indicates Alexa647, red indicates rhodamine, green indicates CF. Images: transmitted light DIC (left), Alexa 647-labeled PEG 5 kDa fluorescence (center), overlay of rhodamine-DOPE and CF-PEG-DSPE fluorescence images (right). T = 4 °C. Scale bar is 10 \textmu m.

After ATPS/GV formation at 37 °C, samples were cooled to 4 °C. At this temperature, the aqueous polymer solution phase separated both in the bulk and in the GV interiors. The resulting ATPS-containing GVs (ATPS/GVs) were collected from the
bulk ATPS interface and placed into a sucrose solution on the microscope slide. Confocal fluorescence images of an ATPS/GV are shown in Figure 2-1. The top panel shows an ATPS-containing GV in isotonic external solution. Two coexisting aqueous microvolumes corresponding to the PEG-rich and dextran-rich phases of the ATPS can be seen inside the GV both in the differential interference contrast (DIC) transmitted light image (left) and in the Alexa647-PEG 5 kDa fluorescence image (center). PEG-rich and dextran-rich aqueous phases can be distinguished in DIC images due to slight differences in refractive index and the optical activity of the dextran polymer; the dextran-rich aqueous phase appears “thicker” in the DIC images. Unambiguous assignment of aqueous phase identities was achieved by addition of fluorescently-labeled PEG 5 kDa (Figure 2-1, center). For this vesicle, the outer PEG-rich aqueous phase contacted the membrane and completely surrounded the inner dextran-rich phase. This morphology is typical for ATPS/GV with PEG-modified lipids.\(^{16}\)

The right-hand panel of Figure 2-1 shows the location of fluorescently labeled lipids; red indicates rhodamine-DOPE and green indicates CF-PEG-DSPE. The clear separation of red and green regions indicates that our addition of PEG-DPPE and CF-PEG-DSPE did not inhibit the formation of micrometer-scale domains; a slight orange tint to the red regions is due to some green emission in these areas. Partitioning of the rhodamine- labeled DOPE into one of the membrane domains, which we assign as the less ordered, DOPC-rich \(L_d\) domain based on the known partitioning of this dye,\(^{27,28}\) appeared to be essentially complete: no rhodamine-DOPE signal could be detected from the other domain. The CF-labeled, PEGylated lipids partitioned into the DPPC/cholesterol-rich, more ordered domain of the lipid membrane (\(L_o\)). Fluorescent
labels that partition into \( L_o \) are uncommon; even molecules with saturated acyl chains (e.g., Texas Red-DPPE) often partition into \( L_d \) due to the presence of the dye moiety on the headgroup.\(^{27}\) The partitioning of CF-PEG-DSPE into the \( L_o \) domain observed here is presumably enabled by the long, flexible PEG chain (2000 Da, 45 monomers) that separates the CF from the lipid headgroup. For this vesicle (Figure 2-1, top), CF-PEG-DSPE was present in both \( L_o \) and \( L_d \), with \(~3.5\times\) higher intensity observed in \( L_o \). We note that CF is notoriously sensitive to local environment and can self-quench at high concentrations.\(^{29}\) However, the amount of dye used here is low, and since the CF is separated from the headgroups by the PEG chain, it may not experience a difference in local environment. Our data are consistent with the expected preference of DSPE tailgroups for the \( L_o \) membrane domain and with reduced partitioning due to steric repulsions between PEG 2 kDa headgroup labels. Based on the anticipated equal areas for \( L_o \) and \( L_d \) domains for this lipid composition,\(^{30}\) and assuming identical partitioning between \( L_o \) and \( L_d \) for our 2.25 total mol % (labeled + unlabeled) PEG 2 kDa modified lipids, we can estimate from the apparent CF-PEG-DSPE partitioning that the molecule fraction PEG-lipid is \(~3.9\%\) in \( L_o \) and \(~1.1\%\) in \( L_d \). Lipids with polymers such as PEG grafted to their headgroups can exist in two basic regimes based on headgroup density.\(^{31,32,33}\) At low densities, the polymers are not close enough to interact with each other, and behave as independent random coils (“mushrooms”) extending from the membrane. At higher densities, polymers are close enough to interact with each other and more fully coat the surface, and hence take on a brushlike morphology, extending out further from the membrane. The mushroom-to-brush transition for PEG 2 kDa lipids is at mole fractions of 0.014 (1.4 mol %).\(^{32}\) Thus, our estimated PEG headgroup densities
suggest that PEGylated headgroups in the $L_o$ domain exist as a continuous brush, while those in $L_d$ are in the mushroom regime.

After acquiring the image shown in Figure 2-1 (top), budding of the ATPS/GV was induced by addition of a sucrose solution of higher osmolarity than the ATPS used to prepare the vesicles. We recently described osmotically induced budding in ATPS/GVs that lack membrane domains (prepared using DOPC/DOPG and DOPC/PEG lipids). Although the interior ATPS is not necessary to osmotically induce GV budding, it does impact the final budded geometries. Budding morphologies were observed when ATPS/GVs were dehydrated via osmotic shock, concentrating the ATPS (which increases the interfacial tension at the aqueous/aqueous boundary, a driving force for minimizing the contact of the PEG-rich and dextran-rich phases) and decreasing the GV volume (increasing excess membrane area, and facilitating formation of higher surface area budded morphologies). Importantly, budded ATPS/GV structures have both the PEG-rich and the dextran-rich interior aqueous microvolumes in contact with the lipid membrane, while spherical structures generally do not. In Figure 2-1 (top), the initial ATPS/GV had the PEG-rich aqueous phase completely surrounding the dextran-rich phase, such that only the PEG-rich volume contacts both $L_o$ and $L_d$ membrane domains prior to the budding event.

In the budded ATPS/GV (Figure 2-1, bottom), the CF-PEG-DSPE rich $L_o$ membrane domain was localized around the PEG-rich aqueous phase, and the rhodamine-DOPE rich lipid domain ($L_d$) surrounded the dextran-rich aqueous phase. Inside the vesicle, partitioning of the Alexa647-labeled PEG into the PEG-rich aqueous phase improved from $K = 5 \pm 1$ to $8 \pm 2.5$ upon budding, where the partitioning
coefficient, $K$, is the concentration ratio of solute in the PEG-rich to dextran-rich aqueous phase. This is due to increased ATPS concentration with osmotic shrinkage; we have previously reported on the use of osmotic shrinkage to improve $K$ for solutes such as proteins in the interior of budded ATPS/GVs.\textsuperscript{15,16} For this GV, we observed a 22% volume loss; for the 8% PEG 8 kDa/8% dextran 10 kDa ATPS used here, this would result in concentration to $\sim 10$ wt % of each polymer, which according to the PEG/dextran/water phase diagram cannot be converted back to a single aqueous phase by moderate heating (i.e., $< 50 \degree C$).\textsuperscript{38}

**Figure 2-2:** Confocal optical microscopy images of interior aqueous polymer and external lipid asymmetry in budded, ATPS-containing giant vesicles. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled dextran 10 kDa (A) or Alexa-labeled PEG 5 kDa (B, C). Images have been false colored: blue indicates Alexa647, red indicates rhodamine, green indicates CF. Panels: transmitted light DIC (left), Alexa 647-labeled polymer fluorescence, (center), overlay of rhodamine-DOPE and CF-PEG-DSPE images (right). $T = 4 \degree C$ in (A) and (B), and $T = 6 \degree C$ in (C). All scale bars are 10 µm.
Additional examples of budded ATPS/GVs with $L_o/L_d$ coexistence are shown in Figure 2-2. The GV in Figure 2-2A was labeled with Alexa647-dextran 10 kDa (0.008%), while those in Figure 2-2B,C were labeled with Alexa647-PEG 5 kDa (0.02%). The partitioning of dextran 10 kDa is not as strong as that of PEG 5 kDa in these ATPS; this can be seen in the partitioning of the Alexa647 PEG 5 kDa in Figure 2-1 and 2-2B,C, as compared with that for dextran 10 kDa partitioning for the vesicle in Figure 2-2A. DIC and fluorescence images indicate that the dextran-rich phase has a smaller volume than the PEG-rich aqueous phase for the GV in Figure 2-2A,B, and the dextran-rich aqueous microvolume was larger in Figure 2-2C.

The structures described here differ in two important ways from previously reported liposome structures comprising lipid domains with polymer-free aqueous interiors.\textsuperscript{10,39–41} First, they contain coexisting interior aqueous microcompartments, each contacting different regions of the lipid membrane, such that not only the membrane, but also the model cytoplasm is chemically asymmetric. Second, the overall shape of the ATPS-filled GVs is governed primarily by the internal aqueous phases rather than by the line tension between, or differences in mechanical properties of, the lipid domains. This can be observed in Figure 2-2B, as well as the lower panel of Figure 2-1, where the (red) $L_d$ lipid phase is larger than required to encapsulate the dextran-rich bud, and also partially coats the PEG-rich GV body. The degree to which this occurs depends in part on the relative sizes of aqueous to lipid domains, which can be altered via the lipid and polymer compositions during preparation. We anticipate that line tension between, and mechanical properties of, $L_o$ and $L_d$, as well as interfacial tension at the ATPS boundary between the dextran-rich and PEG-rich aqueous phases also impact the observed
morphologies. For the recipes used here, the ATPS composition leads to ~2:1 volume ratio of PEG-rich to dextran-rich aqueous phase volumes in bulk, and similar mean volume ratio for GV-encapsulated ATPS, although the relative sizes of the aqueous phases varies for individual vesicles within a batch. \textsuperscript{15} Because the lipid composition used leads to roughly equal \( L_d \) and \( L_o \) domain areas,\textsuperscript{30} we generally see \( L_d \) phase domains that are larger than needed to coat the dextran-rich internal microvolume.

Possible explanations for the observed positioning of \( L_o \) and \( L_d \) lipid membrane domains around the PEG-rich and dextran-rich interior aqueous microvolumes include (1) differences in mechanical properties of the two \( L_d \) and \( L_o \) membrane domains, such that \( L_d \), which has lower bending rigidity,\textsuperscript{42,43} prefers to surround the higher curvature (i.e., smaller volume) dextran-rich bud; (2) interactions between lipid headgroups and the interior aqueous polymer solutions, e.g., PEGylated headgroups with PEG and dextran polymers; or (3) greater deformability of the \( L_d \) membrane domain, such that when budding occurs by expulsion of the interior dextran-rich microvolume from the body of the vesicle, the bud protrudes from this more flexible portion of the membrane. We will evaluate each of these possibilities below.

Two of the three possible explanations for the observed pinning of \( L_o \) and \( L_d \) membrane domains around PEG-rich and dextran-rich interior aqueous phases involved differences in mechanical properties between \( L_o \) and \( L_d \) lipid phase domains. The \( L_d \) domain is known to be more readily deformed than the \( L_o \) domain for DOPC/DPPC/cholesterol membranes;\textsuperscript{42} however, the effect of adding PEGylated headgroups to these ternary mixtures has not been evaluated. Our hypotheses (1) and (3) above assume no major effect on the relative rigidities of \( L_o \) vs \( L_d \) from the 2.25 mol %
PEGylated lipids. We note that this ignores any impact the presence and nonuniform distribution of PEGylated headgroups may have on $L_d$ vs $L_o$ mechanical properties. PEGylated headgroups are known to effect membrane mechanical properties, inducing membrane area expansion and altering bending elastic moduli.\textsuperscript{44–46} We have found that osmotically induced budding in ATPS/GVs with single-domain DOPC/DOPG membranes is favored by addition of PEGylated lipid, with increasing % budded vesicles as the mushroom-to- brush transition was approached.\textsuperscript{16} However, addition of 40% cholesterol, which increases membrane stiffness,\textsuperscript{9,47} to the DOPC/DOPG membranes inhibited budding.\textsuperscript{16} Thus, the increased flexibility provided by PEGylated lipids can be expected to affect $L_d$ domains to a greater degree than the cholesterol- rich $L_o$ domains, and therefore we anticipate that for our PEG- decorated membranes, $L_o$ remains more rigid than $L_d$.

Hypothesis (1). Can the observed structures be explained as the $L_o$ domains coating the region of lower curvature, which due to its larger volume is generally the PEG-rich vesicle body? Coexisting $L_d$ and $L_o$ lipid membrane phase domains are known to result in budded GV structures where buds are composed of different lipid domains than the vesicle body; these structures arise due to differences in mechanical properties between the two domains and from the line tension between them.\textsuperscript{39–41} In the ATPS/GV described here, the buds clearly form via a different mechanism, since they do not necessarily correspond to the different lipid domains, but rather to the interior polymer-rich microdomains of the encapsulated ATPS. This can be seen, for example, in Figure 2B, where the $L_d$ domain is larger than required to coat the dextran-rich bud, and thus partially coats the PEG-rich body of the ATPS/GV. The more flexible $L_d$ membrane
domain\textsuperscript{42} might be expected to localize onto the dextran-rich buds due to their greater curvature (smaller volume). However, our data exclude this explanation; when the relative sizes of PEG-rich and dextran-rich aqueous microvolumes are reversed, L\textsubscript{d} still coats the dextran-rich interior volumes, while the PEGylated L\textsubscript{o} membrane domain still coats the interior PEG-rich domain (Figure 2-2C).

The data in Figures 2-1 and 2-2 are consistent with interpretation (2) above, i.e., that chemical interactions between the PEG-rich interior aqueous phase and the PEGylated lipid headgroups concentrated in the L\textsubscript{o} membrane domain drive the observed positioning of membrane domains. In addition to the positioning of the PEGylated L\textsubscript{o} membrane domain around the PEG-rich aqueous bud, the apparent localization of the CF-PEG-DSPE lipid into L\textsubscript{o} improved nearly 2-fold upon budding (from a fluorescence intensity ratio for the CF-PEG-DSPE in L\textsubscript{o}/L\textsubscript{d} of $K_{\text{PEG-DSPE}} = 2.2 \pm 0.4$ before to $4 \pm 1$ after budding). This increase in apparent CF-PEG-DSPE lipid partitioning into the L\textsubscript{o} membrane domain can be interpreted as a reaction to the change from both domains (L\textsubscript{o} and L\textsubscript{d}) contacting only the PEG-rich interior aqueous microvolume prior to budding, to these domains contacting primarily the PEG-rich and dextran-rich microvolumes, respectively, after budding. In bulk PEG/dextran ATPS, incorporation of DSPE-PEG in small unilamellar vesicles (~120 nm) results in strong partitioning of the PEGylated SUVs into the PEG-rich aqueous phase.\textsuperscript{48} In our ATPS/GVs, we cannot entirely rule out the possibility that the decrease in membrane area (~9\%) upon budding itself contributes to the change in $K_{\text{PEG-DSPE}}$. Although the separation of the CF moiety from the lipid headgroup by the PEG 2 kDa presumably shields the dye itself from large changes in local environment, steric interactions between adjacent PEGylated headgroups may be
impacted by this change in membrane area, which could alter the distribution of CF-PEG-DSPE.

These data cannot rule out possibility (3) above, where buds exit the body of the vesicle at the point where mechanical deformation of the membrane is most facile (i.e., L_d domains). We evaluated this possibility by moving the PEGylated headgroups from the L_o to the L_d domain. By incorporating PEG2000-DOPE and omitting the PEG2000-DPPE and CF-PEG2000-DSPE used in the experiments described above (Figures 2-1 and 2-2), it was possible to localize the L_d phase domain around the PEG-rich aqueous microvolume. Figure 2-3 shows DIC and conventional fluorescence optical microscope images for a budded ATPS/GV in which PEGylated headgroups were included on lipids targeting the L_d membrane phase domain. Here, green fluorescence indicates FITC-labeled dextran polymer in the aqueous phase, and red fluorescence indicates DOPE-rhodamine, labeling the L_d domain of the lipid membrane. When the L_d domain lipid presents PEG moieties, it is localized to the (unlabeled) PEG-rich microvolume. Thus, despite the more facile deformability of the PEGylated L_d domain, the dextran-rich bud in this vesicle is surrounded by the L_o membrane domain. We note that both the higher PEG headgroup density and the mechanical properties of L_d without PEGylated lipid suggest that the PEGylated L_d domain is more readily deformed; the dextran bud nonetheless occurs at the L_o domain in this experiment. These data argue against membrane mechanical properties, and for polymer-polymer interactions, in determining the locations of L_d and L_o membrane domains with respect to interior PEG-rich and dextran-rich aqueous microvolumes.
Figure 2-3: Optical microscope images for ATPS/GV where Ld lipid domain harbors the PEG2000 functionalization. GVs were prepared with 1:1 DOPC: DPPC + 30% cholesterol, and 2.2% DOPE-PEG2000, and imaged at 7 °C. The Ld phase of the vesicles was stained with 0.1% DOPE-rhodamine and is false colored red in the fluorescent images; the Lo phase was not labeled and appears dark. We also added 0.05% FITC-labeled dextran 500 kDa (green) to facilitate identification of the dextran-rich aqueous phase. The DOPE-PEG2000 is targeted to the Ld phase (red); the Ld phase coated the non-labeled PEG enriched bud and the Lo phase the dextran bud. Scale bar is 10 μm.

