Molecular Gymnastics: Interplay between Segregative and Associative Liquid-Liquid Phase Separation

by

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To all members of the Parikh Lab, past, present, and future,

as well as

Siddharth Deshpande and Chang Chen.

Abstract

The interior of a cell is highly crowded with different macromolecules. In order for these macromolecules to interact in meaningful ways they need to be organized into compartments. Some are confined inside membrane-bound organelles, while others form membraneless organelles (MLOs) through liquid-liquid phase separation (LLPS). Compartments formed through LLPS arise either as densely packed coacervates or segregated aqueous two-phase systems (ATPSs). While LLPS and ATPSs have been studied for decades, coacervates and MLOs in cells are a relatively new field of study and few have studied how coacervates act within an ATPS. This thesis aims to combine these two fundamentally antagonistic forms of LLPS, with the hypothesis that it will elucidate methods for molecular distribution within cells.

This study combined an ATPS of PEG and dextran with a coacervating system of PLL and ATP. Their interplay was studied using cell-free models, primarily water-in-oil microfluidic droplets. Observations were collected using optical and fluorescence microscopy. The location of coacervates and their individual components were strongly influenced by the ATPS, with both PLL and ATP/PLL coacervates partitioning into the dextran-rich phase. It was further found that dextran was sequestered inside of the coacervates, potentially leading to the ATPS outside the coacervates to mix. Further testing in artificial vesicles may lead to pathways for cycles of confinement and dissolution as well a method for artificial cell transport.

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Chapter 1

Introduction

1.1 Informal Overview

1.1.1 Compartments in life

Life requires compartments. From organs to cells to organelles, living things need organization to maintain homeostasis[1–5]. On the cellular level, the need for organization is largely driven by the high variety and total volume of macromolecules. The cell's cytosol is crowded with a concentration of macromolecules up to 400 mg/mL[6,7], varying between cell types. Macromolecules take up not only physical space, but additional volume around them is excluded from due to interactions between unalike molecules[6,8]. Compartments allow for order within these dense seas of macromolecules. These hierarchical levels of organization confine processes, maintain local concentrations and environmental conditions, and regulate transport on multiple levels[6,9–11].

When considering compartments within a cell, first thoughts often go to membrane-bound organelles. The membranes defining these organelles prevent unregulated interactions between their internal environment and the surrounding cytoplasum[7]. Levels of organization can be achieved by encapsulating smaller vesicals[7]. However, a large volume of macromolecules still exists within the cytoplasm. This aqueous phase is not directly controlled by membranes, yet still must have some form of order. This is where membraneless organelles come in.

Membraneless organelles (MLO) appear in the cell as condensates. These organelles can be densely packed with molecules, more so than the surrounding cytoplasum[6], allowing the surrounding aqueous phase to be less crowded. The formation, function, and properties of these condensates in cells is a relatively new area of study[12], but many have been found to form through liquid-liquid phase separation (LLSP)[6–8] and exhibit liquid-like characteristics[10]. Their lack of a membrane results in constant physical and chemical interactions with their environment[2,7,8]. MLOs also interact with different molecules in different ways, preferentially including some molecules in the phase while excluding others[5–7,10]. Closer packing of specific molecules within MLOs forces molecular interactions[8], affecting both structure and processing rates[7], largely shifting towards enhanced function[6].

1.1.2 LLPS

LLPS occurs when an aqueous system demixes from one homogenous phase into two partially miscible phases[13,14]. This process can be broadly categorized as either associative or segregative[7]. In an associative system, the demixed state consists of a dilute phase and a dense phase[7,10,13]. The dense phase, commonly referred to as a coacervate[15], is enriched in molecules from the system. In a segregative system, the system forms two aqueous phases, each rich in separate molecules. This type of LLPS system is commonly referred to as an aqueous two-phase

system (ATPS)[6,7]. While the driving forces behind these two forms of LLPS may differ, they both result in phases with distinct compositions.

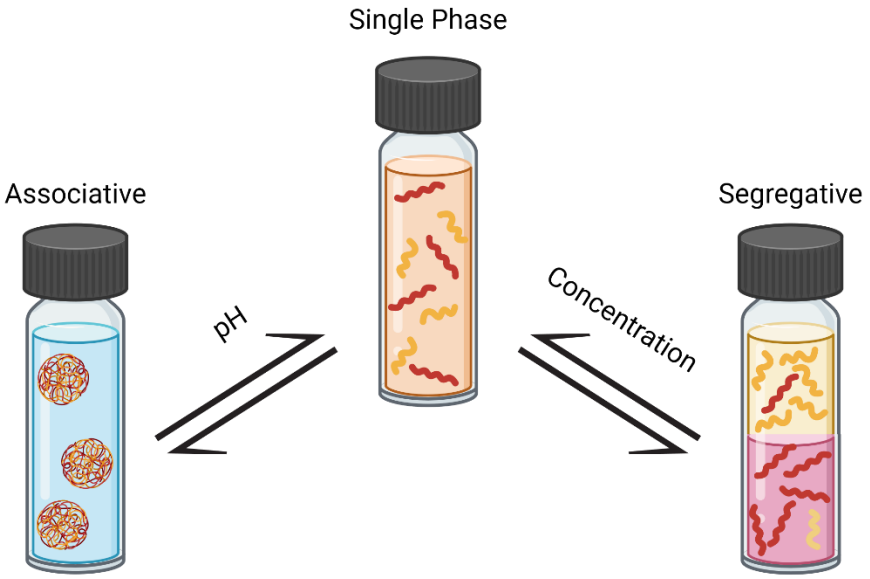

Figure 1 Diagram of associative and segregative LLPS. Created with BioRender.com

The formation and dissolution of coacervates in a system is predominantly driven by pH changes in the environment[2,5,16–18], although it can be affected by other factors such as temperature[19], concentration, and enzymes[10]. The molecules in this system carry charge, which can become unfavorable in some environments. To combat this, oppositely charged molecules group together to form a condensed, coacervate phase. It's also possible for a single molecule to form a coacervate on its own, referred to as a "simple coacervate"[10]. In this paper however, it can be assumed that all coacervates mentioned are multi-component complex coacervates unless otherwise stated.

ATPSs are primarily controlled by changes in concentration, but are also affected by other environmental factors, such as tempurature[7]. Due to entropically unfavorable interactions between unalike molecules, they will partition into phases, each rich in only one of the aqueous

components[20,21]. Added components in the system can also partition between the two phases, including molecules, nano particles, and vesicles/micelles[7]. When confined within a liposome, ATPSs can cause the lipid membrane to phase separate[22,23].

While the structure of these coacervates can be affected by their environment[2], they're most often studied while in their liquid-like phase as it better mimics MLOs. They can also interact with other molecules in their environment, allowing some to pass through while others are preferentially taken up by the coacervates or excluded[6,10,13,18,20,24,25]. While not identical to MLOs in living cells, coacervates are a manageable substitute in synthetic biology studies[10].

1.1.3 Droplet Microfluidics

Microfluidics is a relatively new field of study, emerging in the latter half of the 20th century[26,27]. The sub-field of droplet microfluidics arose in the early 2000's with a focus on droplets formed from immiscible or partially immiscible fluids[28,29]. These droplets can be produced in either a series of glass capillaries[19,30] or patterned microfluidic chips[7,27–29]. These chips are commonly made of either etched glass[31,32] or polydimethylsiloxane (PDMS) formed via soft lithography[5,16,33]. The droplets formed through microfluidics are highly monodisperse[7,28,29], increasing control and consistency in experiments.

The size of the droplets is adjusted through flow rates[27], and different combinations of fluids lead to different structures. The simplest form of droplet consists of two fluids forming a single emulsion[28], such as water-in-oil (w/o) or water-in-water (w/w)[32]. The controlled addition of additional fluids allows for the creation of multiple emulsions[29]. Flowing two partially immiscible fluids, such as an ATPS, into an oil phase creates a more complex version of W/O

droplets[5,19,30,31]. By carrying lipids in an octanol phase, Deshpande et. al. created artificial liposomes through double emulsion[33].

1.2 Summary of Main Results

In this project, an ATPS was combined with a coacervating system to create a four-component system. This system was tested using a combination of bulk mixtures, water in oil droplets, and artificial liposomes. Our results show that PLL preferentially partitions into the dextran-rich phase of a PEG and dextran ATPS, while ATP partitions relatively equally. ATP/PLL coacervates follow the partitioning of PLL and prefer to sit in the dextran-rich phase. Using only labeled dextran, we are able to see fluorescence accumulate in the coacervate phase, showing that dextran is taken up by the coacervates. This movement of dextran from the bulk phase to the coacervates reduces the concentrations of the ATPS below the binodal, leading to mixing.

