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The Graduate School
Eberly College of Science

DYNAMIC AND ASYMMETRIC PROTEIN MICROCOMPARTMENTATION
IN AQUEOUS TWO-PHASE VESICLES

A Thesis in
Chemistry
by
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ABSTRACT

Macromolecular crowding and microcompartmentation are fundamental features of the cell cytoplasm. Aqueous two-phase vesicles (ATPS GVs) are primitive “artificial cells” that exhibit both of these features and retain the activity of encapsulated proteins. I demonstrate dynamic organization of the aqueous compartment within ATPS GVs in response to small changes in temperature or osmotic pressure, the latter being useful for inducing asymmetric protein microcompartmentation. I demonstrate the use of colloidal gold scaffolds as a general method to dramatically increase protein partitioning within bulk aqueous two-phase systems (ATPS) with the retention of a significant level of activity. Additionally, I demonstrate complex temperature- and salt-dependent phase behavior of bulk ATPS and explain how this behavior can be rationalized through polymer hydration and conformation.
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Chapter 1

Macromolecular Crowding as a Fundamental Driving Force for Microcompartmentation in the Cellular Cytoplasm

This thesis describes the application of immiscible aqueous two-phase systems (ATPS) within giant vesicles (GVs) as “synthetic cells (Figure 1-1).” The desired cellular function ATPS GVs are intended to mimic is dynamic intracellular microcompartmentation (microscopic segregation), a fundamental feature exhibited by all living cells (1, 2). A brief overview of the motivations for this thesis work, relevant background, and highlights of experimental results presented follow.

Microcompartmentation and volume exclusion: Two critical parameters for proper cellular function.

Microcompartmentation is the subcellular segregation of DNA, proteins, and small molecules and ions within the cytoplasm and nucleus of living cells (1,2). It is often observed even in the absence of an intervening membrane (1,2). There are many examples of this phenomenon within both prokaryotes and eukaryotes (1-7) (Figure 1-2). Prokaryotes lack a nuclear membrane, yet their DNA does not diffuse evenly throughout the cytoplasm (3). It is instead localized into discrete regions through the assistance of proteins that also regulate DNA transcription (3). Protein kinase A (PKA) is localized
Figure 1-1. Confocal micrographs of an aqueous two-phase system (ATPS) within giant phospholipid vesicles (GVs), the structures described in this thesis as primitive functional analogs of cells. Under these particular experimental conditions, the outer domain is enriched in poly (ethylene glycol) (PEG) and the inner domain is enriched in dextran. A, Left column: Transmitted light (differential interference contrast, DIC) images. Two aqueous domains are evident. DIC amplifies differences in refractive index and displays these differences artificially in relief. The inner dextran-enriched domain has the higher refractive index because dextran has a higher density than PEG and polarizes light. B, Middle column: Lipid (rhodamine) fluorescence. It is evident that there is no physical barrier (lipid membrane) at the ATPS phase boundary. C, Right column: Polymer (or protein) fluorescence. These images enable one to quantify the degree of partitioning of either a fluorescently-tagged polymer or protein between the PEG-enriched and dextran-enriched phases. Top: PEG (fluorescein) fluorescence; bottom: dextran (Alexa488) fluorescence. T = 5°C, bar = 10 µm.
Figure 1-2. Cartoon of microcompartmentation in a cell through different mechanisms.  
A. A small phospholipid vesicle within the cell encapsulates protein molecules, preventing diffusion from the vesicle through a physical barrier.  
B. Proteins are anchored to a cytoskeletal filament.  
C. Proteins are anchored to the cell membrane.  
D. Microcompartmentation achieved through aggregation in a signaling complex.  
E. Proteins are segregated within a budding cellular region.
within numerous regions throughout the cell (4-6). The concentration of calcium ions is higher in the nucleus and subsarcolemmal regions of smooth muscle cells (7). Proteins are often encapsulated within small phospholipid vesicles for transport to other locations in the cell (8). Loaded dopamine molecules are preferentially stored within the halo regions of dense core vesicles within PC12 cells as opposed to being associated with the vesicle matrix (9). Several proteins involved in DNA repair are known to localize preferentially and reversibly at the cell poles of living Bacillus subtilis cells upon the uptake of DNA (10,11). Several proteins involved in the coordination between cellular morphology and the progression of cell division in Caulobacter crescentus bacteria are localized to specific subcellular regions depending on the stage of the cell cycle (12,13). As these selected examples illustrate, microcompartmentation is widespread and is not unique to a specific biomolecule or cell type.

Microcompartmentation has functional consequences in living cells (14). Several examples are the following. Deliberately localizing a substrate for protein kinase A such that it is in close proximity to the enzyme leads to preferential phosphorylation of that substrate over others in the same general vicinity (15). The enzymes required for glycolysis are bound to the membrane of plant cell mitochondria (16), which has potential consequences for the function of the glycolytic pathway such as altered reaction rates and on/off control over the enzyme sequence. Enzymes involved in signaling pathways can be arranged in clusters, allowing for efficient signal transduction and rapid modification of the complex in response to a dynamic intracellular environment (17).

The underlying motivation of this thesis work is to understand and mimic some of the fundamental physical forces that may influence and dictate microcompartmentation
within the cytoplasm of a living cell, specifically the impact of high protein concentrations on cellular organization and function. The cytoplasm is often represented in the literature as a dilute aqueous medium. While much useful information can be gained from work performed in dilute aqueous solutions, on a fundamental level this does not mimic the internal environment of cells (18). The cellular cytoplasm is instead comprised of a high concentration of proteins. This is not insignificant as it leads to macromolecular crowding (volume exclusion) (19-22) wherein the space occupied by the solute is non-negligible relative to the space occupied by the solvent. In other words, protein molecules occupy considerably more volume than small molecules (18). The concentration of an individual protein in the cytoplasm may be low, but the collective concentration of proteins is much greater and they may be considered “background” volume-excluders (Figure 1-3) (22). Some specific examples are the estimated 340 g/L of total protein and RNA present within the cytoplasm of Escherichia coli (20) and the greater than 300 g/L of hemoglobin present within erythrocytes (21). Macromolecular crowding applies to cells in general; the percent of total volume occupied by macromolecules within living cells is estimated to range from 10 to 50 weight percent depending on the cell type (22).

Volume exclusion has many important implications for cellular structure and function. While simple background volume-excluding polymers by definition do not participate directly in cellular reactions, steric crowding induced by volume exclusion nevertheless has a strong impact on cytoplasmic and cytoskeletal structure and function based on many theoretical and experimental studies both in vitro and in vivo (17-88)
**Figure 1-3.** Cartoon of macromolecular crowding. Generic proteins are illustrated as white spheres. “Background” proteins are illustrated as black spheres. While the concentration of the generic protein is low, since there are many different proteins within the cytoplasm the total concentration of proteins present is much higher. Additionally, since the space occupied by the background protein molecules is unavailable for occupation by the generic protein molecules, the effective concentration of generic protein molecules is significantly higher than that suggested from the total volume of the solution.
(elaborated upon in more detail in Tables 1-1 – 1-3). It is possible to use experimental data regarding the effects of crowding on reaction rates to elucidate complex enzymatic reaction mechanisms (89-91).

It is important to note that biochemical observations made in dilute aqueous media are significant and can in fact shed light on the importance of volume exclusion on cellular function. Some proteins that spontaneously fold in dilute buffer do not fold in volume-excluding media and require the assistance of chaperonins (protein complexes that function in the maintenance of protein stability under conditions of stress) to do so under such conditions (92). Some intrinsically disordered proteins are still disordered even in PEG solutions (93). Additionally, a high polymer concentration can bring with it a large increase in solution viscosity. This may compete with crowding and result in difficulties when attempting to predict reaction kinetics within volume-excluding media (94).

In addition to the steric crowding effect, volume exclusion also promotes collisions and electrostatic interactions among the proteins within the cytoplasm, thereby impacting solute diffusion, especially of large (95) and highly charged species (96). Diffusion of the neutral carbohydrate polymers dextran and Ficoll in the cytoplasm and nucleus of MDCK epithelial cells and 3T3 fibroblasts as measured through fluorescence recovery after photobleaching (FRAP) is reduced relative to within water, but conflicting reports exist as to whether this reduction in diffusion is dependent on polymer molecular weight (97-100). A molecular weight dependent segregation of dextran into various cellular compartments was observed (99), which may explain this confusion since different compartments would thereby appear to exert differing effects on diffusion. The
Table 1-1. Selected examples from the literature of the broad effects of volume exclusion in cells.

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diffusion of DNA microinjected into HeLa (cancer) cells is much more severely restricted than that of dextran of comparable size (100); this restriction in diffusion is dependent on molecular weight for DNA, but is not for dextran (100), possibly explaining the molecular weight dependence of dextran microcompartmentation mentioned previously (99).

Actin restricts the diffusion of microinjected GFP by an estimated 53% within Dictyostelium cells (101). Analogous, but simpler, experiments can be performed in bulk solution; dextran and Ficoll restrict the rotational and translational diffusion of tobacco mosaic virus in bulk solution (102). Reduced Ficoll diffusion is not observed within bulk solutions of ovalbumin or BSA (10 – 26 w/w%) (98), suggesting that the presence of and association with volume-excluding polymers is not the sole determinant of restricted macromolecular diffusion in cells (103). Among the other likely determinants is nonspecific electrostatic attraction to and repulsion from other proteins in the cytoplasm (1). Note, however, that macromolecular crowding does play a major role in restricted diffusion since disrupting the cytoskeleton and organelles of HeLa cells does not eliminate the observed anomalous diffusive behavior (104). Similarly, the stage of the cell cycle has no effect (104).

Volume exclusion is widely observed in cells and plays important and wide-ranging roles in cellular function (18-22). Much insight into cellular function has been gained from the use of inert polymers as substitutes for the crowded environment that is inherent to the cytoplasm (18-22). As the next section illustrates, it is the broad goal of this thesis work to apply the principles of macromolecular crowding towards cytoplasmic organization (105).
**Aqueous two-phase systems: Biocompatible volume-excluding media.**

Microcompartmentation and volume exclusion have been demonstrated to be functionally important in living cells. A fundamental and open question posed by the experimental demonstration of microcompartmentation is how it might be maintained *in vivo* (1,62,106). The goal of this thesis work has been to investigate whether the physical effect of volume exclusion can be used to address this question (106). A simpler but useful model system can be found in bulk solution. A bulk aqueous volume-excluding solution comprised of two or more chemically dissimilar polymers (or a polymer and certain salts) is typically able to undergo phase separation, the segregation of the polymer species into distinct domains (107-110). It has been experimentally determined that whether or not aqueous phase separation occurs and the degree of this segregation is dictated by many factors. Among these factors are fundamental properties of the polymers (such as molecular weight, weight percent, conformation, charge, hydrophobicity, and polydispersity), degree of vicinal water, solution temperature, and salt concentration (107). Many biomaterials can be partitioned within an ATPS, the degree of which is often determined experimentally. It is for these reasons that developing a basic thermodynamic theory of aqueous phase separation and partitioning within ATPS has been pursued extensively, but has also proven to be extremely challenging (111-118). Despite these challenges, bulk immiscible aqueous two-phase systems (ATPS) have long been used as non-denaturing media (109); they typically contain approximately 60-90 w/w% water (109) and can be prepared in high concentrations of buffer (109). Further, ATPS can be used to exploit fundamental
properties of the polymers comprising the ATPS for the separation and purification of biomaterials such as proteins, nucleic acids, organelles, and intact cells (107-110) as well as inorganic nanoparticles (119-122). ATPS have been especially useful tools beyond separation media to analyze and quantify fundamental properties of biologically relevant materials such as DNA hybridization (123), the isoelectric point of proteins (124-128) and cells (129,130), and the chemical composition of membranes (131) as a function of the ATPS and biomaterial composition. ATPS are also known to stabilize proteins and DNA under otherwise denaturing conditions (49). Their biocompatibility is well-illustrated through cultivating bacteria within them and collecting an enzyme from these bacteria in one of the two phases; xylanase can be harvested from *Penicillium janthinellum* in this manner (132).

The most commonly reported ATPS in the literature are comprised of poly(ethylene glycol) (PEG), dextran, and water, but as mentioned previously it is also possible to prepare ATPS from a wide variety of polymer combinations, even PEG/salt ATPS such as PEG/phosphate (107-110), although in this case large concentrations of phosphate are required (109). While in most cells no individual protein is in high enough concentration to undergo aqueous phase separation with another protein, the total protein concentration in the cytoplasm is similar to the total polymer concentrations used in bulk ATPS (20,107-110). Of particular importance for the work presented herein is the fact that on a fundamental level, ATPS exhibit a fundamental physical feature of the cellular cytoplasm: both are volume-excluding media capable of biomolecular segregation to specific domains (ie, microcompartmentation).
**Primitive biomimetic architectures through synthetic phospholipid assemblies.**

Aqueous two-phase systems can be used as cytomimetic media, but by themselves are not easily addressable on the microscopic level. This thesis focuses on aqueous phase separation within giant vesicles (GVs), although aqueous phase separation can be observed within micrometer-scale volumes without entrapment within vesicles (133,134). Phospholipid monolayers, bilayers, and vesicles have long been used as primitive analogs of the cell membrane. GVs are widely used (135-148) due to their large size, allowing for manipulation and observation through light microscopy (149), and ease of preparation through a variety of methods including hydration, electroformation, and size extrusion (140-148). High salt concentrations can be introduced within GVs through either electroformation (140,141) or the hydration method (143).

The ease of GV assembly, modification, and manipulation has applications towards this thesis work and future directions. GVs encapsulate the surrounding medium in which they were prepared; this thesis work describes the preparation of GVs in an ATPS in order to encapsulate the ATPS (Figure 1.1). This property can be used to encapsulate large biomolecules such as DNA (150), but it is also possible to entrap biomolecules within an individual GV through microinjection (151,152) and more elaborate methods such as specific molecular transporters (153). Alternatively, exchange of small molecules with the surrounding solution after formation can be achieved under certain conditions as long as the molecular weight is at or above 4 kDa (140,141). GVs can be fused, inducing mixing of their contents (154). GVs can be easily surface-functionalized for various purposes such as micron-scale surface patterning (155,156).
The composition and properties of GVs can be tailored for different applications. Besides phospholipids, GVs can also be prepared from polypeptides (157, 158). GVs with a high mechanical strength can be prepared from block copolymers (polymersomes) (159-163) or polymer-lipid hybrids (164). It is possible to include membrane proteins in GVs through a number of different methods, further expanding their similarity to cell membranes (165) which may possess a protein content of up to approximately 85 w/w% (166). Negatively charged GVs containing the protein zein within the vesicle membrane exhibit growth and shrinkage as a response of the concentration of smaller vesicles added, solution pH, and concentration of sodium dodecyl sulfate (SDS) surfactant (166). Functional bacteriorhodopsin (BR) and ATPase can be incorporated within GV membranes through fusion of GVs with smaller vesicles that are covalently bound to BR (167, 168). The addition of sucrose to the smaller vesicle suspension before dehydration and rehydration dramatically improves the retention of protein activity, approaching 100% activity retention for a number of proteins such as DNA-binding proteins, channels, and transporters (169). Interestingly, it has been proposed that the heterogeneity within polymersome membranes facilitates the incorporation of proteins within the membranes despite their long length, in some cases exceeding 7 nm (159, 170). Limited success has been achieved through the fusion of liposomes with polymersomes in an attempt to combine the benefits of biocompatibility with mechanical strength (164).

Similarly to the use of an ATPS to primitively mimic the cell cytoplasm, GVs are often used to explore fundamental cell membrane properties on a primitive level. This is noteworthy since the GV membrane may play a critical role in surface effects exhibited by ATPS GVs. Local membrane curvature can be physically manipulated to induce lipid
phase separation in GVs (171,172). The effect of unsaturation-induced curvature has been quantified (173) and the effect of local curvature on lipid mixing in monolayers can be investigated (174). GVs, bilayers, and monolayers containing lipids with different phase transition temperatures may be prepared, allowing for fundamental theoretical and experimental investigations of lipid phases (hypothesized to be present but to date not directly visualized within living cells) within microscopically visible model systems (175-183). Lipid phase separation is often studied through fluorescence (181). The use of quantum dots (184-187) within membranes (187) brings with it further possibilities toward quantifying lipid phase localization (187) such as inherent stability within nonpolar environments (187) and reduced photobleaching (184). The fundamental effects of lipid membrane mechanics [in isolation from the effects of proteins (188,189)] on exocytosis in GVs (190) have been investigated. The assembly and dynamics of GVs is highly complex, exemplified by the recent report (191) that small changes in the phospholipid headgroup, specifically the absence of an acetyl group and presumably a change in spontaneous curvature of the lipids (191), can be used to induce the formation of vesicles from what is otherwise a random network of lipids (191). The authors propose that this general phenomenon may explain the formation of the first cell (191).

There are therefore many options for GV assembly in terms of the primary scaffold (lipids or polymers) and the constituents incorporated within the membrane (proteins). This allows one to focus on manipulating the aqueous environment of the vesicle, a field that has not been well studied, without necessitating a focus on developing new methods for GV assembly from scratch because much work has focused on this aspect of GV assembly.
ATPS GVs: Addressing fundamental questions of cytoplasmic organization and function.

This thesis presents ATPS GVs as a primitive class of “synthetic cells” (192-196). While other research has focused on demonstrating such processes as enzymatic activity within GVs, such as transcription and translation within an encapsulated functional cytoplasmic extract (197), expression of GFP (198), mRNA synthesis (199) and self-cleavage (200), enzymatically-induced budding and vesicle formation from a larger vesicle (201), hydrolysis of fluorescein diphosphate (202,203), and lipase activity (204), this thesis presents an alternative approach to the synthetic cell, specifically the application of ATPS GVs for dynamic and directed microcompartmentation as a function of either temperature or osmotic pressure. The focus on the cytoplasm of cells rather than on the membrane distinguishes this work from most studies performed with GVs.

Several complimentary approaches for inducing dynamic microcompartmentation within GVs have been reported recently (205-209), including inducing sol-gel transitions in GVs (205,206) and mixing within both GVs and living cells (207-209). However, none of them demonstrate the dynamic and reversible segregation and mixing of proteins in response to an external stimulus as is demonstrated in this thesis (210).

Volume exclusion has been hypothesized to be a fundamental driving force for a wide variety of fundamental processes such as DNA condensation in bacteria (61-63), protein and DNA microcompartmentation in the cytoplasm (1,50,106), microfilament organization within the cytoskeleton (34), and protein folding in cells (68). The only examples of aqueous phase separation in cells are within lens cells (1); phase separation of the proteins within the cytoplasm of lens cells can be achieved either through changes
in temperature (1) or PEG concentration (211). If aqueous phase separation were present in the cytoplasm, it should be comprised of many phases since there are many different types of proteins within the cell; this would be difficult to visualize and isolate, especially if it occurs on a scale smaller than that directly observable by light microscopy. Multiple synthetic aqueous phases, such as would be expected within living cells, have been prepared and visualized in bulk solution; Albertsson has observed a bulk solution comprised of 18 phases (109). This thesis does not present an opinion on the hypothesis of in vivo aqueous phase separation; it is not claimed herein that aqueous phase separation is either present within cells or affects cellular function. Instead it is demonstrated that ATPS GVs are useful as physically addressable microscopic media that mimic some of the basic structural and functional characteristics of cells (dynamic and directed microcompartmentation of proteins within the cytoplasm and regulation of cellular volume).

In addition, ATPS GVs may be useful as primitive structures to understand some of the fundamental, nonspecific forces that may contribute to cytoplasmic structure and function beyond phase separation of the proteins within the cytoplasm (1,106,211). As one example, signaling complexes have been observed to form microcompartments in cells (212), but less is known about how volume exclusion may impact the assembly of these microcompartments (17). Additionally, it is known that histones and other proteins are important in maintaining DNA microcompartmentation in prokaryotes and eukaryotes (3), but less is known about the nonspecific effects of volume exclusion and (possibly) aqueous phase separation may have on this process (50,61-63,109). It is likely that volume exclusion impacts DNA compaction in vivo. High concentrations of PEG
solutions induce compaction of DNA in bulk solution (56-59). It has been demonstrated that volume exclusion stabilizes DNA in cells (49) and has been hypothesized to be a driving force for DNA condensation in vivo (61-63). ATPS GVs offer the possibility of addressing such questions on a primitive level.

The goals of this thesis work have been fulfilled through several major results. I have demonstrated dynamic protein microcompartmentation within ATPS GVs through small changes in temperature (101, 210, 213) or osmolarity. This microcompartmentation can be either symmetric or asymmetric, the latter being a primitive model of polarity in cells (214-216). I have quantified the impact of membrane elasticity on asymmetry and the changes in membrane surface tension, membrane surface area, and vesicle volume upon dynamic asymmetric protein microcompartmentation. I have increased protein partitioning in bulk ATPS through adsorption onto colloidal Au and have quantified the impact of both the nanoparticle scaffold and the ATPS on partitioning, as well as demonstrating the retention of significant levels of activity. I have also investigated the impact of temperature and salt concentration on aqueous phase separation and have demonstrated that the observed behavior can be rationalized through vicinal water about the polymer chains as well as the conformation of the polymer chains.
References.


103. Restrictions of the diffusion of small molecules, macromolecules, and nanoparticles observed in bulk volumeexcluding solution appear to be independent of the analyte as determined through fluorescence correlation spectroscopy (FCS) (217).


128. A limitation is that the salt must not induce a conformational or other change in the protein that affects partitioning (218).


131. An ATPS can be used to differentiate between inside-out and right side-out red blood cells (219).


141. This work (140) is particularly interesting in that it surmounts difficulties typically associated with preparing GVs in high salt concentrations through introducing salt *after* formation. This likely only works with the electroformation method of GV assembly; the mechanism of solute exchange is thought to occur through lipid tubules that attach the GVs to the electrode surface (140).


183. The role of proteins in membrane organization (220,221) and the existence of lipid rafts in living cells (221) are under debate. It has been speculated that heterogeneity of the lipids within cell membranes may be partly due to the influence of adsorbed polymers (222) such as cytoskeletal filaments.


Chapter 2
Dynamic Protein Microcompartmentation in Aqueous Two-Phase Vesicles

A fundamental feature exhibited by living cells is microcompartmentation (1), the subcellular segregation of proteins, DNA, and other molecules within the cytoplasm. This feature is widely observed in cells and strongly impacts cellular function, whether induced artificially through binding the substrate of an enzyme in close proximity to the enzyme (2) or naturally observed such as in proteins targeted to the nucleus (3), glycolytic enzymes bound to the mitochondrial membrane, (4) or aggregates of proteins arranged in a signaling complex (5). Microcompartmentation is maintained through a number of different mechanisms, among them physical barriers such as a membrane and direct binding to a membrane or cytoskeletal filament (6). Mechanisms such as these are readily observed experimentally. For example, membranes can be directly visualized under a microscope and binding events can be detected through fluorescence resonance energy transfer (FRET) (7). It is reasonable to suspect that cells also exhibit other less obvious mechanisms of microcompartmentation that are less tractable (8), but it is less clear what these mechanisms may be.