Further support for the role of internal aqueous polymer composition on membrane domain positioning comes from the behavior of these ATPS/GVs when exposed to changes in temperature. Figures 2-4 and 2-5 show the effect of temperature. The vesicles in Figure 2-4 show the typical morphology for the ATPS/GV recipes used here, with a smaller volume dextran-rich bud and a larger PEG-rich vesicle “body”; the dextran-rich phase appears denser in the DIC transmitted light images. Here, different representative vesicles from the same sample are shown at each temperature. The vesicle in Figure 2-5 was labeled with Alexa647 PEG 5 kDa so that polymer partitioning between the aqueous phases could be monitored, and was followed as the temperature was changed (this is the same vesicle as shown in Figure 2-2C). Initial images at low temperature, 4 and 11 °C for Figures 2-4 and 2-5, respectively, show two coexisting lipid domains, Lo (green) surrounding the PEG-rich aqueous compartments and Ld (red) surrounding primarily the dextran-rich aqueous buds. Heating led first to the
fragmentation of the $L_o$ and $L_d$ domains into smaller patches that were no longer pinned to the interior aqueous microvolumes (**Figures 2-4B and 2-5B**). At higher temperatures ($49 \, ^\circ{C}$) and longer incubation times (75 min), complete lipid miscibility was observed (**Figure 2-5C**).

**Figure 2-4:** Effect of temperature on membrane domain localization in ATPS-containing GV. Different regions of the sample are shown initially at $4{\circ}C$ (left), after heating to $35{\circ}C$ (center), and subsequent cooling to $4{\circ}C$ (right). Top panel is transmitted light, DIC; bottom panel is overlay of rhodamine-DOPE and CF-PEG-DSPE fluorescence. Fluorescence images have been false colored: red indicates rhodamine, and green indicates CF. Scale bar is $50 \, \mu{m}$.
Figure 2-5: Effect of temperature on membrane domain localization in a single ATPS-containing GV. Fluorescence images have been false colored: green (left panels) indicates CF-PEG-DSPE, red (center panels) indicates rhodamine-DOPE, and blue (right panels) indicates Alexa647 PEG 5 kDa. Scale bar is 10 µm.

For the DOPC:DPPC:cholesterol ratio used here, the miscibility temperature ($T_{\text{mix}}$) is $32 \pm 1 ^\circ C$; however, our ATPS/GVs retained some phase separation after ~10 min at 35 °C. Our membrane composition also includes 2.25% PEGylated lipid, which can be expected to impact $T_{\text{mix}}$ and/or slow the phase transition by reducing lipid diffusion rates in the membrane. Literature reports on PEGylated lipids added to single
component DPPC membranes indicate that while the PEG moieties can lead to slight reductions in the chain melting temperature, when the PEGylated lipid has a longer acyl chain length than the major lipid component (e.g., 18-carbon DSPE vs 16-carbon DPPC), the melting temperature is instead slightly increased. To our knowledge, the effect of PEGylated lipids on ternary DOPC:DPPC:cholesterol mixtures such as were used here has not been investigated. However, minor component lipid impurities can alter $T_{\text{misc}}$. For example, Keller and co-workers have found $\sim 3 \, ^\circ\text{C}$ increase in $T_{\text{misc}}$ for a ternary DOPC:brain sphingomyelin:cholesterol system upon addition of 1% ganglioside GM1, which partitions into the $L_o$ domain. Additionally, when impurities accumulate at phase boundaries, they can reduce line tensions and enable domain persistence at elevated temperatures.

Although as noted above, the internal ATPS does not revert to a single phase upon heating because the polymer solution was concentrated during budding and no longer corresponds to the composition during the GV formation process, some reduction in polymer partitioning might be anticipated at elevated temperature. We therefore monitored the fluorescence intensity ratio of Alexa647-PEG 5 kDa in the PEG-rich and dextran-rich aqueous phases. For the ATPS/GV shown in Figure 2-5, any change in $K$ for the Alexa647 PEG with temperature was not statistically significant, given the error of our measurement ($K = 11 \pm 4$ at 11 °C and $9 \pm 2$ at 49 °C).

In order to quantify the change in localization of the PEGylated $L_o$ domain to the PEG-rich interior aqueous microvolume that occurred as a function of temperature, we calculated an intensity ratio for CF-PEG-DSPE in the membrane surrounding the PEG-rich vs dextran-rich aqueous phases ($R_{\text{PEG}}$). $R_{\text{PEG}}$ was determined by measuring the mean
fluorescence intensity for CF-PEG-DSPE in the membrane surrounding the PEG-rich vesicle bodies and dividing by the mean intensity around the dextran-rich buds. This value is not a partitioning constant, but rather an estimate of PEGylated lipid location with respect to the interior aqueous compartments. Twenty ATPS/GVs were analyzed at 4 and 35 °C, from the sample shown in Figure 4. We found $R_{\text{PEG}} = 4 \pm 1$ initially at 4 °C and $R_{\text{PEG}} = 1.0 \pm 0.1$ after heating to 35 °C. Because even at 35 °C, $L_o$ and $L_d$ membrane domains remained, and most of the CF-PEG-DSPE was concentrated in the $L_o$ domains, $R_{\text{PEG}}$ also indicates the position of the $L_o$ domains with respect to interior PEG-rich vs dextran-rich volumes. At 4 °C, part of the $L_d$ membrane domains, which are localized around the dextran-rich buds, extend onto the PEG-rich vesicle bodies as a consequence of the size mismatch between internal volumes and the membrane domains. At 35 °C, $L_o$ and $L_d$ patches were distributed evenly around the budded GVs. This suggests that the interaction between PEGylated lipids concentrated in the $L_o$ regions and the free PEG polymer molecules concentrated in the PEG-rich phase of the vesicle interior is of relatively low affinity, and is overcome by heating to 35 °C. Subsequent cooling back to 4 °C, restored the $L_o$ and $L_d$ membrane domains to their positions around the PEG-rich and dextran-rich internal aqueous micro-volumes, respectively, with $R_{\text{PEG}} = 3.5 \pm 0.4$. The reversible pinning of lipid domains by the internal aqueous domains demonstrates that lipid domain position is not random, and does not result from the differences in mechanical properties of the $L_o$ and $L_d$ membrane domains but rather is controlled by internal aqueous phase position.
2.4 Conclusion

We have demonstrated the interrelationship of interior aqueous and exterior lipid organization in this very simple cytomimetic model. Here, the “pinning” of lipid domains to internal aqueous compartments was caused by nonspecific interactions between PEGylated lipid headgroups and a PEG-rich internal aqueous phase. More complex and/or specific biorecognition schemes could also be used to control lipid phase location with respect to interior aqueous components. Although living cells may not contain simple micrometer-scale aqueous or lipid domains like those used here, the cytoplasm and plasma membranes are characterized by heterogeneity, which via specific or nonspecific interactions with lipid headgroups, glycolipids, or membrane proteins could be expected to organize similarly to what we observed here with this very simple model system.
2.5 References


24. Abbreviations: DOPC, 1,2-dioleoyl-<i>sn</i>-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-<i>sn</i>-glycero-3-phosphocholine; rhodamine-DOPE, 1,2- dioleoyl-<i>sn</i>-glycero-3-phosphoethanolamine-<i>N</i>-lissamine rhodamine B sulfonyl; PEG-DPPE, 1,2-dipalmitoyl-<i>sn</i>-glycero-3-phospho-ethanolamine-<i>N</i>-methoxy(polyethyleneglycol2000]; CF-PEG-DSPE, 1,2- distearoyl-<i>sn</i>-glycero-3-phosphoethanolamine-<i>N</i>-[poly(ethylene glycol)2000-N-carboxyfluorescein].


38. An additional change upon osmotically induced shrinkage in Figure 1 is worth noting: the interior lipid material seen in the top panel has condensed and moved to the ATPS interface in the lower panel. This is consistent with increased interfacial tension at the aqueous/aqueous phase boundary due to concentration of the ATPS, increasing the driving force for particle collection. Additionally, increased macromolecular crowding within each of the aqueous phases may play a role in condensation of the lipid material. Collection of lipid material at the ATPS interface within preformed ATPS/GV may provide a route to separating the two aqueous microcompartments, a step toward a primitive model of cell division.


Chapter 3

Complete Budding and Asymmetric Division of Primitive Model Cells to Produce Daughter Vesicles with Different Interior and Membrane Compositions


3.1 Introduction

An individual biological cell is the smallest living entity, and yet even the simplest living cells are overwhelmingly complex. Model cells designed to mimic one or more key aspects of their biological counterparts are therefore very attractive as a route to understand the chemical and physical basis of cell structure and function. Lipid vesicles have long been used as models for the membranes of biological cells.\(^1\)-\(^2\) They are simple to prepare and enable the lipid composition to be varied as desired for fundamental studies of membrane biophysics. Giant vesicles (GVs), which are defined as those having diameters greater than a micron,\(^1\)-\(^5\) are of particular interest because they are on the same scale as living cells and are amenable to fluorescence optical microscopy. Important insights into the role of lipid composition in, e.g., transmembrane diffusion, membrane mechanical properties, and lipid phase separation have been gained from such studies.\(^2,6,7\) Remarkable morphological transformations including vesicle fusion, budding, and fission have been observed in these model membranes.\(^8\)-\(^15\)
A wide variety of molecules and materials have been encapsulated within the aqueous interior of lipid vesicles. These range from simple sugars incorporated to enhance image contrast during microscopy to polymers, enzymes, hydrogels, smaller vesicles, or complex collections of molecules such as functional transcription and translation machineries.\textsuperscript{2,16-23} With very few exceptions,\textsuperscript{18,19,24-28} these encapsulated materials have been uniformly distributed throughout the interior volume of the GVs. In contrast, biological cells display intracellular organization including not only organelles and the cytoskeleton, but also less obvious microcompartments such as multienzyme complexes and heterogeneous local protein concentrations\textsuperscript{29}. Intracellular microcompartmentation is dynamic, with changes in local concentrations of various molecules occurring throughout the cell cycle and in response to stimuli\textsuperscript{30-32}. For example, the enzymes of the de novo purine biosynthetic pathway co-localized only when purines were not provided in cell growth media\textsuperscript{31}, and the assembly of glycolytic enzymes onto erythrocyte membranes is thought to be regulated by phosphorylation and oxygenation\textsuperscript{32}. The bacterium \textit{C. crescentus} was recently shown to generate intracellular gradients of protein phosphorylation and consequently DNA replication prior to asymmetric division. The resulting daughter cells differ markedly in morphology and behavior, with one remaining attached to the underlying surface via a stalk and the other using a flagellum to swim away.\textsuperscript{33}

Asymmetric division of living cells, in which the resulting daughter cells inherit different biochemical compositions, is crucial for cell differentiation and development in multicellular organisms and also common in unicellular organisms such as yeast and \textit{C. crescentus}.\textsuperscript{34-36} Additionally, the asymmetric inheritance of degraded proteins has been
implicated in aging\textsuperscript{37}, and malfunctions in asymmetric division are thought to play a role in cancer\textsuperscript{35}. Mechanisms for asymmetric division in living cells can involve external gradients supplied by the cell’s surroundings and/or the asymmetric intracellular distribution of molecules that act as cell fate determinants.\textsuperscript{38} A large number of genes have been implicated in generation of biochemical polarity and facilitation of division into nonidentical daughter cells.\textsuperscript{38} In addition to the genetic component of cellular asymmetry, a spatial, biophysical component seems likely to serve as the initial cue for the polarity axis.\textsuperscript{39} Since newly-formed cells arise by division of existing cells, the membrane is inherited from the pre-division (“mother”) cell, as are the cytoplasm and intracellular contents. An attractive hypothesis is that this inherited material, by providing the cellular architecture in which the genes act, plays a crucial role in the inheritance of polarity. For example, an inherited patch of distinct membrane composition might provide a physical location at which to anchor the cascade of polarity-related molecules and events governed by gene expression in the daughter cells.\textsuperscript{39}

We have developed simple model cells that encapsulate a synthetic “cytoplasm” capable of intracellular compartmentation and (bio)chemical polarity.\textsuperscript{24,25,27} Our models cells are based on aqueous phase separation in giant lipid vesicles.\textsuperscript{26} An aqueous two-phase system (ATPS)\textsuperscript{40-43} containing PEG and dextran polymers serves as a primitive model for the cytoplasm, providing macromolecular crowding\textsuperscript{44} and distinct microcompartments formed by the two aqueous phases. Differences in local protein concentration can be maintained spatially within individual vesicles by partitioning into the PEG-rich or dextran-rich aqueous phase, and modified by changes in temperature, osmotic pressure, or pH.\textsuperscript{24,25,28,40-42} These very simple model cells contain no nucleic
acids or enzymes, just this cytoplasm-mimicking polymer solution, the membrane lipids, and some fluorescent proteins added to demonstrate biomolecule compartmentalization. Membrane heterogeneity in the form of coexisting lipid phase domains has been incorporated by using ternary lipid compositions that give rise to liquid disordered (L$_{d}$) and liquid ordered (L$_{o}$) regions having different lipid composition.$^{8,45-47}$

Herein, we report complete budding and asymmetric fission of these model cells to form nonidentical daughter vesicles that differ in their “cytoplasmic” and membrane compositions, and in some cases are themselves polarized. The ATPS-containing GVs investigated here adopted budded geometries due to osmotic stress, as had been observed previously.$^{25,27}$ As external osmolality was increased further, fission of these vesicles occurred, producing nonidentical daughter vesicles. Fission occurred at the aqueous-aqueous phase boundary, resulting in one daughter vesicle that contained the PEG-rich aqueous phase and another that contained the dextran-rich aqueous phase. In some cases fission was incomplete, with the two daughter vesicles remaining connected by a lipid nanotube; this morphology has been termed “complete budding”.$^{13,45}$ When coexisting lipid membrane phase domains were also present, these were also inherited unequally, with the PEGylated L$_{o}$ domain surrounding the PEG-rich aqueous phase while the L$_{d}$ domain surrounded the dextran-rich aqueous phase. Fluorescent proteins incorporated in the dextran-rich phase of the aqueous interior and bound to the L$_{o}$ membrane domain were also asymmetrically inherited by the daughter vesicles. When the available surface area of the L$_{o}$ and L$_{d}$ domains did not match the volumes of the PEG-rich and dextran-rich aqueous phases, one of the daughter vesicles inherited both L$_{o}$ and L$_{d}$ domains. This daughter could then be exposed to further osmotic stress to generate a second aqueous
phase separation and provide asymmetric localization of the newly formed interior aqueous phases.

3.2 Experimental Materials and Methods

3.2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (DPPE-PEG 2000), 1,2-distearoyl-sn-glycero-phosphoethanolamine-N-[biotinyl(polyethylene glycol)2000] (DSPE-PEG 2000-biotin), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol)2000-N'-carboxyfluorescein] (DSPE-PEG 2000-FITC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-rhodamine), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] were purchased as chloroform solutions from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was from Supelco (Bellefonte, PA). The polymers, poly(ethylene glycol) (PEG) 8 kDa, dextran 10 kDa, and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO). Alexa Fluor 647-conjugated lectin SBA, Alexa488-labeled streptavidin, Alexa 647-conjugated dextran 10 kDa, and the press-to-seal silicone spacers were from Invitrogen (Eugene, OR). Water used in these experiments was purified to a resistivity of
≥ 18.2 MΩ with a Barnstead NANOPure Diamond system from Barnstead International (Dubuque, IA).

3.2.2 Preparation of Giant Vesicles with co-existing fluid phases encapsulating an Aqueous Two-Phase System

Lipid vesicles were formed using the gentle hydration method, as previously described\textsuperscript{[85]}, with slight modifications\textsuperscript{[24,25,27]}. Briefly, a 1:1 molar ratio of DOPC/DPPC + 30% cholesterol was prepared by the addition of 34% DOPC, 34% DPPC, 30% cholesterol, 2.0% DPPE-PEG 2000, 0.09% DOPE-rhodamine, and 0.08% DSPE-PEG2000-biotin to a test tube (10 x 75 mm, Durex borosilicate glass, VWR, Int., West Chester, PA) containing ~100 μL chloroform. The lipid solution was then dried under Ar (g) to produce a thin, lipid film. Residuals of chloroform were removed by placing the test tube under vacuum desiccation for approximately 2 hours. During this time, a bulk ATPS solution consisting of 7 wt % PEG 8 kDa and 10 wt % dextran 10 kDa in water was prepared and incubated at 43°C (Figure 3-1 shows the phase diagram and the temperature-dependence of phase separation in this system). Next, 990 μL of warm, single-phase polymer solution and 10 μL of Alexa Fluor 647-lectin SBA (2 mg/mL) were added along the wall of the test tube and the lipids were hydrated at 43 °C for approximately 48 hours. The same procedure was followed for the preparation of GVs with ratios of 1:1.5 (22.7 mol % DOPC, 45.5 mol % DPPC) and 1:2 DOPC/DPPC + 30% cholesterol (27.2 mol % DOPC, 40.7 mol% DPPC).
Figure 3-1: A portion of the phase diagram for PEG 8 kDa/dextran 10 kDa ATPS. The concentration used to prepare ATPS-containing GVs in this work was 7 wt % PEG 8kDa and 10 wt% dextran 10 kDa, which lies just below the coexistence curve at 37 °C, such that the solution exists as a single phase at this temperature. At 5 °C, this composition is above the coexistence curve and exists as two distinct aqueous phases. Points were determined by cloud point titration, and lines are a polynomial fit included to guide the eye.