Chapter 2

Two Component LLPS

2.1 Aqueous Two-Phase system

Our ATPS was comprised of polyethylene glycol (PEG) (8 kDa) and dextran (9-11 kDa) purchased from Sigma Aldrich. Stock solutions were prepared of PEG and dextran (560 mg/mL and 400 mg/mL respectively) in milli-Q water. 5.6 g of PEG or 4 g of dextran were weighed out and added to a 10 mL volumetric flask. A starting amount of milli-q water, typically ~ 6 mL, was added to the flask and its top was sealed off with parafilm. The PEG or dextran was then dissolved into the water using a combination of vortex and water bath sonication. Once the powder was fully dissolved, additional water was added to the flask to reach the 10 mL mark. The solution was then mixed again before being transferred to a plastic centrifuge tube and stored at 4 °C.

PEG and dextran are commonly used individually as inert crowders[6], and together as an ATPS[22,23,34]. Peg specifically has been used to study biologically relevant systems since 1955[7,35]. The transition between the one-phase and two-phase state of an ATPS can be tracked on a binodal curve[14,31]. Because these polymers can vary from batch to batch, the exact binodal curve must be calculated from scratch for each batch of PEG and dextran. In order to create a binodal curve for our PEG and dextran, we used a variation of cloud-point titration[22,30,35–37]. While typical cloud-point titration methods measure the amount of solution added at each step by weight, ours was measured by volume to allow for mg/mL concentrations instead of the typical

weight percent. A known volume of stock solution for one of the molecules was added to a small glass vial. A small known volume of the second solution was then added and the mixture was stirred. This process was repeated until the resulting mixture reached its cloud-point and became turbid. Milli-Q water was then added in small volume increments to the mixture, followed by mixing, until the mixture became clear again. Alternating volumes of the second molecule's stock and milli-Q water were titrated into the mixture, crossing above and below the cloud-point, until the cloud point could not be reached or a significantly large volume of the second molecule was required. The process was then repeated, switching which molecule is used at the start and which is being titrated into the mixture. The concentration of each molecule was calculated for each crossing over point and graphed to create the binodal curve [fig. 2].

Binodal curves were also made to test how adding other components would affect the ATPS. The first addition was fluorescently labeled dextran (Alexa-fluor 488). The color of the label made it difficult to determine if the system was cloudy or clear at higher concentrations of dextran, but preliminary data showed no change in the binodal. The next set of data added pH buffers [fig. 2, B] that would become necessary in later experiments involving coacervates. The same mM concentration of tris-HCL pH buffer was added to both stock solutions and the water used to dilute the system, ensuring the concentration of buffer remained constant throughout the process. The process was done at pH 3.85, 7.37, and 9.67. Neither the addition of a buffer nor the change in pH resulted in a shift. Lastly, a binodal curve was made with 15 vol% glycerol [fig. 2, C], a necessary addition for GUV production in microfluidics. The glycerol was added to all components just as the pH buffer had been, remaining constant at all steps in the process. The addition of glycerol caused phase separation to happen slower, which may have had some effect

on the equilibrium points recorded due to human error. The slight shift in the binodal was determined to be within experimental error.

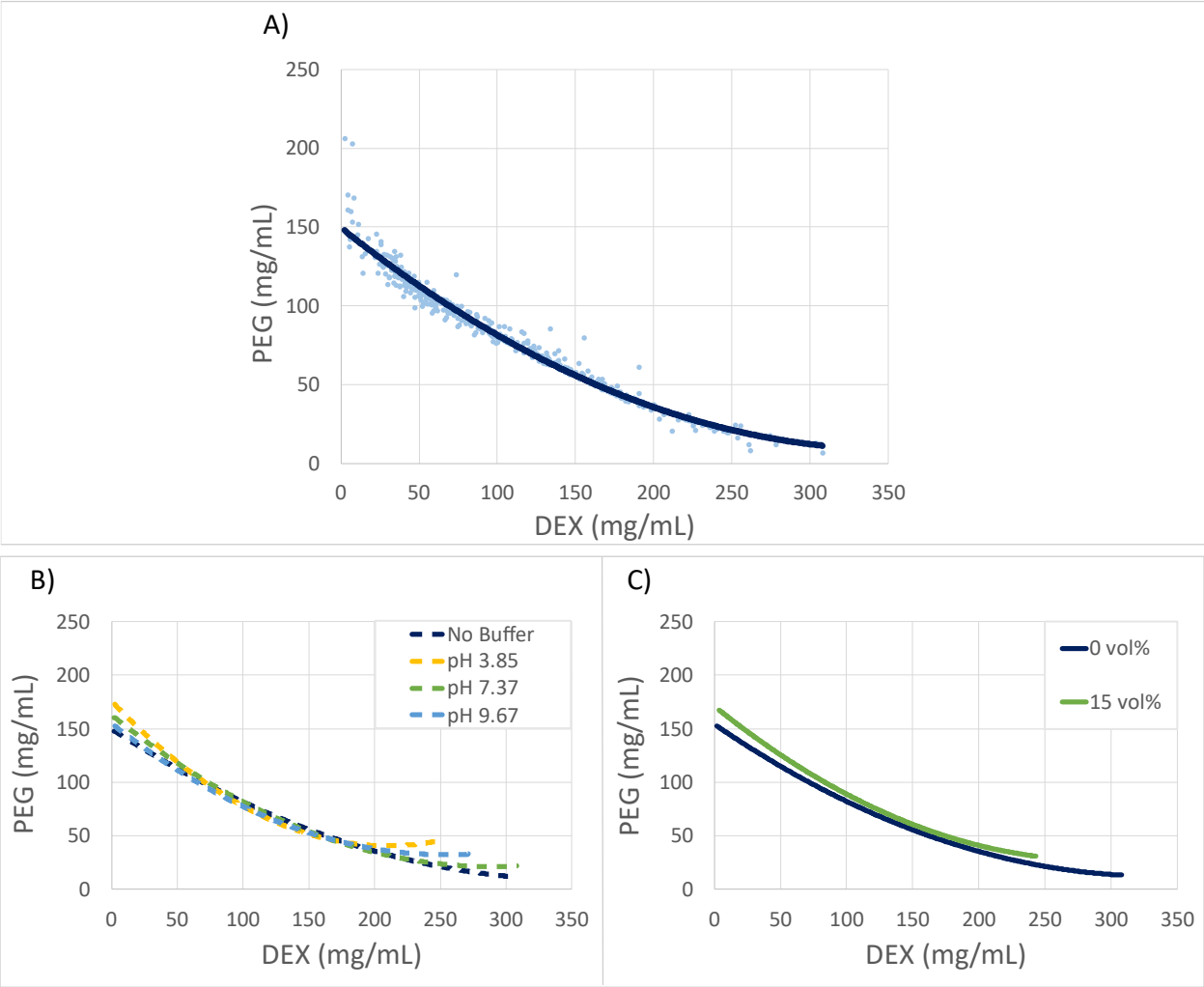

Figure 2. *Binodal curves for PEG and dextran made using a volume-based cloud-point titration method.* ***A)*** *Binodal curve for PEG and dextran in milli-Q water. Light blue points are cloud-point measurements, and the bark blue line shows the trend for the data.* ***B)*** *Comparison of trend lines for cloud points in buffers of varying pH, no significant differences.* ***C)*** *Comparison of binodal curves for 15 vol% glycerol vs no glycerol. Slight deviation is within experimental error.*

2.2 Coacervating System

Our coacervating system consisted of ATP and poly-L-lysine (PLL) (15-30 kDa), both purchased from Sigma Aldrich. This system has been used to form coacervates by others and information on how it coacervates was based on their work, specifically pH values[16,17].

Experiments were performed with the coacervates to see how they would react to different crowding agents. To do this, coacervates were formed in a glass bottom well plate using ATP, PLL, and FITC-PLL. A solution containing either PEG or dextran was then added to the well and the solution was observed over time. The addition of both crowding agents increased the amount of coacervation, however there were some differences in how the coacervates responded. In dextran, the coacervate phases stayed smaller, and tended to sit near the bottom [fig. 3, A-B]. When formed in PEG, the coacervates were more likely to coalesce into larger phases and were dispersed throughout the solution [fig. 3, C-D]. Others have also observed crowders increasing coacervation rates as well as affecting the size of resulting phases[38,39]. These results suggest Peg and dextran may interact with ATP/PLL coacervates beyond inert crowding.