Another fundamental feature exhibited by living cells, the high concentration of proteins within the cytoplasm (9-11), provides some clues towards such a potential mechanism. This feature is known as macromolecular crowding (volume exclusion) (12) and strongly impacts protein and DNA structure and activity based on many in vitro and in vivo studies. The cytoplasm is often mimicked in bulk solution through substituting
“background” proteins with an equivalent concentration of other polymers such as PEG and dextran (12). Cytoskeletal proteins are known to polymerize in bulk solution depending on the concentration of poly(ethylene glycol) (PEG) or dextran present (13); the intrinsically disordered protein FlgM gains structure within living cells and within bulk volume-excluding media (14); DNA spontaneously condenses in PEG solutions (15), suggesting that volume exclusion may serve as one of the driving forces for DNA condensation in living cells (16,17). Solutions containing both PEG and dextran are particularly interesting in that they are known to segregate into two distinct domains (bulk “microcompartmentation”), even at concentrations less than the typical protein concentration observed within cells (18-20). Importantly, proteins and DNA often exhibit a nonspecific affinity for one aqueous phase over the other (18-20), although partitioning can be dramatically increased through binding to one of the phase-forming polymers (21,22).

This chapter presents the use of volume-excluding media to induce dynamic protein microcompartmentation within the aqueous interior of giant vesicles (GVs) (23). Others have reported experiments on mimicking the cytoplasmic environment within GVs through different mechanisms and often with a different focus. Three broad subgroups will be discussed briefly: those that aim to demonstrate enzymatic activity within GVs, mimic the physical environment of the cell cytoplasm, and induce mixing within GVs.

Enzymes and other biologically active macromolecules can be encapsulated within the aqueous compartment of GVs with the retention of activity. Yoshikawa and coworkers have demonstrated the production of green fluorescent protein (GFP) within
GVs (24) while Oberholzer and coworkers have demonstrated the production of mRNA within GVs (25). Kinnunen and coworkers have demonstrated the activity of sphingomyelinase on GVs, resulting in either exocytosis or endocytosis depending on whether the enzyme is added outside and next to the GV or inside the GV (26). Orwar, Zare, and coworkers have demonstrated the activity of alkaline phosphatase in GVs (27,28). Noireaux and Libchaber have pursued a more complex strategy, encapsulating cellular cytoplasmic extract within GVs with the retention of enzymatic activity (29).

The physical environment of the cytoplasm has been mimicked in GVs. Jesorka and coworkers microinject poly(N-isopropylacrylamide) (p-NIPAAm) into GVs and use these structures to induce reversible sol-gel transformations as a function of temperature (30). While they demonstrate dynamic structuring of the aqueous compartment within GVs, they do not demonstrate dynamic protein microcompartmentation. Viallat and coworkers microinject an agarose gel within GVs and use osmotic stress to induce shape deformations of the GVs (31). The gel serves as a primitive analog of the cellular cytoskeleton (31) and is also used to change the physical properties of the aqueous interior of GVs.

Mixing of the aqueous interior of GVs and even living cells has been demonstrated. Ferrer and coworkers use a laser to selectively disrupt the membrane of a specific organelle to induce peptides encapsulated within the organelle to localize to the cytoplasm or nucleus of the cell (32). Vogel and coworkers encapsulate smaller vesicles within GVs and use the differential lipid phase transition temperature of the smaller vesicles to initiate release of carboxyfluorescein from the smaller vesicles into the surrounding GV upon a change in temperature (33). Orwar and coworkers have used
lipid nanotubes to initiate mixing of solutions from the nanotube within GVs (34). Kulin and coworkers use UV light to fuse two GVs, resulting in chelation of a fluorophore from one GV with calcium ions from the other GV (35). While all of these techniques can be used to induce mixing of the contents within microscopic volumes, they do not offer the possibility of inducing segregation once again after mixing has been completed.

This chapter presents the encapsulation of an aqueous two-phase system (ATPS) (18-20) within (GVs) to serve as a primitive functional analog of the cell cytoplasm. Proteins that bind to one of the phase-forming polymers are used to initiate protein segregation to one of the aqueous domains. Aqueous phase separation and protein microcompartmentation are reversible through small changes in temperature, thereby offering dynamic control over local protein concentrations within individual vesicles. ATPS GVs offer more control over their local aqueous structure than is possible in a corresponding bulk ATPS (36).

Interestingly, aqueous phase separation has been hypothesized to be present in the cytoplasm of cells due to its high protein content (37). It is difficult to experimentally verify this hypothesis in vivo; to date the only direct observation of aqueous phase separation in cells has been in lens cells in response to temperature (6) or volume exclusion (38). Primitive analogs of in vivo aqueous phase separation within micron-scale environments are needed to test its potential consequences on cellular structure and function on a basic level. ATPS GVs serve this purpose, offering the possibility of serving as directly observable and physically addressable structures that exhibit aqueous phase separation. They are furthermore primitive analogs of cells in that they exhibit the basic cellular features of microcompartmentation and volume exclusion.
Experimental.

Materials.

PEG 4600 Da (lot 00727D1 and 11608EB) and 8000 Da (lot 092K0156); dextran 10,000 Da (lot 101K1115 and 093K0709) and 505,000 Da (lot 112K1492); biotin-conjugated dextran 10,000 Da, and sodium phosphate (monobasic and dibasic) were purchased from Sigma-Aldrich. Fluorescein-conjugated PEG 5000 Da NHS ester (fluorescein-PEG 5000 Da) and biotin-conjugated PEG 5000 Da were purchased from the Shearwater Corporation (Huntsville, AL), now a part of Nektar Therapeutics. Lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 Da] (DOPE-mPEG 2000 Da), and 1,2-dioleoyl-sn-glycero-2-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-rhodamine) were purchased in chloroform from Avanti Polar Lipids (Alabaster, AL). Alexa488-conjugated glucose-binding lectins (39) concanavalin A (Con A), soybean agglutinin (SBA), and phytohemagglutinin (PHA-L) were purchased from Molecular Probes (Eugene, OR), now a part of Invitrogen. Fluorescein-conjugated streptavidin was purchased from Pierce (Rockford, IL). Deionized water (≥ 18.2 MΩ) was obtained from a Barnstead NANOpure unit.
**Bulk ATPS binodals.**

Bulk ATPS binodals were determined according to the cloud point titration method (19,20). Briefly, PEG was added dropwise to a stirred dextran solution until turbidity (the cloud point), after which time an aliquot of buffer was added to achieve one phase. This sequence was repeated to achieve points along the binodal. Data sets were collected as a function of temperature (Figure 2-1) and were independently verified by preparing solutions both above and below the experimental binodals and ensuring that these solutions behaved as the binodals predict. More detail is presented in chapter 3 since it is in that chapter that an elaboration and discussion of the complex phase behavior observed in ATPS is given. Polydispersity between different polymer batches, particularly dextran, leads to differences in the binodals from batch to batch that are important when utilizing an ATPS near a binodal. An ATPS near a binodal that is two phases in one batch of dextran at that given temperature may be one phase in another batch of dextran at the same temperature. It is for this reason that new binodals were determined for each polymer lot number. Additionally, dextran was used for at most one month after opening the bottle and polymer solutions were prepared fresh from the dry polymers each day due to potential degradation of dextran by bacteria.

**Bulk ATPS partitioning and relative aqueous phase volumes.**

Bulk partitioning of polymers and proteins between the PEG and dextran phases was determined through fluorimetry using a Jobin Yvon Horiba FL3-21 instrument and a
Figure 2-1. Left: Sketches of generic ATPS binodals. Weight percent compositions above a binodal are two phases and those below a binodal are one phase. The crosshatched region illustrates those weight percent compositions that are two phases at 5°C and one phase at 25°C. Right: Experimentally-determined binodals of the PEG 4600 Da/dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer ATPS at 5°C and 25°C.
1 cm quartz cell. In a typical experiment, 30 µL of a fluorophore-conjugated polymer or protein (0.1 w/w% stock solution, additionally containing 0.01 w/w% sodium azide) was added to 2970 µL of an ATPS at 25°C (one phase). This solution was thoroughly mixed and allowed to phase separate overnight at 3°C. The next day (typically approximately 24 hours later), 200 µL aliquots of the top and bottom phases from each sample were obtained for analysis. The slits were adjusted to a bandwidth of 5 nm and two consecutive spectra (of four separate samples) were averaged with a step size of 1 nm and a 1s integration time. The solutions were excited at 494 nm and the emission intensity was measured at the emission maximum. Bulk relative ATPS phase volumes were determined through allowing an ATPS to phase separate for approximately 24 hours at 3°C in a graduated cylinder.

**ATPS GV assembly.**

ATPS GVs were prepared similarly to protocols for GV assembly published elsewhere (40-43). A key difference between this ATPS GV preparation procedure (43) and that reported previously (42) is that I did not image ATPS GVs in sucrose solutions and instead imaged them in the top phase of the bulk ATPS. The top phase of the bulk ATPS was used since it is known to be isotonic with the ATPS (Table 2-1) as measured through a Vapro vapor pressure osmometer (Wescor, model 5520). No (few) vesicles were observed when vesicle suspension was dispersed in the bottom phase of the ATPS, possibly due to vesicle aggregation. Negatively charged vesicles are known to aggregate
Table 2-1. Osmolality of the top phase, bottom phase, and the ATPS itself, 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da ATPS. The top and bottom phases were obtained through phase separation of the ATPS at 3ºC for approximately 24 hours.

<table>
<thead>
<tr>
<th>Material</th>
<th>Osmolality (mmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPS</td>
<td>176 ± 1.15</td>
</tr>
<tr>
<td>top phase</td>
<td>167 ± 2.00</td>
</tr>
<tr>
<td>bottom phase</td>
<td>211 ± 3.06</td>
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in dextran, a process that is more energetically favorable with decreasing charge density within the vesicle membrane (44). The ATPS itself was not used as the imaging medium because it phase separates on cooling, resulting in rapid vesicle translation across the field of view of the microscope.

Briefly, lipid solutions were added to a 10 x 75 mm glass test tube (VWR) along with 100 µL of chloroform and swirled to ensure homogeneity. In the preparations described in this chapter, the lipids were always 44:1 mol ratio DOPC/DOPE-mPEG 2000 Da and 0.048 mol% DOPE-rhodamine. This concentration of PEGylated lipid in the membrane is above that of the mushroom-to-brush transition in bilayers (45,46), leading to an extended polymer conformation (45,46) that does not prevent proteins from interpenetrating the PEG chains and directly binding to nearby phospholipid headgroups (47). The brush PEG conformation is more strongly hydrated than the mushroom conformation (48). The test tube was rapidly rotated by hand under a stream of Ar to form a thin lipid film, which was desiccated under vacuum for at least 2 hours to remove all of the chloroform. Argon was bubbled through water and gently blown over the lipid film for 5 minutes to prehydrate the lipids. Afterwards, 990 µL of an ATPS (one phase) and 10 µL (0.1 or 0.5 w/w%) of fluorophore-conjugated polymer or protein were added to the test tube and the solution was allowed to stand for approximately 24 hours at 37°C. The ATPS always contained 5 mM pH 7.0 sodium phosphate buffer for protein partitioning experiments. This buffer concentration does not impact ATPS binodals. For confocal microscopy experiments, the concentration of the fluorophore stock solution was typically 1 w/w% in order to increase the signal to noise ratio within the ATPS GVs; depending on the size of the polymer or protein conjugate and the degree of labeling, 0.1
w/w% is typically not enough to give a strong signal above that of the background in confocal microscopy. Afterwards, the solution was allowed to stand for approximately 10 minutes at room temperature, then for at least 1 hour at 3°C to phase separate. After this time, vesicles collected at the bulk ATPS interface (Figure 2-2).

**Microscopy.**

Samples were enclosed by a 20 x 5 mm silicone spacer from Molecular Probes sandwiched between two glass microscope slides (Fisher Scientific). ATPS GV-containing vesicle samples were prepared by adding a 5 µL aliquot of lipid suspension from the bulk ATPS interface to 165 µL of the top phase from the original bulk polymer solution (lipid- and fluorophore-free).

Optical and fluorescence images were collected on a Nikon TE 200 or TE 300 inverted microscope using a Nikon Plan Fluor 100X objective (N.A. = 1.3). Light was collected with a CoolSNAP HQ CCD camera (Photometrics, Tucson, AZ), 1392 × 1040 pixels or this other model (1024 x 1024 pixels) and acquired with Image Pro Plus software v4.5 (MediaCybernetics, San Diego, CA). Transmitted light (Nikon tungsten halogen source) was used for differential interference contrast (DIC) images, and either a 175 W Xe lamp (Sutter Instruments, Novato, CA, Lambda 10-2 filter housing) or a Hg lamp was used for fluorescence imaging. Excitation and emission of rhodamine-labeled lipids was performed using a Nikon wide green filter cube (no. EF-4 G-2A); fluorescein, fluorescein isothiocyanate (FITC), and Alexa488 excitation and emission were performed
Figure 2-2. Schematic of the ATPS GV assembly procedure. Bar = 10 µm.
with a Nikon FITC cube (no. 96107). Heating and cooling was controlled through a
Linkam PE-100 microscope stage using a Linkam PE-94 control unit (± 0.1°C). The
exact temperature of the microscope stage was measured using a microprobe (Harvard
Apparatus, model IT-21, ± 0.1°C) and a Physitemp BAT-12 readout unit. The
heating/cooling rate was 10°C per minute. The microscope stage was connected to a
VWR Scientific circulating water bath, model 1160A, filled with distilled water set to
4°C. Final images were processed with Adobe Photoshop v7.0 (Adobe Systems, San
Jose, CA).

Confocal microscopy was performed on an Olympus IX-70 inverted microscope
using a PlanApo 60X objective (N.A. = 1.4). Fluorescein, FITC, and Alexa488
fluorescence were measured using an Ar laser source (488 nm, 10 mW). Rhodamine
fluorescence was measured using a HeNe laser source (543 nm, 1 mW). Images were
collected and processed with Fluoview v4.3. An average fluorescence background was
subtracted from PEG-rich phase and dextran-rich phase intensities ("subtraction
method") prior to calculating the partition coefficient, K, the average intensity in the PEG
phase divided by that in the dextran phase. Alternatively, to eliminate background
fluorescence, the vesicle suspension could be collected from the bulk ATPS and then
added to a fluorophore-free ATPS of the same composition as that used during vesicle
preparation ("washing method"). Vesicles collected from this ATPS after phase
separation and dispersed as normal for microscopy exhibited no background
fluorescence. Further, the partition coefficients obtained afterwards were the same within
one standard deviation using both techniques; for example, in 44.3:1 mol ratio
DOPC/DOPE-mPEG 2000 GVs prepared in the 7.5 w/w% PEG 8000 Da (lot
034K02201)8 w/w% dextran 10,000 Da (lot 083K12931)/5 mM pH 7.0 sodium phosphate buffer ATPS, K (dextran 10,000 Da) = 0.45 ± 0.11 (17 ATPS GVs) using the subtraction method and K = 0.49 ± 0.038 (12 ATPS GVs) using the washing method. The washing method was found to save much time relative to the subtraction method. The temperature of the microscope slide was maintained at 5°C using the before-mentioned microscope stage and attached water bath. Additionally, a lipid membrane was not present within any of the ATPS GVs used for quantitation; neither was there extraneous lipid material present within these ATPS GVs.

NIH Image v1.63 was used to measure relative aqueous phase volumes, the volume of the inner aqueous phase relative to that of the entire ATPS GV at 5°C. Only concentric ATPS GVs were used for these measurements. The same requirements were followed for extraneous lipid material as in the before-mentioned confocal microscopy quantitation measurements of partitioning. The intensity changes across the maximum width of the ATPS GV (4 pixel line width) were determined through DIC, which allowed for determination of both the vesicle border as well as that of the aqueous-aqueous interface. DIC images were only obtained after the inner phase diffused to the center (z-direction) of the ATPS GV. Typically, the inner phase was not aligned in the xy-center of the vesicle, in which case the measurement process was repeated for the inner phase. The pixel values were used to determine relative aqueous phase volumes by converting standardized scaling values at 100X magnification. Histograms were prepared from multiple data sets from separate ATPS GV preparations. Average values did not vary significantly from batch to batch.
Results and Discussion.

Aqueous phase separation within ATPS GVs.

Aqueous phase separation can be induced within ATPS GVs as long as the ATPS weight percent composition is one phase at 25°C or 37°C (ideal for ATPS encapsulation) and two phases at 5°C (ideal for ATPS phase separation). These compositions can be determined by experimentally quantifying ATPS binodals at the relevant temperatures (Figure 2-1). Relative aqueous phase volume measurements suggest that ATPS GVs do not encapsulate identical contents (Figure 2-3), although aqueous phase transitions are observed in ATPS GVs regardless of relative aqueous phase volumes (see later). Another possibility is that different ATPS are encapsulated within ATPS GVs, but they are all along the same tie line. A brief discussion of this point follows.

In a bulk ATPS, relative aqueous phase volume measurements are the same from experiment to experiment, i.e., a measured value of 37 v/v% dextran phase is reproducible in multiple samples of the same polymer weight percent composition at the same temperature within the experimental error of a graduated cylinder. This is not the case in ATPS GVs; there is some degree of variability in relative aqueous phase volumes from vesicle to vesicle (Figure 2-3). This is not entirely due to experimental error; at least some ATPS GVs in the same batch obviously have differing relative aqueous phase volumes. This may lead one to speculate that the polymer weight percent compositions in such ATPS GVs significantly differ. However, aqueous phase transitions are possible within ATPS GVs regardless of relative aqueous phase volumes, a property only possible in bulk solution within a limited range of polymer weight percent compositions (Figure
Figure 2-3. Typical distribution of volume percent dextran phase within ATPS GVs. Average percent dextran phase: bulk, 16 v/v%; ATPS GVs, 23 ± 14 v/v%. ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer. T = 5°C.
A possible explanation of these results is that a given ATPS GV may not have the same overall polymer weight percent composition as another ATPS GV, but the ATPS within each GV is along the same tie line. In a bulk ATPS, all polymer weight percent compositions along a tie line have the same weight percent compositions within the PEG and dextran phases, although differing total weight percent compositions and differing relative aqueous phase volumes. This would also explain why polymer and protein partitioning within ATPS GVs is not dramatically variable from vesicle to vesicle (Figure 2-4).

Dynamic aqueous phase separation and protein microcompartmentation within ATPS GVs.

Aqueous phase separation can be induced within ATPS GVs through either cooling the vesicles or concentrating the ATPS within the GVs through dispersion in hypertonic media (Figure 2-5). Aqueous phase separation can be rendered dynamic (reversible) within ATPS GVs dispersed in isotonic media through small changes in temperature (Figure 2-6) (43). The typical 2- to 1-phase transition is for the inner phase to slowly merge with the outer phase; the typical 1- to 2-phase transition is for multiple inner phases to appear and coalesce into one larger inner phase (Figure 2-6). Such results are typically observed in ATPS GVs independent of the polymer molecular weights as long as the polymer weight percent composition is within the region of the phase diagram discussed beforehand (Figure 2-7, Figure 2-8). The typical length of time to complete an aqueous phase transition is on the order of minutes to approximately
**Figure 2-4.** Typical distribution of K within ATPS GVs. ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer. T = 5°C.
Figure 2-5. Aqueous phase separation in ATPS GV s dispersed in (A) the top (PEG-enriched) phase (isotonic media) and (B) hypertonic sucrose. The latter forces phase separation by concentrating the ATPS within the GVs. Columns, from left: transmitted light (DIC), lipid fluorescence, dextran fluorescence. T = 5ºC, bar = 10 μm.
Figure 2-6. DIC images of typical aqueous phase transitions within an ATPS GV. ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da. Bar = 10 µm.
Figure 2-7. DIC images of aqueous phase transitions within an ATPS GV. ATPS: 4.5 w/w% PEG 8000 Da/2.25 w/w% dextran 505,000 Da. Bar = 10 µm.
Figure 2-8. DIC images of aqueous phase transitions within an ATPS GV. ATPS: 7.875 w/w% PEG 8000 Da/7.875 w/w% dextran 10,000 Da. Bar = 10 µm.
one hour (Figure 2-6) (49). The aqueous phase transition temperature is approximately that of the bulk ATPS taking into consideration some probable localized heating of the sample from the objective (50). Multiple aqueous phase transitions are possible within ATPS GVs (Figure 2-9); phase separation and phase mixing can be reproduced multiple times within an individual ATPS GV.

Note that an ATPS GV preparation is not completely comprised of ATPS GVs. I observe variation both within the vesicle membranes, common for GVs prepared through the hydration method, as well as variation within the ATPS (Figure 2-10). Some structures observed beyond multilamellar vesicles and one-phase vesicles are ATPS GVs in which there is a lipid membrane at the ATPS interface (Figure 2-10); in these structures, the PEG phase can either be the outer or inner phase (Figure 2-11). Since there is no ATPS phase boundary within such structures, it is reasonable that there is also no relative aqueous phase position preference. This result is independent of the ATPS and the GV membrane composition. On rare occasions I have observed three-phase vesicles in which an inner PEG phase is surrounded by a dextran phase that is itself surrounded by another PEG phase (Figure 2-12). Aqueous phase separation within these structures is dynamic (reversible) as well (Figure 2-13).

Proteins that bind to one of the phase-forming polymers can be reversibly partitioned along with the aqueous phase (Figure 2-14) (43). Glucose-binding lectins were chosen to bind to dextran (dextran is a polymer of glucose) and streptavidin was targeted to either the PEG phase or the dextran phase through the concomitant addition of excess biotin-functionalized PEG or dextran. This binding to the polymers was necessary to increase protein partitioning. Bulk ATPS partitioning work is usually done well within
Figure 2-9. DIC images of 6 aqueous phase transitions within an ATPS GV. ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da. Bar = 10 µm.
Figure 2-10. Some typical vesicles from an ATPS GV preparation. From top, a one-phase ATPS GV, a multilamellar GV, an ATPS GV, and an ATPS GV containing extraneous lipid membranes within the aqueous compartment. Columns, from left: transmitted light (DIC), lipid fluorescence, and protein (Con A) fluorescence. T = 5°C, bar = 10 µm.
**Figure 2-11.** ATPS GV s that have a lipid membrane at the ATPS interface can have either a PEG phase or a dextran phase as the inner phase. This is presumably due to the lack of an ATPS phase boundary, therefore eliminating aqueous-aqueous interfacial tension effects in determining relative aqueous phase position preferences. ATPS: 4.125 w/w% PEG 8000 Da/2.625 w/w% dextran 511,000 Da. Columns, from left: DIC, lipid fluorescence, dextran fluorescence. T = 5°C, bar = 10 µm.
Figure 2-12. A vesicle that contains three phases within the aqueous compartment, rare within ATPS GV preparations. ATPS: 7.5 w/w% PEG 8000 Da/8 w/w% dextran 10,000 Da. Columns, from left: DIC, lipid fluorescence, dextran fluorescence. T = 5°C, bar = 10 µm.
Figure 2-13. A three-phase vesicle can undergo aqueous phase transitions. ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da. Bar = 10 µm.
Figure 2-14. A. DIC images of aqueous phase transitions within an ATPS GV. ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da. Bar = 10 µm. B. Micrographs illustrating dynamic aqueous phase separation and protein microcompartmentation within the same ATPS GV. Columns, from left: DIC, lipid fluorescence, lectin SBA fluorescence. Bar = 10 µm.
the two-phase region of the ATPS. Such results are not possible to reproduce in thermo-responsive ATPS GV's due to the inherent limitation of ATPS GV assembly, specifically using polymer weight percent compositions near a binodal (a limitation to be addressed further in chapters 4 and 5).