3.2.3 Preparation of ATPS/GV samples for confocal microscopy

After vesicle formation at 43 °C, sample vials were transferred to 5 °C, a temperature below the ATPS transition, to drive phase separation both in the bulk solution and vesicle interior. Vesicles accumulated at the interface of the phase-separated bulk ATPS, from which 1-2 µL of vesicles were removed and transferred to a shallow well made from placing a silicone spacer on a microscope coverslip (24 x 60 mm, VWR Int., West Chester, PA). Vesicles were first diluted with 10 µL of 5°C PEG-rich top phase, and then an aliquot (10-30 µL) of 130 mM sucrose and 0.5 µL of Alexa 488-streptavidin (9.25 µM) were added to the sample solution. An Anodisc 25 membrane (0.2
µm diameter pores) (Whatman International Ltd., Maidstone, England) was placed on top of the press-to-seal silicon spacer to facilitate addition of further aliquots of sucrose solution with minimal disturbance of the vesicles under observation (when solution is pipetted directly in rather than through the membrane, flow often results in loss of the vesicles from the field of view). A sucrose solution was added every 10-15 minutes, each time increasing the external solution concentration by approximately 13%, until fission occurred. The 10-15 minute delay provided sufficient time for morphological changes; no further changes occurred after 15 minutes unless additional changes in osmolality were provided. The amount of sucrose needed to achieve fission varied from vesicle to vesicle due to variability in the PEG and dextran encapsulation and in vesicle volume.

3.2.4 Quantification of protein partitioning in ATPS-containing vesicles

Protein (Alexa Fluor 647-lectin SBA) and polymer concentrations (Alexa Fluor 647-dextran 10 kDa) in the PEG-rich and dextran-rich phases were determined from their fluorescence intensities by taking a line scan across the PEG-rich and dextran-rich compartments in the vesicle. Solute concentrations were determined directly from the confocal fluorescence intensities using a calibration curve of the labeled protein at different concentrations also acquired on the confocal microscope under identical imaging conditions. Partitioning was calculated as the partition coefficient, K, defined as $K = \frac{C_p}{C_d}$ where $C_p$ is the concentration of the solute in the PEG-rich phase and $C_d$ is its concentration in the dextran-rich phase.
3.2.5 Instrumentation and Software

ATPS GV confocal images were acquired using an Olympus IX-70 laser scanning confocal inverted microscope (LSCM) (Nikon Plan Apo 60X 1.4 NA objective) or an LSM-5 Pascal Laser Scanning confocal microscope from Carl Zeiss, Inc. (Oberkochen, Germany) with a Plan-Apochromat 63 x oil immersion objective (1.4 NA) and Pascal Software as previously described.\textsuperscript{25,27} A temperature-controlled PE-100 microscope stage, a PE-94 control unit (both from Linkam, ± 0.1ºC), and a circulating water bath from VWR (model 1160A) were used to control GV suspension temperature. A microprobe (model IT-21) from Harvard apparatus and a PhysitempBAT-12 readout unit (± 0.1ºC) were used to directly measure GV suspension temperature. A VAPRO vapor pressure osmometer (model 5500) from Wescor, Inc. was used to measure solution osmolality at 25ºC.

3.3 Results and Discussion

Model cells were prepared by encapsulating a PEG 8 kDa/dextran 10 kDa aqueous two-phase system during formation of giant lipid vesicles by gentle hydration as previously described.\textsuperscript{24-28} Briefly, the polymer solution was heated to 42 ºC, where it exists as a single phase, during vesicle formation and subsequently cooled to induce phase separation (5 ºC). The ATPS-containing giant vesicles were then collected from the bulk ATPS interface and placed in a sucrose solution for observation under the confocal microscope. Due to the preparation protocol which led to some concentration of the solution due to evaporation, in the work described here most of the vesicles had
already adopted a budded morphology prior to observation.\textsuperscript{48,49} An example of the budding transition is shown in Figure 3-2. We will first describe the morphology of model cells with a single-domain membrane composed primarily of DOPC, with 29 mole% cholesterol and small amounts of both DOPE-PEG-2K and DOPE-rhodamine, followed by those with micron-scale coexisting L\textsubscript{o} and L\textsubscript{d} membrane domains. It should be noted that the precise osmolality required to achieve a particular morphology depends on the concentrations of PEG and dextran polymers inside the vesicles in both a straightforward way and also by impacting the composition of each phase and value of the ATPS interfacial tension. Variability in the contents of different individual vesicles within a batch is expected based on our previous studies of polymer encapsulation.\textsuperscript{16} We increased the osmolality as needed to force morphological changes in the ATPS-containing vesicles explored here. A sucrose solution was added every 10-15 minutes, each time increasing the external concentration by approximately 13% until complete budding or fission occurred. Measurements were performed to determine the osmolality of the starting and ending solutions, which was not additive for these non-ideal solutions.
Figure 3-2: An ATPS-containing GV before (left panels) and after (right panels) budding was induced by exposure to a hypertonic sucrose solution. Fluorescence images have been false colored: blue indicates Alexa647, red indicates rhodamine, and green indicates carboxyfluorescein (CF). Images: transmitted light DIC (top), overlay of rhodamine-DOPE and CF-PEG-DSPE fluorescence images (center), and Alexa647-labeled PEG 5 kDa fluorescence (bottom). T= 4°C. Scale bar is 10 µm.

The effect of osmotic stress on a budded, ATPS-containing GV is shown in Figure 3-3. The initial budded structure had two coexisting aqueous microcompartments corresponding to the two aqueous phases, one enriched in PEG and the other enriched in dextran. The dextran-rich aqueous phase was stained with Alexa 647-labeled dextran 10
kDa for visualization. Addition of sucrose eventually increased the osmolality of the surrounding solution from 122 ± 1.5 mmol/kg to 163 ± 2.6 mmol/kg$^{50,52}$, resulting in the transformation of the initial budded geometry (left) to two spheres connected by a narrow neck (middle panels), and finally to two separate, spherical vesicles that are no longer connected (right). These morphological transformations can be understood in terms of the osmotic pressure difference between the interior and exterior of the vesicles, which resulted in water loss, reducing their volume and concentrating the interior polymer solutions. This provided both excess membrane area over what was required to coat the now smaller volume of the vesicle, and increased interfacial tension between the now more concentrated PEG-rich and dextran-rich aqueous phases, driving fission of the mother vesicle.

**Figure 3-3:** Fission of an ATPS-containing GV in response to osmotic stress. Osmolality increases from left to right. Confocal fluorescence images have been false-colored: red indicates lipid fluorescence (DOPE-rhodamine) and blue indicates Alexa 647-conjugated dextran 10 kDa. The Alexa647 signal decreased over time due to photobleaching; the blue channel has been adjusted to make the partitioning of Alexa 647-conjugated dextran 10 kDa for each timepoint more apparent. T = 5°C. Scale bar is 10 µm.
Fission resulted in chemically distinct daughter vesicles, one containing the dextran-rich aqueous phase and the other containing the PEG-rich aqueous phase of the initial aqueous two-phase system from the mother vesicle (Figure 3-4A). The inheritance of distinct aqueous phase volumes provided chemical asymmetry between the daughter cells because the PEG and dextran polymers were present at different concentrations in the two aqueous phases. Partitioning is quantified in terms of the partition coefficient, $K$, which is the concentration ratio of solute in the PEG-rich phase, $C_p$, as compared to the dextran-rich phase, $C_d$: $K = C_p/C_d$. To determine the solute concentration in the aqueous compartments, line scans were performed across both the PEG-rich and dextran-rich aqueous phases in the GV, and results were compared with calibration curves. For the budded vesicles in Figure 3-3, $K = 0.60$ for the fluorescent dextran, indicating 1.7$\times$ higher concentration in the dextran-rich phase. After fission, the dextran-rich daughter vesicle contained a correspondingly higher concentration of fluorescent dextran than the PEG-rich daughter vesicle.

Although vesicle fission has been predicted and observed previously in GVs containing homogeneous aqueous interiors, the presence of the microcompartmentalized model cytoplasm is an important distinction. Fission of vesicles with simple aqueous interiors has been induced by various external stimuli including laser illumination, heat, changes in phase transitions, and by the addition of phospholipase A$_2$ and various single-long chain amphiphiles. For each of these examples, fission was symmetric: each daughter vesicle contained the same interior aqueous solution. In contrast, Figure 3-3 shows that for ATPS-containing vesicles,
fission occurs at the aqueous-aqueous phase boundary such that daughter vesicles have different internal aqueous compositions (Figure 3-4A).

**Figure 3-4:** Schematic showing asymmetric fission of model cells.

Daughter vesicles having different lipid membrane compositions but the same aqueous interior contents have been generated by fission of vesicles with coexisting lipid phase domains (e.g., L_o and L_d). Asymmetric membrane inheritance, where L_d membrane goes to one daughter and L_o to another, occurs when vesicles are exposed to osmotic...
shock or heated. In these systems, budding and fission are driven by the reduction of line tension at the liquid-liquid phase boundary between the $L_o$ and $L_d$ membrane domains.\textsuperscript{13,47} This mechanism fixes the location of fission at the $L_o/L_d$ boundary, such that each daughter vesicle inherits only one lipid phase domain. This can be seen as a two-dimensional membrane analogue of how the three-dimensional aqueous phase domains were split between the daughter vesicles in Figure 3-3.

We next formed model cells that combined interior aqueous phase separation with membrane $L_o/L_d$ phase separation. This was accomplished by incorporating a ternary lipid composition selected to provide lateral phase separation based on the phase diagrams from the Keller lab,\textsuperscript{7,46} with a few modifications to adapt it for use in our work. Specifically, lipids having PEGylated headgroups were added to provide preferential wetting of the PEG-rich aqueous interior phase with a PEGylated $L_o$ membrane domain; this preferential wetting and the altered temperature-dependence of $L_o/L_d$ phase separation in our ternary lipid mixture has been explored in a previous publication.\textsuperscript{27} Here, we also incorporated greater biomolecular complexity in these model cells by adding fluorescently-labeled proteins to the interior and exterior of the vesicles. Soybean agglutinin (SBA) labeled with Alexa647, was added to the ATPS; this protein partitions into the dextran-rich aqueous compartment. A small amount of biotinylated lipid, DSPE-PEG-2K-biotin, which partitions into the $L_o$ membrane phase domain, was added during vesicle formation. Streptavidin labeled with Alexa488 was added after vesicle budding to stain the $L_o$ domain (Figure 3-5). Figure 3-6 (leftmost panels) shows the distribution of these molecules in the budded model cells. The SBA was found as anticipated in the aqueous phase wetted by the $L_d$ membrane; this is consistent with the dextran-rich phase
based on known partitioning of SBA in PEG/dextran ATPS\textsuperscript{24,25} and with our previous work with L\textsubscript{o} and L\textsubscript{d} membranes wetting PEG/dextran ATPS.\textsuperscript{27} For the initial vesicles in Figure 3-6A and B (left), the local lectin concentration was $\sim 4\times$ higher in the dextran-rich phase as compared to the PEG-rich aqueous phase (e.g., for the vesicle in Figure 3-6A, $C_p = 64 \pm 5$ nM, $C_d = 264 \pm 13$ nM).

\textbf{Figure 3-5:} ATPS/GV before (top) and after (bottom) the addition of a membrane-bound protein. Alexa488-streptavidin was added to the external solution, upon which it bound to the biotinylated headgroups (DSPE-PEG 2000-biotin) in the L\textsubscript{o} phase. Confocal images are as follows: DIC (top), DOPE-rhodamine (red, middle) and Alexa488-streptavidin (green, middle left) and Alexa647 fluorescence (bottom). T= 5°C. Scale bar = 10 μm.
Figure 3-6: Effect of osmotic stress on two ATPS-containing GVs (A and B) in which lipid membrane phase coexistence was present. The membrane composition for both vesicles was 1:1 DOPC/DPPC + 30% cholesterol, with 2.4% DPPE-PEG-2K, 0.09% DSPE-PEG-2K-biotin, and 0.4% DOPE-rhodamine. Osmolality increases from left to right. Confocal fluorescence images have been overlaid and false-colored: red is DOPE-rhodamine, indicating the L_d membrane domain, and green is streptavidin-Alexa488, bound to DSPE-PEG-2K-biotin, which is partitioned into the L_o membrane domain. Blue indicates lectin SBA-Alexa 647. Arrows on the far right indicate the location of lipid nanotubes between the daughter vesicles. T = 5°C. Scale bars are 10 µm.

Approximately every 15 minutes after initial image acquisition, sucrose was added to increase the osmotic pressure of the external solution, from 122 ± 1.5 mmol/kg initially to 142 ± 1.5 mmol/kg for the vesicle shown in Figure 3-6A. As was observed for the single-phase membranes shown in Figure 3-3, separation into two spherical or quasispherical daughters occurred in response to the osmotic stress. Here, the dextran-rich daughter vesicles contained higher internal protein (SBA) concentrations and were surrounded by L_d membrane, and the PEG-rich daughter vesicles were in contact with the
L_o membrane, on which the membrane-bound protein (streptavidin) was localized. Thus, separation resulted in asymmetric inheritance of both the interior composition, including a soluble protein, and of the membrane lipids with their associated protein. For example in Figure 3-6A, the concentration of “cytoplasmic” protein was 5-fold higher in the dextran-rich daughter vesicle as compared to the PEG-rich daughter vesicle ($K = 0.19 \pm 0.02$). Fluorescence signal from the labeled streptavidin was only associated with the L_o phase domain and hence a partitioning coefficient for this protein in the membrane cannot be calculated; it appears to have been inherited exclusively by the PEG-rich daughter vesicles. Similar results were obtained for the vesicle in Figure 3-6B, for which osmolality was increased from $108 \pm 2.6$ mmol/kg initially to $216 \pm 6.5$ mmol/kg in the panel to the far right. Due to differences in vesicle volume vesicle-to-vesicle variability in the internal concentrations of PEG and dextran polymers, the external osmolality required to induce these morphological changes was not identical for all vesicles.

In some cases a lipid nanotube could be seen connecting the daughter vesicles (see arrows in far right panels, Figure 3-6A and B). Such structures are not uncommon in the vesicle literature; when a force is applied to a vesicle, the membrane can deform to produce lipid tubes (tethers), as a way to protect the integrity of the membrane. Complete budding is distinct from fission in the presence of a shared lipid nanotube and its aqueous contents. Membrane tethers have been produced by means of hydrodynamic flow, micropipettes, optical tweezers, and kinesin motor proteins. Li et al recently reported the generation of many lipid nanotubes inside ATPS-containing giant vesicles in response to osmotic stress. There, nanotubes accumulated at the aqueous/aqueous interface and in effect served as a storage site for excess membrane
area, which could be pulled back into the main membrane by increasing membrane tension.\textsuperscript{61} We presume that nanotube formation similarly occurred here as a result of excess membrane area upon volume loss, and the accumulation of this lipid material at the aqueous/aqueous phase boundary may have facilitated both the fission and/or complete budding transitions and the formation of nanotube tethers that often connected the daughter vesicles. For the two vesicles shown in Figure 3-6, the connecting nanotubes did not break even at the highest osmolalities tested (142 ± 1.5 mmol/kg for Figure 3-6A, and 216 ± 6.5 mmol/kg for Figure 3-6B).