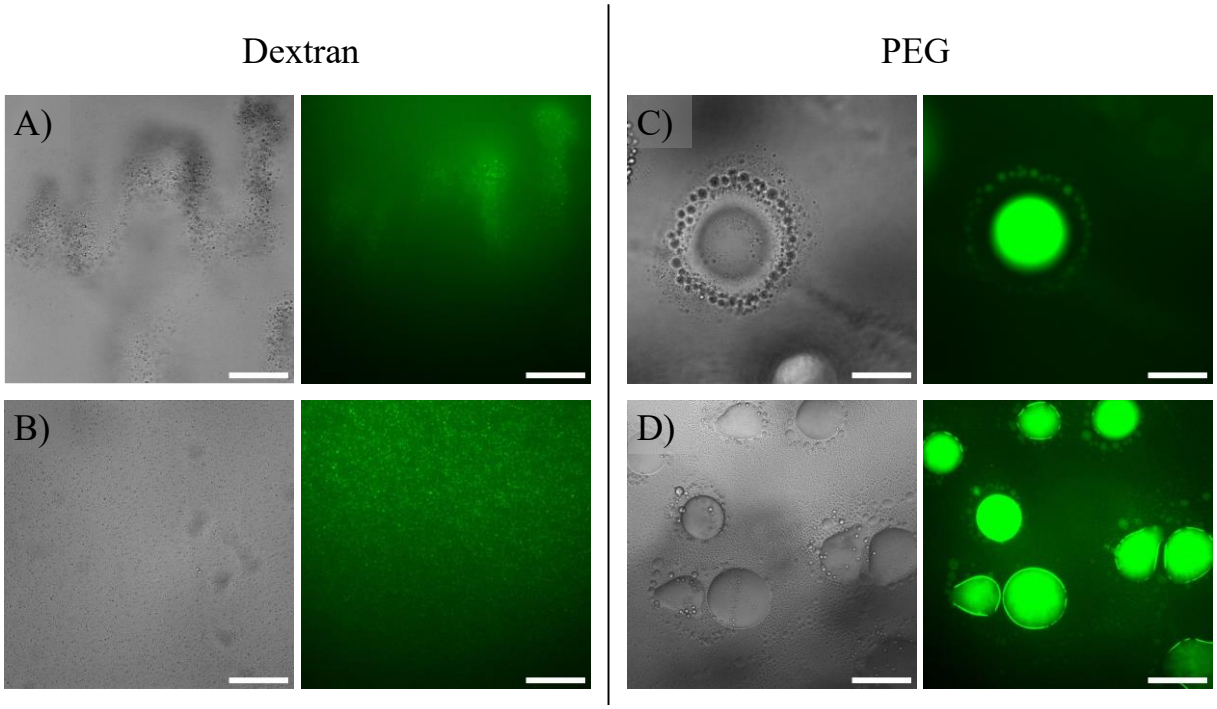

Figure 3*. ATP/PLL coacervates were made in a glass bottom well plate with fluorescently labeled PLL. A crowding agent was added to the well, increasing the amount of coacervation, but with different affects on growth.* ***A-B)*** *Dextran was added to 100 mg/mL. The coacervate phases stayed small and hardly coalesced.* ***C-D)*** *PEG was added to 124 mg/mL. The coacervates coalesced into larger phases, often surrounded by a ring of smaller phases.* ***B and D*** *show the bottom of the well plate while* ***A and D*** *are higher up, suspended in the solution. Grey images are bright field, while green are artificially colored fluorescent images, showing the location of FITC-PLL. All scale bars are 100µm.*

The dissolution of coacervates in different solutions was also explored. This was done by mixing PLL, ATP, and a crowder together in a glass bottom well plate at pH 7 and letting it sit for ~10 minutes for coacervates to form. A pH 2 buffer was then added, lowering the solutions pH to 3.7, below the range for coacervation[17]. A control was also done with coacervates formed in the absence of a crowder. When the pH was lowered in the control, the coacervate phase dispersed in seconds [fig. 4], matching what's been reported by others[2,16]. A second control was done where an equal volume of pH 7 buffer was added instead of pH 2. Very little change was seen, confirming the dissolution was due to the lowered pH and not the change in volume.

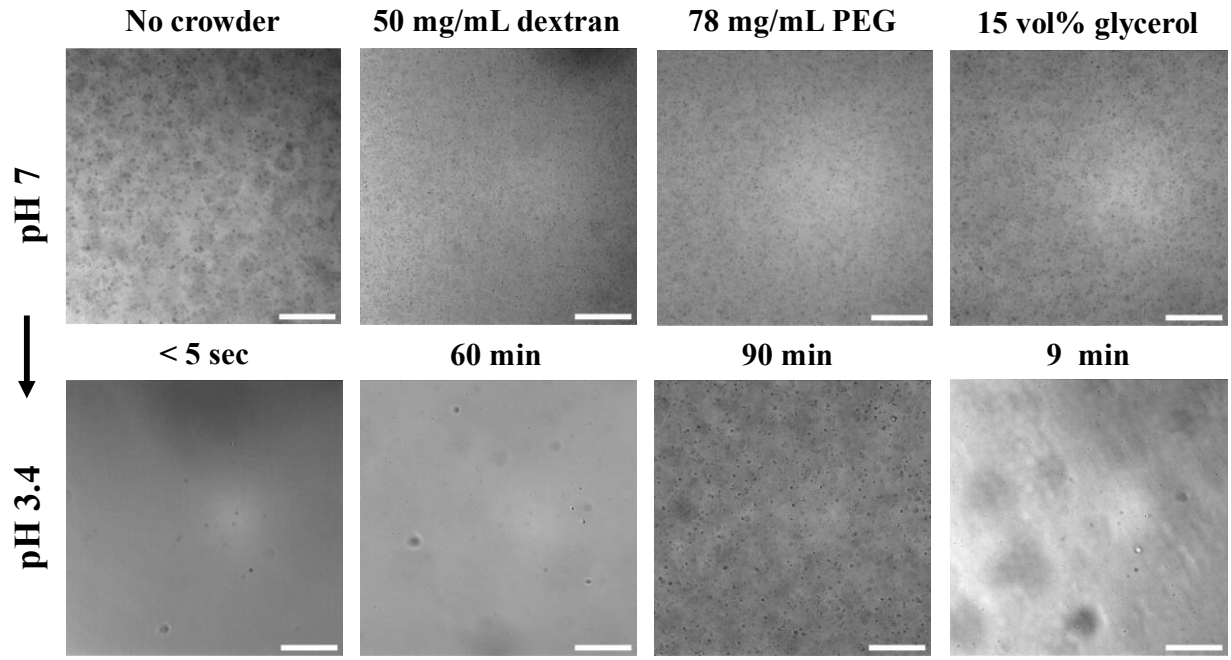

Figure 4. *ATP/PLL coacervates before (top) and after (bottom) having their pH lowered outside of the conditions needed for coacervation. In just DI, the phases broke up instantly. When coacervates formed with a crowder (Dextran, PEG, or glycerol), the phases took longer to disperse. Higher concentrations of crowder increased the time for the phases to disperse. At high enough concentrations of PEG and dextran, the coacervate phases remained for over an hour without noticeable change. External mixing with a pipette caused the coacervates at lowered pH to disperse instantly, regardless of crowder concentration.*

When this same process was repeated on coacervates formed in a PEG or dextran, the dissolution took much longer. While the effect was small in lower concentrations, as more crowder was added the solution took longer and longer to become clear. At the highest

concentrations of PEG or dextran tested, the solution remained unchanged for over an hour. Glycerol was also used as a way to compare how viscosity alone affected this process. While glycerol did slow the process, the stability was not as long lasting as the high concentrations of PEG or dextran [fig. 4]. In every case, mixing the solution with a pipette after the pH was lowered would immediately disperse the coacervates.

This is still an ongoing point of research, but these preliminary results suggest that PEG and dextran have the ability to stabilize the coacervate phase, either temporarily or long term, and that this stabilization extends beyond that of inert crowding. While studies have been done to stabilize LLPS phases, the focus tend to be on preventing coalescence[7,40], rather than remaining stable in an unfavorable pH. These phases can be stabilized by a membrane or coating, made with a large variety of structures, including amphiphiles, macromolecules, and nanoparticles[20,41,42]. No papers were found to use PEG, dextran, or similar crowders to stabilize coacervates, but they may form similar membranes. Alternatively, the crowding agent may be further condensing the coacervate phase such that the diffusion of ions between the coacervate and solution is slowed, slowing the change in charge within the coacervate and therefore preventing/ slowing their dissolution.

Chapter 3

Partitioning and Sequestering

3.1 Partitioning of single components in ATPS

It's known than molecules can partition within an ATPSs[7,23,30], so our exploration into combining these systems began with bulk tests to see if and how PLL and ATP partition between PEG and dextran. For these experiments, vials were mixed with 152 mg/mL PEG and 11.7 mg/mL dextran. One component of the coacervating solution was then added, either 7.8 mM ATP or 2.2 mg/mL PLL (10:1 weight ratio of PLL to FITC-PLL). Labeled ATP was not used initially as it's much more costly. I control vial was also made with just PEG and dextran. The vials were vortexed to mix and left in the fridge overnight to equilibrate. The separated vials were then held against a white background to observe the phases [fig. 5].

In the control vial [fig. 5, A] a clear division between the two phases can be seen due to light diffraction. Because the dextran used had a higher molecular weight than the PEG, we concluded that the bottom phase was dextran-rich. In the vial with PLL, the majority of the bright yellow FITC-PLL was concetrated within the bottom of the vial [fig. 5, A]. We concluded

that the PLL had mixed with the dextran-rich phase and not made a new third phase as there was still only one diffraction layer within the vial.