Quantification of partitioning.

Binding to the phase-forming polymers provides an easy check for the retention of protein activity within ATPS GV's through measuring the degree of partitioning. If the function of a protein is to bind to one of the polymers comprising the ATPS, partitioning is directly related to activity. Polymer and protein partitioning within ATPS GV's has been quantified (Table 2-2) (43). The partition coefficient, $K$, is defined as the amount of analyte in the PEG phase divided by that in the dextran phase. As such, values of $K$ greater than 1 indicate partitioning to the PEG phase and those less than 1 indicate partitioning to the dextran phase. Although it is possible to achieve a 3-fold to 7-fold degree of protein partitioning in ATPS GV's, microcompartmentation in ATPS GV's is much less than that within a corresponding bulk ATPS (43).

These differential partitioning results were of special concern since protein partitioning was achieved through binding to one of the phase-forming polymers. Since protein partitioning is reduced in ATPS GV's, a reasonable first approximation would suggest that protein binding is less and therefore the proteins are denatured within ATPS GV's. However, these differences in protein partitioning are due to differences in
Table 2-2. Partitioning of phase-forming polymers and proteins within an ATPS in bulk and within GVs. Each data point in the bulk K column represents the average of at least 4 independent measurements. The numbers in parenthesis within the right-hand column are the number of ATPS GVs quantified to arrive at the reported value of K. In the case of streptavidin, biotin-functionalized polymer was added in equal weight percent to the streptavidin (excess mol% since the molecular weight of streptavidin is approximately 60,000 Da). ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer.

<table>
<thead>
<tr>
<th>polymer/protein</th>
<th>K bulk (3°C)</th>
<th>K GVs (5°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 5000 Da</td>
<td>4.8 ± 0.11</td>
<td>1.7 ± 0.37 (22)</td>
</tr>
<tr>
<td>dextran 10,000 Da</td>
<td>0.28 ± 0.0050</td>
<td>0.40 ± 0.11 (32)</td>
</tr>
<tr>
<td>streptavidin (excess biotin-PEG 5000-Da)</td>
<td>39 ± 3.7</td>
<td>6.7 ± 2.9 (22)</td>
</tr>
<tr>
<td>streptavidin (excess biotin-dextran 10,000 Da)</td>
<td>0.035 ± 0.0034</td>
<td>0.27 ± 0.086 (16)</td>
</tr>
<tr>
<td>streptavidin</td>
<td>1.7 ± 0.12</td>
<td>1.8 ± 0.40 (15)</td>
</tr>
<tr>
<td>lectin PHA-L</td>
<td>0.048 ± 0.0033</td>
<td>0.26 ± 0.038 (31)</td>
</tr>
<tr>
<td>lectin SBA</td>
<td>0.072 ± 0.0089</td>
<td>0.35 ± 0.13 (15)</td>
</tr>
<tr>
<td>lectin Con A</td>
<td>0.032 ± 0.0096</td>
<td>0.33 ± 0.12 (20)</td>
</tr>
</tbody>
</table>
polymer partitioning, not protein denaturation. When the polymer weight percent is manipulated such that the bulk polymer partitioning approaches that previously observed within ATPS GVs, bulk protein partitioning also approaches that observed previously within ATPS GVs (Figure 2-15 and Figure 2-16) (43). Potential reasons for this differential polymer partitioning are elaborated upon in chapter 4.

Heating a bulk ATPS containing lectins to 37°C overnight, then cooling to 3°C overnight to initiate phase separation, does not change the partition coefficient within one standard deviation. This suggests that heating to 37°C does not denature the lectins in an ATPS under these conditions, and especially not streptavidin, which is stable under a wide range of temperatures and pH values. The mechanism of lectin partitioning involves binding to dextran since adding glucose to a bulk ATPS brings the partition coefficient closer to 1 as a function of increasing glucose concentration (Figure 2-17) (51). Con A binding to dextran is known to be reversible with the addition of excess glucose (52). Collectively, these results suggest that protein activity is retained within ATPS GVs.
Figure 2-15. Effect of PEG partitioning on streptavidin (in excess biotin-PEG 5000 Da) partitioning in a bulk ATPS. The PEG partitioning was manipulated through changing the polymer w/w% within the ATPS. ATPS: PEG 4600 Da/dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer. T = 3°C. Each data point represents the average of at least 4 independent measurements in both axes.
Figure 2-16. Effect of dextran partitioning on lectin PHA-L and streptavidin (in excess biotin-dextran 10,000 Da) partitioning in a bulk ATPS. The dextran partitioning was manipulated through changing the polymer w/w% within the ATPS as in Figure 14. ATPS: PEG 4600 Da/dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer. T = 3°C. Each data point represents the average of at least 4 independent measurements in both axes.
Figure 2-17. Effect of adding glucose to an ATPS on the partitioning of lectin SBA. ATPS: 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer ATPS with additional glucose as noted in the figure. T = 3°C. Each data point represents the average of at least 4 independent measurements.
Conclusions.

Aqueous phase separation can be induced within giant vesicles. There are distributions in the degree of partitioning and relative aqueous phase volumes from vesicle to vesicle that do not appear to hinder dynamic aqueous phase separation. Aqueous phase separation appears to be dynamic (reversible) irrespective of the ATPS weight percent composition as long as the external medium is isotonic and the preparation yields ATPS GVs. Protein microcompartmentation can be achieved within ATPS GVs through proteins that bind to one of the phase-forming polymers. These proteins retain activity as demonstrated through comparison of partitioning in bulk relative to that within vesicles. Dynamic temperature-induced aqueous phase separation and partitioning qualitatively approximates that of a corresponding bulk ATPS.
Future Directions.

A fundamental function of living cells is the selective transport of molecules across the cell membrane. Biomolecular transport across ATPS GV membranes has applications both in delivery of extravesicular molecules to within the vesicle as well as controlling the chemical environment within the vesicles. Microcompartmentation is achieved within the cellular cytoplasm through a variety of mechanisms; developing other methods to initiate the effect within ATPS GVs beyond temperature or osmolarity (43) will expand their functionality and bring them closer to the complexity observed within living cells. Certain salts are known to affect bulk ATPS phase behavior; it is conceivable that aqueous phase behavior within ATPS GVs can be controlled through the dynamic, selective transport of ions across the membrane. Small molecules such as water diffuse readily across phospholipid bilayers. The transport of many molecules, however, becomes more difficult with increasing size and polarity of the molecule. This problem can potentially be addressed through incorporating channels of either natural or synthetic origin within ATPS GV membranes.

Protein channels can conceivably be incorporated within ATPS GV membranes to allow for nondestructive changes in the composition of the vesicle interior as well as serving as a mechanism of delivery of extravesicular molecules that would not otherwise cross the membrane. α-Hemolysin (53,54), a toxin that forms transmembrane channels in cells, is an attractive candidate. The internal dimensions of this and similar pores can be non-destructively determined (55), important when investigating the potential of mutated forms for selective transport (56). α-Hemolysin has been site-selectively
modified (57-59) such that it opens in response to a protease (60) and UV light (61) and switches between both the open and closed state in response to divalent cations (62). Similar techniques have allowed for the introduction of ion-binding sites and size-selective transport through functionalization with cyclodextrins (63,64). The versatility of analogous modifications has been demonstrated through the detection of single molecules in biomembranes (65,66), single-base mismatched DNA (67), and the simultaneous detection of divalent cations (68). Other naturally occurring channels exist that serve in protein transport across membranes (69).

The use of channels is not limited to those of natural origin. Size-selective peptide nanotubes have been synthesized that allow for the transport of glucose (70) and glutamic acid (71). The ion transport propensity and selectivity of such channels can be modified (72). Both interior and exterior surface functionalization of peptide nanotubes is possible (73), which should allow for tailoring their transport properties and ease of incorporation within membranes. Peptide nanotubes have been shown to self-assemble, some types in solution and some within lipid bilayers (74-79). Channels based on self-assembling dendrimers have also been prepared (80).

Channels can be used to transport both small ions and larger molecules. Synthetic transporters of potassium (81) and chloride (82-84) have been synthesized. A novel synthetic molecule has been recently reported that adopts a conformational change upon binding adenosine 5-triphosphate (ATP) (85), shielding the ATP molecule from the nonpolar environment of the lipid bilayer and allowing for the selective transport of ATP over glutathione (GSH) (85). Cooperative detection of ions at the lipid membrane is possible as well; a synthetic membrane-bound receptor has been recently reported that
complexes copper (II) (86). Whether or not a channel is large enough for a molecule to pass through depends on the degree of vicinal water associated with the molecule and not simply the diameter of the channel (87).
References.


36. Examples include directed aqueous phase localization and budding, both presented in chapter 4.


49. Some variability is expected; the completion of a 1- to 2-phase transition within a large vesicle with a small inner phase is expected to take longer than that within a small vesicle with a large inner phase if the phase transition is diffusion-limited.

50. The bulk phase transition temperature of the 9.375 w/w% PEG 4600 Da (lot 00727D1)/9.375 w/w% dextran 10,000 Da (lot 093K0709) ATPS is between 16°C and 17°C measured through a circulating water bath (temperature verified through a microprobe). The phase transition temperature within 44.3:1 mol ratio DOPC/DOPE-mPEG 2000 Da ATPS GVs prepared in this same ATPS is somewhere between 7°C and 15°C, not taking into consideration localized heating of the sample from the objective. Assuming this additional heating is
approximately 5°C, the bulk ATPS phase transition temperature is the same as that within corresponding ATPS GVs within experimental error.

51. Lectin Con A activity is known to require calcium and magnesium ions (39); unfortunately, difficulties arose when attempting to prepare ATPS GVs in the presence of small concentrations of these cations. However, dextran is known to contain divalent cation impurities (20). Bulk partitioning of Con A in an ATPS prepared in 5 mM pH 7.0 sodium phosphate buffer is the same within one standard deviation as that observed in the same ATPS prepared in 5 mM MOPS buffer additionally containing 1 mM each of calcium and magnesium salts, suggesting that the lack of added calcium and magnesium ions to the ATPS does not affect Con A activity.


56. Possessing dimensions on the order of the channel is not the only necessary requirement for the transport of a given molecule due to such factors as the interaction of water with the channel (87).


Chapter 3

Binodal Crossing in Bulk and Microscopic Aqueous Two-Phase Systems

Chapter 2 has implied that the only method to induce phase mixing of a PEG/dextran ATPS is through heating and conversely that phase separation is induced only on cooling. This is not true for all polymer weight percent compositions, i.e., it is possible to induce phase separation in an ATPS on cooling, phase mixing on heating, and phase separation once again on further heating (binodal crossing). This chapter explores this unexpected discovery, specifically some of the potential driving forces behind the observed behavior and its application towards controlling aqueous phase separation and protein microcompartmentation in ATPS GVs.

Aqueous two-phase systems (1) have long been used as non-denaturing media for the purification of a wide variety of biomaterials, from enzymes and DNA to intact cells (1-4). These solutions have further proven their utility towards measuring and applying the fundamental properties of biological polymers and surfaces such as the isoelectric point of proteins (5-7), DNA hybridization (8), and the chemical composition of cell membranes (9). The differential effects of salts on the partitioning of proteins as a function of ATPS pH can be quantitatively utilized to determine the isoelectric point of a protein (6). Double-stranded DNA extracted from bacteria partitions to a different phase than the corresponding denatured, single-stranded DNA (8). The sensitivity of partitioning in an ATPS to analyte properties such as charge can be exploited to separate very similar vesicle species (9).
Consequently, much work has focused on predicting and applying the factors determining the phase behavior of and partitioning within an ATPS (1-3). The goal of such work is to reduce the amount of experimental work that must be completed to determine the most appropriate ATPS for a given separation. Towards this goal much research has therefore focused on developing a theoretical basis for aqueous phase separation and partitioning based on experimental data (1-3, 10-18). An important and fundamental requirement of such a theory is prediction of the ATPS binodal, a graph that is used in part to determine when an ATPS phase separates and when it does not. It has proven challenging to quantify and determine the relative importance of the large number of complex variables involved in determining the polymer weight percents at the binodal as a function temperature and salt concentration as well as the degree of partitioning within the ATPS (1-3, 10-18). Among these variables are polymer/polymer interactions, macromolecular conformation, polymer polydispersity, charge, and vicinal water (1-3, 10-18). It is the focus of this chapter to document the effects of temperature on ATPS binodals and qualitatively rationalize the behavior through some of these variables with an emphasis on water associated with the polymer chains.

There is widespread confusion regarding the effect of temperature on the polymer weight percents over which an ATPS phase separates (1,19,20). Since the question of under what polymer concentration and temperature an ATPS phase separates is fundamental, this confusion should be addressed. Widely differing effects of temperature have been proposed (1,19,20). A simplistic first approximation would be that increasing temperature increases the entropy of the polymer chains, therefore favoring phase separation at higher polymer weight percents with increasing temperature. Some report
that increasing temperature favors phase separation above 20°C (19). Still others report that temperature has little effect on phase behavior below 50°C (1) or 90°C (20).

However, I have unexpectedly discovered that bulk ATPS phase behavior is more complex than any of these propositions. I suspect that the reason for the previously mentioned erroneous assumptions is that most experimentally determined PEG/dextran ATPS binodals reported in the literature almost exclusively include primarily ATPS weight percent compositions containing a high concentration of one of the phase-forming polymers and extrapolate through intermediate polymer weight percent compositions (1). This is likely due to the fact that the most commonly used method to determine ATPS binodals is the polarimetry/refractometry method, representative example given in (21), in which based on personal experience it is difficult to experimentally determine intermediate polymer weight percent compositions along the binodal except through extrapolation. This intermediate range should not be overlooked because it is here that unusual temperature-dependent phase behavior is observed. These intermediate polymer weight percents along ATPS binodals can, however, be accurately determined through the cloud point titration method. I demonstrate the utility of this method of binodal determination herein, and also demonstrate why even small changes in ATPS binodals with respect to temperature can be useful.

This chapter reports on the temperature-dependent binodal crossing of PEG/dextran, PEG/polyacrylamide (PAAm), and PEG/Ficoll ATPS. Whether or not binodal crossing is observed as a function of salt concentration depends on the salt in question. PEG/phosphate binodals shift down with increasing temperature. A rationale for these results as related to the degree of vicinal water (structured water closely associated with
the polymer chains, to be discussed later) and hydrogen bonding within the ATPS as well as its inter-relationship with polymer conformation is presented. While many have proposed that phase separation in PEG/dextran ATPS is driven by a disruption of hydrogen bonding (1,20), a large body of experimental and theoretical evidence has accumulated that demonstrates an extensive vicinal water network about PEG chains and its specific impacts on PEG chain conformation. While the disruption of the vicinal water network in ATPS is surely not the only determinant of temperature- and salt-dependent phase behavior, I focus on vicinal water since this topic has been extensively studied in relation to PEG and it is an aspect of ATPS that can be discussed in qualitative terms.

As an application of these results, the induction of aqueous phase separation and protein microcompartmentation within ATPS GVs at 5°C, phase mixing at 25°C, and phase separation and protein microcompartmentation again on further heating to 50°C is demonstrated. Limitations of such experiments are addressed. Further, results are presented which suggest that the relative volume occupied by the dextran phase in PEG 8000 Da/dextran 500,000 Da ATPS GVs at 5°C is independent of the polymer weight percent composition, a property that is in stark contrast to that exhibited by a corresponding bulk ATPS. Again, such experiments are difficult as discussed in more detail later. However, these results are of interest for future work regarding the control of enzymatic activity within ATPS GVs as a function of local enzyme concentrations as discussed in chapter 5.
Experimental.

Materials.

Poly (ethylene glycol) (PEG), dextran, Ficoll, and sodium phosphate (monobasic and dibasic) were obtained either from Sigma-Aldrich or Fluka. Polyacrylamide (PAAm) was obtained from Scientific Polymer (Ontario, New York). Polymer structures are given in Figure 3-1. Lipids 18:1 (9-cis) 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 18:1 (9-cis) 1,2-dioleoyl-sn-glycero-3-(phospho-rac-[1-glycerol]) (sodium salt) (DOPG), and 18:1 (9-cis) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (DOPE-rhodamine) were obtained from Avanti Polar Lipids (Alabaster, AL). Alexa488-conjugated proteins streptavidin and lectin concanavalin A (Con A) were obtained from Molecular Probes (Eugene, OR). Fluorescein-conjugated streptavidin was obtained from Pierce (Rockford, IL). Biotin-conjugated PEG 5000 Da-NHS was obtained from the Shearwater Corporation (Huntsville, AL), now known as Nektar Therapeutics. Note that polymer weight percent compositions as defined here include any water already present in the polymers as received. “Dry” dextran, for example, is known to contain approximately 5-10 w/w% water (3).

Instrumentation and software.

Instrumentation and software utilized was the same as noted in chapter 2.
**Figure 3-1.** The chemical structures of poly(ethylene glycol) (PEG), dextran, and polyacrylamide (PAAm).
ATPS GV assembly.

Modifications of standard literature protocols were used for ATPS GV assembly (22-24) as in chapter 2. The mol ratio of DOPC to DOPG was approximately 10:1.

Bulk ATPS binodals.

Bulk ATPS binodals were determined according to the cloud point titration method (2,3,22). The basic concept is to add one polymer solution dropwise into the other until turbidity (the cloud point) is observed, then add water (or buffer) beyond the point at which the solution turns clear again. This procedure is repeated in sequence. The polymer weight percent compositions at which the ATPS is cloudy are points along the binodal of the ATPS (3).

It is very important to not add either a highly concentrated polymer solution or large solution volumes during the titrations such that the weight percent compositions at the cloud point are inaccurately estimated. I have determined the most appropriate conditions for a given ATPS through extensive experimental work. To determine the binodals of a PEG 8000 Da/dextran 500,000 Da ATPS, I have found it convenient to start with 1 g of 15 w/w% dextran in an approximately 15 g vial and into it titrate 15 w/w% PEG. Twenty-five weight percent of each polymer species is convenient to determine the binodals of a PEG 5000 Da/dextran 10,000 Da ATPS. To determine how much PEG to initially add to the dextran solution for the first cloud point, it is convenient to add several drops of PEG at a time, and once the approximate amount is known one can reproduce
the results more slowly to arrive at a more accurate determination. For the additions of water or buffer after the cloud point has been reached such that the ATPS is once again in the one-phase region, it is helpful to add a small amount of water after the first cloud point and to add progressively more after subsequent cloud points since total polymer weight percent compositions are less affected by the addition of a given weight of water or buffer as the total volume of the ATPS increases.

Since there are differences in the molecular weight distribution and possibly water content between different batches of polymer (3), ATPS binodals typically shift slightly when different lot numbers are utilized. These shifts are typically small, but enough such that it impacts ATPS GV assembly (Figure 3-2). Independently prepared ATPS solutions of polymer weight percent compositions predicted to be either above or below the experimentally determined binodals were utilized to ensure that the predicted binodals agreed with the observed behavior (visually demonstrated shortly). Solutions exhibited the predicted phase behavior over at least one hour of equilibration at the temperature being evaluated. This suggests that the cloud point titration method, although not a thermodynamic measurement, is accurate (5 separate experiments yielded the same binodal). Temperature control of ATPS solutions was achieved through immersing the aforementioned vial-enclosed ATPS solutions in a circulating temperature-controlled water bath. A microprobe from Harvard Apparatus confirmed the accuracy of the temperature readings given by the water bath within several tenths of a degree C. For high-temperature work, the vial containing the ATPS solution was only opened to add more polymer or water after cooling in an ice bath to prevent evaporation of water from the sample.
Figure 3-2. Impact of polymer lot number on the binodals of PEG 8000 Da/dextran 10,000 Da ATPS. A: PEG lot 71K0150, dextran lot 101K1115; B: PEG lot 044K0092, dextran lot 084K1226; C: PEG lot 034K02201, dextran lot 083K12931. T = 25°C.
Results and Discussion.

Examples of bulk ATPS phase behavior.

As discussed in Chapter 2, an ATPS is either one or two phases as a function of polymer weight percent. Figure 3-3 gives representative examples of ATPS binodals. These are plots of the transition boundary between the one-phase and the two-phase region as a function of polymer weight percent. Compositions below a binodal are one phase and those above it are two phases. The weight percent compositions comprising the binodal are dependent on factors such as polymer molecular weight, as shown in Figure 3-3 and (3), salt concentration, and temperature. Over large weight percents of PEG, the binodals shift up with increasing temperature. In contrast, over lower weight percents of PEG, phase separation is favored both with decreasing temperature below 25°C and with increasing temperature above 25°C; that is, the binodals determined at different temperatures cross. Figures 3-4 - 3-7 illustrate typical phase diagrams for bulk PEG/dextran, PEG/PAAm, and PEG/Ficoll ATPS as a function of temperature. Figure 3-8 is a photograph of an ATPS that is two phases at 5°C, one phase at 25°C, and two phases at 50°C, providing a visual illustration of the binodal crossing effect in a bulk ATPS. Interestingly, polymer partitioning becomes worse over this range (Figure 3-9).

To the best of my knowledge such crossing phase behavior is unique to ATPS, although related behavior is observed (25). The most closely related behavior reported in the literature is the multiple cloud points exhibited by a fluorinated surfactant (25); however, the phase diagrams reported exhibit a one-phase region embedded within a two-
Figure 3-3. A. ATPS binodals of dextran 10,000 Da (lot 084K1226) and different molecular weights of PEG. 1: 4600 Da (lot 14316AC); 2: 8000 Da (lot 044K0092); 3: 12,000 Da (lot 396030); 4: 20,000 Da (lot 390136); 5: 35,000 Da (lot 454839). B. ATPS binodals of PEG 8000 Da (lot 044K0092) and different molecular weights of dextran. 6: 10,000 Da (lot 084K1226); 7: 40,000 Da (lot 124K1343); 8: 70,000 Da (lot 025K0625); 9: 150,000 Da (lot 114K0238); 10: 500,000 Da (lot 123K0671). T = 25°C for all of the binodals.
Figure 3-4. Binodals of the PEG 35,000 Da (lot 419735)/dextran 40,000 Da (lot 22K1194) ATPS at 5°C, 25°C, and 50°C.
Figure 3-5. Binodals of the PEG 8000 Da (lot 034K02201)/dextran 10,000 Da (lot 083K12931) ATPS at 5°C, 25°C, 50°C, and 60°C.
Figure 3-6. Binodals of the PEG 35,000 Da (lot 419735)/PAAm 10,000 Da (lot 02) ATPS at 5°C, 25°C, 37°C, and 50°C.
Figure 3-7. Binodals of the PEG 8000 Da (lot 044K0092)/Fc 70,000 Da (lot 46326) ATPS at 5°C, 25°C, and 50°C.
Figure 3-8. Digital photograph of a PEG 8000 Da/dextran 10,000 Da ATPS at 5°C, 25°C, and 50°C, after approximately 24 hours at their respective temperatures. The ATPS was stained with 20-nm Au nanoparticles that partition to the PEG phase in order to aid visualization of the ATPS binodal crossing effect.
Figure 3-9. Effect of temperature on polymer partitioning in the PEG 8000 Da (lot 044K0092)/dextran 500,000 Da (lot 123K0671) ATPS at 5°C, 25°C, 37°C, 50°C, and 60°C. Each data point represents the average of at least 4 independent measurements.
phase region instead of binodal crossing as in ATPS (24). Binodal crossing is characteristic of all of the more than 50 PEG/dextran, PEG/PAAm, and PEG/Ficoll ATPS I have investigated. General trends are to be discussed shortly.