Co-existing L\textsubscript{o} and L\textsubscript{d} domains can facilitate fission of tubes pulled from GVs.\textsuperscript{62,63} For example, Allain et al. demonstrated that breakage, or fission, occurred in membrane nanotubes with co-existing L\textsubscript{o}/L\textsubscript{d} domains, and was not observed in homogenous lipid membrane tethers.\textsuperscript{62} For the vesicles in Figure 3-6, the nanotubes appear to be a single phase. The daughter vesicles in Figure 3-6A are connected by a nanotube composed of lipid in the L\textsubscript{o} phase (see also Figure 3-7) while the nanotube in 3-6B appears to be entirely composed of L\textsubscript{d} lipids. The nanotube in Figure 3-6B appears to display pearling; pearling instabilities have been reported when tubular structures were destabilized by optical tweezers\textsuperscript{64}, induced curvature,\textsuperscript{65} anchored polymer\textsuperscript{66}, and nanoparticle binding\textsuperscript{67} and observed in axons in which pearling was driven by osmotic perturbations\textsuperscript{68}. 
Figure 3-7: Response of an ATPS-containing GV to osmotic stress. Here, the PEG-rich and dextran-rich daughter vesicles remained connected by a lipid nanotube. Osmolality increases from left to right. Fluorescence images have been false colored. Blue indicates Alexa647, green indicates carboxyfluorescein (CF), and red indicates Cy3 fluorescence. Images: overlay of DOPE-CF and streptavidin-Cy3 (bound to lipid DSPE-PEG-2000-biotin) fluorescence (top), and lectin SBA-Alexa647 fluorescence (bottom). For clarity, the color channels are displayed as upper and lower panels for the same timepoint. T = 5°C. Scale bar is 10 µm.

It should be noted that streptavidin binding has also been shown to cause nanotube formation. Protein-membrane interactions can alter the membrane’s curvature through anchor insertion, and can induce nanotube formation through a number of ways including: the protein’s structure; through protein assemblies; and by altering the surface charge of the membrane. It is not difficult to imagine that the deformation of the membrane and production of tubular structures, via streptavidin-binding, may participate in the morphological changes observed in our system. However, streptavidin was not required for fission (see Figure 3-3), and nanotube formation still occurred in the absence of streptavidin. For example, Figure 3-8 shows a model cell with no protein bound to its membrane, which still divided asymmetrically to produce daughter vesicles originally connected by a membrane tether, which then ruptured.
Figure 3-8: Confocal fluorescence images collected during asymmetric division of ATPS-containing GV presenting micron-scale lipid domains (lipid composition was 1:1 DOPC/DPPC + 30% cholesterol, with 2.2% DPPE-PEG-2K, 0.08% DSPE-PEG2K-carboxyfluorescein, and 0.08% DOPE-rhodamine). Osmolality increases from left to right (130 ± 1.5 mmol/kg to 238 ± 5.5 mmol/kg). Fluorescence images have been false colored: red indicates DOPE-rhodamine in the L_d membrane domain, and green indicates DSPE-PEG 2000-carboxyfluorescein, in the L_o membrane domain, and blue indicates Alexa 647-lectin SBA. The Alexa647 signal decreased over time due to photobleaching; the blue channel has been adjusted to make the partitioning of SBA apparent for each timepoint. T = 5°C. Scale bar is 10 µm.

For the vesicle in Figure 3-8, we used fluorescently labeled lipid, DSPE-PEG 2000-carboxyfluorescein, in place of DSPE-PEG 2000-biotin–streptavidin-AF488, for tracking of the L_o phase. As before, the PEG-rich bud was initially surrounded by the PEGylated L_o membrane, and the dextran-rich bud was in contact with the L_d phase. Osmotically driven asymmetric fission resulted in separate daughter vesicles: a PEG-rich daughter vesicle with L_o membrane, and a dextran-rich daughter surrounded by L_d membrane. Similar to the fission events in Figure 3-6, there was a 4-fold difference in
local lectin concentration in the dextran-rich daughter vesicle as compared to the PEG-rich daughter vesicle \( (C_p = 18 \pm 4 \text{ nM}, C_d = 71 \pm 12 \text{ nM}) \). A membrane tether of primarily \( L_o \) lipid (green), with some \( L_d \) (red), initially connected the two daughter vesicles (3\textsuperscript{rd} panels from right), but ultimately broke to release the daughter vesicles (last panels). To verify that the nanotube connecting the daughter vesicles had indeed broken rather than simply moved out of the focal plane, we added water to the external solution to reduce osmolality and induce vesicle swelling. Rather than a reversal of the fission event, we observed an increase in the distance between the PEG-rich and dextran-rich daughter vesicles until they were no longer visible in the same focal plane. Dilutions performed for other daughter vesicles usually yielded similar results: addition of water led to nanotube breakage due to fluid flow, facilitating the completion of the vesicle fission events rather than reversing them. Fusion of the daughter vesicles and retraction of the bud to form a single, spherical vesicle was never observed, however in some cases the nanotubes persisted rather than breaking. Vesicles connected by a nanotube ruptured approximately 50\% of the time. Breakage of the membrane tether was usually observed as a result of fluid flow from the addition of sucrose or deionized water to the external solution, including collisions with other vesicles in the suspension due to this flow.

In Figures 3-6 and 3-8, and for the majority of vesicles we observed for these membrane compositions, each daughter vesicle inherited only \( L_o \) or \( L_d \) phase lipid compositions, i.e., only green or red membrane (Figure 3-4B). This is similar to what has been observed for daughter vesicles generated by line tension-driven fission of GVs that lack an ATPS, and suggested that line tension at the \( L_o/L_d \) phase boundary was important in our system despite the fact that it was not required for achieving fission (see
Our ATPS-containing vesicles also have an interfacial tension at the boundary between the PEG-rich and dextran-rich aqueous phases. Interfacial tensions for PEG/dextran ATPS are on the order of $5 \times 10^{-3}$ dyne/cm for the composition initially encapsulated and can be expected to increase by one or more orders of magnitude with the increased total polymer concentration that occurs when vesicle volume is osmotically decreased by as much as half.$^{25,75}$

When the mole ratio of DOPC to DPPC was 1:1, the relative surface areas of the $L_o$ and $L_d$ phases were approximately equal, and matched the relative volumes of the interior aqueous phases. This can be seen in the budded ATPS GV$s$ (Figures 3-6 and 3-8), in which the $L_o/L_d$ phase boundary coincides with the PEG/dextran interface. Upon division, the PEG-rich daughter vesicle is surrounded entirely by $L_o$ phase and the dextran-rich daughter vesicle only contains membrane in the $L_d$ phase. However, phase boundary mismatch had been observed in ATPS-containing GV$s$ for which the relative phase volumes were ~2:1, resulting in more $L_d$ phase than required to coat the dextran-rich bud, and thus partial contact of the $L_d$ phase with the PEG-rich bud.$^{27}$ We therefore prepared model cells in which $L_o$ and $L_d$ membrane areas were mismatched with respect to the interior aqueous phase volumes in order to examine the consequences of membrane/interior phase mismatch on fission.

**Figure 3-9** shows how a mismatch in membrane domain area and interior aqueous phase volume results in inheritance of both $L_o$ and $L_d$ membrane domains by one of the two daughter vesicles (Figure 3-4C). The left-hand panels of **Figure 3-9A** and **B** show budded vesicles in which the interface of the $L_o/L_d$ domains does not coincide with the interior aqueous-aqueous phase boundary. In **Figure 3-9A** (left), the surface area of
the interior dextran-rich bud was smaller than the available $L_d$ membrane area, such that the $L_d$ membrane also coats part of the PEG-rich aqueous phase. The opposite situation is observed in Figure 3-9B (left), with part of the $L_o$ lipid phase domain coating the dextran-rich aqueous phase bud. Addition of sucrose to the external solution resulted in invagination of the membrane at the aqueous-aqueous interface between the PEG-rich and dextran-rich phases (middle). A further increase in external osmolality resulted in complete budding and/or fission (far right panels). Here, one of the two daughter vesicles inherited the larger-area membrane domain and the other inherited both $L_o$ and $L_d$ membrane domains. Which daughter ended up with the dual-phase membrane depended on the initial mismatch of internal aqueous volumes and membrane domain surface areas. This was controlled by varying the ATPS composition to achieve different relative volumes of the PEG-rich and dextran-rich phases or by varying the lipid composition to achieve different relative areas of $L_o$ and $L_d$ membrane domains. For example, the ratio of DOPC to DPPC ratio was increased to 1:2 for the vesicle shown in Figure 3-9B. Additional examples, in which 1:1.5 DOPC:DPPC + 30% cholesterol were used, are shown in Figure 3-10. These data indicate the primacy of the aqueous-aqueous phase boundary in determining the site of vesicle division: division always occurred at the aqueous-aqueous phase boundary but only sometimes at the $L_o/L_d$ phase boundary. The resulting daughter vesicles each contained one of the two aqueous phase volumes in its entirety, and were coated by whatever membrane composition was necessary to enable this. As for the 1:1 lipid composition used above, when these 1:2 and 1:1.5 DOPC:DPPC lipid ratio vesicles formed daughter vesicles connected by a nanotube after osmotic stress, they subsequently lost this connection, converting to full fission events,
approximately half of the time. We note that in some cases, even nanotubes that appeared to contain only the $L_d$ lipid domain ruptured during the course of our experiments. Whether a given nanotube remained or ruptured when water was added in an attempt to reverse the budding event appeared to depend more on forces exerted on the structures by fluid flow than on their $L_o/L_d$ composition.

![Figure 3-9: Division of ATPS-containing GVs with excess area of either $L_d$ or $L_o$ membrane domain. Membrane compositions were: 1:1 DOPC/DPPC + 30% cholesterol (A), 1:2 DOPC/DPPC + 30% cholesterol (B). Osmolality increases from left to right. Fluorescence images have been overlaid and false-colored. Blue indicates lectin SBA-Alexa 647, red indicates $L_d$ domain lipid (DOPE-rhodamine), and green indicates $L_o$ domain streptavidin-Alexa488 (bound to lipid DSPE-PEG–2K-biotin). T = 5°C. Scale bar is 10 µm.](image)
Figure 3-10: Membrane inheritance in daughter vesicle upon complete budding (top) and fission (bottom) of ATPS-containing vesicles for which the membrane domain areas and aqueous domain volumes are mismatched. Osmolality increases from left to right. Fluorescence images have been false-colored: blue indicates Alexa647, red indicates rhodamine, and green indicates Alexa488. Images: overlay of DOPE-rhodamine and streptavidin-Alexa488 (bound to lipid DSPE-PEG-2000-biotin) fluorescence (top), and lectin SBA-Alexa647 fluorescence (bottom). For clarity, the color channels are displayed as upper and lower panels for the same timepoint. T = 5°C. Scale bar is 10 µm.

The ability to produce daughter vesicles in which membrane asymmetry is inherited from the mother vesicle is interesting as a primitive model of polarity inheritance in biological cells. An important question in cell biology is how polarity cues are inherited during cell division. In addition to genetic inheritance, membrane type and polarity are also continuous through generations.39,76 One hypothesis suggests that the membrane, which is passed on directly from the mother cell, may play an important role in polarity initiation in daughter cells, serving as a landmark for localization of a cascade.
of biochemical events that generate polarity in the daughter cells.\textsuperscript{39,76} Such a cascade could occur, for example, by a patch of membrane recruiting molecules from the cell interior to form a microdomain on or near that membrane patch to initiate the polarity cascade by providing spatial organization to the many gene products known to be involved in polarity.

![Figure 3-11: Second-generation aqueous phase separation and budding in a daughter vesicle. Membrane composition was 1:2 DOPC/DPPC + 30% cholesterol. Osmolality increases from left to right. Panels top to bottom are transmitted light (DIC), membrane fluorescence, and interior protein fluorescence. Confocal fluorescence images have been overlaid and false-colored. Red indicates L_d domain lipid (DOPE-rhodamine), and green indicates L_o domain lipid (streptavidin-Alexa 488, bound to DSPE-PEG 2000-biotin), and blue indicates the lectin, SBA-Alexa 647, which is partitioned into the dextran-rich interior aqueous phase. T = 5°C. Scale bar is 10 µm.](image)

We observed several instances in which an inherited patch of L_o or L_d membrane in one of the daughter vesicles formed a bud due to additional phase separation of the
encapsulated aqueous volume under osmotic stress. This process led to polarity in the membrane, the aqueous interior, and the distribution of internal and external proteins (i.e., SBA, which was partitioned into the dextran-rich aqueous phase, and streptavidin, which was bound to the biotinyated \( L_o \) membrane domain). An example is shown in Figure 3-11. Initially in a 122 ± 1.5 mmol/kg solution, the \( L_d \) phase of the mother vesicle is contacting the PEG-rich bud (top panel). Fission, which was associated with an approximately 20-25\% further loss in volume at a final osmolality of 157 ± 8.5 mmol/kg solution, resulted in daughter vesicles connected by a membrane tether (middle panel). The PEG-rich daughter vesicle inherits both the \( L_o \) domain, on which the streptavidin-AF488 is localized, and a portion of the \( L_d \) domain, which was present in excess over what was required to coat the dextran-rich daughter vesicle. Inside the vesicles, the PEG-rich daughter vesicle contained approximately 3.5x less protein (12 nM) than the dextran-rich daughter vesicle, which had 43 nM SBA. Further exposure to osmotic stress caused the PEG-rich daughter vesicle to bud (right hand panel). This was possible because the PEG-rich aqueous phase contains both PEG and dextran polymers, which upon concentration due to osmotic dehydration formed a new aqueous two-phase system, albeit with a smaller volume dextran-rich phase than in the original mother vesicle. This is apparent in the transmitted light (DIC) image, as well as in the blue channel showing the location of SBA, which has partitioned into the new dextran-rich phase bud, with a 2-fold difference in local “cytoplasmic” protein concentration between the bud and body of the vesicle (\( C_d = 27 \text{ nM, } C_p = 12 \text{ nM; } K = 0.42 \)).

Phase separation in what was formerly the PEG-rich phase of the ATPS can be understood in the context of the compositions of the two phases. Based on partitioning
measurements, the PEG-rich phase of an encapsulated ATPS with an intended composition 7 wt % PEG 8 kDa and 10 wt % dextran 10 kDa contains on the order of 2\times as much PEG and 0.5\times as much dextran as the dextran-rich phase.\textsuperscript{24} In general, significant concentrations of both polymers are present in each phase of an ATPS, with the relative concentrations and relative volumes determined by the ATPS composition relative to the binodal and tie lines.\textsuperscript{40,43} The composition of each phase lies on the binodal curve that separates single-phase solutions from two-phase coexistence. Hence, in our system once the two phases are split from each other by vesicle fission any further increase in polymer concentration due to volume loss can cause the individual solutions to phase separate within the daughter vesicles. In some cases, we observed phase separation in both the PEG-rich and dextran-rich daughter vesicles. An example is shown in Figure 3-12.\textsuperscript{77} This vesicle was exposed to a higher external sucrose concentration than those discussed above (osmolality was increased to 452 \pm 0.5 mmol/kg).
Figure 3-12: Aqueous phase separation in each of resulting vesicles after complete budding to form two daughter vesicles connected by a lipid nanotube. Membrane composition was: 1:1 DOPC/DPPC + 30% cholesterol. Osmolality increases from left to right. Top row is transmitted light (DIC). Fluorescence images have been overlaid and false-colored. Green indicates $L_d$ domain lipid (DOPE-CF), red indicates $L_o$ domain (streptavidin-Cy3, bound to lipid DSPE-PEG 2000-biotin), and blue indicates lectin SBA-Alexa 647 (note that the red and green dyes are reversed as compared with previous figures). Arrows highlight the location of newly-formed aqueous phases within each of the daughter vesicles. $T = 5^\circ C$ for the first three panels and 32 $^\circ C$ for the last three panels. Scale bar is 10 µm.

Phase separation in the PEG-rich daughter vesicle is apparent in the second panel of Figure 3-12, with the tiny dextran-rich phase first appearing as a droplet fully surrounded by the PEG-rich phase in the second panel. Phase separation in the dextran-rich daughter vesicle can be seen in the 3rd panel, with the tiny new phase surrounded by the larger phase. At this point deionized water was added to test whether the daughter vesicles were still attached by a nanotube; they were still attached and moved closer together in response to this decrease in external osmolality. The sample was also heated from 5 $^\circ C$ to 32 $^\circ C$ between panels 3 and 4 of Figure 3-12; heating did not result in
breakage of the nanotube but did facilitate budding of the newly-formed dextran-rich aqueous phase from the PEG-rich daughter vesicle (5th and 6th panels).