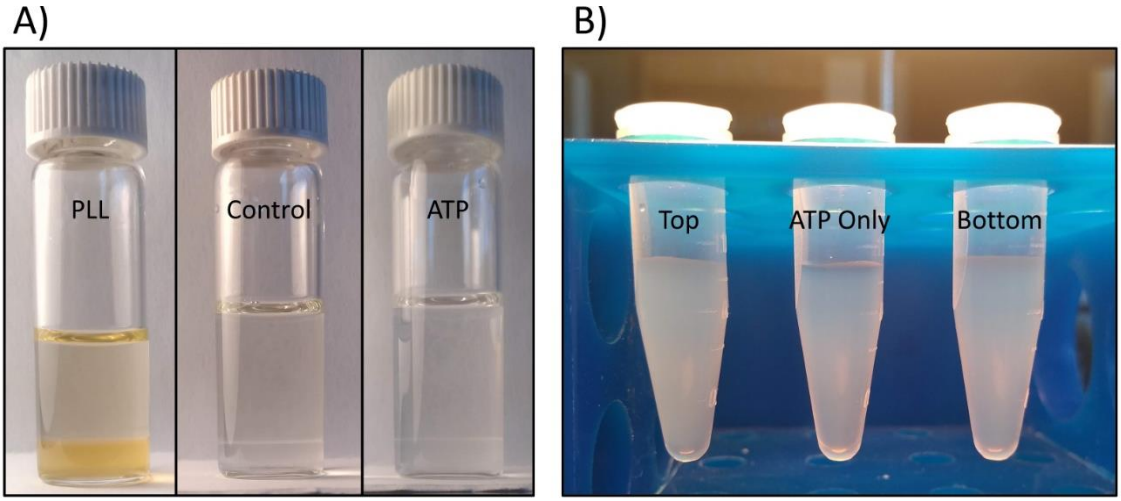

Figure 5. A) *Vials showing the partitioning of PLL (left) and ATP (right) in an ATPS. The center control contains only PEG and dextran at the same concentrations. The divide between PEG and dextran can be see due to light diffraction, and the bottom phase was determined to be dextran based on its higher molecular weight. The FITC used to label the PLL allows its location to be seen, while the clear ATP gives no information.* ***B)*** *Vials of coacervates formed by adding PLL to solutions allocated from the top PEG-rich (left) and bottom DEX-rich (right) phases in the ATP vial, as well as a control with only ATP and no crowder. No clear differences were observed.*

In bulk, the ATP solution was not visable to the unaided eye [fig. 5, A]. The seperated solution appeared identical to the control vial, indicating that the ATP had not formed a third phase and was mixed into at least one of the two phases. An attempt was made to measure partitioning of ATP between these two phases by forming coacervates. The idea behind this was that if the ATP had partitioned as strongly as the PLL there would be a clear difference in the amount of coacervates formed in each vial. Vials were prepared with an allocated volume from either the top PEG-rich phase or bottom dextran-rich phase as well as a third control vial containing a solution of ATP without PEG or dextran. An equal volume of PLL solution was then added to each vial, forming coacervates [fig. 5, B]. While the control vial was slightly less cloudy due to the lack of a crowding agent, the other two vials had no noticable difference so no preferance could be determined.

These preliminary bulk experiments were followed up with the more quantitative approach of measuring the partitioning of fluorescent ATP and PLL within the ATPS. Nine different solutions of PEG and dextran were mixed with varying concentrations. Peg was varied between 218, 163, and 108 mg/mL and dextran was varied between 176, 117, and 58 mg/mL. All nine of these concentrations resulted in a two-phase mixture above the binodal, with the exception of the lowest which sat very close to the binodal line. The third component, either 2.5 mg/mL PLL (10:1 wt PLL:FITC-PLL) or 0.8 mM ATP (1000:1 mM ATP: Cy3-ATP) , was added in equal amounts to each PEG and dextran solution. These values were chosen to reflect later experiments done in GUVs. The solutions were then mixed and left in the fridge overnight to

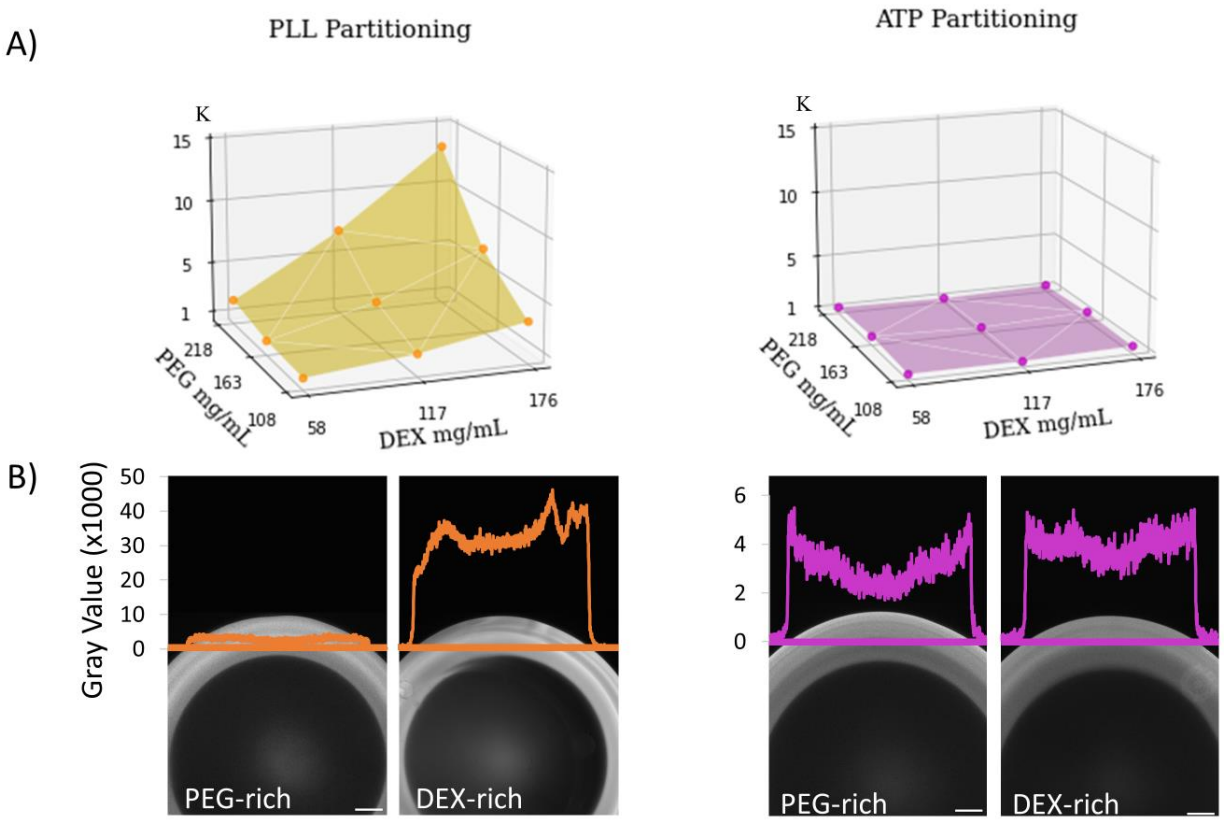

Figure 6. A) *Partitioning coefficient (K) is calculated as the fluorescent intensity of FITC-PLL (left) or Cy3-ATP (right) in the dextran-rich phase over that of the PEG-rich phase and graphed as the Z axis, with the varying PEG and dextran concentrations as X and Y.* ***B)*** *Examples of the droplets used to calculate K, from samples with the highest concentrations of PEG and dextran. Fluorescent intensity was collected from along a line and graphed. The line and graph are overlayed on the images to show the difference in fluorescence between the PEG- and dextran-rich domains. A bright ring can be seen due to the wetting angle. PLL has a clear pattern of partitioning into the dextran-rich phase while ATP remains close to K = 1, indicating no preference. Scale bars 100 μm.*

settle. After they had separated, 5 µL was taken from either the top PEG-rich or bottom dextran-rich phase of the solution and placed onto a covered hydrophobic glass slide to form a droplet. The droplet was then quickly imaged around all sides using fluorescent microscopy. The high wetting angle resulting from the hydrophobic slide slowed evaporation but also resulted in a ring of higher intensity at the edge of the droplets [fig. 6, B]. To keep the data consistent, all images were taken on the plane of contact between the droplet the slide. This process was repeated with 3 droplets each from the top and bottom of each vial at consistent microscope conditions and on three separate occasions.

In ImageJ, the bright ring was isolated from the rest of the droplet using the clear function and the threshold was adjusted so only the ring would be measured and the mean intensity was recorded. The intensity was normalizing to the background value, collected before isolating the ring. A partitioning coefficient (K) was calculated by dividing the intensity of the dextran-rich phase by that of the PEG-rich phase [fig. 6]. The partitioning of PLL aligned with the bulk experiments, with PLL strongly preferring the dextran-rich phase. As the concentrations of PEG or dextran were moved farther from the binodal, the partitioning of PLL became stronger. ATP had no clear trend or significant partitioning, confirming that ATP has no preference for either phase.