It is important to emphasize that clear data presenting temperature-dependent ATPS binodal crossing is, to the best of my knowledge, absent from the literature despite extensive documentation of temperature-dependent ATPS phase behavior elsewhere (1,3). However, binodal crossing in polyvinylalcohol (PVA)/dextran ATPS as a function of urea concentration (a protein denaturant) has been clearly documented elsewhere (1,21).

**Reason for emphasis of binodal crossing discussion on PEG.**

I propose that the ATPS binodal crossing effect discussed previously is a manifestation of hydrogen bonding and the vicinal water network that is associated with the polymer chains. This subject has been extensively studied in relation to PEG and structurally similar polymers. As will be discussed shortly, PEG is strongly hydrated. This is an important observation in that its properties of conformation and hydrophilicity are strongly dependent on its hydration, both of which are important determinants of phase behavior. This discussion therefore focuses on PEG because the topic of PEG and vicinal water has been studied extensively.
PEG is strongly hydrated.

As I have mentioned, I hypothesize that ATPS binodal crossing is a consequence of polymer hydration in ATPS. I present a qualitative explanation of the bulk ATPS phase behavior documented in this chapter that involves the degree of vicinal water associated with the phase-forming polymers, specifically focusing on PEG since the subject of vicinal water and PEG has been extensively studied. It is known that water molecules participate in direct/vicinal hydrogen bonding to PEG. The number of water molecules this involves is typically reported as being approximately two or three for every monomer (26-39), up to 16 per monomer (28). The specific arrangement of water molecules about the chain has in fact been hypothesized to be the basis for the water-solubility of PEG. This is in contrast to the much lesser degree of water solubility exhibited by structurally similar polymers such as poly(methylene glycol), poly(propylene glycol), and poly(isopropylene glycol) (26,27). The vicinal water associated with PEG chains in aqueous solution is important when addressing its consequences on PEG chain conformation and the bulk phase separation of PEG in an ATPS. This is especially evident if the vicinal water associated with the PEG chains has the effect of restricting the degree of conformational freedom of the polymer (36).

The effect of structured water on PEG chain conformation will be discussed after a brief summary of the wide range of techniques that have been used to detect and quantify vicinal water about PEG chains and the highlights of the experimental results. Such a discussion is included herein in order to emphasize that the effects of vicinal water on
PEG chain properties is not purely speculative and has been given extensive quantitative treatment.

A very wide range of techniques have been used to detect and quantify vicinal water in PEG solutions, among them nuclear magnetic resonance (NMR) spectroscopy (29-32), calorimetry (33-35), interfacial force microscopy (IFM) (36), the extended surface forces apparatus (eSFA) and quartz crystal microbalance (QCM) techniques (37), Raman spectroscopy (30), acoustic interferometry (38), light scattering (38), and measured dielectric constants (39). These studies have been performed on PEG and PEO (note close similarity between PEG and PEO as described in reference 4) in solution as well as self-assembled monolayers of PEG on surfaces. Monolayers of PEG are themselves of interest as structures that resist the adsorption of proteins (40-43).

NMR spectroscopy has been used to detect the mobility (rotation, translation, etc) of water molecules in PEG solutions (29) as well as that of the polymers themselves (29). This mobility is related to the degree of vicinal water binding to the polymer chains. The vicinal water network is disrupted and is rebuilt continuously on the picosecond time scale, which has the effect of slowing the rotational reorientation of the molecules in solution (29). NMR suggests that one (29) or three (32) molecules of water are present for every PEG monomer and that vicinal water affects monomer rotation within PEG chains (30,31). Differential scanning calorimetry (DSC) has been used to measure the change in enthalpy of PEG solutions on melting (35). A change in enthalpy that is similar in magnitude but opposite in sign is not observed on freezing due to the slow formation of the vicinal water network (35). Calorimetry has been used to determine that there are at least three molecules of vicinal water per monomer of PEG 2000 Da (33), 2.7
per monomer of PEG 3510 Da (34), between approximately 1.75 to 5 per monomer over the PEG molecular weight range of 200 Da to 2000 Da (38), and between approximately 2.3 and 2.8 per monomer over the PEG molecular weight range 150 Da – 100,000 Da (35). Studies using IFM, a technique that measures the repulsive force acting on a tip as a function of distance from the sample, determined that monolayers of PEG on surfaces contain a 5 nm thick layer of vicinal water (36) that is six orders of magnitude more viscous than bulk water (36). Both eSFA (similar in concept to IFM) and QCM (measures changes in vibration of a quartz crystal sensor) studies demonstrate that PEG monolayers contain 83 volume percent water (37) that induces a high degree of organization within the monolayer (37). The amount of water lost upon compression is in approximately 2- to 3-fold excess of that required to hydrate the PEG chains (37).

Raman spectroscopy, the spectra from which are highly sensitive to polymer conformation, illustrates that PEG chains lose helical structure on heating (31). Acoustic interferometry, which has been used to measure sound velocity in PEG solutions that is itself indirectly dependent on the degree of structuring in the solution, indicates that PEG loses approximately 20% of its vicinal water content on heating from 5°C to 70°C (38). Light scattering through photon correlation spectroscopy (PCS) has been used to deduce a decrease in PEG hydrated radius on heating from 20°C to 60°C (38). Light scattering has also been used to investigate the diffusion of PEO in dilute solutions (44-47). This technique suggests that PEO chains may be entangled in aqueous solution as a function of the restriction placed upon the chains by vicinal water (44). The technique also illustrates that PEO occupies a larger volume in water relative to methanol, an effect not induced by PEO aggregation (45,46). This uptake of water within the polymer chains is inferred
from the unexpectedly large hydrodynamic radius and radius of gyration of PEO (47). Dielectric constants, which can be used to detect the mobility of water molecules, has been used to estimate that there are from 2.2 to 6.0 water molecules per monomer of PEO over the 3300 Da to approximately 600,000 Da molecular weight range (39).

A brief summary of these results follows. Vicinal water is associated with PEG chains in aqueous solution. The degree of vicinal water is usually given as being from one water molecule per PEG monomer up to six water molecules depending on the experimental technique. Whatever the exact amount of water per PEG monomer, the vicinal water both impacts PEG aqueous solubility and restricts the conformation of the monomer subunits within the polymer chain as elaborated upon in the next section.

**Vicinal water impacts PEG chain conformation.**

Important to the phase behavior of PEG-containing ATPS is the helical conformation of PEG in water (26,48-50). This will be elaborated upon shortly. It is possible that the previously mentioned larger volume of PEG in water relative to organic solvents (45,46) is due to PEO adopting a helical conformation in water similarly to PEG (26,48-50) and not water packing in PEO chains (45). Regardless, water is closely associated with PEO chains. The solubility of PEO at elevated temperature has been shown both theoretically (51) and experimentally (52) to be dependent on PEO chain conformation (53). The conformation and degree of hydration of PEG in water are in fact inter-related. PEO chains can contract on heating (37,54), likely due to the loss of vicinal water from the
chains. The conformation of PEG monolayers on surfaces strongly influences the degree of hydration of the PEG chains both theoretically (55,56) and experimentally (57,58).

Collectively, the previously described experimental and theoretical results demonstrate that the degree of hydration of PEG chains is correlated with PEG chain conformation (59), both of which are important for phase separation in an ATPS (1-3). Note that all observations of PEO solutions cannot be automatically attributed to PEG solutions; anomalous behavior is observed in aqueous PEO solutions due to the hydrophobic chain ends (60-62), which are instead hydroxyl groups in PEG chains (4). Interestingly, this chain end effect may be the source of clustering in solutions of PEO (60-62); other proposed possibilities include impurities in the polymer solution (46,47). Although clusters have been reported in solutions of PEO (60-62), maximum diameter less than 3 mm (62), clusters are unlikely to be present in PEG solutions if the chain ends are indeed the source of the clustering behavior exhibited in PEO solutions (60,61).

This discussion has demonstrated that vicinal water is associated with PEG in aqueous solution and that it impacts the solubility and conformation of PEG. A discussion of the importance of PEG chain conformation on its bulk ATPS phase behavior follows after the next section on the morphology and degree of hydration of dextran and Ficoll.

**Morphology and hydration of dextran, Ficoll, and PAAm.**

Similarly to PEG, the conformation and degree of hydration of dextran, Ficoll, and PAAm has consequences for their phase behavior in ATPS as elaborated upon shortly.
This section summarizes what is known about the conformation and degree of hydration of these three polymers. The effect of salts on the properties of dextran has been studied as well and will be discussed in a later section.

The morphology of dextran and Ficoll have been studied to a lesser extent than PEG, especially in relation to their degree of hydration. Dextran is a polymer of glucose; the type used in this work is from *Leuconostoc mesenteroides*, which is important to note since the properties of dextran have been shown to be highly dependent upon the source (63). Ficoll is a polymer of sucrose. There is agreement that dextran is approximately spherical at and above 10,000 Da molecular weight (64-67). There is also agreement that dextran is much more highly branched over the 100,000 Da molecular weight range than below this range (68), otherwise possessing an approximate 5% degree of branching (64,69,70), and that Ficoll is a highly branched polymer (66). There is disagreement, however, relating to the spherical nature (71) of dextran as compared to Ficoll. Some report that dextran is more elongated than Ficoll (65) because, over the same effective diameter range, the elliptical nature (71) of dextran increases much more greatly than Ficoll (65). This is in general agreement with the observation that dextran is a highly flexible polymer (64). Others report that dextran is more spherical than Ficoll of comparable effective diameter (66) and that the conformation of Ficoll is intermediate between that of a random coil and a sphere. Dextran exhibits some intramolecular hydrogen bonding (68) although the molecular weight dependence of this hydrogen bonding is, to the best of my knowledge, unknown (68).

The degree of hydration of dextran appears to decrease with increasing effective diameter (64,65,72,73) while that of Ficoll is approximately constant or increases only
slightly (66). A report that the effective volume occupied per gram of dextran increases with increasing dextran molecular weight and therefore the degree of hydration similarly increases (67) appears to be inconsistent with the observation that the root mean square radius of dextran increases more slowly relative to molecular weight (70). Further insights into dextran hydration can be gained through crystal structures. Chain packing is tighter in crystallized dextran when the polymer is heated relative to the anhydrous variant (74), implying that hydration leads to a more compact polymeric morphology. The main effect of hydration appears to be the separation of different chain segments and not other more drastic effects on polymer morphology (74). There are approximately 1 to 1.5 water molecules for every two glucose monomers in the hydrated dextran crystal structure (74).

PAAm exhibits intramolecular hydrogen bonding within the polymer due to hydrogen bonding within the amide groups (75). In contrast, intermolecular hydrogen bonding is typically seen in high molecular weight PAAm (76), higher than the 10,000 Da molecular weight evaluated in this work, which leads to gelation (76). PAAm strongly absorbs water, more strongly than either PEG or dextran on a per monomer basis (77,78). Disruption of water structure is observed to increase with polymer molecular weight and degree of crosslinking as observed for other polymers (77,78).

Now that the degree of hydration and conformation of PEG, dextran, Ficoll, and PAAm in aqueous solution have been discussed, the effects of the disruption of the vicinal water network of the polymer chains and its impact on ATPS phase behavior will be given.
**Effect of heating on PEG vicinal water and polymer chain conformation.**

It is reasonable to presume that as PEG/polymer solutions are heated, the vicinal water network about the polymer chains discussed previously will begin to break (79). Such an effect would also be expected to disrupt any potential intramolecular hydrogen bonding in dextran (68), Ficoll, and especially in PAAm, which possesses many amide bonds (75, 76). This disruption of the vicinal water network will have two effects. One effect is that the PEG chains will possess more flexibility (12, 79), allowing them to intervene within other polymer chains (such as dextran) more easily (12). This facilitated intermixing of the polymers comprising the ATPS favors phase mixing and will shift the ATPS binodals up (increasing polymer weight percent required for phase separation) with increasing temperature. The other effect is that the PEG chains will become more hydrophobic (51, 53), increasing the chemical dissimilarity of the PEG chains relative to dextran, especially since the solubility (and presumably hydrophilicity) of dextran increases with increasing temperature (3). This facilitates segregation of the polymer species and favors phase separation, therefore shifting the ATPS binodals down (decreasing polymer weight percent required for phase separation) with increasing temperature. A combination of these effects gives temperature-dependent crossing of bulk ATPS binodals.

**Molecular weight dependence of ATPS phase behavior.**

Further documentation of binodal crossing for various polymer molecular weights is given in Figures 3-10 – 3-14. Two general trends may be noted with respect to polymer
Figure 3-10. Binodals of the PEG 4600 Da (lot 00727D1)/dextran 40,210 Da (lot 22K1194) ATPS at 5°C, 25°C, and 50°C.
Figure 3-11. Binodals of the PEG 12,000 Da (lot 396030)/dextran 10,000 Da (lot 101K1115) ATPS at 5°C, 25°C, and 50°C.
Figure 3-12. A. Binodals of the PEG 35,000 Da (lot 454839)/dextran 10,000 Da (lot 083K12931) ATPS at 5°C, 25°C, and 50°C. B. Binodals of the PEG 35,000 Da (lot 419735)/dextran 40,210 (lot 22K1194) ATPS at 5°C, 25°C, and 50°C. Note the differences in scale in the y-axis.
Figure 3-13. Binodals of the PEG 20000 Da (lot 390136)/PAAm 10,000 Da (lot 03) ATPS at 5°C, 25°C, and 50°C.
Figure 3-14. Binodals of the PEG 8000 Da (lot 71K0150)/PAAm 10,000 Da (lot 02) ATPS at 5°C, 25°C, 37°C, and 50°C.
molecular weight. One is that ATPS containing higher molecular weights of dextran tend to give less exaggerated crossing behavior (compare Figures 3-12A and 3-12B, remembering that there are differences in scale in the y-axis). This is likely due to the lesser degree of hydration with increasing dextran molecular weight mentioned previously (64,65,72,73). Another trend is that increasing molecular weights of PEG tend to give more exaggerated crossing behavior (compare Figures 3-6, 3-13, and 3-14). This may be due to the fact that there is more vicinal water per polymer molecule with increasing polymer molecular weight, serving to exaggerate the effect of the disruption of the vicinal water network with increasing temperature, and therefore an exaggeration of the binodal crossing effect is observed.

It is also noteworthy that much higher weight percents of PEG are required for phase separation with dextran 70,000 Da than Ficoll 70,000 Da (Figure 3-15). It has been difficult to elucidate the impact of PEG chain conformation on phase separation due to the concomitant increase in hydrophobicity with increasing temperature as discussed above (51,53). Similarly, it is difficult to isolate the impact of protein conformation on partitioning in PEG/dextran ATPS with increasing temperature due to the resulting change in protein hydrophilicity of denatured proteins relative to the native state (80). However, by similar arguments as those made in previous sections, if Ficoll is indeed less globular than dextran (66), perhaps this imparts flexibility within Ficoll polymer chains such that it is easier for a Ficoll molecule to accept a PEG chain into its volume more easily than is possible for a dextran molecule (12).
Figure 3-15. Binodals of the PEG 8000 Da/dextran 70,000 Da (lot 064K0613) and PEG 8000 Da/Ficoll 70,000 Da (lot 46326) ATPS at 25°C.
Effect of salts on PEG vicinal water network disruption and polymer conformation.

Qualitatively similar behavior as the application of heat is observed as a function of sodium chloride concentration at 25°C (Figures 3-16 and 3-17), i.e., binodal crossing is observed both as a function of temperature and NaCl concentration. This is a reasonable result since water-structure forming or breaking salts would be expected to disrupt vicinal water bonding to the PEG chains. The sodium and chloride ions are approximately in the middle of the Hofmeister series. In contrast, the addition of pH 7 sodium phosphate buffer shifts PEG 8000 Da/dextran 10,000 Da and PEG 8000 Da/PAAm 10,000 Da ATPS binodals down when the temperature is held constant at 25°C (Figure 3-18 and 3-19). This behavior is based on two effects. One is that PEG phase separates from sodium phosphate [Figure 3-20 and (3)], but not from sodium chloride. This is possibly due to the position of phosphate in the Hofmeister series (81-83). It has been postulated that the origin of PEG/salt phase separation is the inability of multivalent anions possessing a large charge density to form a complex with the oxygen atoms of PEG chains (81,83). Additionally, since phosphate is a small molecule, increasing temperature to disrupt the vicinal water network around PEG chains (also increasing chain flexibility) will not provide a driving force for phase mixing (unlike as observed with dextran and other polymers, hypothesized above to contribute to shifting up ATPS binodals), resulting in only a down shift of PEG/phosphate ATPS binodals with increasing temperature. The salt will compete with PEG for water hydrogen bonding (84).

The second factor to consider is the gelling of dextran in high concentrations of NaCl (85). The fundamental mechanism of gelation in dextran as a function of salt additives is,
Figure 3-16. Binodals of the PEG 8000 Da (lot 034K02201)/PAAm 10,000 Da (lot 03) ATPS in 0M, 1M, 2M, and 3M, NaCl at 25ºC.
Figure 3-17. Binodals of the PEG 8000 Da (lot 71K0150)/dextran 505,000 Da (lot 32K1454) ATPS in 0M, 1M, 2M, and 3M NaCl at 25ºC.
Figure 3-18. Binodals of the PEG 8000 Da (lot 034K02201)/PAAm 10,000 Da (lot 03) ATPS in 5 mM, 150 mM, and 300 mM pH 7.0 sodium phosphate buffer at 25°C.
Figure 3-19. Binodals of the PEG 8000 Da (lot 71K0150)/dextran 10,000 Da (lot 101K1115) ATPS in 0 mM, 100 mM, 200 mM, 300 mM, and 400 mM pH 7.0 sodium phosphate buffer at 25ºC.
Figure 3-20. Binodals of the PEG 35,000 (lot 419735)/2M pH 7.0 sodium phosphate buffer ATPS at 5°C, 25°C, 37°C, and 50°C.
to the best of my knowledge, unknown (86). Although it has been reported that potassium preferentially induces dextran gelling over other alkali earth cations (85,86) due to a hypothetical induced fit of potassium ions between different segments of the dextran chain (74,85), the maximum salt concentration investigated other than KCl was 1.5M (85). I have observed that large concentrations of NaCl (evaluated up to 3M) greatly increase the viscosity of dextran 500,000 Da. The conformation of dextran should be more restricted when it gels and therefore will be less able to accept PEG chains into its volume (12). This will favor phase separation and will contribute to shifting PEG/dextran ATPS binodals down. Further, PEG has been demonstrated to complex certain monovalent cations under certain conditions in water (87,88), potentially impacting PEG phase behavior in bulk ATPS if this impacts PEG conformation (89).

Dynamic protein microcompartmentation in giant vesicles.

I have shown that binodal crossing in bulk ATPS gives some fundamental insights into the mechanism of phase separation, specifically giving added weight to the hypothesis that polymer conformation, itself impacted by vicinal water, plays a prominent role. Binodal crossing of bulk ATPS can further be applied towards dynamic temperature-dependent protein microcompartmentation within giant vesicles, specifically through inducing aqueous phase separation both on heating and cooling depending on the location along the bulk ATPS binodal. Figures 3-21 and 3-22 illustrate two PEG 8000 Da/dextran 511,000 Da ATPS GVs that are two phases at 5°C, one phase upon heating to
Figure 3-21. A. Transmitted light (DIC) images of dynamic aqueous phase separation in an ATPS GV prepared in the 4.125 w/w% PEG 8000 Da/2.625 w/w% dextran 511,000 Da/5 mM pH 7.0 sodium phosphate buffer ATPS, 9.75:1 mol ratio DOPC/DOPG. B. The same ATPS GV as in A, illustrating dynamic protein microcompartimentation. The columns, from left to right, are DIC, lipid (rhodamine) fluorescence, and streptavidin (FITC) (in excess biotin-PEG 5000 Da) fluorescence. Bars = 10 µm.
Figure 3-22. A. Transmitted light (DIC) images of dynamic aqueous phase separation in an ATPS GV prepared in the 4.125 w/w% PEG 8000 Da/2.625 w/w% dextran 511,000 Da/5 mM pH 7.0 sodium phosphate buffer ATPS, 9.75:1 mol ratio DOPC/DOPG. The same ATPS GV as in A, illustrating dynamic protein microcompartmentation. The columns, from left to right, are DIC, lipid (rhodamine) fluorescence, and streptavidin (FITC) (in excess biotin-PEG 5000 Da) fluorescence. Bars = 10 μm.
29°C or 34°C, and phase separate again on cooling to 5°C. This behavior is analogous to bulk ATPS behavior at this particular weight percent composition along the binodal. **Figures 3-23** and **3-24** illustrate two PEG 8000 Da/dextran 511,000 Da ATPS GV s that phase separate at 5°C, mixes to one phase at 25°C, and phase separate again on continued heating to 50°C. This behavior is analogous to bulk ATPS behavior at this particular weight percent composition along the binodal.

Challenges in utilizing binodal crossing in ATPS GV s include the low ATPS GV yield at 5°C in ATPS within the binodal crossing region (possibly due to vesicle aggregation induced by a large weight percent of dextran) and my unwillingness to heat an ATPS GV beyond 50°C due to the risk of damage to the microscope optics. I have always observed GV quality to be poor within the binodal crossing region of the ATPS.