Fluorescence from the SBA was concentrated into the smaller phase volume of both daughter vesicles, which was unexpected since the smaller phase within the dextran-rich daughter should be the newly-formed PEG-rich phase. The transmitted light DIC images for this vesicle also suggested to us that the smaller phase was indeed the PEG-rich phase, contrary to the expected (and routinely observed) partitioning of this protein into the dextran-rich phase. These data indicate that the high local concentration of SBA in the dextran-rich daughter vesicle, coupled with macromolecular crowding from the polymers, may have caused protein aggregation that resulted in accumulation of the SBA either in the PEG-rich phase or at the aqueous-aqueous interface in this daughter vesicle. We have previously observed both accumulation of protein aggregates at the aqueous-aqueous phase boundary and partitioning of denatured proteins into the PEG-rich phase of GV-encapsulated ATPS. SBA can also be seen accumulating at the aqueous/aqueous interface of the Ld daughter vesicle (shown in red) after phase separation (3rd panel). For the vesicles shown in Figures 3-11 and 3-12, the occurrence of a second phase separation event in the daughter vesicles was possible only after they had become separate structures; when PEG-rich and dextran-rich phases are in the same container, a loss of volume results in a change in the composition of the phases but does not generate additional phases. Although the nanotube in Figure 3-12 persisted even after dilution and heating, the two aqueous volumes do not appear to be in communication on the timescale of these experiments (tens of minutes).
3.4 Conclusion

Polarized “mother” vesicles divided to produce chemically distinct daughter vesicles, each inheriting different membrane and interior compositions as well as different concentrations of soluble and membrane-bound proteins. This was possible by taking advantage of an aqueous two-phase system as a model cytoplasm that provided several important features: macromolecular crowding, protein sorting via partitioning between the aqueous phases, “pinning” the location of the PEGylated L₀ membrane to the PEG-rich aqueous phase, and fixing the location for the division plane. Although biological cells do not contain simple ATPS, the cytoplasm of living cells is compartmentalized, allowing for differences in local concentration. Aqueous phase separation is biophysically reasonable in the macromolecularly crowded intracellular milieu, and has in rare cases been observed in living cells.⁷⁻⁸ Likewise, biological membranes are known to exhibit spatial heterogeneity, which was modeled here by simple liquid phase co-existence. Although multiple divisions were precluded here by the limited amount of membrane area available, self-replicating vesicles have been reported based on, e.g., addition of surfactant or fatty acid precursors to existing fatty acid or lipid vesicles.⁸²,⁸³ Retention of encapsulated molecules through multiple cycles of growth and reproduction has been reported.⁸⁴ It may ultimately be possible to couple such an approach with the compartmentalized membranes and interiors used here to produce additional “generations” of asymmetrically dividing vesicles. We have introduced a simple, nonliving experimental model system for asymmetric fission, which underscores the apparent complexity of behaviors that can result from simple chemical and physical
interactions such as self-assembly, phase separation, and partitioning. Additionally, this work supports the possibility that spatial/organizational cues, in addition to genetic signals, could be important for achieving and maintaining polarity through cell division cycles.
3.5 References


48. Due to some variation in the specific polymer compositions encapsulated by each vesicle, we see some variability in partitioning as well as in the osmolality required for budding and fission for individual vesicles even within a single batch.

49. The osmolality of PEG-rich phase was 115 ± 2.9 when freshly prepared and increased to 136 ± 1.2 after incubation under parafilm at 42 °C for 2-3 days to form vesicles, thus even without addition of sucrose the external solution was hypertonic with respect to the solution encapsulated within the vesicles.

50. Vapor pressure osmometry measurements were performed at room temperature (25°C). For the PEG-rich phase of the ATPS, measurements at this temperature will overestimate the osmolality under our experimental conditions (4°C) by on the order of 10% or more; thus, these values are most useful in a comparative rather than absolute sense.

52. The initial addition of 130 mM sucrose led to a decrease in measured osmotic pressure, due to the nonadditive effect of multiple solutes on osmolality.\(^5\) This did not alter the budded geometry of the vesicles, presumably because the total interior polymer concentration was on average lower in the ATPS from which the vesicles were formed.\(^6\) Further aliquots of sucrose at increasing concentrations led to increased osmolality and the observed morphological changes leading to fission.


77. In the initial images (far left panels), green fluorescence corresponding to the labeled streptavidin on the L, lipid is visible between the PEG-rich and dextran-rich ends of the mother vesicle. We see membrane accumulation between the two aqueous phases under hypertonic conditions for some vesicles, and cannot be sure whether this indicates a barrier to diffusion between the two aqueous domains. Work by Li and Dimova suggests that this material is lipid nanotubes, which would not seal off the two aqueous compartments. Our data do not enable us to make a distinction for this vesicle.


Chapter 4

Differential Segregation of Denatured Proteins and Preferential Accumulation in One Daughter Vesicle Upon Asymmetric Division of Model Cells

4.1 Introduction

The differential segregation and asymmetric distribution of protein aggregates in dividing cells, such that only one cell inherits accumulated protein damage, has consequences for cellular aging, but examination of possible mechanisms by which this segregation occurs has been hindered by the complexity of living cells. Giant lipid vesicle (GV, > 1μm) cell models provide an alternative means for testing hypothesis in cellular biology. Indeed, they have been used as membrane models, as micro-volumes for complex reactions (i.e., transcription and translation), and as artificial cells. Here, our interest is in using GVs to examine differential segregation of denatured and aggregated protein in asymmetrically dividing model cells, to gain insight into possible mechanisms for age asymmetry in living systems.

During asymmetric division, biological cells preferentially segregate subcellular components to opposite poles in order to generate progeny with different cell fates. In budding yeast, the mother vesicle undergoes a finite number of divisions to produce daughter vesicles, a process known as the replicative life span (RLS), before cell death.
During this process, senescence factors accumulate in the mother, which reduce its RLS. A number of (bio)molecules have been identified as possible senescence factors including extrachromosomal rDNA circles (ERCs), oxidative damage to cellular components, and denatured protein aggregates.\(^2\)\(^-\)\(^4\) Protein aggregation is a prominent marker of aging, and has been linked to a number of age-related diseases.\(^{24}\)\(^-\)\(^26\) Studies examining asymmetric segregation of protein aggregates have found that during division the mother, but not the daughter, inherits aggregates.\(^2\)\(^-\)\(^4\)\(^,\)\(^27\)\(^,\)\(^28\) Asymmetric inheritance has also been observed in \textit{Drosophila melanogaster} cells, which divide asymmetrically to produce one daughter cell containing protein aggregates and one daughter cell free of aggregated protein.\(^1\) Malfunctions in segregation result in the breakdown of asymmetry and inheritance by both cells, which has consequences for their proper function.\(^{2,29}\) Liu et. al. recently identified a group of proteins important in polarity, termed the polarisome, as playing an essential role in the retention of damaged and aggregated proteins in mother cells.\(^{27}\) Still the mechanism by which senescence factors are asymmetrically distributed is not well understood.\(^2\)\(^-\)\(^4\)\(^,\)\(^24\)\(^-\)\(^29\) One hypothesis is that by way of a passive mechanism, aggregates are partitioned in a biased fashion resulting in a “old pole” and a “young pole” prior to division, thus generating cells with different ages.\(^{24,30}\)

We have developed a simple model of polarity that is capable of complex processes, including asymmetric distribution of “cytoplasmic components” and division, in the absence of complex machinery.\(^18\)\(^-\)\(^21\) Our model system is based on polyethylene glycol (PEG)/dextran aqueous two-phase systems encapsulated in GV.\(^{16,17}\) ATPS are composed of two or more dissimilar polymers in water or buffer that mix to form one phase or phase-separate in accordance with a phase diagram.\(^{31}\)\(^-\)\(^34\) When encapsulated in
GVs, the polymer solution mimics crowding\textsuperscript{35} and the PEG-rich and dextran-rich phases mimic the chemical heterogeneity\textsuperscript{36} of biological cytoplasm. Biomolecules such as proteins or DNA can be localized to sub-regions of the vesicle interior by partitioning between the aqueous phases, and the degree of localization can be modified by external stimuli, such as osmotic pressure, temperature, and pH.\textsuperscript{16-21,31-34} Proteins will partition based on their structural conformation; denatured proteins will partition to the PEG-rich phase due to exposed amino acid residues, while native proteins will partition to the more hydrophilic dextran-rich phase, and aggregates potentially to the interface.\textsuperscript{31,32,37-40} Exposure of ATPS/GVs to hypertonic conditions results in asymmetrically budded vesicles in which the internal polymer phases are segregated to opposite ends of the vesicle.\textsuperscript{18,19} Further exposure to hypertonic solution drives GV fission to produce chemically distinct daughter vesicles that are different in interior and membrane composition as well as any proteins they contain.\textsuperscript{21}

In this work, we are interested in examining the unequal distribution of denatured proteins in asymmetric GV fission. Denaturation of a protein is the process in which the native three-dimensional structure is changed without breaking of peptide bonds.\textsuperscript{41} A number of conditions can disrupt the native conformation including heat, pH, and chemicals such as urea or guanidine hydrochloride.\textsuperscript{41} Most proteins are stable at neutral pH, and can undergo structural changes at acidic or basic pH.\textsuperscript{41} Previously, it was demonstrated that human serum albumin (HSA) localizes to the dextran-rich phase near neutral pH (pH 5-8), and to the PEG-rich aqueous phase at denaturing pH 4.1 and at basic values above pH 8.\textsuperscript{20,32,37,38} Partitioning of HSA in ATPS is consistent with structural changes,\textsuperscript{42} as confirmed by circular dichroism.\textsuperscript{20} Similarly, fibrinogen, a blood clotting
protein, partitioned to the PEG-rich phase in ATPS GVs formed at acidic pH, as a result of partial denaturation. To encapsulate denatured proteins in GVs, we formed vesicles at pH 4.1 where the proteins were partially acid unfolded, and examined their distribution in dividing vesicles.

4.2 Experimental Materials and Methods

4.2.1 Chemicals and Materials

L-α-Phosphatidylcholine (egg PC), 1,2-dioleoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (sodium salt) (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-rhodamine), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DOPE-PEG-2kDa) were purchased as chloroform solutions from Avanti Polar Lipids, Inc. (Alabaster, AL). The polymers, poly(ethylene glycol) (PEG) 8 kDa, dextran 500 kDa, sucrose, and 2-(N-morpholine)ethanesulfonic acid (MES) buffer salts were purchased from Sigma Chemical Co. (St. Louis, MO). Alexa Fluor (AF) 488 and AF 647 labeling kits, AF 647 and AF488 carboxylic acid, succinimidyl esters, AF647-fibrinogen, Amino dextran 10 kDa and the press-to-seal silicone spacers were purchased from Molecular Probes, Inc. (Eugene, OR). Amino PEG 5 kDa was purchased from Nektar Therapeutics (Huntsville, AL). Human Serum Albumin was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Water used in these experiments was purified to a resistivity of \( \geq 18.2 \, \text{M} \Omega \) with a Barnstead NANOPure Diamond system from Barnstead International (Dubuque, IA).
4.2.2 Preparation of Giant Vesicles encapsulating an Aqueous Two-Phase System

Lipid vesicles were formed using the gentle hydration method, as previously described. Briefly, a 3:7 molar ratio of egg PC/DOPG with 0.09% DOPE-rhodamine was added to a test tube (10 x 75 mm, Durex borosilicate glass, VWR, Int., West Chester, PA) containing ~100µL chloroform. The lipid solution was then dried under Ar (g) to produce a thin, lipid film. Residuals of chloroform were removed by placing the test tube under vacuum desiccation for approximately 2 hours. During this time, a bulk ATPS solution consisting of 3.9 wt % PEG 8 kDa and 4 wt % dextran 500 kDa in 1mM pH 4.1 MES buffer was prepared and incubated at 37°C. Next, 970µL of warm, single-phase polymer solution and 20 µL of Alexa Fluor 488-HSA (26µM) or 5µL of Alexa 647-fibrinogen (1.5mg/mL), and either 20 µL of Alexa Fluor 647-PEG 5 kDa or 10 µL of Fitc-dextran 10 kDa were added to a microcentrifuge tube, mixed, and placed in the 37 °C incubator for ~ 15 min. The solution was then added along the wall of the test tube and the lipids were hydrated at 37 °C for approximately 48 hours. The same procedure was followed for the preparation of GVs containing ~1.8 mol % DOPE-PEG-2k Da. Vesicles in Figures 4-7 and Figures4-8 did not contain PEGylated lipid. To form vesicles with native proteins (under non-denaturing conditions), we prepared vesicles in Barnstead DI water.

4.2.3 Preparation of ATPS/GV samples for confocal microscopy

After vesicle formation at 37 °C, sample vials were transferred to 5 °C, a temperature below the ATPS transition, to drive phase separation both in the bulk
solution and vesicle interior. Vesicles accumulated at the interface of the phase-separated bulk ATPS, from which 0.5-2 µL of vesicles were removed and transferred to a shallow well made from placing a silicone spacer on a microscope coverslip (24 x 60 mm, VWR Int., West Chester, PA). Vesicles were first diluted with 10 µL of 33 mM sucrose. A sucrose solution was added every 5-10 minutes, each time increasing the external solution concentration by approximately 13%, until fission occurred.

4.2.4 Quantification of protein partitioning in ATPS-containing vesicles

Protein (Alexa Fluor 488-HAS and AF647-fibrinogen) in the PEG-rich and dextran-rich phases were determined from their fluorescence intensities by taking a line scan across the PEG-rich and dextran-rich compartments in the vesicle. Solute concentrations were determined directly from the confocal fluorescence intensities using a calibration curve of the labeled protein at different concentrations also acquired on the confocal microscope under identical imaging conditions. Partitioning was calculated as the partition coefficient, K, defined as $K = \frac{C_p}{C_d}$ where $C_p$ is the concentration of the solute in the PEG-rich phase and $C_d$ is its concentration in the dextran-rich phase.

4.2.5 Instrumentation and Software

ATPS GV confocal images were acquired using a Leica TCS SP5 laser scanning confocal inverted microscope using a 63x oil objective (Leica Microsystems). A VAPRO
vapor pressure osmometer (model 5500) from Wescor, Inc. was used to measure solution osmolality at 25 °C.

4.3 Results and Discussion

We began by verifying that HSA was denatured and partitioned to the PEG-rich compartment of the ATPS GV. Figure 4-1 shows representative images of ATPS/GVs containing Alexa Fluor 488-HSA (AF488-HSA) formed at neutral and acidic pH. At neutral pH, HSA partitioned to the dextran-rich compartment (Figure 4-1 A), while at pH 4.1 the protein localized to the PEG-rich phase (Figure 4-1 B). Protein localization is shown in green. Fluorescent PEG 5 kDa (blue) partitioned to the PEG-rich aqueous phase and was added for unambiguous assignment of the aqueous micro-compartment.

The partitioning of the denatured HSA can be quantified in terms of the partition coefficient, $K$, which is the concentration ratio in the PEG-rich phase, $C_p$, as compared to the dextran-rich phase, $C_d$, $K = C_p/C_d$. To determine the protein concentration in the aqueous compartments, line scans were performed across both the PEG-rich and the dextran-rich aqueous phases in the GV, and results were compared to calibration curves. In Figure 4-1 B, $K = 3.83$ for the fluorescent protein, indicating a preferential partitioning to the PEG-rich aqueous compartment ($C_p = 786$ nM, $C_d = 205$ nM).
**Figure 4-1:** pH-dependent partitioning of HSA. Fluorescently labeled HSA and PEG 5 kDa in PEG 8 kDa/dextran 500 kDa ATPS/GVs formed in neutral pH (A) and pH 4.1 (B). Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right). Scale bar is for both images and is 10 µm.

In **Figure 4-1**, we showed examples of a spherical and an asymmetric, or budded, vesicle. Both geometries were observed at this initial osmolality (18.3 ± 1.5 mmol/kg). In both examples lipid material was observed at the PEG/dextran aqueous-aqueous phase boundary (red channel of **Figure 4-1**). Accumulation of lipid material at the PEG 8 kDa/dextran 500 kDa interface has previously been reported. Dominak et. al examined pH-dependent partitioning of HSA in PEG 8 kDa/dextran 500 kDa GVs formed at pH 4.1 and observed membrane particulate at the aqueous-aqueous phase boundary at the same initial osmolalities used here. However, for the vesicles in **Figure 4-1**, PEGylated lipid was incorporated into the bilayer to increase membrane flexibility to facilitate vesicle budding and fission. Li et. al. demonstrated membrane nanotube formation inside ATPS/GVs, whose membranes contained GM1 or PEG-modified lipids, upon
The molecular mechanism for the formation of nanotubes was hypothesized to be the reduction in vesicle free energy to minimize the asymmetric spontaneous curvature of the inner and outer membrane leaflets, which may have been caused by interactions between parts of the “cytoplasmic” aqueous polymers and the protruding moieties on the lipids (sugar groups of GM1 and PEG moieties, respectively). We believe that nanotube formation similarly occurred here. The presence of PEGylated lipid and higher osmolalities used in our system may have resulted in the formation of more nanotubes as compared to the work performed by Dominak et. al.

We found that aggregation of denatured HSA could be triggered in situ by exposure of vesicles to further osmotic stress. The vesicle shown in Figure 4-2 was formed at pH 4.1 to induce protein denaturation, as demonstrated by the partitioning of AF488-HSA in the PEG-rich phase (K= 7.75). At this low osmolality, membrane particulate has already collected at the PEG/dextran phase boundary. The addition of a hypertonic sucrose solution to the external media in which the vesicle was dispersed resulted in protein aggregation and accumulation at the aqueous-aqueous phase boundary (Figure 4-2B). Protein aggregation occurs as a result of the coagulation of misfolded or partially denatured proteins that non-specifically interact due to the hydrophobic surfaces that are normally buried in the native protein. Aggregation as shown here was due to the high local concentration of denatured AF488-HSA in the PEG-rich phase, combined with macromolecular crowding from the polymers. The presence of protein aggregates can be seen more clearly at the phase boundary in Figure 4-2 C. This data is consistent with previous work examining HSA at denaturing pH in ATPS GVs.
**Figure 4-2:** Effects of increasing osmolality on denatured protein in an ATPS GV. Panels A-C represent increasing external osmolality. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right).