3.2 Partitioning of coacervates in ATPS

The next step was to find how ATP/PLL coacervates partitioned within the PEG/dextran ATPS. We hypothesized that because PLL preferred dextran and ATP had no preferences, the coacervates would also prefer the dextran-rich phase. This hypothesis was confirmed through various experiments. Within vesicles, if the ATPS was triggered first, the coacervates would form

in the dextran-rich phase. If coacervation was triggered first, the coacervates partitioned into the forming dextran-rich phase. Similar results were seen by allowing a droplet with coacervates and homogenous PEG and dextran to evaporate. In w/o droplets where PEG and dextran are phase separated the coacervates once again form preferentially in the dextran-rich phase [fig. 7].

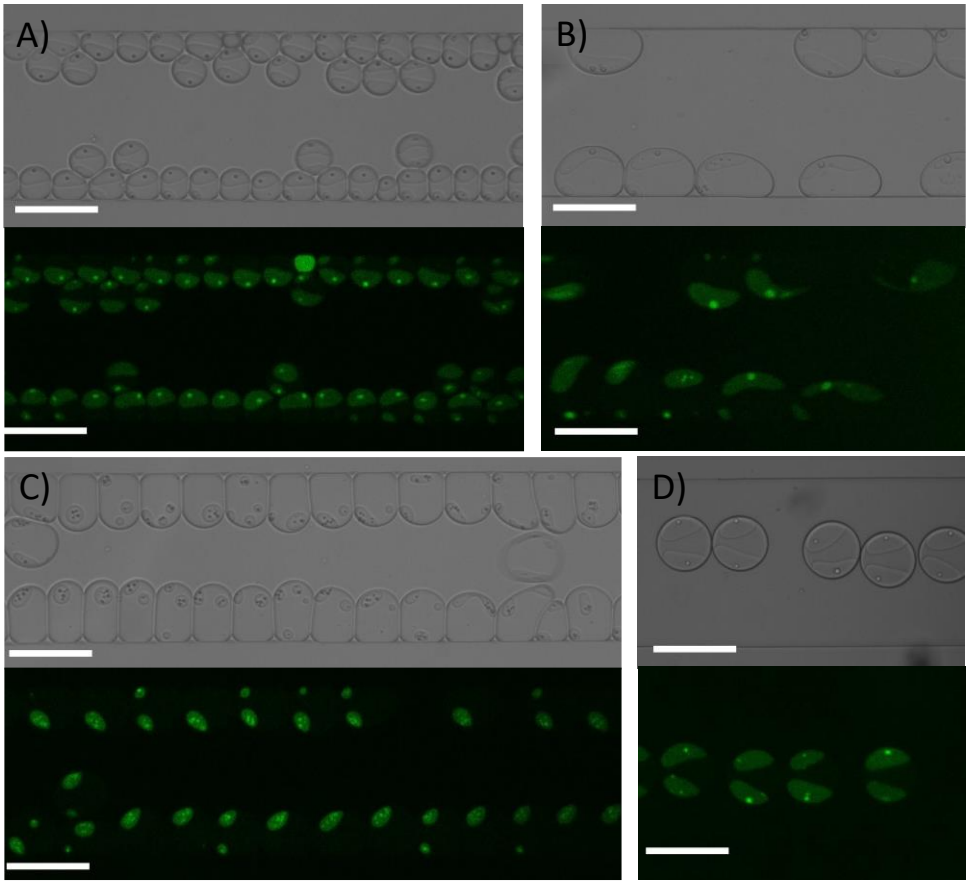

Figure 7. *Images of droplets containing PLL, ATP, PEG, and labeled dextran. Top half of each image is brightfield, bottom half is fluorescent images taken shortly after. The condensed coacervate phases (seen as small dots in the droplet) partition into the fluorescently labeled dextran-rich phase. Scale bars are 100 µm.*

Knowing that the coacervates and PLL both partition into the same phase, the idea arose that coacervation could be controlled through the partition of PLL. While concentration is not the main driving factor behind coacervation, it still affects the amount of coacervates and the size of the coalesced phases[43]. A hypothesis was formed that in a system with an excess of ATP but a

very small concentration of PLL, the presence of an ATPS would lead to a local increase in PLL concentration, inducing or increasing coacervation.

This hypothesis was tested using w/o droplets. Droplets were made with a small concentration of PLL (0.25 – 0.4 mg/mL) and excess of ATP (5 mM) suspended in either an ATPS with PEG (5 mM) *and* dextran (5 mM) [fig. 8, A] or a control of PEG (10 mM) *or* dextran (10 mM) [fig. 8, B]. The addition of one component of the ATPS to the control was to ensure that the difference between the two set ups was the localization of PLL, not the presence of a crowding agent.

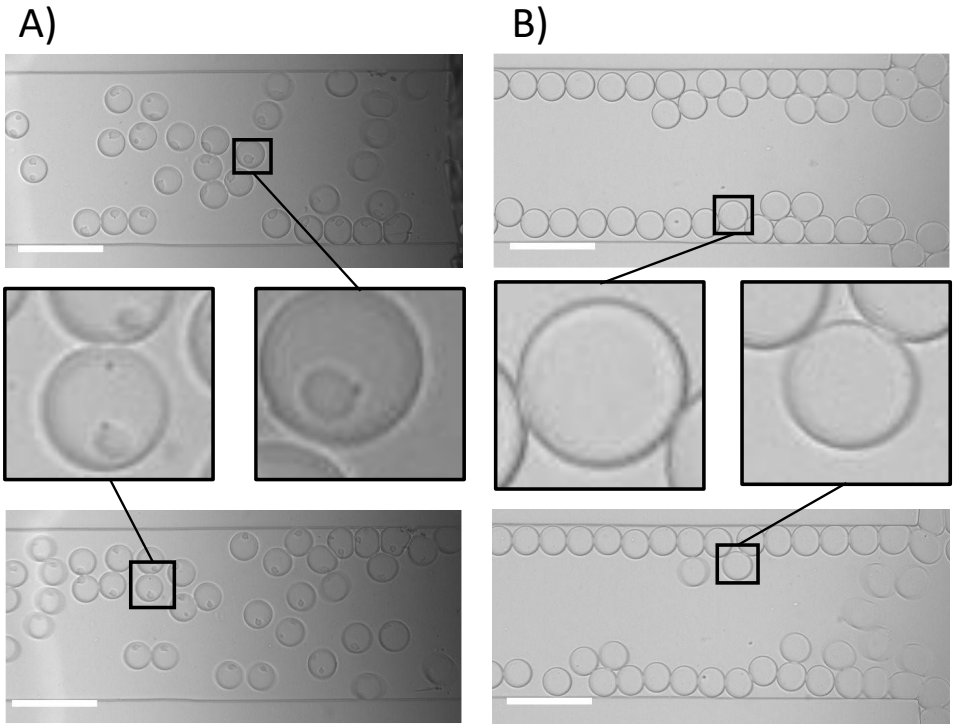

Figure 8. *Images of droplets made under the same conditions in terms of flow rates and concentrations of PLL, ATP, crowder, and pH buffer. The concentration of PLL in each droplet is very small while ATP is in excess.* ***A)*** *Droplets were made with both PEG and dextran such that it forms an ATPS. Small coacervates can be seen at the edge of the dextran-rich phase in almost every droplet.* ***B)*** *Droplets were made with only Peg as a crowder instead of an APTS. Only a handful of droplets had a coacervate. All scall bars are 100 µm.*

The experiments confirmed our hypothesis. At the lowest concentrations of PLL used (0.25 mg/mL), there was no visible coacervation in the control and small coacervates visible with

the ATPS. At slightly higher concentrations of PLL some coacervates could be seen in the single-phase droplets, but the two-phase droplets had more consistent coacervation and larger coacervate phases. These results were consisted regardless of which crowder was used in the control.

3.3 Sequestering of dextran in coacervates

While studying our system we noticed an interesting trend. Not only was the fluorescent label of PLL largely overlapping that of dextran, fluorescently labeled dextran was accumulating at the coacervates. To ensure this wasn't simply signal bleeding over from the PLL or ATP label, coacervating solutions were made where only dextran was labeled. Even with no fluorescent markers on the coacervates' components, they still had a strong fluorescent signal [fig. 9]. It's known that coacervates can sequester molecules from the system[10,13,18,20,24,25], and we hypothesized that this was the case with dextran and our coacervates.