**ATPS relative phase volumes: Bulk and GV s.**

A long-term goal of this ATPS GV work is to use them to manipulate enzymatic activity within an individual vesicle. As elaborated upon in chapter 5, an enzyme partitioned to one of the phases within an ATPS GV will be of a higher concentration than if the enzyme were distributed throughout the vesicle interior. Therefore, aqueous phase transitions offer a method to dynamically control local enzyme concentrations within ATPS GV s and therefore dynamically manipulate enzymatic activity. In order for such a process to be maximally effective, the enzyme should partition strongly to one of
Figure 3-23. A. DIC images of dynamic aqueous phase separation in an ATPS GV prepared in the 1.875 w/w% PEG 8000 Da/9 w/w% dextran 511,000 Da/5 mM pH 7.0 sodium phosphate buffer ATPS, 9.75:1 mol ratio DOPC/DOPG. B. The same ATPS GV as in A, illustrating dynamic protein microcompartmentation. The columns, from left to right, are DIC, lipid (rhodamine) fluorescence, and streptavidin (FITC) (in excess biotin-PEG 5000 Da) fluorescence. Bars = 10 µm.
Figure 3-24. **A.** DIC images of dynamic aqueous phase separation in an ATPS GV prepared in the 1.875 w/w% PEG 8000 Da/9 w/w% dextran 511,000 Da/5 mM pH 7.0 sodium phosphate buffer ATPS, 9.75:1 mol ratio DOPC/DOPG. **B.** The same ATPS GV as in A, illustrating dynamic protein microcompartmentation. The columns, from left to right, are DIC, lipid (rhodamine) fluorescence, and streptavidin (FITC) (in excess biotin-PEG 5000 Da) fluorescence. Bars = 10 µm.
the phases and the volume occupied by that phase should be substantially less than that occupied by the other phase.

Of interest within this broad context is my observation that relative aqueous phase volumes in ATPS GVs at 5°C appear to be independent of that within a corresponding bulk ATPS. At low weight percent of dextran in a PEG 8000 Da/dextran 500,000 Da ATPS, where the PEG phase occupies more volume than the dextran phase in a bulk ATPS, the same trend is observed in ATPS GVs (Figure 3-25). Conversely, at higher weight percent of dextran, where the PEG phase occupies less volume than the dextran phase in a bulk ATPS, the PEG phase still occupies more volume than the dextran phase in ATPS GVs (Figure 3-25). Relative aqueous phase positions are not affected. Similar results were obtained in different polymer lot numbers. The most likely explanation for these unexpected results is a different encapsulated polymer weight percent within ATPS GVs relative to the corresponding bulk ATPS, but I was still able to obtain dynamic aqueous phase separation in these structures (approximately 50% of the time). A major impediment to investigating this result in more detail is the low ATPS GV yield observed when they are prepared in an ATPS to the right of the ATPS critical point. This low vesicle yield within the binodal crossing region of the ATPS is independent of polymer molecular weight. ATPS GVs prepared from DOPC/DOPE-mPEG 2000 Da cannot be utilized for these experiments since they decompose on heating to 50°C.
Figure 3-25. Relative aqueous phase volumes in ATPS GV at 5°C. All ATPS GV were 9.75:1 mol ratio DOPC/DOPG prepared in a PEG 8000 Da (lot 090K0910)/dextran 511,000 Da (lot 023K0675)/5 mM pH 7.0 sodium phosphate buffer ATPS at 37°C. A. 4.125 w/w% PEG/2.625 w/w% dextran. Average volume percent dextran phase, bulk: 22; GV: 19 ± 13 (29 ATPS GV measured). B. 1.875 w/w% PEG/9 w/w% dextran. Average volume percent dextran phase, bulk: 86; GV: 11 ± 7.6 (35 ATPS GV measured).
Conclusions.

PEG/dextran, PEG/PAAm, and PEG/Ficoll ATPS binodals cross with increasing temperature. PEG/dextran/NaCl and PEG/PAAm/NaCl ATPS binodals at constant temperature cross with increasing concentration of NaCl. PEG/dextran/phosphate and PEG/PAAm/phosphate ATPS binodals at constant temperature shift down with increasing phosphate concentration. PEG/phosphate ATPS binodals shift down/left with increasing temperature. Qualitative rationalizations of this behavior have been presented that involve the water structure about and the conformation of the polymer chains present in the ATPS. This has given insight into some of the driving forces for phase separation in an ATPS. Binodal crossing of bulk ATPS can be applied towards inducing dynamic aqueous phase separation and protein microcompartmentation within giant vesicles, both on cooling and heating with judicious choice of an ATPS. The internal aqueous environment within ATPS GVs may exhibit useful behavior that is different from a corresponding bulk ATPS.
Future Directions.

Future work should involve using the experimental results described herein towards developing a theoretical basis for the mechanism of aqueous phase separation, particularly in PEG/dextran ATPS. The temperature-dependent phase behavior I have documented, illustrating that hydrogen bonding and polymer conformation can be used to provide qualitative rationalizations of the observed behavior, should provide insight towards this goal. I have shown that this behavior exists, and have given qualitative rationalizations that can now be pursued more rigorously in theoretical terms. Ultimately, such insights will aid in predicting which ATPS is most suitable for a given biomolecular separation, a task that is typically empirical at present (3). Further investigations regarding the effects of different salts (different charge, size, etc) on ATPS binodals should be pursued.
References.


4. A point of wide confusion in the literature and therefore worthy of note is that, strictly speaking, poly (ethylene glycol) (PEG), HO-(CH2CH2O)n-OH, is not the same as poly (ethylene oxide) (PEO), HO-(CH2CH2O)n-H. PEG has two terminal hydroxyl groups while PEO has one (90).


7. A limitation is that the salt concentration used in the cross-partition experiment must not induce a conformational or other change in the protein (91).


25. Yan, P.; Huang, J.; Lu, R.-C.; Jin, C.; Xiao, J.-X.; Chen, Y.-M. Two cloud-point phenomena in tetrabutylammonium perfluorooctanoate aqueous solutions:


50. There are approximately five monomers per turn in PEG helices (27).


53. Precipitation of PEG from the ATPS does not occur at 50ºC. Insolubility of PEG in water normally occurs at much higher temperatures (92).


59. Interestingly, vicinal water, present in living cells (93), is also strongly correlated with protein and DNA structure and activity (94-101). Studying the effects of vicinal water on PEG chain properties may therefore in some instances provide fundamental insights into the effect of vicinal water on protein properties.


83. Note, however, that strong evidence has recently been reported which suggests that the ordering of the Hofmeister series is more likely related to van der Waals forces rather than hydration forces (102).


89. Numerous authors report that PEO, in water (no organic solvents), does not complex monovalent ions such as KI (103-105). This is not in contradiction with reference 87 because cation complexation appears to depend upon the anion present (88,105).


Chapter 4

Dynamic Asymmetric Protein Microcompartmentation in Aqueous Two-Phase Vesicles

Previous chapters have demonstrated that changes in temperature can be used to dynamically control aqueous phase separation and protein microcompartmentation within ATPS GVs. Temperature was chosen as the stimulus primarily because it is an easy factor to control. However, viewing an aqueous phase diagram suggests an easier method of initiating aqueous phase separation within an ATPS GV. Since phase separation is favored at high polymer weight percents, a logical approach is to concentrate the ATPS within the GV after formation. This can be achieved readily by preparing ATPS GVs in a one-phase ATPS and afterwards dispersing them in a hypertonic solution. This forces water out of the GVs through osmosis, concentrating the ATPS within, thereby forcing phase separation.

It was not readily apparent how to use this principle to easily achieve aqueous phase transitions within an individual ATPS GV without latching onto the vesicle such as with a micropipette (1); without immobilization of the ATPS GV, the application of additional solution typically results in rapid translation of the vesicle outside the field of view. This chapter presents such a method along with some unexpected results regarding the initiation of ATPS GV asymmetric protein microcompartmentation (budding) as well as control over relative aqueous phase positions solely due to differences in the composition of the phospholipid headgroups. These results have strong biological implications in that the budding reported herein presents a method to dynamically control
asymmetric protein microcompartmentation within ATPS GVs in response to a realistic stimulus experienced by living cells.

It has been demonstrated that macromolecular crowding (2,3) strongly impacts cellular function based on both theoretical and experimental *in vitro* and *in vivo* observations. Among such cellular processes are the folding of proteins (4-7), condensation and assembly of DNA (8-11), polymerization of cytoskeletal proteins (12,13), reaction kinetics (14), regulation of cellular volume (15), and the assembly of signaling complexes (16). Many factors contribute to the organization within and the function of living cells, of which macromolecular crowding is only one. It is not clear how to only change the degree of macromolecular crowding without disrupting every other process within the cell (17). An illustrative example follows.

A particularly challenging focus of much work has been on exocytosis (18,19). One open question is the relative importance of the lipids and proteins within the cell membrane in the vesicle fusion process (20-22). Such a question is challenging, but is experimentally addressable. Particularly useful are experimental model systems that are designed to exhibit budding (23,24) or exocytosis (20,21) but under which the experimental conditions can be varied much more easily and comprehensively than within a living cell (20,21). For example, it has been demonstrated that exocytosis can be mimicked in a system comprised of a vesicle and a lipid nanotube, the latter serving as a fusion pore (20,21). Injecting a fluorophore solution into the nanotube results in the formation of a small vesicle that migrates along the length of the nanotube, merges with the larger vesicle membrane, and releases the fluorophore into the surrounding environment (20,21). Factors governing exocytotic events such as flow rate through the
nanotube can be controlled (20,21). Such experiments suggest the importance of lipid mechanics, in isolation from the effects of proteins, on the fundamental driving forces involved in vesicle fusion to the cell membrane, the final stage of exocytosis (20,21). A complimentary question to the one mentioned previously regarding the mechanism of budding in cells is the possible influence of macromolecular crowding. There is neither an obvious method of addressing such a question within a living cell in the absence of other important considerations (17) nor a mechanism through which such control of cellular organization and morphology may be exerted.

It is in this light that ATPS GVs strongly demonstrate their utility as primitive models of cells. This chapter demonstrates asymmetric protein microcompartmentation within ATPS GVs in response to small changes in macromolecular crowding. This budding occurs with increasing frequency with increasing osmotic pressure. The presence of budding depends on the elasticity of the GV membrane. Asymmetric protein microcompartmentation can be rendered dynamic (reversible) through judicious changes in osmolarity. During such an event, the ATPS GV volume changes considerably, but the membrane surface area does not change appreciably. Both polymer and protein partitioning within the ATPS GVs increase with increasing osmolarity. Further, relative aqueous phase positions within an individual ATPS GV can be manipulated through appropriate changes in the phospholipid headgroups.

These results have significant biological ramifications in that they collectively suggest a fundamental mechanism (25-27) that may contribute to polarity (asymmetric microcompartmentation) within living cells, important in a wide range of functions from cell division to exocytosis (25,26). Recent examples have been reported in *Bacillus*
*subtilis* (28,29). Depending on the stage of the cell cycle, this bacteria can uptake DNA from the environment and recombine it with its own DNA (28). This is an important function in that it offers the bacteria a mechanism of acquiring drug resistance (28). It has been shown that certain proteins in *B. subtilis* involved in the uptake of environmental DNA are preferentially localized to the bacterial cell poles upon this uptake (28,29). While the mechanism of microcompartmentation is unclear, the functional significance is apparent. Understanding how the microcompartmentation of these DNA-uptake proteins is maintained may offer a mechanism to inhibit it and therefore offer insight into how to inhibit the development of drug resistance in bacteria. As discussed later, the cytoplasm of a living cell is comprised of proteins, not PEG and dextran, but ATPS GVs nevertheless offer model systems through which the basic mechanisms serving to drive asymmetric microcompartmentation within living cells may be elucidated.
Experimental.

Materials.

Polymers poly (ethylene glycol) (PEG) 8000 Da (lot 034K02201 and 044K0092), dextran 10,000 Da (lot 083K12931 and 084K1226), sucrose, sodium phosphate (monobasic and dibasic), and biotin-conjugated dextran 10,000 Da were purchased from Sigma-Aldrich. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Fluorophore-conjugated polymers and proteins were purchased either from Molecular Probes (Eugene, OR), (now a part of Invitrogen) or the Shearwater Corporation (Huntsville, AL) (now a part of Nektar Therapeutics). Distilled water was purified to a resistivity of $\geq 18.2 \text{ M}\Omega$ with a Barnstead NANOPure Diamond system. Silicone spacers from Molecular Probes were used to enclose ATPS GV dispersions for imaging.

Instrumentation and software.

Instrumentation and software was the same as described in chapter 2.

Bulk ATPS binodals and partitioning, ATPS GV assembly, and dynamic budding.

Bulk ATPS binodals and partitioning were evaluated as described in chapter 2. ATPS GVs were prepared similarly as described in chapter 2, a key difference being that
after formation ATPS GVs were typically dispersed in a hypertonic sucrose solution that was itself prepared in 5 mM pH 7.0 sodium phosphate buffer. Polymer weight percent within ATPS GVs was therefore increased by removing water from the GVs after formation; for comparison, polymer weight percent was increased in a bulk ATPS by increasing the total weight percent of PEG and dextran while keeping the weight percent ratio of PEG to dextran constant (Figure 4-1). A plot of osmolarity against total polymer weight percent was therefore used to determine bulk conditions that approximated those within ATPS GVs under osmotic stress (Figure 4-1). All ATPS GVs were prepared in a 7.5 w/w% PEG 8000 Da/8 w/w% dextran 10,000 Da/5 mM pH 7.0 sodium phosphate buffer ATPS (osmolality = 113 ± 2.52 mmol/kg) unless explicitly noted. The lipid mol ratios were either 44:1 DOPC/DOPE-mPEG 2000 Da, 10:1 DOPC/DOPG, or 44:10:1 DOPC/DOPG/DOPE-mPEG 2000 Da.

I was able to initiate dynamic budding of individual ATPS GVs through changing the osmolarity of a vesicle solution without the assistance of micropipettes for immobilization of the vesicle through the following procedure. A 1 µL vesicle sample was added to 40 µL of 201 ± 1.00 mmol/kg sucrose/buffer on an open microscope slide. Under such conditions in the ATPS studied, many budding ATPS GVs are observed. Evaporation of the solution from the microscope slide due to localized heating by the objective was minimized through the use of the temperature-controlled microscope stage (3ºC). Forty microliters of DI water was added close to, but not touching, this sample. Adding water directly to the vesicle sample would result in undesired rapid vesicle translation from the field of view. A budding ATPS GV that was relatively stationary was then centered in the field of view. A further volume of DI water was gently added
Figure 4-1. Bulk ATPS osmolality as a function of the total polymer w/w% composition of the ATPS. The weight ratio of PEG to dextran was held constant at 7.5 w/w% PEG:8 w/w% dextran. Each data point represents the average of at least 3 independent measurements.
between the two solutions, allowing the two solutions to mix by diffusion. I therefore estimate that this process diluted the ATPS osmolarity within the GVs by a factor of approximately 2. The ATPS GV did move as a result of the addition of water, but typically not so quickly that the vesicle could not be followed within the field of view by moving the sample stage. The result was a transition from a budding morphology to a spherical morphology. A similar procedure was followed with a further addition of between 5 and 40 µL of 411 ± 2.08 mmol/kg sucrose/buffer, resulting in a transition from a spherical to a budding morphology. These morphological transitions were typically rapid, on the order of approximately 1 to 10 minutes.

Changes in surface area and volume in ATPS GVs from the budding to the spherical state were estimated through calculations performed with measurements from NIH Image v1.63. Calculation of the volume and surface area of a spherical vesicle is straightforward. The volume and surface area of a budding vesicle was calculated through measuring that of both phases, including the overlap region, and then subtracting the overlap region. I determined the volume and surface area of the interfacial region through the measured length and width of this region and approximating its shape as that of two pyramids. Five ATPS GVs were used to arrive at these values.
Results and Discussion.

Asymmetric microcompartmentation in ATPS GVs.

It was documented in chapter 2 that aqueous phase separation can be induced within ATPS GVs through dispersal of pre-formed ATPS GVs in a hyperosmotic solution. Partitioning can also be increased through this process (30). My motivation for following such a procedure in this work was to not only increase partitioning, but also to investigate the mechanism of the switch in relative aqueous phase positions in response to osmotic stress previously reported (30). My hypothesis was that changing aqueous-aqueous and aqueous-membrane interfacial tensions within the ATPS GVs are these driving forces.

I have discovered that it is possible to induce budding within ATPS GVs under hypertonic conditions. This kind of budding (asymmetric microcompartmentation) has not been observed previously within ATPS GVs, the closest morphology being that of small “daughter” vesicles attached to a larger vesicle dispersed in approximately 20X hypertonic sucrose (31). This morphology of small vesicles attached to a larger vesicle is itself quite commonly observed in GVs under hyperosmotic stress (and therefore less noteworthy) as discussed later. Small changes in the osmolarity of the ATPS GV dispersion were observed to induce a transformation from concentric structures to budding morphologies in which one aqueous phase was extending from the other (Figure 4-2). When ATPS GVs were dispersed in the PEG phase of the ATPS (isotonic), the majority of the vesicles were concentric (30). In contrast, asymmetric microcompartmentation in DOPC/DOPE-mPEG 2000 Da ATPS GVs occurred when the
Figure 4-2. Confocal micrographs illustrating the response of 44:1 mol ratio DOPC/DOPE-mPEG 2000 Da ATPS GVs to osmotic stress. Top row: spherical ATPS GV. Bottom three rows: budding ATPS GVs. These ATPS GVs were dispersed in 152 ± 1.00 mmol/kg sucrose/buffer. Columns, from left: transmitted light (DIC), lipid fluorescence, lectin SBA fluorescence. T = 5°C, bar = 10 µm.
ATPS GVs were dispersed in a hypertonic solution (32) (Figure 4-2). Such vesicles consist of two aqueous phases not bordered by a membrane, one phase extending from the rest of the vesicle. Lectin SBA, a protein that binds to glucose, was partitioned to the dextran phase to achieve asymmetric protein microcompartmentation within these ATPS GVs (Figure 4-2).

Some general characterizations of the resulting budding ATPS GVs are as follows. Budding occurred with increasing frequency as the extravesicular solution tonicity increased, i.e., more of the ATPS GVs were budding with increasing osmolarity of the vesicle dispersion. This was expected because increasing polymer weight percent composition increases aqueous-aqueous interfacial tensions and therefore increases the drive to minimize contact area between the phases. Additionally, multiple budding phases were commonly observed as the hypertonicity increased (Figure 4-2). It is difficult to be more quantitative with respect to these two observations due to large variability between experiments, even from the same original vesicle preparation, but the general trends noted above were consistent. Since I define the budding phase as the smaller of the two phases, the budding phase was typically the dextran phase since it was typically the smaller of the two phases (Figure 4-2). The dextran phase is typically the smaller phase under isotonic conditions as well, noted in chapter 2. However, the budding phase was sometimes the PEG phase instead (Figure 4-3). This approximates bulk ATPS behavior at the particular polymer weight percent composition I used in which the dextran phase was the smaller of the two phases (33).

A particularly important result was that the budding aqueous phase was enriched in proteins preferentially partitioned to that phase, illustrating that protein distribution
Figure 4-3. Confocal micrographs illustrating that the budding aqueous phase in ATPS GVs can be the PEG phase if it occupies a smaller volume relative to the dextran phase (this is atypical). **A:** Dispersed in $165 \pm 2.00$ mmol/kg sucrose/buffer. **B:** Dispersed in $248 \pm 0.58$ mmol/kg sucrose/buffer. Columns, from left: transmitted light (DIC), lipid fluorescence, lectin SBA fluorescence. $T = 5^\circ C$, bar = 10 $\mu$m.
was also manipulated asymmetrically within the ATPS GVs (Figures 4-2 and 4-3). The generality of these results is demonstrated by the fact that budding was also observed in hypertonically-dispersed GVs prepared in ATPS comprised of other polymer molecular weights (Figure 4-4) and lipids within the membrane (Figure 4-5), the latter set within limitations to be discussed in more detail later. ATPS GVs could be successfully dispersed in media of osmolality in excess of 800 mmol/kg. With large osmolarities such as this, however, it is difficult to determine which ATPS GVs are unilamellar due to presence of a large number of small “daughter” vesicles attached to the periphery of the main vesicle.

Hypertonically-stressed ATPS GV membranes were not flaccid. Although excess membrane surface area is present (34), reports of stable shape deformations of GVs (stable as a single lipid phase, at constant temperature, over a broad range of hypertonic stress, no vesicle fission) are unusual (34,35); transient shape deformations are typical (35-39). Many reports of GVs under hypertonic conditions report shrinkage (31,34-42) and the formation of smaller “daughter vesicles” from the original GV (31,40,41). Noteworthy methods to osmotically-deform GVs are summarized in Table 4-1; other GV deformations (43-85) are given in Table 4-2. The questions I discuss next pertain to why and how budding can occur in ATPS GVs.

The two variables in this work are the impact of the ATPS and that of the membrane on budding. DOPC/DOPE-mPEG 2000 Da ATPS GVs typically did not withstand dispersion in hypertonic media unless the aqueous phase contained a polymer such as PEG. The degree of membrane flaccidity of such GVs prepared in PEG solutions was somewhat dependent on the amount of PEG in the aqueous phase; more PEG led to
Figure 4-4. ATPS GV budding is possible in other ATPS besides the PEG 8000 Da/dextran 10,000 Da ATPS when dispersed in hypertonic solutions. Shown here is an ATPS GV prepared in a 9.375 w/w% PEG 4600 Da/9.375 w/w% dextran 10,000 Da ATPS, the same lipid ratios as in Figure 4-2, and dispersed in slightly hypertonic sucrose/buffer solution. Columns, from left: transmitted light (DIC), lipid fluorescence, lectin con A fluorescence. T = 5°C, bar = 10 μm.
Figure 4-5. Confocal micrographs illustrating the differential effects of osmotic stress on ATPS GV morphology as a function of the lipids in the GV membrane. A: 44:1 mol ratio DOPC/DOPE-mPEG 2000 Da GV, dispersed in 133 ± 2.08 mmol/kg sucrose/buffer; B: 10:1 mol ratio DOPC/DOPG GV, dispersed in 816 ± 3.00 mmol/kg sucrose/buffer; C: 44:10:1 mol ratio DOPC/DOPG/DOPE-mPEG 2000 Da GV, dispersed in 289 ± 1.00 mmol/kg sucrose/buffer. Columns, from left: transmitted light (DIC), lipid fluorescence, lectin SBA fluorescence. T = 5°C, bar = 10 µm.
Table 4-1. Noteworthy examples from the literature of techniques or materials that have been used to shrink or deform GVs through hyperosmotic stress.

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>reversible shape deformations of GVs through the use of a special chamber that allows for control over osmotic pressure</td>
<td>34</td>
</tr>
<tr>
<td>agarose gel utilized to induce stable shape changes in GVs under hyperosmotic stress</td>
<td>35</td>
</tr>
<tr>
<td>many “daughter vesicles” clearly observed in hypertonically-stressed GVs</td>
<td>40</td>
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</tbody>
</table>
**Table 4-2.** Selected examples from the literature of techniques or materials that have been used to shrink or transiently deform GV membranes other than through hyperosmotic stress.