Interestingly, the vesicle shown above did not bud more with increasing amounts of sucrose, even at the highest osmoalities observed (124.3 ± 5.9 mmol/kg). This may be due to the presence of membrane and/or aggregated protein at the interface. We were not able to acquire images in the z-plane for this image due to vesicle movement in the aqueous solution, and thus, we cannot confirm that the membrane/aggregates span the entire interface serving as a boundary between the two aqueous microcompartments. However, an important driving force for budding and fission in ATPS/GVs upon osmotic deflation has been reduction of the interfacial tension at the PEG-dextran interface, facilitated by excess membrane area.\(^{21}\) The presence of aggregates completely covering the phase boundary could reduce the interfacial tension between these two polymer-rich phases, removing the driving force for vesicle shape changes, and could also impede
membrane closure making it difficult to completely divide. Although the vesicle did not bud, the wettability of the polymer-rich aqueous phases changed from panels A-C in Figure 4-1. Wetting transitions of PEG/dextran ATPS encapsulated within GVs depends on the interfacial tensions at the interfaces between the PEG-rich phase and the membrane, the dextran-rich phase and the membrane, and the PEG-rich and dextran-rich phases. Here, the interfacial tension between the dextran-rich phase and the membrane is higher than that between the PEG-rich and dextran-rich phases, and thus the wettability of the dextran-rich phase increased with polymer concentration48, as observed in Figure 4-2 C. Higher dextran/membrane tensions also favor nanotube formation.48

Protein aggregation was also observed in polarized vesicles containing damaged protein, as presented in Figure 4-3. Initially in 18.3 ±1.5 mmol/kg osmolality, the vesicle has segregated the denatured protein into the PEG-rich phase, and has created a new compartment, or bud, that is essentially free of “cellular” damage (K= 5.04, C_p= 1.6μM, C_d = 326 nM). The presence of aggregates is visible in the green channel (Figure 4-3 A). As the external osmolality is increased by the addition of higher concentrations of sucrose, protein aggregates form in situ (Figures 4-3 B-D). Additional increases in external osmolality result in the formation of more aggregates and subsequent accumulation at the interface until almost no protein is observed in the PEG-rich compartment (3.8 x less than Figure 4-3A; C_p = 431nM) (Figure 4-3 D).
Figure 4-3: Effects of increased sucrose on a denatured protein in polar mother vesicle. Osmolality increases from A-D. GV composed of egg PC/DOPG with 1.8 mol % PEGylated lipid encapsulating PEG 8 kDa/dextra 500 kDa formed at pH 4.1. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right). Scale bar = 10 µm, T= 25°C.

In some cases, exposure to increased osmotic stress did not result in accumulation of aggregates at the interface, but instead resulted in asymmetric division such that denatured protein accumulated in the PEG-rich daughter vesicle. Figure 4-4 demonstrates asymmetric segregation of denatured protein by the PEG-rich daughter vesicle upon division. Denatured AF488-HSA is preferentially partitioned to the PEG-rich phase of the GV with a K= 10.03 (Cp= 2.5 µM, Cd= 254 nM) (Figure 4-4 A). As the concentration of the external sucrose solution is increased to 106.3 ± 4.9 mmol/kg,
division occurs at the aqueous-aqueous phase boundary resulting in 9.5 x more denatured protein in the PEG-rich daughter as compared to the dextran-rich daughter vesicle ($C_p = 3.2 \mu M; C_d = 345.84 \text{nM}$) (Figure 4-4 B-F).

Fission may have been facilitated by membrane at the interface, which in this case may have served as a complete barrier separating the two aqueous compartments, and providing enough excess membrane to divide upon further osmotic deflation. Osmotic-stress induced budding and fission of ATPS GVs has previously been demonstrated, and was a result of the excess membrane area coupled with the increased PEG/dextran interfacial tension due to water loss. $^{18,19,21}$
Figure 4-3: Inheritance of denatured protein by PEG-rich daughter vesicle upon asymmetric division. The external osmolality is increased from Panels A-F. Polymer partitioning in the interior ATPS was visualized by Alexa647-labeled PEG 5 kDa. Images have been false colored: red indicates rhodamine, green indicates Alexa 488, and blue indicates Alexa 647. Panels: rhodamine-DOPE membrane (left), Alexa 488-labeled HSA fluorescence (center), Alexa 647-polymer fluorescence (right). Scale bar = 10\( \mu \)m, temperature = 25°C.

Although in the above example, asymmetric denatured protein inheritance was demonstrated, osmotic deflation-driven division of these vesicles was rare. For the vesicles shown in Figure 4-2 and Figure 4-3 the division of the “mother” GV to produce
daughters with different concentrations of denatured protein did not occur, even at the highest osmolalities examined. We hypothesized that this might be due to the accumulation of denatured HSA and membrane at the interface, hindering GV fission. Albumin proteins are able to bind lipids to form stable protein-lipid complexes, and are capable of removing lipid monomers from bilayer membranes.\textsuperscript{49-53} Specifically, albumin has been shown to interact with and bind to phosphatidylcholine\textsuperscript{49}, a lipid used in this work. Thus we were concerned that the accumulation at the interface may be a result of the membrane-protein interactions and not due to protein aggregation alone. To examine this in more detail, we performed bulk experiments in the absence of lipid material, as demonstrated in Figure 4-5. For this experiment we chose an ATPS composition that was 3x more concentrated than the initial ATPS concentration, in order to induce aggregation and to force any aggregates that formed to the interface. In Figure 4-5A the dextran-rich phase is observed as small droplets in transmitted DIC (left), and the PEG-rich phase appears bright due to the presence of Alexa647-labeled PEG 5 kDa (middle). The far-right hand panel shows the accumulation of AF488-labeled HSA aggregates at the PEG/dextran aqueous-aqueous phase boundary and the presence of denatured protein in the PEG-rich phase. The aggregates can be seen more clearly in the enlarged view shown in Figure 4-5B. This work verifies that as HSA denatures the aggregates localize to the ATPS interface independent of any protein-lipid interactions that may occur.
Although HSA aggregates accumulate at the interface in the absence of membrane material, we were interested in examining “cellular” strategies for controlling the removal of protein aggregation by production of daughter vesicles. The presence of lipid material, degraded protein, and any lipid-protein complexes formed at the interface may preclude or hinder fission events. Additionally, albumin proteins can destabilize bilayer membranes causing leakage from vesicles, which may have facilitated the fission event that occurred above.\textsuperscript{51,52} Thus, we chose to examine a protein without lipid binding properties, in order to remove any protein aggregation and/or localization effects that may have been caused by albumin-lipid interactions.

We chose fibrinogen; a 340 kDa blood coagulating protein that does not interact with membrane lipids.\textsuperscript{54} Fibrinogen undergoes partial unfolding at acidic pH, which
exposes hydrophobic amino acid residues that are normally buried in the native conformation. Localization of fibrinogen to the PEG-rich phase in a PEG/dextran ATPS at acidic pH has previously been demonstrated. We verified acid-induced aggregation by examining the partitioning of fibrinogen in bulk ATPS. Figure 4-6 shows the localization of AF467-labeled fibrinogen in a bulk PEG 8 kDa/ dextran 500 kDa ATPS formed in 1mM MES pH 4.1 buffer. The fluorescent labeling shown here is different than in previous examples- the polymer is green and the protein (fibrinogen) is blue. The dextran-rich phase appears as droplets in DIC (left) and appears green in the middle panel due to partitioning of FITC-dextran 10 kDa, which was added for clear assignment of the phases (middle). The accumulation of fluorescently labeled fibrinogen at the interface is observed in the far right panels of Figures 4-6 A and B. Thus, we would expect this protein to behavior similarly when encapsulated within ATPS GVs.

**Figure 4-6:** Fibrinogen aggregates at Bulk ATPS interface. The ATPS composition used here is: 11.7% PEG 8 kDa, 12.0 % dextran 500 kDa in 1mM MES buffer at 25°C. From left to right: transmitted DIC, polymer fluorescence (middle), protein fluorescence (far right). Blue represents Alexa 647-fibrinogen, and FITC-dextran 10 kDa, partitioned to the dextran-rich phase, is shown in green. Scale bar = 10 µm.
Asymmetric accumulation of denatured fibrinogen in a polarized vesicle is demonstrated in Figure 4-7. Initially, the denatured protein is preferentially localized to the PEG-rich compartment with a K = 3.33 (C_p=6.88nM, C_d=2.06nM), (Figure 4-7A, far right panel), with aggregates visible at the PEG/dextran interface (Figure 4-7 A). Increasing the hypertonicity of the external solution induced aggregation, as observed in the blue channel of Figure 4-7 B. Heating resulted in vesicle fission, to produce a PEG-rich daughter vesicle, which contained 2.3 x more fibrinogen than the dextran-rich phase (C_p=6.28nM, C_d=2.73nM), as well as the aggregated protein. Failure to segregate damaged protein in biological cells has been observed in a number of diseases and as a source of aging.2,29

Figure 4-7: Asymmetric inheritance of degraded protein. Egg PC/DOPG vesicles with 3.9 wt% PEG 8 kDa/4 wt% dextran 500 kDa formed in 1mM MES, pH 4.1 buffer. Panels A-C represent increasing osmolality. From left to right: lipid membrane fluorescence, polymer fluorescence (middle), protein fluorescence (far right). Red represents rhodamine, Fitc-dextran 10 kDa partitioned to the dextran-rich phase is shown in green, and blue represents Alexa 647-fibrinogen. Scale bar = 10 µm. T=25°C.
The data presented above demonstrated a single vesicle fission resulting in the accumulation of denatured protein in one of two daughter vesicles; however, biological cells are capable of undergoing numerous cell divisions. To examine if vesicles could divide more than once we subjected a polarized mother vesicle containing asymmetrically segregated denatured protein, and possessing a compartmentalized cytoplasm mimic to increased osmotic stress and heat (Figure 4-8). In Figure 4-8A, the denatured protein is ~7x more concentrated in the PEG-rich bud than the dextran-rich bud ($C_p=6.16\text{nM}$, $C_d= 0.84\text{nM}$), and aggregates have localized and accumulated at the interface between the two daughter vesicles. As the external sucrose solution and temperature is increased, the daughter vesicles detach, resulting in asymmetric inheritance of the denatured protein by the PEG-rich daughter vesicle (Figure 4-8C). Further increases in external sucrose and heat induce polarity of the PEG-rich daughter vesicle, which results in the accumulation of denatured protein at the body/bud interface. Here, the bud contains ~3x less denatured protein, which presumably upon division would remain in the PEG-rich daughter vesicle due to non-specific interactions. Accumulation of protein aggregates at a specific pole, as shown here, has been hypothesized as a possible mechanism in which biological cells may generate aggregate-free daughter cells.$^{24,30}$
Figure 4-8: Asymmetric inheritance of degraded protein and second budding of ATPG GV. Egg PC/DOPG vesicles with 3.9 wt% PEG 8 kDa/4 wt% dextran 500 kDa formed in 1mM MES, pH 4.1 buffer. Panels A-E represent increasing osmolality and heat. From left to right: lipid membrane fluorescence, polymer fluorescence (middle), protein fluorescence (far right). Red represents rhodamine, Ftc-dextran 10 kDa partitioned to the dextran-rich phase is shown in green, and blue represents Alexa 647-fibrinogen. Scale bar = 10 µm.
4.4 Conclusions

We have demonstrated the differential segregation of denatured proteins between daughter vesicles upon asymmetric GV fission based on aggregate formation and non-specific interactions. To encapsulate denatured protein in ATPS GV s we formed vesicles at pH 4.1 where the protein is partially acid unfolded. Conformational changes resulted in the preferential partitioning of the denatured protein to the more hydrophobic PEG-rich phase of the PEG/dextran ATPS encapsulated within a GV. Exposure of the vesicle to a hypertonic sucrose solution resulted in protein aggregation and accumulation at the aqueous-aqueous phase boundary. The accumulation of protein at the interface as observed in this work is similar to the assembly of the cytoplasmic FtsZ protein in prokaryote cells prior to division. FtsZ, a tubulin homolog, localizes to the future division site in bacteria, where it polymerizes into a ring-like structure and serves as a scaffold for additional division proteins. 27-30 This vesicle provides a simple model of the localization of a cytoplasmic protein to the future division site. Although division did not occur in this example, previous research has demonstrated ATPS GV fission occurs at the aqueous-aqueous phase boundary. 31 In some instances, further heating and exposure to osmotic stress drove asymmetric GV fission, which resulted in a PEG-rich daughter vesicle harboring higher concentrations of denatured protein as compared to the dextran-rich daughter vesicle. Although much simpler than the asymmetric clearance mechanisms observed in biological cells, this work demonstrates a simple means of mother-biased segregation of “cellular damage”. Sorting based on aggregate formation and non-specific interactions, as shown here, may similarly occur in biological cells.
4.5 References


Chapter 5

Characterization of Multicomponent Polymer Solutions by Vapor Pressure Osmometry

5.1 Introduction

Knowledge of polymer solution thermodynamics is of fundamental importance for understanding solution properties ranging from osmotic pressure to phase behavior. Experimental determination of solution osmolality can provide thermodynamic information in the form of the dependence of solvent chemical potential on composition. The addition of one or more solutes to a solvent affects solvent-solvent interactions, which changes solution colligative properties such as boiling point, freezing point, vapor pressure, and osmotic pressure. In ideal solutions, solute-solute, solute-solvent, and solvent-solvent interactions are equivalent, and the experimentally determined osmolalities would equal the calculated molalities based on the amount of solute added to the solution. However, real solutions deviate from colligative ideality. For single solute solutions, the interactions between identical solutes, as well as solute-solvent and solvent-solvent interactions, must be considered. In multicomponent solutions, intermolecular interactions between the different solutes must also be taken into account. For this reason, the osmolality of the multicomponent solution is not simply the sum of the individual contributions from each solute. A number of methods exist for measuring solution osmolality including freezing point depression and vapor pressure or membrane osmometry.
The thermodynamics of polymer solutions, in particular those that form aqueous two-phase systems (ATPS), have been extensively studied due to their widespread use in bioseparations.\textsuperscript{12-15} ATPS are solutions composed of chemically different polymers (or a polymer and a salt) in water or buffer that can phase separate or mix in accordance with a phase diagram.\textsuperscript{12-15} Predicting the osmolality of these multicomponent solutions is difficult due to the interactions between the macromolecules and the solvent. Water as the solvent can interact with these polymers in a number of non-covalent ways, which results in local water structure around a given macromolecule that is different from that in the bulk (i.e., the ratio of polymer to water molecules is either higher (accumulated) or lower (excluded) in the vicinity of the surface of the second polymer).\textsuperscript{14} This has consequences for mixing and separation. For example, phase separation can result in an ATPS where each phase is enriched in a different polymer as discussed above; however, another possibility is that of a co-existing system consisting of a polymer-rich phase containing both polymers and a polymer-poor phase containing the solvent.\textsuperscript{14} Temperature and additives such as salts can influence phase separation.\textsuperscript{15}

We are interested in knowing the osmotic pressure of these two-polymer solutions. Solution osmotic pressure can be determined from osmolality by the following equation:

$$\Pi = RTc$$  \hspace{1cm} (1)

where \(\Pi\) is the osmotic pressure, \(R\) is the ideal gas constant, \(T\) is the temperature, and \(c\) is osmolality in moles/kg. Measuring the osmolality of the resultant ATPS is important for understanding solution behavior and is directly related to our artificial cell work. For example, the osmotic pressure of the polymer/sucrose solution in which GVs
are often dispersed is important for determining vesicle behavior in response to osmotic stress, as demonstrated in Chapter 2 and 3 of this thesis. However, the data is not always straightforward. As discussed in Chapter 3 of this thesis, when measuring the osmotic pressure of the external PEG solution, the osmolality initially decreased after the addition of a hypertonic sucrose solution. This can be explained by the non-additive effects of multiple solutes on solution osmolality. Of equal importance is measuring the resultant osmotic pressure of two polymers (e.g., PEG and dextran) in an aqueous solution, as our cytoplasm mimic is comprised of an ATPS. In this chapter, we are interested in measuring the osmotic pressure of two-polymer solutions and examining the effects of temperature on solution osmolality. We chose to measure the osmolality of PEG/dextran and PEG/PAAm polymer solutions for this initial study because they form ATPS that responded differently from each other with respect to temperature and salts. Thus, we were interested in examining how the polymer-polymer interactions differed in these systems and what impact that had on osmolality for solutions containing both polymers. Additionally, we were interested in determining whether information about the polymer-polymer interactions, relative to their interactions with water, could be extracted for these systems from osmotic pressure data, as has been done by Record et. al for a polymer and a small solute. However, due to the viscosity of solutions containing two polymers, as compared to aqueous solutions containing a small solute and a single polymer, obtaining interaction information may prove difficult.