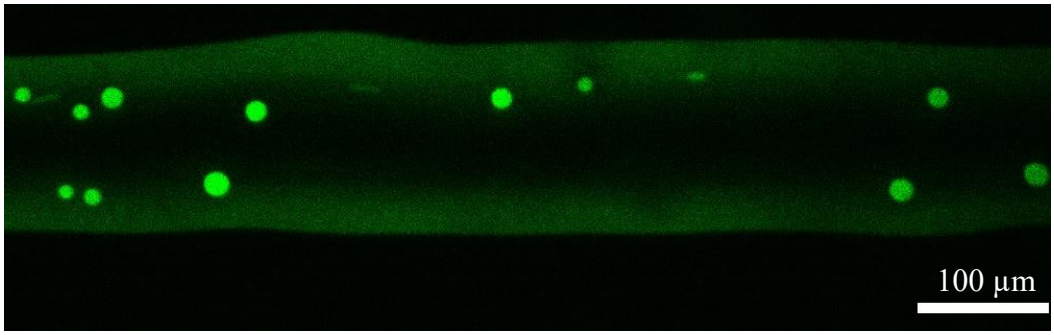

Figure 9. *Fluorescent image of PEG, dextran, PLL, and ATP mixing in a microfluidic channel. The aqueous flow is bordered by oil, but the pressure was lowered so droplets would not be pinched off. Channel is imaging Alexa Fluor 488 labeled dextran. PLL and ATP are both unlabeled, but the coacervate phases can still clearly be seen. Image is artificially colored green.*

Fluorecent images only confirmed the sequestering of labeled dextran, so testing was needed to confirm if unlabeled dextran was also sequestered. This was done by looking for changes in the ATPS binodal, as the sequestering of dextran into coacervates would lower the

concentration in the bulk below the binodal. Cloud point titration and bulk experimentation were attempted but did not work as the coacervates are cloudy and block the eye from seeing if PEG and dextran have formed one phase or two. Instead, tests were done with w/o droplets to compare droplets with an ATPS with and without the addition of coacervates.

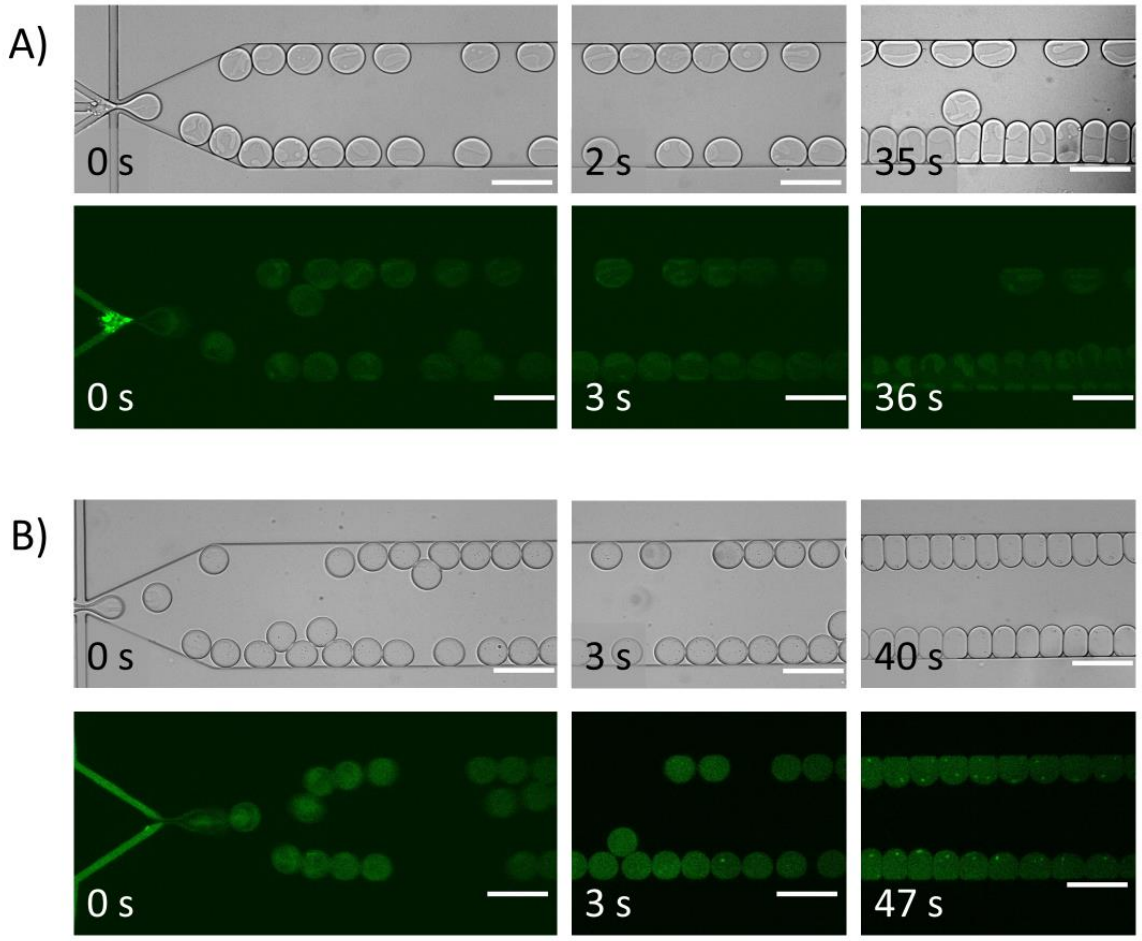

Figure 10. *W/O droplets containing* ***A)*** *PEG and labeled dextran or* ***B)*** *PEG, labeled dextran, PLL, and ATP. All droplets have the same amount of PEG and dextran based on initial solutions and flow rates. In the droplets with PLL/ATP coacervates, PEG and dextran quickly mix while the droplets without coacervates maintain an 2-phase system. Fluorescent images artificially colored green. All scale bars 100 µm.*

As a point of reference, droplets were made containing PEG and dextran with ratios near the binodal. These were then compared to droplets with the same concentrations of PEG and dextran but now ATP and PLL were added. All droplets stabilized as either two distinct phases, one homogenous phase, or sometimes a mix of the two with some forming a streaky in between

phase indicating concentrations on the binodal. The addition of ATP/PLL coacervates slowed stabilization slightly. By slightly adjusting the flow rate of solution into the chip, the concentration can be changed slowly. This was done for both set ups, adjusting the PEG channel to see at what point the system shift from 2 phases to a transition phase, and then to 1 phase. Comparisons afterwards found that for certain flow ratios, the PEG and dextran would mix in the presence of coacervates but form a 2-phase system on their own [fig. 8]. This suggests that the presence of the coacervates shifted the binodal curve and aligns with the hypothesis that dextran is being sequestered inside the coacervates.

Seeing this done in a vesical could further cement this conclusion, as the ATPS can be stabilized before coacervates are formed, removing any errors that could arise by comparing two separate experiments. We don't yet have an explanation as to why dextran is sequestered inside of the coacervates. We've also yet to test if PEG is accumulated, excluded, or unaffected by ATP/PLL coacervates.

Chapter 4

System in Confinement

4.1 Microfluidic set up

The w/o droplets and vesicles used to study this system were made using microfluidics. The pattern for these microfluidic chips, as well as the silicon wafers with said pattern [fig. 11], were provided by our collaborators at WUR[17,33]. The polydimethylsiloxane (PDMS) chips used in our microfluidics system are made using soft lithography. To do this, the elastomer components are combined in a tube and centrifuged to remove bubbles. A square of tinfoil is wrapped around the wafer to create a cup shape [fig. 11] and 15 grams of PDMS elastomer is poured onto the wafer. It's then placed in a desiccator to remove any bubbles before baking for at least 3 hours at 70 °C. The PDMS can then be removed from the wafer [fig. 11] and the chips are cut from the pattern. Glass slides are also spin coated with PDMS elastomer and baked. The cut PDMS and coated chips are then cleaned with plasma and nitrogen before being stuck together and placed back in the oven for 1-2 hours. In order for the chips to make vehicles, the outermost

channels need to be treated with PVA to become hydrophilic. This and must be just after the bonded chips have been baked. This step is not needed for w/o droplets.

Figure 11. *Images of PDMS microfluidic chip making process. Shows the silicon wafer, tinfoil wrap, removal of PDMS from wafer, and the finished PDMS chip on bonded to a slide.*

Once the chips have been bonded and optionally coated, tape is placed over the top to keep the input and exit holes clear of dust [fig. 11]. After 3 days the PDMS has finished curing and the chips are ready to be used. To use the chips, tubes are placed in the inlet holes to connect the chip to vials of solutions. The solutions are pushed through the tubes by air pressure, supplied by a microfluidic pressure controller from ElveFlow. By changing the air pressure, the flow rate of the solutions within the chip can be regulated. This set up is also used for PVA coating the chips. The set up for what solutions go into which inlet depends on the end product goal.

4.2 Water-in-Oil droplets

For the w/o droplets, the aqueous phase is suspended in a mixture of HFE 7500 oil[5,31] and 2 w/w% FluoSurf C surfactant purchaced premixed from Darwin Microfuidics. This oil phase is flowed through the outermost channels, while the inner two carry the aqeous phase(s) [fig. 12]. By dividing components of LLPS systems into two different inflow channels that meet at the junction, phase seperation before the droplet forms can be created. This allows the ATPS to have relativly consisten ratios in each droplet while still remaining at concentrations above the

binodal[31]. It also prevents the formation of coacervates through the chip, which can clog the channels.