<table>
<thead>
<tr>
<th>method</th>
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</tr>
</thead>
<tbody>
<tr>
<td>surfactant</td>
<td>43-47</td>
</tr>
<tr>
<td>enzyme (lipase)</td>
<td>48</td>
</tr>
<tr>
<td>salt</td>
<td>49,50</td>
</tr>
<tr>
<td>electric field</td>
<td>51-53</td>
</tr>
<tr>
<td>light</td>
<td>54,55</td>
</tr>
<tr>
<td>temperature</td>
<td>56-62</td>
</tr>
<tr>
<td>pH</td>
<td>63,64</td>
</tr>
<tr>
<td>lipid properties and distribution within bilayer</td>
<td>60,61,64</td>
</tr>
<tr>
<td>amphiphilic polymer</td>
<td>65,66</td>
</tr>
<tr>
<td>actin</td>
<td>67-71</td>
</tr>
<tr>
<td>protein (C1q, bacteriorhodopsin)</td>
<td>72,73</td>
</tr>
<tr>
<td>enzyme (ceramide, phospholipase A2)</td>
<td>74,75</td>
</tr>
<tr>
<td>magnetic tweezers</td>
<td>76</td>
</tr>
<tr>
<td>lipid phase separation</td>
<td>24,75,77-83</td>
</tr>
<tr>
<td>transient adhesion to a surface (vesicle “flickering”)</td>
<td>84,85</td>
</tr>
</tbody>
</table>
less flaccidity within the membrane. The most important conclusions to be gained from these experiments are that the presence of polymer within the GVs aids in the preservation of the GVs under hypertonic stress and that GVs containing a polymer-grafted membrane (no ATPS within the vesicles) can be flaccid under hyperosmotic stress. Regarding the second variable, the membrane, I discovered that membrane surface area does not change on budding as discussed shortly. This and other more quantitative evaluations of budding ATPS GVs are presented in the following three sections, specifically the impact of membrane elasticity on budding, changes in membrane surface strain and vesicle surface area and volume during dynamic budding, and the impact of osmotic stress on partitioning within hypertonic (budding and spherical) ATPS GVs.

**Effect of membrane elasticity on asymmetric microcompartmentation in ATPS GVs.**

Dispersing ATPS GVs in a hypertonic solution concentrates the ATPS within the GVs. Presumably membrane elasticity, itself dependent on the conformation of the grafted PEG chain in PEGylated GVs (86,87), plays a major role in the occurrence of budding within hypertonic ATPS GVs. An easy method to investigate the impact of membrane elasticity on budding in ATPS GVs is to incorporate cholesterol within DOPC/DOPE-mPEG 2000 Da ATPS GV membranes (88,89). Increasing cholesterol content should increase membrane rigidity (88,89). Membrane rigidity is a primary determinant of membrane strength (90), along with membrane thickness (90). Little
effect of cholesterol was observed until 40 mol% was added, at which point budding was reduced by a factor of approximately 2 to 3. These results suggest that the incorporation of large amounts of cholesterol into polymer-grafted ATPS GV membranes is required to significantly impact membrane elasticity.

I decided that it was necessary to use ATPS GV without a polymer grafted within the membrane. I predicted that ATPS GVs not containing a polymer-functionalized headgroup would have less membrane elasticity (86,87) due to the increase in elasticity imparted by the polymer-functionalized headgroup (86,87). I experienced difficulties preparing ATPS GVs with only DOPC. I therefore decided to pursue two different approaches towards this problem. One approach was to use another literature GV assembly procedure through the hydration method that uses a charged lipid (91) instead of lipid-conjugated PEG (92,93) to induce GV formation. The inclusion of a charged lipid does not change GV elasticity (94), but it does significantly decrease membrane stability against rupture (as measured by micropipet aspiration), even at only 10 mol% charged lipid, as is the case in the work described herein (94,95). In contrast to DOPC/DOPE-mPEG 2000 Da ATPS GVs, budding was not observed within DOPC/DOPG GVs prepared in the same ATPS (Figure 4-5); these latter structures are typically spherical. Interestingly, in the DOPC/DOPG ATPS GVs, dextran was the outer phase; this relative phase position reversal will be discussed in more detail later. I prepared ATPS GVs containing both the polymer-conjugated lipid as well as the charged lipid and observed that they undergo budding under osmotic stress as well (Figure 4-5). This was a reasonable result since incorporating a charged lipid has no effect of
membrane elasticity (94) while grafted polymers can instead have a significant impact (86,87).

The second more quantitative approach was to systematically vary the mol% of DOPE-mPEG 2000 Da within this DOPC/DOPG ATPS GV preparation. Decreasing the mol% of PEGylated lipid within the GV membrane, while keeping the DOPC/DOPG mol ratio constant, decreased the percentage of ATPS GVs that exhibited budding when dispersed in hypertonic media (Figure 4-6). These results collectively demonstrate two facts. One is that membrane elasticity is a strong determinant of whether or not budding occurs in hypertonic ATPS GVs. The other is the surprising conclusion that cholesterol does not strongly impact polymer-grafted membrane elasticity until the inclusion of 40 mol% cholesterol.

**Dynamic asymmetric microcompartmentation in ATPS GVs.**

Importantly, aqueous phase budding can be rendered dynamic (reversible) through judicious changes in the osmolarity of an ATPS GV suspension. Decreasing hypertonicity resulted in a transition from a budding to a spherical morphology; subsequently increasing hypertonicity similarly resulted in a reversion to a budding morphology again (Figure 4-7). Proteins could also be reversibly partitioned to the budding phase (Figure 4-8). Dynamic asymmetric microcompartmentation is observed whether the budding (smaller) phase is either PEG or dextran (Figure 4-7 and Figure 4-9).
Figure 4-6. Percentage of budding, relative to spherical, ATPS GVs within a given preparation of DOPC/DOPG/DOPE-mPEG 2000 Da GVs. The mol ratio of DOPC/DOPG was held constant at 10:1, while the mol% of DOPE-mPEG 2000 Da was varied. These ATPS GVs were dispersed in $562 \pm 1.53$ mmol/kg sucrose/buffer prior to imaging. $T = 25^\circ$C. Each data point represents the average of at least 20 ATPS GVs.
Figure 4-7. DIC images of dynamic aqueous phase budding within an ATPS GV. This ATPS GV (44:1 mol ratio DOPC/DOPE-mPEG 2000 Da) was originally imaged in a hypertonic sucrose/buffer solution. The times above (below) each frame are the minutes into the process that the images were acquired. A: Transition induced by adding DI water. B: Transition induced by adding hypertonic sucrose. T = 3°C, bar = 10 µm.
Figure 4-8. Micrographs of dynamic asymmetric protein microcompartmentation within an ATPS GV. This is the same vesicle as in Figure 4-7. A: Before dilution. B: After dilution. C: After concentration. Columns, from left: transmitted light (DIC), lipid fluorescence, lectin SBA fluorescence. T = 3°C, bar = 10 µm.
Figure 4-9. DIC images of dynamic aqueous phase budding within an ATPS GV, similar to that in Figure 4-7, except that the PEG phase is the budding phase in this ATPS GV. 
A: Transition induced by adding DI water.  
B: Transition induced by adding hypertonic sucrose.  
$T = 3^\circ C$, bar = 10 $\mu$m.
I have determined that budding membrane surface area, for the ATPS-containing GVs described herein, does not change when the vesicle is diluted in water. The most likely explanation for these results is that the budding phase extends as far as the excess membrane surface area allows upon ATPS concentration such that the membrane surface area does not change overall. I estimate that the change in membrane surface area when a budding ATPS GV reverts to a concentric morphology is approximately 5% or less (Table 4-3). These calculations were performed as described in the experimental section. It is therefore not surprising that surface strain, related to the change in surface area by the formula $\delta \varepsilon = \frac{dA}{A}$ (96) where $\varepsilon$ is the surface strain (96) and $A$ is the surface area (96), induced by budding was minimal (Table 4-3).

The average increase in volume of an ATPS GV from the budding state to the concentric state upon dilution by a factor of approximately 2 was 130% (Table 4-3). Some comparisons to results in the literature follow. One report states that egg lecithin GVs prepared in water could reduce in radius by at least 60% upon the addition of small concentrations of NaCl (41). A reduction in radius by at least 33% was observed in DOPC GVs (prepared in sucrose) (35), at least 40% with other lipids (40). However, the shrinkage was presumably irreversible due to vesicle fission (35,40). Most interestingly, similar GVs containing an agarose gel, under a similar degree of hyperosmotic stress (a factor of approximately 6 or 7) could undergo reversible shape changes when the osmotic pressure was reduced after the initial increase as long as the change in volume was not greater than 80% (35). These results prove the importance of the gel on preventing the
Table 4-3. Surface strain and changes in membrane surface area and volume upon a transition from a budding to a concentric morphology as a result of dilution by a factor of approximately 2. T = 5°C. $\varepsilon$ = surface strain, SA = surface area, V = volume (96).

<table>
<thead>
<tr>
<th>quantity</th>
<th>value</th>
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<tr>
<td>$</td>
<td>\Delta \varepsilon</td>
</tr>
<tr>
<td>$</td>
<td>\Delta SA</td>
</tr>
<tr>
<td>$\Delta V</td>
<td>130 \pm 8.7%</td>
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destruction of the GVs in response to hypertonic stress (35), similar to the effect of the ATPS in the work reported herein.

The main conclusions from these experiments was that asymmetric protein microcompartmentation is reversible in ATPS GVs, membrane surface area and surface strain do not change during this process, and vesicle volume does increase on dilution. Since I have demonstrated that dynamic asymmetric microcompartmentation does not appear to impact vesicle integrity, a logical question to investigate is what effect ATPS concentration has on partitioning within ATPS GVs. This question is addressed in the next section.

**Partitioning within ATPS GVs in response to hypertonic stress.**

I have quantitatively investigated the effects of increasing polymer weight percent composition on partitioning within the budding phase of ATPS GVs. Dispersing ATPS GVs in hypertonic solutions increased both polymer and protein partitioning within these structures. Presumably hypertonic stress forces water out of the vesicles, thereby concentrating the ATPS within. The degree of polymer and protein partitioning is well-known to increase within bulk ATPS as a function of increasing concentration of both polymers (97). This is because the more concentrated ATPS will possess a greater difference between the polymer weight percent compositions of the PEG and dextran phases (97).
The partitioning of dextran and lectin SBA (a glucose-binding protein) (98) increased in ATPS GVs with increasing hypertonicity of the ATPS GV dispersion (Figure 4-10 and Figure 4-11). An increased degree of partitioning by a factor of approximately 4 to 6 in the dextran-enriched phase for both dextran and a dextran-partitioned protein (lectin SBA) was observed with an increase in vesicle dispersion osmolarity of approximately 3 (Figure 4-10). A decreased partition coefficient (less than 1) is indicative of an increased degree of partitioning with respect to polymers or proteins partitioned to the dextran phase. Unfortunately I was unable to perform similar experiments with polymers or proteins partitioned to the PEG phase due to low intensities in the dextran phase that eventually lead to large errors in the plot of the partition coefficient as a function of ATPS GV osmolarity. Histograms of the distribution of lectin SBA partitioning within ATPS GVs under an increasing degree of hypertonic stress clearly illustrate this increasing degree of partitioning (Figure 4-11). The partition coefficient decreases with an increasing degree of hyperosmotic stress (Figure 4-11).

The general trend of increasing partitioning with an increasing polymer weight percent composition was also observed in a bulk ATPS (Figure 4-12 and Figure 4-13). These experiments within bulk ATPS were rendered analogous to those within ATPS GVs through increasing the polymer weight percent composition while keeping the weight percent ratio of PEG to dextran constant at 7.5:8. With increasing polymer weight percent, the partition coefficient of PEG increased and that of dextran decreased (both representing an increased degree of partitioning) (Figure 4-12). These trends held for proteins as well; the partitioning of lectin Con A, lectin SBA, and streptavidin preferentially partitioned to the dextran phase through excess biotin-dextran 10,000 Da
**Figure 4-10.** Partitioning of dextran 10,000 Da and lectin SBA in ATPS GVs (44:1 mol ratio DOPC/DOPE-mPEG 2000 Da) as a function of ATPS GV suspension osmolality. $T = 5^\circ C$. Each data point represents the average of at least 40 ATPS GVs.
**Figure 4-11.** Typical histograms illustrating the distribution of K within ATPS GVs. These ATPS GVs were 44:1 mol ratio DOPC/DOPE-mPEG 2000 Da, and were dispersed in a sucrose/buffer solution prior to imaging at 5°C. The ATPS GVs were imaged in (from top) 112 ±1.00, 133 ± 2.08, 145 ± 1.00, and 165 ± 2.00 mmol/kg sucrose/buffer, respectively.
Figure 4-12. Bulk polymer $K$ as a function of ATPS osmolality. The weight percent ratio of PEG to dextran was held constant at 7.5 PEG:8 dextran. Each data point represents the average of at least 4 independent measurements.
Figure 4-13. Bulk protein $K$ as a function of ATPS osmolality. The weight percent ratio of PEG to dextran was held constant at 7.5 PEG:8 dextran. **A:** lectin Con A; **B:** streptavidin in excess biotin-dextran; **C:** lectin SBA. Each data point represents the average of at least 4 independent measurements.
all increased with increasing polymer weight percent (Figure 4-13). These results suggest that the mechanism of increased partitioning within ATPS GVs under an increasing degree of hyperosmotic stress is due to the increased polymer weight percent induced by loss of water from the vesicles.

**Differential relative aqueous phase positions as a function of the phospholipid headgroups.**

Interestingly, DOPC/DOPG ATPS GVs prepared in a PEG 8000 Da/dextran 10,000 Da ATPS exhibited different relative aqueous phase positions; dextran was the outer phase instead of the inner phase (Figure 4-5). I see two possible mechanisms behind these results: surface coverage of DOPC/DOPE-mPEG 2000 Da ATPS GVs by PEG and the changes in aqueous-aqueous and aqueous-membrane interfacial tensions induced by changes in polymer concentration (osmotic stress). The first mechanism, membrane surface coverage, cannot explain the switch in relative aqueous phase positions on changing the lipid headgroups. 44:1 mol ratio DOPC/DOPE-mPEG 2000 Da ATPS GVs have approximately 10% of their membrane surface area covered by the helical PEG chains (99) from the polymer-conjugated lipid based on an approximately 2.2 nm PEG hydrodynamic radius estimated from the data reported in (100). Based on this one would expect the PEG phase to wet the membrane in this membrane composition, which is observed, but this is always observed under isotonic conditions except in DOPC/DOPG ATPS GV encapsulating an ATPS of low molecular weight dextran.
Importantly, I have shown that this switch in relative aqueous phase positions is dependent on ATPS polymer MW and the phospholipid headgroups (Figure 4-14, 4-15). This is the most important conclusion since the relative phase position reversal noted in (30) was attributed to hyperosmotic stress; I have shown that the lipid headgroups themselves can be used to achieve the same effect. Another important result is that changing the lipid headgroups does not prevent dynamic microcompartmentation, which could be rendered dynamic (reversible) independent of the lipids comprising the membrane as long as the preparation resulted in ATPS GVs (Figure 4-16 - 4-18).

It is much more difficult to obtain interfacial tension measurements within ATPS GVs. Increasing aqueous-aqueous interfacial tensions due to hyperosmotic stress could be extrapolated from bulk ATPS (97,101). It is reasonable to presume that minimization of contact area between the phases is a driving force behind budding in ATPS GVs. However, it is not clear to me how to directly measure aqueous-membrane interfacial tensions within ATPS GVs. Aqueous-membrane interfacial tensions are likely very different with different lipids present in the GV membrane. Besides the obvious differences in relative aqueous phase positions, the partitioning (K) of dextran in DOPC/DOPE-mPEG 2000 Da ATPS GVs prepared in a 7.5 wt% PEG 8000 Da/dextran 10,000 Da ATPS, dispersed in the PEG-enriched phase of the ATPS, is 0.45 ± 0.11 (17 ATPS GVs) at 5°C, while that in DOPC/DOPG ATPS GVs is 0.72 ± 0.041 (3 ATPS GVs), all other conditions identical. Note that although DOPC/DOPG ATPS GV yield is very low under these particular conditions, I have always observed a low degree of polymer partitioning within such vesicles.
Figure 4-14. Representative micrographs of the effect of dextran molecular weight in relative aqueous phase positions in DOPC/DOPG ATPS GV s dispersed in the top phase of the bulk ATPS. A: PEG 8000 Da/dextran 10,000 Da, dextran outer phase. B: PEG 8000 Da/dextran 40,000 Da, PEG outer phase. C: PEG 8000 Da/dextran 70,000 Da, PEG outer phase. D: PEG 8000 Da/dextran 500,000 Da, PEG outer phase. Columns, from left: transmitted light (DIC), lipid fluorescence, outer phase fluorescence (top, dextran; all others, PEG). T = 5°C, bar = 10 µm.
Figure 4-15. Representative confocal micrographs of the effect of the phospholipid headgroups in PEG 8000 Da/dextran 10,000 Da ATPS GV s dispersed in the top phase of the bulk ATPS on relative aqueous phase positions. A: DOPC/DOPE-mPEG 2000 Da, PEG outer phase. B: DOPC/DOPG, dextran outer phase. C: DOPC/DOPG/DOPE-mPEG 2000 Da, PEG outer phase. Columns, from left: transmitted light (DIC), lipid fluorescence, dextran fluorescence. T = 5°C, bar = 10 µm.
Figure 4-16. Aqueous phase transitions in a DOPC/DOPE-mPEG 2000 Da ATPS GV. Note that on cooling, multiple dextran phases coalesce into one larger dextran phase. **A:** Start of heating sequence. **B:** Start of cooling sequence. Bar = 10 µm.
Figure 4-17. Aqueous phase transitions in a DOPC/DOPG ATPS GV. Note that on cooling, multiple PEG phases coalesce into one larger PEG phase. A: Start of heating sequence. B: Start of cooling sequence. Bar = 10 µm.
Figure 4-18. Aqueous phase transitions in a DOPC/DOPG/DOPE-mPEG 2000 Da ATPS GV. Note that on cooling, multiple dextran phases coalesce into one larger dextran phase. **A:** start of heating sequence. **B:** start of cooling sequence. Bar = 10 µm.
Conclusions.

This chapter has demonstrated that changes in volume exclusion within ATPS GVs can lead to asymmetric microcompartmentation and is a function of the elasticity of the membrane. Increasing mol% cholesterol within the membrane had a much less dramatic impact on the presence of budding within a given preparation than decreasing mol% of grafted polymer. Asymmetric microcompartmentation can be rendered dynamic (reversible) through changes in osmolarity. These transitions result in neither a change in membrane surface area nor surface strain, but dilution by a factor of 2 results in a 130% increase in volume under the conditions evaluated. The partitioning of the polymers comprising the ATPS within the GVs as well as that of proteins partitioned to the aqueous phases increases with increasing osmolarity. Relative aqueous phase positions can be switched in ATPS GVs as a function of the phospholipid headgroups in the membrane as well as the polymer molecular weight of the ATPS within the GVs.
**Future Directions.**

This chapter has documented an easy method to achieve a high degree of curvature within the membrane of ATPS GVs. An obvious application of these budding vesicles is lipid phase separation within the membrane. One such experiment is whether a particular lipid can be driven to the region of highest curvature in ATPS GVs (24,78). Membrane topology and elasticity can be manipulated through phase separation in the membrane (76). Curvature within a cell is of wide interest since curvature plays important roles in functions from channel activity (23) to reproduction (102). Another potential but speculative experiment is whether one of the aqueous phases can be directed to wet a particular lipid phase if lipid phase separation were induced within the membrane. Positive results from such an experiment would be of wide interest in that it would suggest that the cell cytoplasm itself might impact lipid phase separation *in vivo*.

An obvious contrast between budding ATPS GVs and the budding observed in living cells is the scale of the budding event. In living cells a budding event may be on the order of tens of nanometers; the budding exhibited by ATPS GVs is instead on a scale observable by light microscopy, facilitating its visualization. This does not render insights gained from ATPS GVs inapplicable to living cells. In a living cell, the proteins within the cytoplasm potentially serve as distinct aqueous phases (103), and since there are many proteins within a given cell, budding driven by one of these protein phases may be on a much smaller scale. Additionally, preferential partitioning to these budding phases offers a potential mechanism of directing other proteins to these budding regions of the cell.
References.


32. *Escherichia coli* can grow in a greater than 100-fold range of osmolarities (17), much less than the osmolarity changes induced within ATPS GVs in our work.

33. The relative volumes of the phases of a bulk PEG/dextran ATPS are defined by a given polymer weight percent composition (show no variability) at a given temperature and salt concentration (97).


95. Extrapolating from the data for POPC/POPG GVs in reference 94, the DOPG may decrease 10:1 mol ratio DOPC/DOPG GV stability by approximately 25%.


Chapter 5

Enzyme-Colloidal Gold Conjugates for Increased Enzyme Partitioning in Aqueous Two-Phase Systems

Previous chapters in this thesis have demonstrated that ATPS GVs are effective cytomimetic media for controlling local protein concentrations through aqueous phase transitions. However, a drawback of these structures is that many proteins do not partition well within them. By necessity of ATPS GV preparation, the weight percent polymer composition of the ATPS to be encapsulated must be relatively close to the binodal at the phase transition temperature. This typically limits the partitioning possible within these structures since partitioning becomes less distinct when the polymer weight percent composition is closer to the one-phase region of the phase diagram (1). This chapter presents protein conjugation to colloidal Au as a method to address this limitation while retaining all requirements for dynamic aqueous phase separation in response to temperature.

Other methods exist to increase protein partitioning in ATPS. The most obvious method is perhaps increasing the total polymer weight percent comprising the ATPS (1). Protein partitioning is known to increase with increasing polymer weight percent (increasing degree of polymer partitioning) (1). However, doing so renders the ATPS two phases regardless of temperature and is therefore unsuitable for application within temperature-responsive ATPS GVs. Affinity partitioning (2-5) is also a widely used technique to improve partitioning within ATPS. This method involves binding of the analyte to one of the phase-forming polymers. For the separation of proteins, either the
protein must have an inherent affinity for one of the polymers, such as the case of glucose-binding lectins and dextran (2, 4, 6), or an affinity tag must be bound to a small percentage of one of the polymers (3-5). Varying the salt (cation or anion) or salt concentration (1, 7) in an ATPS can also dramatically impact the partitioning of proteins (1, 7), but this is typically not suitable for GV assembly. As an extreme example, increasing the NaCl concentration in a PEG/dextran ATPS beyond the point at which dextran gels (several molar salt) gives increased protein partitioning to the PEG phase (7). Varying the pH is another method to improve protein partitioning in ATPS (8) and has been used to determine protein isoelectric points (8), but this may have detrimental effects on enzymatic activity. In contrast to all of these methods, as discussed shortly, the direct adsorption method of proteins to Au nanoparticles is suitable for a wide range of proteins and requires neither high polymer weight percents, affinity molecules, salt additives, nor pH changes in order to achieve a substantial degree of protein partitioning.