Herein, we present osmolality data for three-component solutions (polymer + polymer + water) at different temperatures. The goal of this study was to obtain a fundamental understanding of the factors affecting solution osmolality, as this will enable
us to prepare ATPS polymer solutions with desired osmotic pressures for artificial cell work. Additionally, we wanted to gain an understanding of the polymer-polymer interactions occurring in these solutions, as this may shed light on differences in the behavior of the different polymer solutions, for example the effect of temperature and salts. Finally, we evaluated the potential for vapor pressure osmometry (VPO) as a way to quantify interaction potentials for these systems.

5.2 Experimental Materials and Methods

5.2.1 Materials

Polymers used for these experiments were polyethylene glycol (PEG) 35 kDa and dextran 10 kDa, and were obtained from Sigma-Aldrich. Polyacrylamide (PAAm) 10 kDa was obtained from Scientific Polymer (Ontario, New York). Water in the experiments was purified to a resistivity of $\geq 18.2 \text{ M}\Omega$ with a Barnstead NANOPure Diamond system (Dubuque, IA). Osmolality standards (100, 290, 1000 mmol/kg) were purchased from Wescor Inc. (Logan, Utah).

5.2.2 Sample Preparation

Stock solutions (20 % w/w) were prepared gravimetrically on a Mettler analytical balance by weighing out the desired amount of dry polymer and placing it in a glass vial containing a stir bar. The vial was placed on the balance, zeroed, and DI water was added
to reach the desired weight. Polymer stock solution concentrations were verified by refractometry using polymer standards on a Leica Auto ABBE refractometer.

Two-component solutions containing a single polymer in water (e.g., PEG + water) were prepared by taking aliquots of the original 20 % stock solutions and then diluting to the desired final wt % with DI water (e.g., 1-6 wt % PEG). Three-component solutions containing two polymers in water were prepared as above except that a fixed concentration of the first polymer solution (e.g., 4 wt % PEG) was added to each vial, and then the second polymer solution was added to the same vials in increasing amounts (e.g., 1-6 wt% dextran). The wt % of each solution was then converted to molality. In later experiments, samples were prepared using gravimetric methods on a Mettler analytical balance. Sample solutions were prepared as above except that each aliquot was weighed. DI water was then added to the vial and the weight recorded. The molality of each sample was then determined.

5.2.3 Cloud Point Titration

Cloud point titrations were performed to determine the compositions and experimental conditions at which the PEG/dextran and PEG/PAM polymer solutions exist as a single phase and when phase separation occurs. Briefly, separate 20 % (w/w) stock solutions of PEG and dextran in DI water were prepared gravimetrically as described above. A glass vial with stir bar was obtained and was weighed on a Mettler analytical balance. Approximately 1 gram of the 20 % dextran stock solution was added to the glass vial and the exact weight was recorded. The 20 % PEG stock solution was
then titrated into the dextran solution until the point of turbidity, an indication that the polymer solution is crossing into the two-phase region, and the vial was reweighed. DI water was then added until the solution turned clear, at which point the vial was reweighed. This process was repeated approximately 10-15 times. Cloud point titrations were repeated at 5 °C and 37 °C by placing vials in a water bath set to the desired temperature. Phase diagrams were generated using Igor Pro 6.22A.

5.2.4 Vapor Pressure Osmometry

In this work, solution osmolality was measured using a Wescor Vapro 5520 vapor pressure osmometer at 25 °C and 37 °C (incubation room). Triplicate measurements of osmolality were performed on each sample. The thermocouple was cleaned extensively before each use, and often during the experiments as needed, using 2 M ammonium hydroxide and filtered DI water. The osmometer was calibrated before each use and after each cleaning using Standard solutions of NaCl at osmolalities of 100, 290, and 1000 mmol/kg.
5.3 Results and Discussion

We measured osmolality by VPO as a function of solution composition at different temperatures for PEG 35 kDa/dextran 10 kDa and PEG 35 kDa/PAAm 10 kDa (structures of PEG, dextran and PAAm monomers are provided in Figure 4-1). These polymer pairs were chosen due to their differences in phase behavior as a function of temperature. The phase diagrams shown below were generated to ensure that the solutions examined by VPO were in the one-phase region, which must be experimentally determined for each polymer lot number since variability between polymer batches can affect the position of the binodal. Solution osmolalities for the single-phase two-polymer solutions were then measured at 25 °C and 37 °C.
Figure 5-1: The chemical structures of Polyethylene glycol (PEG), dextran, and polyacrylamide (PAAm). Dextran as shown here is in its simplest form.

5.3.1 ATPS Phase Diagrams

Figure 5-2 shows the phase diagram for PEG 35 kDa/dextran 10 kDa at 5, 25, and 37 °C. Polymer compositions chosen that are above all three binodal lines are in the two-phase region and will phase separate, while those that fall below the line are in the one-phase region and will mix. At high weight % of PEG 35 kDa and low weight % of dextra 10 kDa (far left on the graph) the binodals shift up with temperature such that at 37°C a higher polymer concentration is needed to achieve phase separation than at lower temperatures. However, upon further inspection, we noticed that for low weight % PEG 35 kDa and high weight % dextran 10 kDa (right-hand side of the graph), the opposite is
observed, with higher polymer concentrations necessary for phase separation at lower temperatures than at 37°C. Although the temperature dependence is slight, it is reproducible and when bulk solutions are prepared they follow the predicted temperature dependence of phase behavior.\textsuperscript{22}

**Figure 5-2:** Phase diagram for PEG 35 kDa/dextran 10 kDa ATPS. Experimentally determined binodals at 5(☐), 25 (●) and 37°C(○). Points were determined by cloud point titration, and lines are a polynomial fit included to guide the eye.

Temperature effects can be seen more clearly in the phase diagram for PEG 35 kDa/PAAm 10 kDa (**Figure 5-3**). At high weight % of PEG (≥4 %) and low weight % of PAAm (≤9%) the 37°C binodal is above the 25°C line, indicating that higher polymer concentrations are needed to achieve phase separation at 37 as compared to 25 °C. This is reversed for the low-PEG, high-PAAm side of the phase diagram.
Figure 5-3: Phase diagram for PEG 35 kDa/PAAM 10 kDa ATPS. Experimentally generated binodals at 25°C (●) and 37°C (○). Points were determined by cloud point titration, and lines are included to guide the eye.

Based on Figures 5-2 and 5-3, we selected a range of polymer compositions that fell within the one-phase region to examine using VPO for determination of solution osmolality of these two-polymer solutions.

5.3.1 Osmolality of PEG 35 kDa/dextran 10 kDa

To characterize osmolality for PEG 35 kDa/dextran 10 kDa solutions and examine the effects with temperature, we measured the dependence of osmolality of the two-component solutions [PEG + water] and [dextran + water] on solution composition,
and compared values to data acquired for the three-component [PEG + dextran + water] solutions. **Figure 5-4A** plots osmolality as determined by VPO vs. PEG molality for prepared two-component [PEG + water] solutions at 25 and 37 °C. It is important to note that although a vapor pressure osmometer was used to measure solution osmolality, the data has already been converted from vapor pressure to osmolality values. For an ideal dilute solution we would expect the measured solution osmolality to equal the prepared solution molality, for example, 1 mmole of solute, such as sucrose, would result in 1 osmole (or mmol/kg). For the data shown here, the osmolality of the PEG two-component solution increases with increasing PEG molality. However, the presence of PEG in water deviates from ideality and significantly contributes to the osmotic pressure of the solution. Indeed, the addition of macromolecular solutes to solvent increases the osmolality over that of the ideal solution.\(^1\) This is more significant at 25°C, as observed by the slope of the line. Solution osmolality decreased by ~34% with an increase in temperature (**Figure 5-4B**). This data can be explained by the water structure around the polymers and the interactions occurring in solution. PEG is strongly hydrated; the number of water molecules typically reported is two or three waters per every monomer.\(^{23-27}\) At elevated temperatures, the hydrogen bond interactions of PEG are weakened resulting in an increase in free water content.\(^{28-31}\) Increases in water activity and decreases in water-PEG interactions (osmotic coefficient), with increasing temperature have previously been reported for PEGs of varying molecular weights, including PEG 35 kDa, in this temperature range.\(^{27,32-35}\)
Figure 5-4: Vapor Pressure Osmometry data for PEG 35 kDa two-component [PEG + water] solutions at 25°C (○) and 37°C (●). Osmolality is plotted vs. PEG 35 kDa molality and each point is the average of triplicate readings on identical samples.

Next, the osmolality for a single two-component [dextran + water] solution was examined. For a ~4.4 mmol/kg dextran solution, the osmolality decreased from 40 mmol/kg to 23 mmol/kg, a 42.5% difference with an increase in temperature from 25 and 37°C. Like PEG, dextran is hydrated, there are approximately 1 to 1.5 molecules of water per monomer, and thus this temperature dependence can be explained by hydrogen bonding and hydrophobic effects.  

Figure 5-5A shows experimental osmolality values for PEG 35 kDa/dextran 10 kDa at 25°C, where the molality of PEG 35 kDa was varied and that of dextran 10 kDa was fixed at ~4.4 mmolal. The presence of dextran in the three-component solution increases the osmolality over the PEG two-component solution at all PEG concentrations.
examined, as expected. However, the experimentally determined three-component solution data shows a substantial departure from the additive values of the two-component solutions ([PEG + water] and [dextran + water]), as indicated by the solid line. The three-component system was most similar to the simple additive osmolality prediction at the highest polymer concentrations tested. At low PEG concentrations, contributions from PEG-dextran interactions result in a higher solvent chemical potential and lower osmolality, whereas at higher PEG concentrations the solvent chemical potential is lower due to the large PEG-water interactions, and thus is more similar to the sum of the binary osmolalities.\textsuperscript{26,27}

The effect of temperature on solution osmolality was then examined by repeating this experiment at 37°C (Figure 4-5B). The three-component solutions decreased by an average of 33 %. This data is consistent with the literature and is due to the changes in water structure. Gaube et. al. reported decreased osmotic coefficients (i.e., decreased PEG-water and dextran-water interactions) for ternary PEG-dextran solutions with increasing temperature due to changes in hydration,\textsuperscript{38} and Eliassi and co-workers reported increased water activity (and thus decreased osmolality) for ternary solutions of PEG-dextran-water between 35-55°C.\textsuperscript{39}
Figure 5-5: Vapor Pressure Osmometry data for PEG 35 kDa/dextran 10 kDa at 25°C (A) and 37°C (B). Osmalities of 3-component [PEG + dextran + water] solutions as a function of PEG molality, where dextran was held constant and the molality of PEG was varied (○). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [dextran + water] osmolalities.

In the above experiments, the same samples were measured at both temperatures to avoid variability that may occur with different sample batches. However, since the osmolality of these solutions was quite low and VPO had not previously been used to determine osmolality of two-polymer solutions, we were interested in the repeatability of
this technique and examining how the osmolality of polymer solutions varies from sample to sample. **Figure 5-6** shows osmolality data for PEG 35 kDa/dextran 10 kDa at 25°C, where each graph represents a different sample. Although day-to-day variability was observed, the general trends are the same. In **Figure 5-6A**, osmolality values for low PEG concentrations (<2.5 mmolal) deviate from those performed on different days, however higher PEG concentrations are in good agreement with the other samples. This could be the result of measurement error when preparing the solutions. Additionally, contamination of the thermocouple (TC) head could have occurred. Although care was taken to thoroughly clean the TC head, the polymers, even at low wt % used here, are viscous and can lead to contamination. For **Figure 5-6 B-D**, the osmolality values of all solutions examined are in the same ranges. The additive values overestimate the experimental three-component solutions, with less deviation at higher PEG concentrations. This data shows that vapor pressure osmometry can be useful for obtaining solution osmolality for ternary solutions without significant batch-to-batch variability.
Figure 5-6: Vapor Pressure Osmometry data for PEG 35 kDa/dextran 10 kDa at 25°C on four different days. Osmalities of 3-component [PEG + dextran + water] solutions as a function of PEG molality, where dextran was held constant and the molality of PEG was varied (●). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [dextran + water] osmolalities.
5.3.2 Osmolality of PEG 35 kDa/PAAm 10 kDa

We chose to examine interactions between PEG 35 kDa and PAAm 35 kDa next. PAAm exhibits both inter- and intramolecular hydrogen bonding and is solvated by more water molecules (~ 4) than either PEG or dextran on a per monomer basis. It is interesting to examine how PEG/PAAm interactions compare to those between PEG/dextran. Osmolality data for two-component [PAAm + water] solutions as a function of temperature are presented in Figure 5-7. Here, the PAAm two-component solution slightly increased with temperature at lower concentrations but was essentially the same at higher concentrations. Day et al. reported that in dilute aqueous solution, PAAm prefers to interact with water than with other polymer chains. It is reasonable to presume that intermolecular hydrogen bonds between different PAAm chains could be broken via raising the temperature, resulting in PAAm-water interactions. A combination of the interactions occurring in this system could contribute to the increase in osmolality (decrease in solvent chemical potential) with a slight increase in temperature.
Figure 5-7: Vapor Pressure Osmometry data for PAAm 10 kDa two-component [PAAm + water] solutions at 25°C (○) and 37°C (●). Osmolality is plotted vs. PAAm 10 kDa molality and each point is the average of triplicate readings on identical samples.

Next, we measured the osmolality of [PAAm + PEG + water] three-component solutions. We chose to vary PAAm, instead of PEG to reduce the high solution viscosity that occurs when PEG is the variable solute; varying PAAm and holding PEG constant provides the same thermodynamic information. A single two-component PEG solution, with a concentration of 1.3 mmol/kg had osmolality values of 26 mmol/kg and 27 mmolal/kg at 25 and 37°C, respectively.

Experimental data for [PAAm + PEG + water] is presented in Figure 5-8. Here, the three-component solution increases with PAAm concentration and is close to the sum of the two-component solutions at all concentrations examined. Increasing the temperature to 37°C had little effect on solution osmolality, as demonstrated in Figure 5-
As for the PEG/dextran system described above, we repeated this experiment on different batches of polymers and here, at different temperatures, as demonstrated below.

**Figure 4-8:** Vapor Pressure Osmometry data for PAAm10 kDa/PEG 35 kDa at 25 °C (A) and 37°C (B) Osmalalities of 3-component PAAm/PEG solutions as a function of PAAm molality, where PEG was held constant and the molality of PAAm was varied (●). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [PAAm + water] osmolalities.

**Figure 5-9** shows osmolality data for PAAm 10kDa/PEG 35 kDa at 25°C and 37°C, where each set of graphs represents a different sample performed at both
temperatures (i.e., A and B are a pair, C and D, and E and F). Although the measured values vary slightly from batch-to-batch, the general trend is the same: the osmolality of all solutions remained essentially constant with an increase in temperature. This is consistent with literature data showing that for [PEG + PAAm + water] solutions the PEG-PAAm interactions do not change markedly with temperature. A discrepancy is found in Figure 5-7 E, F where the PAAm two-component solution increases initially, but then remains the same at higher concentrations (i.e., 6 wt% PAAm is 76 mmolal, and 75 mmolal at 25° and 37°C, respectively). Possible reasons for this could be contamination of the thermocouple (TC) head due to the viscosity of PEG/PAAm solutions, or error during sample preparation. Restrictions due to viscosity and choosing compositions that were in the one-phase region limited the range of polymer concentrations that could be examined.
Figure 5-9  Vapor Pressure Osmometry data for PAAm10 kDa/PEG 35 kDa at 25 °C (A, C,E) and 37 °C (B, D, E) on three different days. Osmolalities of 3-component PAAm/PEG solutions as a function of PAAm molality, where PEG was held constant and the molality of PAAm was varied (●). The solid black line represents expected values if the multicomponent solution was the sum of the 2-component [PEG + water] and [PAAm + water] osmolalities.

The data presented above provided information about the contributions from polymer-polymer interactions in two-polymer solutions on the resultant solution
osmolality, as well as the effects of temperature for these two- and three-component solutions. In all cases, the initial osmolality measurements of the three-component solutions were underestimated by the additive data, and they most closely matched the additive values at higher concentrations. This can be explained by the interactions occurring in the system. As the concentration of polymer one (P1) is increased, while the second polymer (P2) remains constant, eventually the amount of P2 that P1 can interact with will be limited, and P1 will interact only with water. Thus, at higher concentrations of P1 the osmolality of the three-component solution is closest to the additive values.