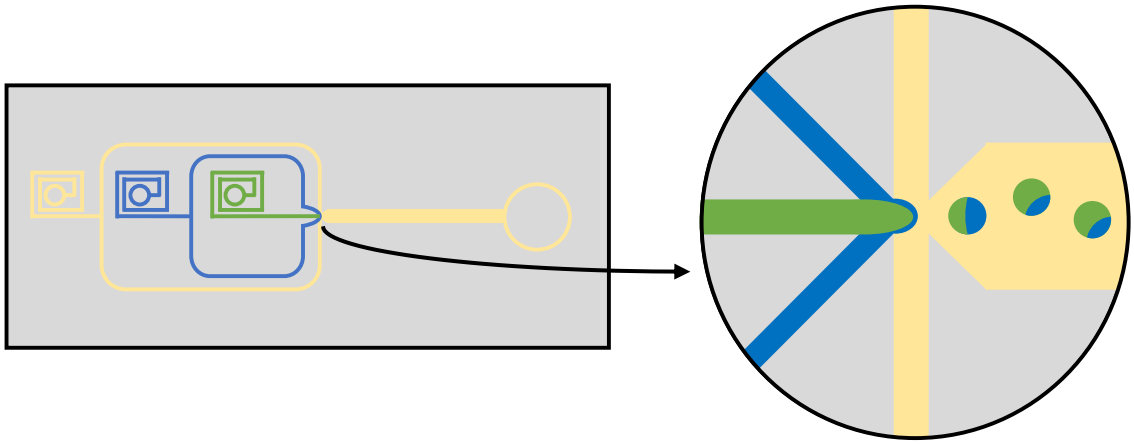

Figure 12. *Schematic of chip for making water in oil droplets. Circled area shows structure at the junction. The yellow channels contain oil and surfactant. The blue and green channels are carrying aqueous solutions that mix at the junction.*

Once droplets are being produced, their size and production rate can be controlled through changes in flow rate. Flow rate can also be used to adjust the ratio between the two aqeous solutions. This allows for quick comparisons between slightly different ratios of aqueos components in stepped increments. This method is fairly simple and allows for quick data collection. However, changing anything about the components once they've been encapsulated is difficult. The resulting interactions between components is driven by their reaction rates and the order cannot be adjusted as all start when the channels meet. In order to change the encapsulated solutions more easily the system needs to be confined in a membrane.

4.3 Octanol Assisted Liposomes

There are multiple ways to assemble liposomes, but microfluidics has proven to be effective for monodisperse production. The process involved creating a double emulsion of water in oil in water, with lipids carried in the oil phase. This is done by flowing the outer aqueous bath solution through the outermost channel, lipids in octanol through the middle channel, and the

inner aqueous solution through the inner most channel [fig. 13]. When all solutions have reached the junction, a layer of lipid carrying octanol is created between the outer aqueous and inner aqueous phases, facilitated by the pattern of PVA coating done previously. The flow of the inner aqueous pushes the lipid layer outward which is then pinched off by the outer aqueous[33], similar to a bubble being blown.

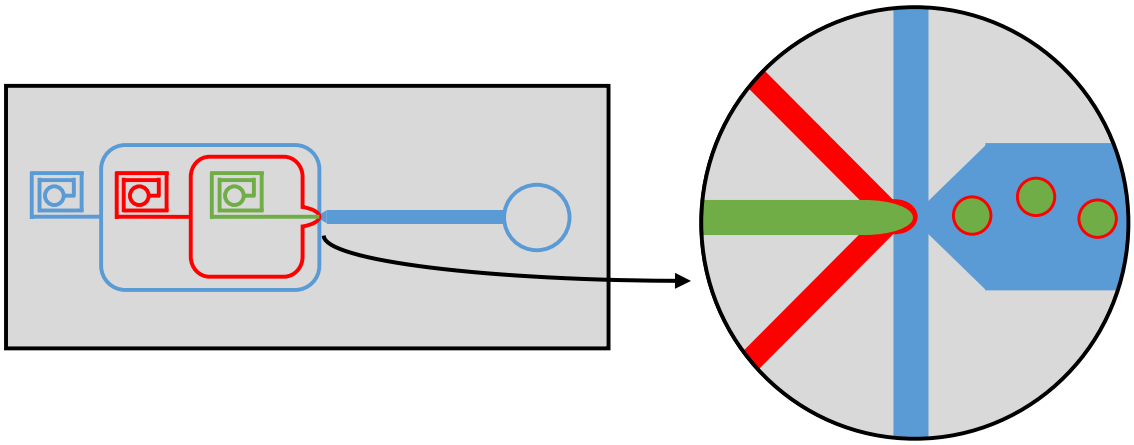

***Figure 13**. Schematic of chip for making liposomes through double emulsion. Circled area shows structure at the junction. Blue channel is the outer aqueous solution. Red channel is lipids in octanol. Green channel is the inner aqueous solution.*

The initial emulsion will have an excess of lipids and octanol, which form an "octanol pocket" that dislocates from the liposome as it flows down the channel with the help of glycerol added to the aqueos solutions. Once in the exit chamber, the lower density droplets of excess octanol and lipid will float to the top while the liposomes sink, allowing them to be removed for testing and imaging.

The use of liposomes allows for water to be moved across the membrane [22]. Additionally, it has been shown that coacervation can be induced in vesicles made using this technique by changing the buffer in the bath[16,17]. These two factors allow the initial inner aqueous solution to be below the concentration and pH ranges needed for phase separation, encapsulating consistent concentrations, and preventing clogging. These changes can also be reversed, and their order can

be altered for more information on how each LLPS system interacts with the other. The membrane also makes collection easier, allowing the vesicles to be moved to a well plate for study. This set up facilitates more complex experiments than W/O droplets but is much more difficult to learn and master. The membrane also adds an additional layer of complexity to experiments. As such, many experiments could be done more easily with w/o droplets, but liposomes will be needed to study the system more complexly.

Chapter 5

Discussion of Future Work

5.1 pH triggered cycle

From our findings, there arose the possibility of a four-component polymer system confined within a lipid membrane wherein, triggered by pH changes, the internal components cycle between a homogenous single phase and a hierarchical multiphase system [fig. 14].

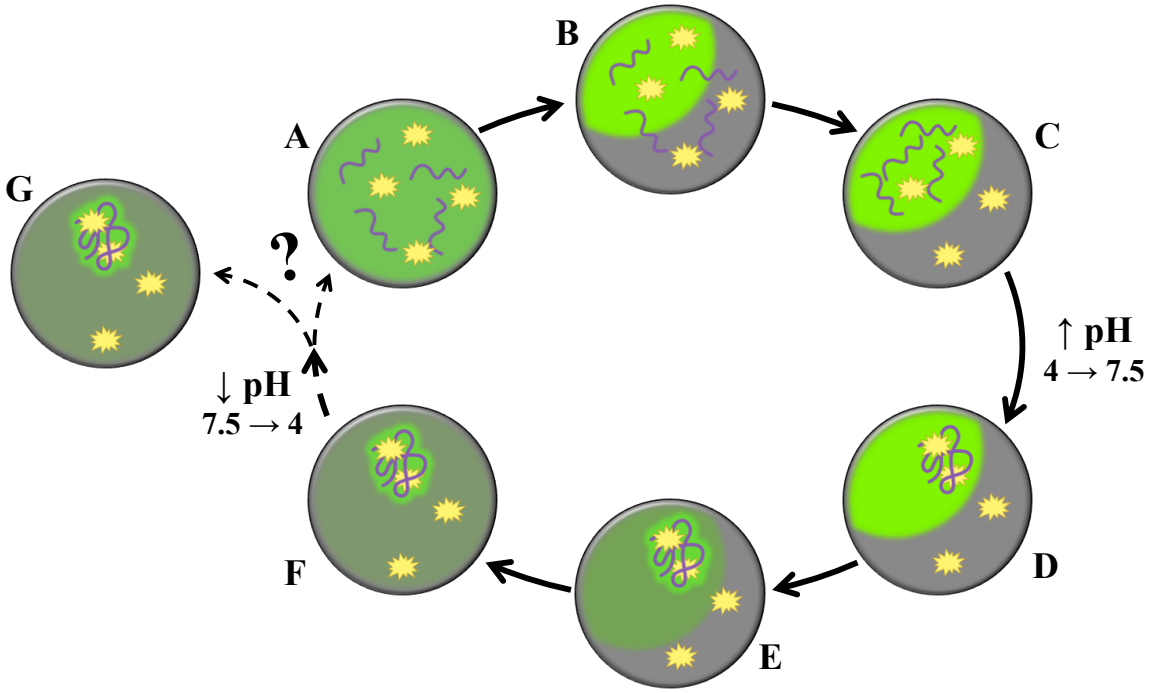

Figure 14. *Diagram of hypothesis for LLPS cycle. In **A**, the initial state consists of PEG (gray), dextran (green), PLL (purple) and ATP (yellow) in one homogenous phase. From **A to B**, PEG and dextran undergo concentration driven phase separation. From **B to C**, PLL partitions into the dextran-rich phase. From **C to D**, pH increases allowing PLL and ATP to coacervate. From **D to E**, dextran is sequestered inside the coacervate phase. **E to F**, the concentration of dextran in the bulk drops below the binodal, leading to mixing. Future work is needed to know how lowering the pH after state F will affect the system. **F to A**, the coacervate phase breaks up, releasing dextran, and returning to the initial state. **G** shows a second option, where the coacervate phase is stabilized by the ATPS, even in unfavorable conditions.*

The set up begins with a GUV that contains dextran, PEG, PLL, and ATP [fig. 14, A]. The concentrations and pH are such that all 4 components are in one, homogenous phase. For our purposes, this would need to be the initial state due to how our GUVs are produced. Having one phase allows for each GUV to have a known concentration. Additionally, coacervates stick to the microfluidic system, so the GUV must initially have a pH of 4 or less.