Colloidal Au has recently been used as a scaffold for biological polymers, such as DNA-Au nanoparticle conjugates that are capable of reversible hybridization through changes in temperature (9-11) and for the conjugation of proteins (12-16) and peptides (17). The direct adsorption method of biomaterial conjugation to colloidal Au generally results in some loss of activity (16), but in some cases bioconjugates have been demonstrated to retain or even increase enzymatic activity as well as increase tolerance to changes in temperature and pH (12, 14, 15). An important aspect to consider when attempting adsorption of a protein to Au nanoparticles is that some portion of the enzyme should be positively charged in order to electrostatically bind to the negatively charged Au surface (12). Lysine residues are positively charged at physiological pH (7.4). Since
many proteins have lysine residues available for binding [e.g., HRP has 6 (18) and bovine serum albumin (BSA) has 59 (19)], the adsorption method is suitable for conjugation to a large selection of proteins. This electrostatic mechanism of protein binding to nanoparticles even occurs away from the protein isoelectric point, at least in the case of BSA (20).

Endoglucanase-Au nanoparticle conjugates at high temperature and pH do not exhibit a rapid drop in activity, typically observed with the native enzyme under these conditions, but the activity does drop to the level of the native enzyme after approximately 150 minutes (14). This demonstrates the retention of enzymatic activity under otherwise denaturing conditions. Pepsin-colloidal Au conjugates, after greater than 1 day, exhibit roughly twice the activity of the native enzymes measured at 4°C, pH 3, measured over the same time intervals up to at least four days (15). This demonstrates that activity is retained longer than observed relative to the native enzyme. Certain peptides capable of targeting to the cell nucleus retain the same function after conjugation to Au nanoparticles (17), offering a method to target materials to the nucleus without infectious agents such as viruses (17). It is possible to retain more enzymatic activity in enzyme-colloidal Au conjugates. Coating a colloidal Au surface with molecules that will specifically bind to histidine-tagged HRP has been shown to prevent nonspecific adsorption onto the nanoparticle surface with the retention of greater than 90% activity (16).

The development of methods to increase the retention of enzymatic activity of proteins conjugated to nanoparticles may be facilitated through understanding the basis of protein denaturation on surfaces (12-17,20-28). One consideration is the identity of the
buffer utilized; the amount and structural retention of BSA adsorbed onto colloidal silica is maximized in phosphate buffer (21); after adsorption, the buffer was observed to be less important (21), suggesting conformational stabilization of the protein. The activity of enzyme-Au nanoparticle conjugates is reduced with increasing particle size due to an increasing percentage of each protein nonspecifically adsorbed onto the surface (22). Similar results have been reported on the basis of protein denaturation on colloidal silica (23,24). Further, an increasing degree of adsorption (or a decreasing strength of adsorption) increases the activity of α-amylase adsorbed onto colloidal silica (25). These results collectively suggest that a general method to retain enzymatic activity of protein-nanoparticle conjugates is to minimize the amount of protein molecule surface area in contact with the surface (21-25).

One method to reduce nanoparticle-protein contact area is to use small nanoparticles (higher curvature). The activity of chicken egg lysozyme adsorbed onto colloidal silica is strongly dependent on the diameter of the particle; larger particles yield conjugates of lower activity than smaller nanoparticles (23). This is likely due to more enzyme surface area adsorbed onto the nanoparticle surface, a hypothesis supported both by a concomitant decrease in enzyme α-helical content with increasing nanoparticle diameter (23). Large changes in secondary structure, consistent with protein denaturation, are also observed with increasing colloidal silica diameter in the binding of human carbonic anhydrase I (HCAI) (24). However, another method to reduce nanoparticle-protein contact area, surface modification with polymers, was observed to decrease the activity of α-amylase adsorbed onto silica nanoparticles to varying extents
depending on the type of polymer (25). This may be due to a stronger degree of protein binding to hydrophobic surfaces relative to hydrophilic surfaces (25-28).

My motivation for binding HRP to colloidal Au was to utilize the high surface area to volume ratio of the nanoparticles in order to prepare conjugates that possess multiple enzymes in one structure. These HRP-colloidal Au conjugates exhibited increased surface interactions with the polymers comprising the ATPS, an effect that often lead to dramatically increased partitioning. An additional requirement was to retain activity in comparison to the native enzyme. The number of enzymes adsorbed per nanoparticle, important for the determination of percent activity retention, was estimated through a flocculation assay and the enzymatic activity in the supernatant solution after centrifugation. The amount of adsorbed HRP estimated through this analysis was in slight excess of the theoretical range of HRP adsorption.

I have determined that protein-colloidal Au partitioning in ATPS is dependent on ATPS composition, polymer molecular weight, Au nanoparticle diameter, and in some instances nanoparticle concentration in the ATPS. Approximately 25% activity retention was observed for 15-nm HRP-Au nanoparticle conjugates. A particularly noteworthy result was that a large degree of partitioning was observed in ATPS close to the binodal at the phase separation temperature and in ATPS with a low degree of polymer partitioning. These results increase the relevance of the adsorption method towards future work within ATPS GVs.
Experimental.

Chemicals.

Lipids were obtained from Avanti Polar Lipids (Alabaster, AL). PEG, dextran, sodium phosphate (monobasic and dibasic), trisodium citrate, HAuCl₄ trihydrate, sodium chloride, bovine serum albumin (BSA), horseradish peroxidase (HRP), fluorescein isothiocyanate (FITC)-conjugated HRP, Sigma-Fast optical density solution (OPD, contains o-phenylene diamine, hydrogen peroxide, and buffer), KCN, and K₃Fe(CN)₆ were obtained from Sigma-Aldrich. Protein A was obtained from Pierce. Fluorescein-conjugated BSA and Alexa488-conjugated protein A were obtained from Molecular Probes. OPD was used within one hour of preparation and was stored in a closed dark bottle when not in use. Hydrochloric acid and nitric acid were obtained from EMD Chemicals and EM Science, respectively. Deionized water (≥ 18.2 MΩ) from a Barnstead NANOpure Diamond unit was used for all experiments. Colloidal Au (31-nm and 50-nm, <8% standard deviation) was purchased from SPI supplies (West Chester, PA); other diameters were prepared as described later.

All proteins were stored in either 1-mg or 2-mg quantities at –80°C until use. In particular, note that HRP must be stored as a dry powder (if activity retention is required) and azide is an inhibitor of this enzyme (material data sheet, Molecular Probes).
**Instrumentation and equipment.**

A Fluorolog Jobin Yvon Horiba FL3-21 fluorimeter was used to determine bulk fluorophore-tagged protein and polymer partitioning. A Hewlett-Packard 8453A diode-array UV/Visible (UV/Vis) spectrometer (1 nm resolution, 1 s integration time) with ChemStation UV/Vis software was used to determine bulk Au nanoparticle and HRP-colloidal Au conjugate partitioning. This spectrometer was equipped with a temperature-control unit and was set at 25°C. A Biofuge Pico centrifuge from Heraeus Instruments was used for the purification of HRP-Au colloid conjugates. Costar microcentrifuge tubes (1.7 mL, number 3620) were used to prepare HRP-Au nanoparticle conjugates.

**Colloidal Au preparation.**

The preparation of Au nanoparticles in-house was according to the citrate reduction method (29-31). Average diameters were determined through transmission electron microscopy (TEM) images on a JEOL 1200 EXII instrument with GATAN Digital Microscopy software (in collaboration with Marcus Helfrich) and NIH Image v1.63. Nanoparticle concentrations were determined through UV/Vis spectroscopy of a 10X dilution. The results of the characterization are as follows: 12.2 nM of 12.5 ± 1.02 nm Au based on 180 particles (in collaboration with Francis Reyes) and 18.8 nM of 15.3 ± 1.33 nm Au based on 120 particles.
Protein-colloidal Au conjugate preparation.

One hundred microliters of 40 mM pH 7.4 sodium phosphate buffer, prepared by titration of HCl into dibasic sodium phosphate, was added to a centrifuge tube, followed by 800 µL of colloidal Au solution. This solution was vortexed for several seconds, followed by an addition of 100 µL of protein stock solution that was prepared in 40 mM pH 7.4 sodium phosphate buffer was added. The concentration of the protein stock solution was 800 µg/mL for absorption onto 13- and 15-nm Au nanoparticles, 4000 µg/mL, 31-nm Au; 8000 µg/mL, 50-nm Au. The conjugate solution was vortexed for several seconds and allowed to equilibrate for 20 min. All of the proteins used in this study are known to bind to Au nanoparticles in at most 15 minutes (32), possibly as little as 1 minute (33). Maximal surface coverage of the protein on colloidal Au can be determined at this point through slight modifications of the standard flocculation assay (33). For coverage of HRP, I varied the initial concentration of added HRP, added 100 µL of 1.5 M NaCl to the 1 mL of conjugate solution, allowed this to sit for > 1 hour, and afterwards diluted by a factor of 10, noting any broadening of the peak at approximately 525 nm. Bare colloidal Au aggregates in high concentrations of salt (33); peak broadening in the UV/Vis spectrum of the nanoparticles is indicative of such aggregation and by inference incomplete surface coverage by the protein of the nanoparticles (33). After the 20 min equilibration noted previously, the conjugate solution was then centrifuged for 40 min at 13.0 krpm for the smaller (13 nm) Au nanoparticles and 20 minutes at 13.0 krpm for the larger Au nanoparticles to separate the unbound protein from the conjugate.
The relative centrifugal force (rcf) of the centrifuge is defined (34) as:

\[ \text{rcf} = 11.17 \times R_{\text{max}} \times (\text{rpm}/1000)^2 \]

Rmax is the maximum distance from the axis of rotation of the centrifuge (in centimeters) and rpm is the number of revolutions per minute (34). The maximum rcf of this instrument is 16,060*g (34) and the maximum speed (rpm) is 13.0 krpm (34). Therefore, Rmax is 8.51 cm and a speed of 13.0 krpm for this instrument is equivalent to an rcf of approximately 16,000*g.

The supernate was removed and the conjugates were resuspended to 100 µL in 5 mM pH 7.4 sodium phosphate buffer. Subsequently, unless otherwise noted, ten such preparations were combined into one centrifuge tube, centrifuged again, and resuspended to 100 µL. If such a concentrated (~ 1.5 mM Au concentration for 15-nm Au-protein conjugates, ~ 9.8 mM for 13-nm Au-protein conjugates) solution was not required (such as for activity quantification and for investigating the effect of conjugate concentration on partitioning), resuspension of one conjugate preparation was to 1 mL, the sample was centrifuged again, and subsequently resuspended to 100 µL (~ 10% the concentration of the more concentrated conjugate solutions). An approximately 100-fold concentration of the conjugate stock solution relative to initial Au nanoparticle concentration was important in order to observe conjugate absorbance at 525 nm in the phase to which the conjugates did not partition by UV/Vis spectroscopy above the background noise level when the degree of partitioning was large. This is because, by the inherent nature of the conjugate partitioning experiments, the conjugates must be diluted by a factor of 100 in the ATPS (see later). In some instances, when the amount of conjugate in the PEG phase was undetectable through UV/Vis spectroscopy, an approximately 1.5 mL aliquot of a
PEG phase sample containing protein-Au conjugate was centrifuged and most of the supernate extracted to obtain distinct peaks in the sample through UV/Vis spectroscopy (see later).

**Partitioning.**

Partitioning of protein-colloidal Au conjugates was determined through UV/Vis spectroscopy, adding 30 µL of the conjugate solution to 2970 µL of ATPS at 25°C. Partitioning of bare Au nanoparticles was determined through substituting a Au nanoparticle stock solution in place of some of the water comprising the ATPS (more detail given later). Partitioning of fluorophore-tagged proteins and polymers was determined through fluorimetry, adding 30 µL of a 0.1 - 0.5 w/w% protein solution to 2970 µL of ATPS at 25°C. To determine the effect of conjugate concentration on polymer partitioning, protein-Au nanoparticle conjugates were added in addition to the fluorophore-tagged polymer; in this case, the Au was dissolved before fluorescence spectral acquisition through adding 70 µL of 0.2 M KCN and 30 µL of 4.8 mM \( \text{K}_3\text{Fe(CN)}_6 \) to 100 µL of top or bottom phase sample (final concentrations: 70 mM KCN, 0.72 mM \( \text{K}_3\text{Fe(CN)}_6 \), ~ 1.6 nM nanoparticles) similarly to a literature protocol (35). The absorbance due to Au determined through UV/Vis spectroscopy disappeared after at most several minutes as previously reported (35).

I chose ATPS that, at 3°C, possessed a dextran phase somewhere between 15-30 v/v% of the total ATPS, except when explicitly evaluating the effect of polymer weight
percent on the degree of partitioning, for two related reasons. One was to ensure that the ATPS was not near the critical point, where the degree of partitioning is typically low due to an approximately even distribution of polymers between the two phases (1). The other was to minimize the impact of relative distance from the critical point on partitioning as a function of polymer molecular weight (1). The relative phase volumes of the ATPS is a measure of distance from the critical point (1); it was hoped that maintaining an approximately constant volume percent dextran phase would remove the impact of tie line length of the degree of partitioning as a function of polymer molecular weight (1). Additionally, all ATPS were also one phase at 25°C, hopefully rendering the results described herein useful towards eventual partitioning within ATPS GVs (4).

In all cases, the ATPS was allowed to sit overnight at 3°C. The next day, 200 µL aliquots of the top and bottom phases were obtained by pipette for analysis. The partition coefficient, K, is the concentration of material in the PEG phase divided by that in the dextran phase (1). All absorbance data points were corrected for the background over the 800-850 nm wavelength range, likely to be more accurate than obtaining a background correction over a single reference wavelength when absorbance intensities are low.

**Activity.**

The general method of HRP activity determination provided by Sigma-Aldrich was followed. A blank spectrum of optical density solution (defined in chemicals section previously) was obtained on the UV/Vis spectrometer. Five hundred nanoliters (0.5 µL)
of HRP (either a 500-fold dilution of a standard concentration of HRP or a 100-fold
dilution of an HRP-Au nanoparticle conjugate solution) was added to a 1.0 cm quartz
cuvette, which was placed in the spectrometer sample holder. Two hundred microliters
of OPD was then added (facilitating more rapid mixing than if the smaller volume were
added to the larger volume) and the acquisition of spectra was immediately started over
30 s intervals for 2.5 min. The product peak was at approximately 445 nm. All
absorbance data points were corrected for the background over the wavelength range
noted beforehand. The temperature was set to 25°C.

**ATPS GV assembly.**

ATPS GVs were prepared as in Chapter 2. The lipids were 44:1 mol ratio
DOPC/DOPE-mPEG 2000 Da.
Results and Discussion.

Determination of HRP maximal surface coverage on colloidal Au.

The maximal surface coverage of HRP on 15-nm colloidal Au was at approximately 60 µg added HRP, determined through a combination of two experiments. The first was a flocculation assay (33), which determined the amount of HRP required to stabilize the conjugates in the presence of high concentrations of NaCl (Figure 5-1). When Au nanoparticles aggregate (12), the maximum peak wavelength in the visible range through UV/Vis spectroscopy is redshifted and the width of this peak broadens (33). On average, no such changes were observed at 60 µg added HRP after the addition of 1.5 M NaCl, indicating that the colloidal Au was not aggregating and therefore covered by HRP. Figure 5-2 illustrates that the absorbance of the conjugates does not change when this or higher HRP concentrations are added. Therefore, 80 µg added HRP is in excess of the amount required to cover the nanoparticles (36) and this amount was subsequently chosen to ensure full coverage of the nanoparticles by the enzyme.

Assuming that 60 µg of HRP covered the colloidal Au and that the molecular weight of HRP is approximately 44,000 g/mol (Protein Data Bank), the flocculation assay suggests that there are approximately (upper limit) 73 HRP molecules per Au nanoparticle. This is in approximate agreement with the literature value of 61 molecules of HRP per 15-nm Au nanoparticle (32). Theoretical surface coverage, based on the dimensions of HRP (3.6 nm, 3.6 nm, 5.6 nm, obtained from data provided at the Protein Data Bank, PDB ID 1HCH), is between 37 and 57 molecules of HRP per nanoparticle dependent upon
Figure 5-1. Examples of the spectra of HRP-colloidal Au conjugates (15 ± 1.3 nm Au) covered with varying concentrations of HRP to the addition of 1.5M NaCl. A, B: 97, 73 HRP:Au (80, 60 µg/mL HRP); C: 61 HRP:Au (50 µg/mL HRP); D: 55 HRP:Au (45 µg/mL HRP); E: 48 HRP:Au (40 µg/mL HRP); F: 30 HRP:Au (25 µg/mL HRP). The Au peak is of the same dilution as that of the conjugates, no NaCl added. The value of 73 HRP:Au is an upper estimate of that required for stability. This results in a slight underestimation of HRP percent activity retention, which is more favorable than overstating this value. The primary source of error resides in the preparation of an HRP solution of precise concentration.
Figure 5-2. Absorbance at 525 nm as a function of the amount of HRP added to 15 ± 1.3 nm Au after the addition of NaCl, plotted from the data in Figure 5-1.
which side of HRP bound to the nanoparticles. My experimental value of approximately 73 HRP per Au is close to this theoretical range, suggesting an approximate monolayer of protein on the nanoparticles (32).

Flocculation experiments can be used to report on the concentration of HRP required to stabilize the nanoparticles, but not how much HRP is actually adsorbed onto the nanoparticles. I therefore centrifuged the conjugate solution and measured the activity within the supernatant solution. Through the activity in the supernatant solution I was able to estimate the concentration of HRP within it. No conjugate was present within the supernatant solution as indicated by the lack of absorbance at 525 nm, demonstrating that any activity within the supernatant solution is of free HRP and not that of the conjugate. These results indicated that approximately 97% of the 73 HRP required to stabilize the nanoparticles are bound. Again, this is in agreement with the experimental observation, through electron microscopy, that many proteins (HRP among them) form only an approximate monolayer on Au nanoparticles (32). This experimental value of HRP bound to colloidal Au is critical in estimating the retention of HRP enzymatic activity after adsorption on Au nanoparticles (to be discussed later).

Partitioning of proteins and protein-colloidal Au conjugates: Effect of polymer molecular weight.

It has generally been observed that the degree of protein partitioning in polymer/polymer bulk ATPS increases with increasing polymer molecular weight discrepancy (37) or increasing protein molecular weight (38). In other words, ATPS
comprised of polymers of similar molecular weight will typically not partition proteins as well as ATPS comprised of polymers of highly differing molecular weights (1,37). This effect was observed for proteins of molecular weight greater than 10,000 Da (37). The issue is, however, much more complicated (39) because changing polymer molecular weight also changes polymer partitioning between the two phases, a major determinant of protein partitioning within the ATPS (1,37,38) as discussed in the experimental section. The main point of this discussion is that changing polymer molecular weight within an ATPS also changes many other variables that also impact partitioning within the ATPS, hindering a direct interpretation of the impact of polymer MW on partitioning.

The partitioning of FITC-conjugated HRP in all of the bulk ATPS studied (close to the 5°C binodal) is close to 1. Not surprisingly, FITC-conjugated HRP partitioning is not noticeable in corresponding ATPS GV (Figure 5-3). However, conjugates of HRP to 15.3 nm colloidal Au exhibited a dramatically increased partitioning in all bulk ATPS studied (Table 5-1) and partitioned to the PEG phase. The absorbance intensity in the dextran phase was low, but enough such that distinct peaks were observed at 525 nm (Figure 5-4). The average retention of Au after the conjugation, centrifugation, and resuspension steps was approximately 80% in the case of the 15.3 nm Au nanoparticles.

An immediate question was why there was such a large decrease in the degree of partitioning in ATPS containing a high molecular weight of dextran (Table 5-1) (40). Insight into the reason for this behavior can be found in the effect of conjugate concentration on partitioning. In the PEG 8000 Da/dextran 150,000 Da ATPS, conjugates of several nM concentration in the ATPS partitioned by a factor of
Figure 5-3. Micrographs illustrating the lack of partitioning of native (FITC-labeled) HRP in ATPS GV's during dynamic aqueous phase separation within the GV. Columns, from left: transmitted light (DIC), lipid fluorescence, HRP fluorescence. T = 3°C, bar = 10 µm.
Table 5-1. Bulk partitioning of HRP-colloidal Au conjugates at 3°C as a function of ATPS molecular weight. These and all other ATPS were prepared in 5 mM pH 7.4 sodium phosphate buffer. The average diameter of the Au nanoparticles was 15 ± 1.3 nm. Each data point represents the average of at least 4 independent measurements.

<table>
<thead>
<tr>
<th>ATPS</th>
<th>K conjugates (bare Au) (3°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 wt% PEG 4600 Da (lot 14316AC)/9 wt% dextran 10,000 Da (lot 084K1226)</td>
<td>150 ± 2.3 (73 ± 3.2)</td>
</tr>
<tr>
<td>7.5 wt% PEG 8000 Da (lot 044K0092)/8 wt% dextran 10,000 Da (lot 084K1226)</td>
<td>130 ± 7.9 (69 ± 2.1)</td>
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<tr>
<td>4.875 wt% PEG 8000 Da (lot 044K0092)/6.5 wt% dextran 40,000 Da (lot 124K1343)</td>
<td>140 ± 1.8 (77 ± 1.1)</td>
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<td>4.75 wt% PEG 8000 Da (lot 044K0092)/5 wt% dextran 70,000 Da (lot 025K0625)</td>
<td>150 ± 7.8 (78 ± 0.65)</td>
</tr>
<tr>
<td>4.125 wt% PEG 8000 Da (lot 044K0092)/5 wt% dextran 150,000 Da (lot 114K0238)</td>
<td>3.6 ± 0.031 (1.6 ± 0.16)</td>
</tr>
<tr>
<td>4.125 wt% PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671)</td>
<td>3.0 ± 0.061 (1.9 ± 0.31)</td>
</tr>
</tbody>
</table>
Figure 5-4. Examples of conjugate absorbance at 525 nm in the PEG and dextran phases in a bulk ATPS. The intensity in the PEG phase was adjusted by a factor of 10 to correct for the 10-fold dilution before acquisition of the spectrum. Partitioning was overnight at approximately 3°C.
approximately 2 greater than that of corresponding conjugates of 10% that concentration in the ATPS. An increased concentration of conjugates may favor increased partitioning to the PEG phase since the PEG polymer chains are smaller than the dextran polymer chains and therefore the PEG phase can more easily accommodate more conjugates within its volume. These results cannot be explained by conjugate structural changes as a function of concentration of the conjugate for two reasons. One is that conjugates prepared by centrifuging one solution exhibited the same partitioning as those prepared by centrifuging ten solutions, centrifuging the combined solution, then diluting this combined solution to a similar extent as the previously mentioned more dilute solution. The other is that conjugate aggregation would be expected to lead to precipitation into the dextran (bottom) phase; this is not observed as evidenced by the increased degree of partitioning within the PEG (top) phase.