Osmolality data for the [PAAm + PEG + water] three-component solutions did not deviate from additive as much as the [PEG + dextran + water] solutions. We interpret this data to mean that PAAm and PEG interact similarly with each other as they do with themselves. This is consistent with literature reporting that the hydrogen bond interactions in PEG/PAAM/water mixtures are approximately equivalent, and PEG and PAAm prefer to interact with water in dilute aqueous solutions.

The slopes of the lines are different for the two polymer pairs examined in this work. The osmolality of the [PEG + dextran + water] three-component solutions increased significantly at 25°C with an increase in concentration, but the change in osmolality with concentration was not as substantial at higher temperatures. This is reasonable due to the increases in polymer-water interactions with an increase in PEG concentration at low temperatures, and the release of water at higher temperatures. The changes in osmolality as a function of PAAm concentration were essentially constant at both temperatures, which is in good agreement with the interactions occurring in this system. The slopes of the two-component solutions also increased with concentration,
as expected, and differences in polymers can be attributed to the different degrees of hydration. Although the slopes of the lines varied for the same polymers from batch to batch, the overall trend was the same. However, examining a larger concentration range or larger concentration intervals could provide a better description of the thermodynamic properties of these aqueous two-polymer pairs. This is difficult due to the increased viscosity of the polymer solutions with increased concentration. In fact, others have reported difficulty in examining increased polymer concentrations due to the high viscosities.\textsuperscript{46}

Overall this technique provided information about the changes in solution osmolality in the presence and absence of an additional polymer. Contributions to solution osmolality strongly depend on the degree of hydration for each polymer, as well as the interactions between the same and different polymers. A quantitative measurement of the interactions of these polymers would provide important information about the degree of hydration (exclusion) or interaction (accumulation) of the polymers in aqueous solution. We next evaluated whether polymer-polymer interactions, relative to water, could be quantified using the measured osmolality data from VPO as demonstrated below.

5.3.1 Quantifying polymer-polymer interactions

Record and coworkers introduced VPO as a novel technique for the characterization of the thermodynamic consequences of interactions between a small
solute and a biopolymer in aqueous solution.\textsuperscript{9,10} The interactions between a solute and a protein have thermodynamic effects that are either more or less favorable than protein-water interactions, and thus the ratio of solute to water molecules is either higher (accumulated) or lower (excluded) in the vicinity of the protein surface as compared to the bulk of the solution.\textsuperscript{9} These solute effects can be quantified by \textit{m}-values, defined as derivatives with respect to solute concentration ($m_3$) of the observed standard free energy change for the process ($\Delta G^\circ_{\text{obs}} = -RT\ln K_{\text{obs}}$), where $K_{\text{obs}}$ is the equilibrium concentration quotient for the process (expressed in terms of concentrations and not thermodynamic activities) and the subscripts 1, 2 and 3 refer to water, biopolymer/model compound, and small solute or second polymer, respectively\textsuperscript{47,48}.

\[
m - \text{value} = \frac{\partial \Delta G^\circ_{\text{obs}}}{\partial m_3} = -RT \frac{\partial \ln K_{\text{obs}}}{\partial m_3} = RT \frac{\partial \ln K_{\gamma}}{\partial m_3} = RT \Delta \left( \frac{\partial \ln \gamma_2}{\partial m_3} \right) = \Delta \mu_{23}.
\]

$\mu_{23}$ can be obtained experimentally by vapor pressure osmometry. By preparing two- and three-component solutions in water, where the two-component solution contains the polymer in water and the three-component solution contains both species of interest in water, the excess osmolality, $\Delta \text{Osm}$, can be obtained ($\Delta \text{Osm} = \text{Osm} (m_2, m_3) - \text{Osm} (m_2, 0) - \text{Osm} (0, m_3)$), providing information only about the polymer-polymer interactions relative to water since contributions from individual polymer-water interactions are subtracted.\textsuperscript{47,48} $\Delta \text{Osm}$ is related to the chemical potential derivative, $\mu_{23}$, by the following equation:

\[
\mu_{23} = RT \frac{\Delta \text{Osm}}{m_2 m_3}.
\]
and is a measure of the favorable or unfavorable interaction of the two components, relative to their interactions with water.\textsuperscript{47,48} Thus, a plot of $\Delta\text{Osm}$ vs. the molality product ($m_2$, $m_3$) of the two species allows for $\mu_{23}$ to be determined.\textsuperscript{47,48}

Record and co-workers examined the effects of small solutes on biopolymers, which had a large osmotic pressure contribution. Highly positive values indicate exclusion, or preferential hydration, of the polymer near the surface of the second polymer.\textsuperscript{47,48} They reported a $\mu_{23}/RT$ of $1.2 \pm 0.1$ for interactions between glycine betaine (GB) and tripotassium citrate, indicating that GB is highly excluded from anionic oxygen.\textsuperscript{47} We were interested in learning if this analysis could be applied to polymer-polymer solutions, despite their low osmotic pressure and the presence of some day-to-day variation in measurements. Here, we used osmometry to quantify the interactions of PEG 35 kDa with dextran 10 kDa at 25 and 37°C. We selected these polymers since the osmolality values examined in this work varied with temperature, as compared to the PAAm/PEG osmolality values, which remained essentially constant with temperature.\textsuperscript{49}

**Figure 4-10** presents interaction data for PEG/dextran at both temperatures. $\mu_{23}/RT$ was obtained from the slope of the line and increased from $1.685 \pm 0.257$ to $2.0516 \pm 0.152$ with an increase in temperature. These values are not statistically different; scatter in the data limits our ability to determine polymer interactions. This technique may not be able to provide quantitative values due to the high polymer viscosities and small concentration ranges examined. Furthermore, polymer polydispersity could be a source of error because the assumption inherent here is that there are only two different solutes, but if each polymer is polydisperse there will be more kinds of interactions. The dextran used here actually contains a range of molecular weights from approximately 9-11 kDa.\textsuperscript{49}
Examination of lower molecular weight polymers, polymers that are monodisperse, or the PEG and dextran monomers, as done by Record et. al. for polyethylen glycol (PEG)/protein interactions, may overcome some of these issues.

**Figure 5-10:** Excess osmolality $\Delta$ Osm from VPO studies of PEG 35 kDa-dextran 10 kDa at 25 °C (A) and 37°C (B). $\Delta$ Osm vs. $m_2m_3$, the product of molal concentrations of dextran 10 kDa and PEG 35 kDa; the slope is $\mu_{23}/RT$. PEG 35 kDa was the variable solute.
5.4 Conclusions

Osmolality data for two-polymer solutions as a function of composition at 25 and 37 °C was presented. For all solutions examined the presence of a second polymer increased the osmolality over that of the two-component solution. We interpret this as contributions from polymer-polymer interactions that were not accounted for in the two-component [polymer + water] solutions. The effect of temperature was different for the different polymer pairs, but was dependent on the degree of polymer hydration, and interactions between the different polymers within a three-component solution. Work aimed at quantifying polymer-polymer interactions proved difficult due to the small concentration range examined, small differences in osmolality values, high polymer viscosity, and possible errors that may have occurred due to the polydispersity of the polymers. Examination of the monomers and/or lower molecular weight polymers may overcome these shortcomings. In this work, we were able to quantify general types of interactions (excluded or accumulated), but scatter in the data prevented meaningful quantitative comparison of the 25 and 37 °C data sets. These experiments provide the first example of determining interaction coefficients by VPO for aqueous solutions of two polymers. In the future, using lower molecular weight polymers or examining the monomer units can alleviate viscosity problems that were encountered during experimentation. The work presented in this chapter provided information about solution osmolality of two-polymer solutions, and will allow for the osmotic pressure of our artificial cells to be accurately controlled.
5.5 References


48. Guinn, E. J.; Pegram, L. M.; Capp, M. W.; Pollock, M. N.; Record, T. M., Jr. Quantifying Why Urea is a Protein Denaturant while Glycine Betaine is a Protein Stabilizer.

49. Dextran10 kDa was supplied from Sigma-Aldrich with a molecular weight distribution provided as 9-11 kDa.

Chapter 6

Conclusions and Future Directions

6.1 Conclusions

This dissertation described the use of ATPS GVs as model cells for the examination of the consequences of phase-separation driven microcompartmentation of the membrane and cytoplasm—namely asymmetric segregation of cytoplasmic components and subsequent GV fission, resulting in chemically distinct daughter vesicles. Work examining malfunctions in polarity, specifically the asymmetric distribution and inheritance of denatured proteins in progeny vesicles, was also presented. Although the overall goal of this thesis was to examine polarity in artificial cells, research analyzing the thermodynamic behavior of two-polymer solutions was also performed; this knowledge will allow for the control of the osmotic pressure of the external solution in which vesicles are dispersed, which is important for controlling their behavior.

In Chapter 2 we presented the first example of model cells possessing heterogeneity in both the “cytoplasmic” interior and plasma membrane. This was achieved by using a lipid recipe capable of phase separating into co-existing micron-scale lipid membrane domains (Lφ/Lδ), and by encapsulating an aqueous two-phase system
within the vesicle interior. Exposure of these model cells to external stimuli, such as a hypertonic sucrose solution, resulted in an asymmetric, or budded, geometry such that the interior polymer-rich phases were in contact with different membrane domains, the location of which was controlled by non-specific interactions between the internal and membrane phases. This work presented a simple model of polarity and demonstrated asymmetric localization of cytoplasmic components in the absence of complex machinery. Although biological cells are much more complex than the artificial cell presented here, it is reasonable to presume that the behaviors demonstrated in this work—phase separation, partitioning, and non-specific interactions—are important factors for polarity maintenance and induction in living systems.¹

Chapter 3 expanded upon this work to examine the effects of osmotic stress on polar artificial cells. Exposure of asymmetric ATPS GVs to hypertonic sucrose solutions resulted in division at the aqueous-aqueous phase boundary to produce two daughter vesicles different in their “cytoplasm” and membrane compositions, as well as any proteins that they contained. Additionally, we demonstrated that when there was more of one type of membrane than was needed to coat the internal aqueous compartment fission resulted in inheritance of that membrane type by one of the two daughter vesicles. The inherited patch of membrane was important for polarity in the daughter vesicle in which it was inherited; upon exposure to a solution with increased osmolality the daughter polarized, becoming asymmetric in the interior, membrane, and protein compositions.
This work provided a simple model of asymmetric cell division and demonstrated that spatial cues, in addition to genetic factors, might be important for polarity initiation in daughter cells.\textsuperscript{2}

Malfunctions in asymmetric segregation and division have been implicated in a number of disease states, including cancer, and the asymmetric distribution of denatured proteins is believed to play a role in cellular aging.\textsuperscript{3,4} In chapter 4, we modeled differential segregation and asymmetric inheritance of denatured proteins upon division of an artificial cell. To encapsulate denatured proteins, we prepared ATPS GVs at pH 4.1, where the protein is partially acid unfolded. Denaturation of the protein resulted in asymmetric distribution within the “cytoplasmic” interior. Protein aggregates were produced in situ by further exposure to osmotic stress. In some instances, exposing vesicles to increased temperature and osmotic stress resulted in inheritance of higher concentrations of denatured protein by one of two daughter vesicles.\textsuperscript{5} Sorting of asymmetric proteins to different cell poles by a passive mechanism, as shown here, is one hypothesis as to how cells may control asymmetric partitioning of cellular damage.

While the overall goal of the work presented in this thesis was to examine the behavior of these synthetic polar cells, this work also led to important findings on the non-ideality of two-polymer solutions, as described in Chapter 5. For both PEG/dextran and PAAm/PEG polymer solutions, we found that the three-component [polymer + polymer + water] solutions showed a substantial departure from the additive values of the
two-component [polymer + water] solutions. The three-component systems were most similar to the simple additive values of the two component [polymer + water] values at the highest polymer compositions examined. Additionally, we determined that changes in osmolality with increasing polymer concentration as a function of temperature depended on the degree of polymer hydration and polymer-polymer interactions for the respective polymers examined. Although much knowledge was gained from these studies, polymer-polymer interactions at different temperatures could not be determined using vapor pressure osmometry (VPO). This work presented the first study to examine two-polymer solutions using VPO to acquire quantitative information about polymer interactions, and provided valuable information concerning solution thermodynamics and the effects of temperature on solution osmolality for these aqueous two polymer systems.

6.2 Future Directions

The model cell presented in this dissertation provided a means for examining hypotheses such as the consequences of phase-separation of the cytoplasm, the role of membrane inheritance in daughter vesicles, differential denatured protein partitioning and asymmetric division. Although this work made important strides towards understanding possible mechanisms of cellular asymmetry, there are still many unanswered questions regarding the induction and maintenance of cell polarity in living systems, which can be
studied using our model system, as discussed below.

Our model cell can build upon this initial work to further investigate multiple division processes. During division in biological cells, the cytoplasm becomes asymmetric and the mother divides; each daughter inherits a portion of the cytoplasm, in which the sub-cellular components are not homogenously distributed. This could be modeled by choosing a budded vesicle in which each polymer-rich compartment has phase separated prior to division (i.e., the PEG-rich “bud” has a PEG-rich phase surrounding a dextran rich phase, as demonstrated in Chapter 4). Heating and subjecting the vesicles to osmotic shock could achieve phase separation in the daughter vesicles. After division, further increasing the temperature and osmotic stress may result in expulsion of this phase from the daughter vesicle to produce a bud, and eventually a second fission event may occur. Additionally, it would be interesting to induce protein aggregation in these vesicles and examine the distribution of degraded protein in daughter cells. Multiple divisions from a single vesicle may also be able to be achieved by increasing the volume ratio of the internal polymer-rich aqueous phases (e.g., the ratio of PEG-rich to dextran-rich phases could be 9:1 instead of ~2:1, which would decrease the size of the dextran-rich phase and possibly make it easier for multiple small buds to pinch off).

In Chapter 4, we demonstrated the asymmetric distribution of degraded protein and subsequent division such that one daughter vesicle inherited higher concentrations of
denatured protein. In future work, the asymmetric distribution of denatured enzyme and native enzyme (e.g., catalase) would allow for the examination of the consequences of denatured protein inheritance on cell function in that daughter vesicle. Additionally, a passive mechanism in which protein aggregates are differentially segregated to an “old pole” and a “young pole” has been hypothesized to be a way in which cells prevent the transmission of damaged protein to daughter cells.\(^6\) This hypothesis could be tested in our model cells by using ATPS GVs composed of a 2:1 mole ratio of L\(_d\): PEGylated-L\(_o\) lipids. This membrane composition would result in excess L\(_d\) membrane that partially contacts the PEG-rich bud, and upon division, the PEG-rich daughter vesicle would inherit both L\(_o\) and L\(_d\) membrane domains. Exposure of the daughter vesicle to hypertonic solution would drive polarity resulting in a dextran-rich “young pole” wetted by L\(_d\), membrane and a PEG-rich “old pole” harboring higher concentrations of denatured protein and in contact with the L\(_o\) membrane. This work would demonstrate a simple “pole-biased” mechanism for protein sorting based on spatial cues and non-specific interactions.

In Chapter 5 two-polymer solutions were difficult to analyze due to their high solution viscosity, and thus we were limited to a small concentration range. Additionally, the operable temperature of the instrument (15-37 °C) limited our ability to examine the effects of multiple temperatures on solution properties for these two-polymer solutions. Strey et. al. used sedimentation equilibrium ultracentrifugation to obtain osmotic
pressure data of polymer solutions containing a single polymer in water over a concentration range of 0-50 wt\% and over a temperature range of 10-40 °C.\textsuperscript{7} This method should also be applicable to two-polymer aqueous solutions, and would allow for examination of higher concentrations and a broader range of temperatures than explored in Chapter 5. When investigating polymer-polymer interactions, one way to overcome the high viscosity would be to examine the monomer units in aqueous solution, as done by Record et. al to obtain information about interactions between Polyethylene glycol (PEG) and proteins.\textsuperscript{8} However, composition gradient static light scattering (GC-SLS), a technique based on Rayleigh light scattering and UV-absorbance of a solution whose composition is varied with time, would be a more suitable method for quantification of polymer-polymer interactions in two-polymer solutions since this method measures thermodynamic behavior directly.\textsuperscript{9} Minton et. al. developed GC-SLS and used it as a technique to obtain information about solute-solute interactions in aqueous solutions containing two macromolecules.\textsuperscript{9} Although this method has to date only been used for protein solutions, this technique should be a viable method to obtain attractive and repulsive information about two polymers in aqueous solutions.
6.3 References


VITA

Meghan Koback

Education
2012 Pennsylvania State University-University Park, PA Ph.D. in Chemistry: Advisor Dr. Christine D. Keating

2005 King’s College-Wilkes-Barre, PA B.S. Chemistry

Peer Reviewed Publications


3. Andes-Koback, M.; Keating, C. D. Differential Segregation of Denatured Proteins and Preferential Accumulation in One Daughter Vesicle Upon Asymmetric Division of Model Cells Manuscript in Preparation


Selected Presentations