By adding a hypertonic solution to the bath, the concentrations of PEG and dextran within the GUV cross over the binodal and phase separate [fig. 14, B]. Alternatively, the temperature could be changed to transition the ATPS from one phase to two. This step is required only to initiate the cycle, because of the initial conditions required to make our GUVs.

As the dextran-rich phase forms, the PLL will partition along with it [fig. 14, C]. By increasing the pH of the bath, coacervation is triggered inside of the liposome[16,17] [fig. 14, D]. The coacervate phase will sit within the dextran-rich phase both because that's where it partitions to and because the majority of the PLL had already partitioned to that phase. As the coacervate phase forms, it will begin to sequester dextran from the bulk [fig. 14, E]. When enough dextran has been moved into the coacervate phase, it's concentration in the bulk will drop below the binodal, causing the bulk phase to mix into one-phase [fig. 14, F].

Future work is still needed to confirm what will happen to the system when the pH is then decreased outside of the range for coacervation. One hypothesis is that the coacervate phase will be broken up and dispersed back into the system. This is what's been observed by others with GUVs containing PLL/ATP coacervates without PEG or dextran[2,16]. If this is the case, when the coacervate is dispersed back into the bulk, it will also release the dextran back into the system[7] [fig. 14, A]. The release of dextran will also lead to the bulk concentration crossing back

over the binodal, leading to phase separation [fig. 14, B-C]. This cycle can then continue so long as the pH is switched back and forth.

However, preliminary testing of bulk coacervates with crowding agents has shown that pH triggered dispersion of the coacervate phases is slowed and potentially even prevented by high enough concentrations of PEG and dextran. The phase is only dispersed by gentle mixing with a pipette tip. It's not yet known how these bulk experiments will translate to GUVs. If the presence of PEG and dextran prevents the coacervate phase from breaking up, then a full cycle may not be possible or may require additional intervention. However, this would still be a significant finding, showing the coacervate phase can be stabilized for some amount of time by PEG or dextran, allowing it to remain in an environment that shouldn't allow for coacervation to occur [fig. 14, G].

5.2 Vesical Polarity and Asymmetric Division

Asymmetric cell division occurs when the components of the mother are distributed unequally to the daughters, resulting in cells with new compositions and concentrations. This process is crucial in living cells and multicellular organisms, as it allows cells to differentiate[23,24].

The processes of asymmetric cell division in model cells using an APTS has already been studied by Andes-Koback and Keating[23]. Their work showed that by osmotically deflating a vesical containing an ATPS of PEG and dextran, the dextran-rich phase will partition into a bud separate from the PEG-rich phase [fig. 15, B – A]. The ATPS within the vesical also lead to phase separation in the lipid membrane[22]. By continuing to remove water from the vesical, Andes-Koback and Keating were able to break the bud off from the mother, resulting in a

daughter that had a different lipid composition and a higher concentration of dextran than the mother[34] [fig. 15, C - D].

By adding a coacervate to the mother vesical, another level of complexity can be added to this system. The daughter will not only have a higher concentration of dextran but also remove the coacervate phase from the mother, as well as a substantial amount of PLL [fig. 15, D].

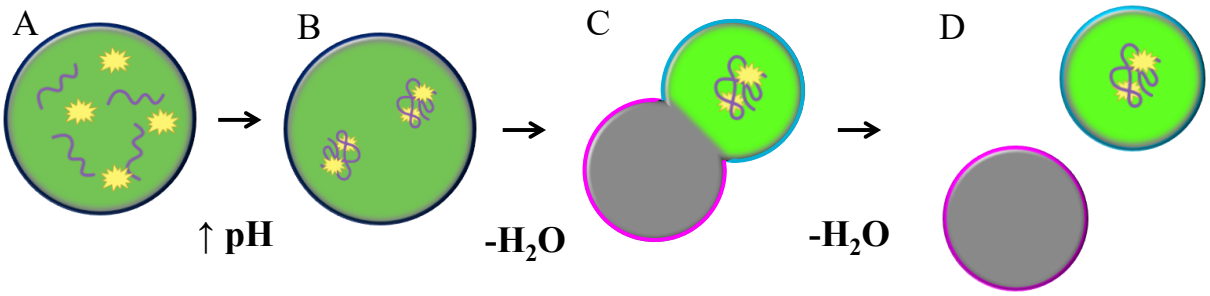

Figure 15. *Scheme of asymmetric division in model GUV. Idea adapted from Andes-Koback and Keating [ref. 36] with the addition of ATP/PLL coacervates.* ***A)*** *GUV with homogenous mixture of ATP, PLL, PEG, and dextran.* ***B)*** *pH is increased to induce coacervation.* ***C)*** *Water is drawn out of the GUV, leading to membrane deformation. ATPS separates into different buds, leading to phase separation in the membrane. PLL and coacervates partition into dextran-rich phase (green).* ***D)*** *Continued osmotic pressure results in asymmetric fission.*

Coacervates are known to sequester various proteins and molecules within the cell[10,13,18,20,24,25]. By adding a coacervate to Andes-Koback and Keating's model for asymmetric division, proteins could be asymmetrically confined to a subset of daughter vesicles.

5.3 Artificial Vesical Transport

As an extension of asymmetric division, if the resulting daughter vesical were merged with a new vesical, the cargo can be transported from the mother to the new vesical. When applied to the system outlined in this thesis, a daughter vesical could be created via asymmetric osmotically driven vesical division [fig. 16]. That daughter would contain a high concentration of dextran, PLL, and PLL/ATP coacervate. After introducing an "empty" GUV to the daughter, the two vesicles can be merged[44], transferring the contents of the daughter into the newly formed vesical [fig. 16, A – D]. Furthermore, if the coacervate phase is carrying additional cargo, that

cargo can be released into the new vesical by lowering the pH[16,17] out of range for coacervation [fig. 16, E].

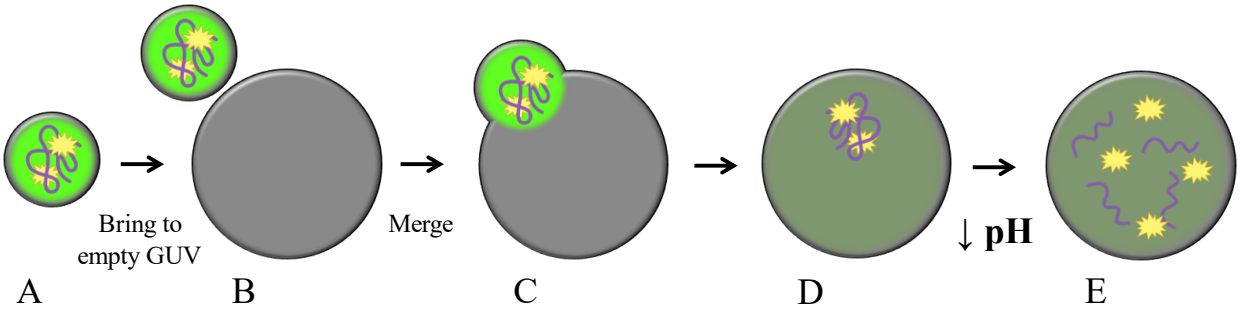

Figure 16. *Scheme for artificial vesical transport.* ***A)*** *Initial GUV is rich in dextran and carries a coacervate.* ***B)*** *A second GUV that does not contain any molecules of interest.* ***C)*** *The two GUVs are merged.* ***D)*** *The dextran and coacervate from the initial GUV are now contained in the new merged GUV.* ***E)*** *pH is lowered, releasing the coacervate's components into the new GUV.*

This form of osmotically induced transport of a coacervate phase between vesicles opens new opportunities in artificial cell research. The addition of a carried protein inside of the coacervate which could be released later on leads to the possibility of this system being used for drug delivery[2].

5.4 Closing Remarks

The goal of these experiments was to add to the fast-growing study of LLPS in artificial liposomes. This work shows that combing segregative and associative LLPS leads to higher levels of organization within the system. Combining these results with the work of others elucidates the possibilities available with only a simplified model of a cell. Further study of these simplified systems allows us to better understand the development of protocells, as well as simplified versions of what goes on in our cells. The study of LLPS in cell like environments will lead to advances in cell therapies and drug delivery systems.

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