Further, the observed results are not due to a change in the partition coefficient of the polymers comprising the ATPS due to the addition of the conjugate for two reasons. One, if the conjugates served as a phase-forming species, the ATPS binodals would shift down. This ATPS is one phase at 25°C both in the presence and absence of the conjugates. Since the ATPS is just below the 25°C binodal, the binodals do not shift down. Two, partitioning fluorophore-tagged PEG and dextran in a PEG 8000 Da/dextran 500,000 Da ATPS containing several nM conjugate concentration within the ATPS and dissolving the Au before determining polymer partitioning gave similar results as when conjugate was not present within the ATPS (Table 5-2).
Table 5-2. Bulk partitioning of polymers at 3°C as a function of the presence of several nM HRP-Au nanoparticle (13 ± 1.0 nm) conjugate in the ATPS. ATPS: 4.125 wt% PEG 8000 Da/3 wt% dextran 500,000 Da (lot 123K0671)/5 mM pH 7.4 sodium phosphate buffer. Each data point represents the average of at least 4 independent measurements.

<table>
<thead>
<tr>
<th>polymer</th>
<th>K (3°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 5000 Da (no conjugate)</td>
<td>2.0 ± 0.043</td>
</tr>
<tr>
<td>PEG 5000 Da (with conjugate)</td>
<td>1.9 ± 0.11</td>
</tr>
<tr>
<td>dextran 500,000 Da (no conjugate)</td>
<td>0.12 ± 0.0014</td>
</tr>
<tr>
<td>dextran 500,000 Da (with conjugate)</td>
<td>0.074 ± 0.0015</td>
</tr>
</tbody>
</table>
A more dramatic effect is observed in the PEG 8000 Da/dextran 500,000 Da ATPS. While the more concentrated conjugates partitioned to the PEG phase, more dilute conjugates instead partitioned to the dextran phase (Figure 5-5). The basic rationale for this switch in the phase to which the conjugates partitioned follows that of the PEG 8000 Da/dextran 150,000 Da ATPS. Large proteins typically partition to the dextran phase in PEG 8000 Da/dextran 500,000 Da ATPS (1,7,37,38). This was only observed for high molecular weights of dextran. A quantification of partitioning of dilute Au-HRP conjugates in ATPS comprised of lower molecular weights of dextran was not possible because of low conjugate absorbance in the dextran phase, a combined effect of low initial conjugate concentration and a high degree of partitioning. However, Au-HRP conjugates in these ATPS all partitioned to the PEG phase as evidenced by the large absorbance in the PEG phase. Au-BSA conjugates were evaluated as discussed later.

Interestingly, the partitioning of bare 15-nm colloidal Au is also dependent on the concentration of nanoparticles added to the ATPS, over a similar range as that used for conjugate partitioning (Figure 5-6). These results were also observed for 13-nm Au nanoparticles (Figure 5-7). The result was not significantly affected by keeping the citrate concentration constant at 3.5 mM, the concentration in the colloidal Au stock solutions, indicating that it is an effect not driven by the small change in citrate concentration (Figure 5-7). The Au nanoparticles partitioned to the dextran phase at lower concentrations and partitioned to the PEG phase at higher concentrations. Since this effect is observed for HRP-colloidal Au conjugates as well as for bare Au nanoparticles, uniquely in the PEG 8000 Da/dextran 500,000 Da ATPS, I speculate that a higher concentration of nanoparticles is excluded from the dextran phase when the
Figure 5-5. Effect of HRP-Au nanoparticle conjugate concentration in the ATPS on their partitioning. ATPS: 4.125 wt% PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671). Au nanoparticle diameter = 13 ± 1.0 nm. T = 3°C. Each data point represents the average of at least 4 independent measurements.
Figure 5-6. Effect of Au nanoparticle concentration in the ATPS on their partitioning. ATPS: 4.125 wt% PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671). Au nanoparticle diameter = 15 ± 1.3 nm. T = 3ºC. Each data point represents the average of at least 4 independent measurements.
Figure 5-7. Effect of Au nanoparticle concentration on their partitioning. A. Citrate concentration not held constant. B. Citrate concentration held constant at 3.5 mM. ATPS: 4.125 wt% PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671). Au nanoparticle diameter = 13 ± 1.0 nm. T = 3°C. Each data point represents the average of at least 4 independent measurements.
nanoparticle concentration is high enough (41-43), but only when the dextran molecular weight is also large. Evidence for this hypothesis includes the large increase in the concentration of Au in the PEG phase of a PEG 8000 Da/dextran 500,000 Da ATPS when increasing concentrations of Au are added to the ATPS, in contrast to the very small increase in the concentration of Au in the dextran phase (Figures 5-8, 5-9) (44). Exclusion is not observed in a PEG 8000 Da/dextran 10,000 Da ATPS (Figure 5-10).

**Partitioning of protein-colloidal Au conjugates: Effect of nanoparticle diameter.**

Other conditions being constant (45), increasing the conjugate diameter was expected to increase protein partitioning within the ATPS. Increasing protein surface area (46,47) is sometimes (46-48) but not always (37,45,46) reported to increase protein partitioning within polymer/polymer ATPS. As an illustration of the complications involved, 16 different hemoproteins partition differently in ATPS in a manner that is not related to protein molecular weight (46). Surface properties such as hydrophobicity (45,49), charge (45,50) and extra amino acid residues (51) as well as more radical changes such as denaturation (52) also impact protein partitioning within ATPS (53).

The size of the conjugate is the source of the increased partitioning of HRP. Conjugates prepared with larger diameters of colloidal Au exhibited increased partitioning in two different PEG/dextran ATPS (Table 5-3, Figure 5-11, Figure 5-12). The effect is striking; a decrease in Au nanoparticle diameter from 30.7 nm to 12.5 nm (a factor of approximately 2.5) decreased the partition coefficient by a factor of greater than
Figure 5-8. Concentration of Au in the PEG and dextran phases of an ATPS with increasing concentrations of Au added to the ATPS. ATPS: 4.125 wt% PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671). Au nanoparticle diameter = 13 ± 1.0 nm. T = 3°C.
Figure 5-9. Concentration of Au in the PEG and dextran phases of an ATPS with increasing concentrations of Au (BSA-Au nanoparticle conjugates) added to the ATPS. ATPS: 4.125 wt% PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671). Au nanoparticle diameter = 13 ± 1.0 nm. T = 3°C.
Figure 5-10. K Au-BSA conjugates as a function of Au concentration in the ATPS. ATPS: 7.5 wt% PEG 8000 Da (lot 044K0092)/8 wt% dextran 10,000 Da (lot 084K1226). Au nanoparticle diameter = 13 ± 1.0 nm. T = 3°C. Each data point represents the average of at least 4 independent measurements.
Table 5-3. Bulk partitioning of HRP-Au nanoparticle conjugates at 3°C as a function of colloidal Au diameter. ATPS: 7.5 w/w% PEG 8000 Da (lot 044K0092)/8 w/w% dextran 10,000 Da (lot 084K1226).

<table>
<thead>
<tr>
<th>nanoparticle diameter (nm)</th>
<th>K (3°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 ± 1.0</td>
<td>60 ± 6.3</td>
</tr>
<tr>
<td>15 ± 1.3</td>
<td>130 ± 7.9</td>
</tr>
</tbody>
</table>
Figure 5-11. $K$ as a function of the diameter of the Au nanoparticles (Au component only) in Au-BSA conjugates. ATPS: 7.5 wt% PEG 8000 Da (lot 044K0092)/8 wt% dextran 10,000 Da (lot 084K1226). $T = 3^\circ$C. Each data point represents the average of at least 4 independent measurements.
Figure 5-12. Ln K as a function of the diameter of the Au nanoparticles (Au component only) in Au-HRP conjugates. All conjugates were of sub-nM concentration in the ATPS. ATPS: 4.125 wt% PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671). T = 3°C. Each data point represents the average of at least 4 independent measurements.
40. This not only illustrates that Au nanoparticle diameter can dramatically increase partitioning, but also demonstrates that conjugates can exhibit significant partitioning in ATPS comprised of high molecular weights of dextran. A qualitatively similar trend was observed for conjugates partitioned in a PEG 8000 Da/dextran 10,000 Da ATPS. Conjugates prepared from large diameters of colloidal Au were low investigated in ATPS comprised of low molecular weights of dextran because the absorbance in the dextran phase would be far below the detection limit of UV/Vis spectroscopy.

**Partitioning of protein-colloidal Au conjugates: Effect of polymer weight percent.**

Polymer weight percents were chosen along the 25°C binodal of an ATPS. This was done to render these results relevant towards future work within ATPS GVs. An increasing degree of conjugate partitioning was predicted with an increasing degree of polymer partitioning (1). This indeed turned out to be the case for HRP conjugated to 15-nm colloidal Au (Figure 5-13). An increasing degree of partitioning was observed in either direction from the critical point. However, the degree of partitioning was large in all cases.

**Activity of HRP and HRP-colloidal Au conjugates.**

A graph of HRP-Au (15-nm) nanoparticle conjugate absorbance against time gives an approximately linear plot, indicative of a first order reaction (Figure 5-14).
Figure 5-13. Conjugate partitioning as a function of PEG partitioning. ATPS: PEG 8000 Da (lot 044K0092)/dextran 10,000 Da (lot 084K1226)/5 mM pH 7.4 sodium phosphate buffer. T = 3°C. Each data point represents the average of at least 4 independent measurements. There is error in both the determination of K PEG 5000 Da and K Au-HRP conjugate. Each data point represents the average of at least 4 independent measurements in both axes.
Figure 5-14. Example of change in absorbance plotted against time of an o-phenylene diamine/hydrogen peroxide/buffer solution in response to added Au-HRP conjugates, indicative of activity. Increasing standard deviations with time are additive effects of different slopes for different trials. T = 25°C. Each data point represents the average of at least 4 independent measurements.
used a molar extinction coefficient of $8.1 \times 10^{-3} \text{ cm}^{-1} \text{ M}^{-1}$ \text{µM}^{-1}$ (Marcus Helfrich). There are large standard deviations in the plot are due to deviations from batch to batch of HRP colloidal Au conjugates, not within an individual trial. The ATPS does not denature HRP, but conjugation does, however, reduce HRP activity to approximately $25\% \pm 15\%$ (Table 5-4). This estimate of percent activity retention assumes approximately 73 HRP per nanoparticle as estimated from the flocculation assay (Figure 5-1, Figure 5-2) and the experimentally determined activity of HRP as a function of HRP concentration (Figure 5-15). Although the flocculation assay by itself cannot be used to determine how much HRP is on the nanoparticles (only how much HRP is in solution), as discussed above the flocculation assay appears to give a reasonable estimate of how much HRP is adsorbed onto the nanoparticles. Since FITC-conjugated HRP adsorption onto Au nanoparticles may be different from that of HRP, there does not appear to be an easy way to directly measure the amount of HRP adsorbed onto the colloidal Au. Since the change in absorbance due to OPD degradation is low over the course of an experiment, the error in this data induced by OPD degradation is low when the level of activity in the sample is not low as well.

**Adsorption to Au nanoparticles is a general method to increase protein partitioning in ATPS.**

A wide range of proteins besides HRP can be adsorbed onto Au nanoparticles (32). In addition to HRP, I have also adsorbed BSA and protein A onto Au nanoparticles.
Table 5-4. Activity of some typical HRP-colloidal Au conjugates. The concentration of HRP in and the percent activity retention of the conjugate were based on the concentration of colloidal Au with the assumption that there were approximately 73 HRP molecules per conjugate (which was estimated from flocculation assays). Each data point represents the average of at least 4 independent measurements.

<table>
<thead>
<tr>
<th>Concentration HRP in conjugate (nM)</th>
<th>Activity (µM/s) (% activity retention), 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0308</td>
<td>0.0943 (30)</td>
</tr>
<tr>
<td>0.0186</td>
<td>0.0703 (44)</td>
</tr>
<tr>
<td>0.0322</td>
<td>0.0447 (13)</td>
</tr>
<tr>
<td>0.0255</td>
<td>0.0284 (12)</td>
</tr>
</tbody>
</table>
Figure 5-15. Activity of HRP as a function of HRP concentration. T = 25°C. Each data point represents the average of at least 4 independent measurements.
The degree of partitioning of BSA and protein A is typically small depending on the ATPS (Table 5-5). The virtually nonexistent degree of partitioning of all proteins evaluated in the PEG 8000/dextran 500,000 ATPS is not due to poor polymer partitioning in this ATPS; PEG partitions in a 2:1 ratio and dextran partitions in a greater than 8:1 ratio, all comparable or better than partitioning in the PEG 8000/dextran 10,000 ATPS evaluated (Table 5-5). The partition coefficient of HRP, BSA, and protein A conjugates of 13-nm Au nanoparticles is given in Table 5-6. The partitioning of BSA and protein A conjugates of colloidal Au is to the dextran phase because these proteins partition to the dextran phase in their native state in low MW ATPS. All of the conjugates exhibited a large degree of partitioning: 60:1 to the PEG phase for HRP conjugates, greater than 550:1 to the dextran phase for BSA conjugates, and greater than 2200:1 to the dextran phase for protein A conjugates. This last value is particularly impressive, given that the ATPS evaluated is close to the 3°C binodal.

Comparison to other methods to increase partitioning of HRP.

To the best of my knowledge, the only reports regarding increasing the degree of partitioning of HRP are from the laboratory of Kula and coworkers (54,55). They bind HRP to an antibody for HRP, increasing HRP partitioning due to the dramatically increased size of the conjugate (54,55). They use this affinity partitioning technique to direct the antibody (and therefore HRP) to either the PEG phase (through conjugating PEG chains to the antibody) or the dextran phase (no modification). The degree of
Table 5-5. Partition coefficients of FITC-HRP, fluorescein-BSA, Alexa488-protein A, fluorescein-PEG 5000 Da-NHS, Alexa488-dextran 10,000 Da, and FITC-dextran 500,000 Da in several ATPS at 3°C. **ATPS 1:** 9 wt% PEG 4600 Da (lot 14316AC)/9 wt% dextran 10,000 Da (lot 084K1226)/5 mM pH 7.4 sodium phosphate buffer. **ATPS 2:** 7.5 wt% PEG 8000 Da (lot 044K0092)/8 wt% dextran 10,000 Da (lot 084K1226)/5 mM pH 7.4 sodium phosphate buffer. **ATPS 3:** 4.125 wt % PEG 8000 Da (lot 044K0092)/3 wt% dextran 500,000 Da (lot 123K0671)/5 mM pH 7.4 sodium phosphate buffer. Each data point represents the average of at least 4 independent measurements.

<table>
<thead>
<tr>
<th>ATPS #</th>
<th>Protein or polymer</th>
<th>K, 3°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HRP</td>
<td>0.76 ± 0.027</td>
</tr>
<tr>
<td>1</td>
<td>BSA</td>
<td>0.16 ± 0.0054</td>
</tr>
<tr>
<td>1</td>
<td>protein A</td>
<td>0.18 ± 0.013</td>
</tr>
<tr>
<td>2</td>
<td>HRP</td>
<td>0.72 ± 0.020</td>
</tr>
<tr>
<td>2</td>
<td>BSA</td>
<td>0.19 ± 0.0027</td>
</tr>
<tr>
<td>2</td>
<td>protein A</td>
<td>0.23 ± 0.0058</td>
</tr>
<tr>
<td>3</td>
<td>HRP</td>
<td>1.1 ± 0.024</td>
</tr>
<tr>
<td>3</td>
<td>BSA</td>
<td>0.95 ± 0.011</td>
</tr>
<tr>
<td>3</td>
<td>protein A</td>
<td>0.98 ± 0.014</td>
</tr>
<tr>
<td>2</td>
<td>PEG 5000</td>
<td>3.5 ± 0.091</td>
</tr>
<tr>
<td>2</td>
<td>dextran 10,000</td>
<td>0.31 ± 0.0036</td>
</tr>
<tr>
<td>3</td>
<td>PEG 5000</td>
<td>2.0 ± 0.043</td>
</tr>
<tr>
<td>3</td>
<td>dextran 500,000</td>
<td>0.12 ± 0.0014</td>
</tr>
</tbody>
</table>
Table 5-6. Partition coefficients of different protein-Au nanoparticle conjugates at 3°C. ATPS: 7.5 wt% PEG 8000 Da (lot 044K1226)/8 wt% dextran 10,000 Da (lot 084K1226)/5 mM pH 7.4 sodium phosphate buffer. The Au nanoparticle diameter was $13 \pm 1.0$ nm. Each data point represents the average of at least 4 independent measurements.

<table>
<thead>
<tr>
<th>protein</th>
<th>$K$, 3°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP</td>
<td>$60 \pm 6.3$</td>
</tr>
<tr>
<td>BSA</td>
<td>$1.8^{3} \pm 1.1 \times 10^{-5}$</td>
</tr>
<tr>
<td>protein A</td>
<td>$4.5 \times 10^{4} \pm 3.7 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
partitioning of HRP through this method was approximately 5:1 or less in all cases (55).
The best degree of partitioning shown herein is greater than 30 times this value (Table 5-1). Affinity partitioning is known to increase the degree of partitioning of proteins in an ATPS up to several orders of magnitude, but is highly dependent on the specific technique utilized as well as the protein in question (56).
Conclusions.

Most proteins do not partition well in isotonic ATPS GV's due to the inherent limitation of using an ATPS near the one-phase region of the phase diagram. Conjugation of HRP to approximately 15-nm colloidal Au can increase HRP partitioning to the PEG phase of a bulk ATPS by a factor of greater than 150 while retaining approximately 25% activity. Other protein-Au nanoparticle conjugates exhibited partitioning by a factor of greater than 2000:1. An increase in partitioning is observed regardless of the ATPS polymer molecular weight studied and occurs to a lesser extent when the ATPS polymer w/w% is on a shorter tie line. The diameter of the Au nanoparticles can dramatically increase the degree of partitioning. The concentration of conjugate also impacts partitioning in high MW ATPS, most dramatically in a PEG 8000 Da/dextran 500,000 Da ATPS in which the conjugates partitioned to different phases depending on the conjugate concentration.
Future Directions.

Manipulating enzymatic activity through proximity.

This thesis has demonstrated that aqueous phase separation within ATPS GVs can be rendered dynamic through small changes in temperature (4). It is also possible to induce an aqueous phase transition through changes in osmolarity (4). These results demonstrate the possibility of dynamic control over local protein concentrations within ATPS GVs. This may be of use in demonstrating on/off control over enzymatic activity.

This general concept is not without precedent. A large number of reports have been published documenting the effect of manipulating local sequential enzyme concentrations to alter the kinetics of the overall reaction (57,58). The goal is to apply such studies towards enzyme complexes in vivo (59) that typically dissociate when isolated and studied in vitro (60) and tend to be difficult to study (61). As one example, the rate of product (intermediate) transfer from yeast mitochondrial citrate synthase (CS) to yeast mitochondrial malate dehydrogenase (MDH) increases and the diffusion of the intermediate throughout the surrounding volume decreases when the enzymes are artificially bound within the same complex (60,62). Although indirect methods of determining that two enzymes may be spatially juxtaposed, such as coprecipitation in PEG (63,64), have been performed, it is difficult to argue that two enzymes are not in spatial proximity to one another when they are artificially bound to the same surface (60,62). Similar results are observed in enzyme channels that are easier to isolate as an intact structure such as tryptophan synthase (TS) (65,66). In this example, there is direct evidence of a physical channel connecting the enzyme subunits and an intermediate in the
reaction trapped within this channel (65,66). As suggested above, such direct evidence of
direct substrate transfer between enzymes or enzyme subunits is the exception rather than
the rule. Interestingly, volume exclusion may promote enzyme complex assembly and
stability (67,68) similarly to the volume exclusion-driven assembly of noncomplimentary
DNA-coated Au nanoparticle conjugates (69).

It should be noted that a physical channel or direct substrate transfer is not
required to manipulate enzymatic activity through local enzyme concentrations. A
cartoon illustrating the induction of dynamic channeling within an ATPS GV through
phase transitions is given in Figure 5-16. Two sequential enzymes will exhibit a faster
rate of product formation when they are in close proximity to one another due to a
decreased extent of substrate diffusion throughout the rest of the vesicle. This induced
proximity can be achieved through localizing them to the inner phase within an ATPS
GV. Since the inner phase can be induced to mix with the rest of the ATPS through a
phase transition, it should be possible to use aqueous phase transitions to effectively turn
the reaction on and off.

Another method to force two sequential enzymes to be in close proximity is to
bind them to the same nanoparticle (Figure 5-17) (70). These structures should exhibit
greater activity than the scenario in which the sequential enzymes are bound to separate
nanoparticles. Additionally, it may be possible to include self-complimentary DNA
strands within conjugates coated with one of the sequential enzymes to initiate on/off
control over enzymatic activity without utilizing ATPS GVs or aqueous phase transitions.
**Figure 5-16.** Cartoon of dynamic control over enzymatic proximity within an ATPS GV through aqueous phase transitions. The rate of product formation of the sequential enzymatic reaction will be greater when enzyme 1 (white spheres) is in close proximity to enzyme 2 (black spheres). This is the case in a phase-separated ATPS GV (left) when both enzymes are partitioned to the inner phase. The rate of product formation will be less when the enzymes are distributed throughout the vesicle volume. This is the case in a one-phase ATPS GV (right). Aqueous phase transitions therefore offer the possibility of on/off control over sequential enzymatic activity within an ATPS GV.
Figure 5-17. Cartoon of control over enzymatic proximity within a population of enzymes conjugated to colloidal Au. E1 and E2 are two sequential enzymes. If both E1 and E2 are bound to the same particle, the rate of product formation should be greater than if the enzymes are bound to separate particles.
Non-quenching nanoparticles for conjugate preparation.

A limitation of using colloidal Au as the scaffold for HRP is that Au nanoparticles quench fluorescence. This hinders quantification of partitioning within ATPS GVs and may inhibit the detection of fluorescent products from enzymes conjugated to the nanoparticles. These problems may be circumvented through the replacement of colloidal Au with colloidal silica. While bare colloidal silica is not stable at much less than pH 8 (71), possibly due to decreasing negative surface charge (71), the surface can be functionalized with aluminate to give it stability and a negative charge down to approximately pH 3 (71). Another potential option to minimize fluorescence quenching by Au in enzyme conjugates is to coat the nanoparticles with long or large spacers (72). Alternatively, quantum dots (73) could be used as inherently fluorescent nanoparticles, the stability of which has recently been enhanced through reversible encapsulation within chaperonin proteins (74). Polystyrene beads are also biocompatible; they have recently been bound to Chlamydomonas reinhardtii algae cells to demonstrate directed and reversible transport of cargo via phototaxis (75).
References.


36. Further proof that 80 µg of added HRP is well in excess of that required for 15-nm colloidal Au stabilization under the conditions described herein is that after this amount of HRP is adsorbed onto the nanoparticles, the conjugate can be centrifuged, pelleted, and fully resuspended back into solution. The nanoparticles would instead irreversibly aggregate if they were not fully covered by HRP.


40. Increasing the molecular weight of PEG within the ATPS aggregated the conjugates when the PEG molecular weight was 12,000 Da or greater.


44. This effect is not observed in ATPS of low dextran molecular weight.


53. A trivial example is that resuspending HRP-Au nanoparticle conjugates in DI water resulted in random partitioning of the conjugate, in many instances aggregation and precipitation into the dextran phase.


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Publications: