UNDERSTANDING INTRACELLULAR ORGANIZATION USING
AQUEOUS PHASE MODEL SYSTEMS

A Dissertation in
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by
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ABSTRACT

The intracellular milieu is a heterogeneous environment composed of many different organelles and compartments that perform specific functions simultaneously in spatially confined areas. This environment is incredibly complex, which can make studying enzyme reactions inside cells challenging. The aqueous phase model systems described here aim to provide insight as to the mechanisms used by cells to control enzyme activity, compartment formation, and compartmentalization of biomolecules. Chapter 1 provides a general background of intracellular organization in biological cells, followed by a discussion of fundamental principles governing biomolecule activity within a nonideal solution. The types of aqueous phase separation that appear in later chapters are discussed, as well as a review of related aqueous phase-separated model systems.

Chapter 2 describes complex coacervation of a long poly(uridylic acid) RNA (poly U, a polyanion) and short cationic peptide (RRASLRRASL) that undergo reversible phase separation in response to the phosphorylation state (and therefore charge state) of the peptide. Phosphorylation was controlled enzymatically by a kinase and phosphatase pair. This demonstrates that phosphorylation could be a viable means to control compartment formation and dissolution in vivo. Additionally, the poly U/RRASLRRASL coacervates were capable of concentrating solutes up to 1150×. In Chapter 3, sequential enzyme activity is studied in a polyethylene glycol (PEG)/sodium citrate biphasic system. The enzymes glucose oxidase (GOX) and horseradish peroxidase (HRP) and substrate Amplex Red partitioned to opposite phases and required mass transport across an interface for product formation. A mathematical model was developed to describe the
complex kinetics in the system, and the model was validated when it accurately described other experimental conditions. Chapter 4 looks at the reaction of HRP with two different substrates that differed in their relative hydrophobicity. Catalysis by HRP was highly dependent on solution composition because attractive interactions between the more hydrophobic substrate and more hydrophobic crowders and cosolutes (different molecular weights of PEG) caused a larger decrease in enzyme activity. This was confirmed by diffusion nuclear magnetic resonance (NMR) measurements. This is significant because it is the first systematic study of substrate-crowder interactions within the macromolecular crowding community. In Chapter 5, enzymes and substrates of the purine biosynthesis pathway were partitioned to the dextran-rich phase of a PEG/dextran biphasic system. The mathematical model developed in Chapter 3 was used in this phase system to describe the kinetics and mass transport under conditions that were difficult to study experimentally. The results suggest that significant advantages of enzyme colocalization may only be observed if essentially all of the enzyme is restricted to compartments, rather than only being weakly partitioned. Finally, Chapter 6 offers some general conclusions from this work, as well as some future outlook on using aqueous phase systems as model cells.
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<th>Definition</th>
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<tbody>
<tr>
<td>10-/THF</td>
<td>10-formyltetrahydrofolic acid</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>ASL</td>
<td>adenylosuccinate lyase</td>
</tr>
<tr>
<td>ATIC</td>
<td>AICAR transformylase/IMP cyclohydrolase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPS</td>
<td>aqueous two-phase system</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CCC</td>
<td>critical coacervate concentration</td>
</tr>
<tr>
<td>D</td>
<td>diffusion coefficient</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>heavy water</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAP</td>
<td>2,3-diaminophenazine</td>
</tr>
<tr>
<td>dex</td>
<td>dextran</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOSY</td>
<td>diffusion ordered spectroscopy</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FAICAR</td>
<td>5-formamidoimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>GOX</td>
<td>glucose oxidase</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine monophosphate</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
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<tr>
<td>K</td>
<td>partitioning coefficient</td>
</tr>
<tr>
<td>$k_{\text{cat}}$</td>
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<tr>
<td>$K_i$</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>$K_M$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>$\lambda$-PP</td>
<td>lambda protein phosphatase</td>
</tr>
<tr>
<td>log D</td>
<td>logarithmic distribution coefficient</td>
</tr>
<tr>
<td>LSCM</td>
<td>laser scanning confocal inverted microscope</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cutoff</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel(II)-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>ODE</td>
<td>ordinary differential equation</td>
</tr>
</tbody>
</table>
OPD  $o$-phenylenediamine

PDDA  poly(diallyldimethylammonium chloride)

PDE  partial differential equation

PEG  poly(ethylene glycol)

PFG-NMR  pulsed field gradient – nuclear magnetic resonance

pI  isoelectric point

$pK_a$  logarithmic acid dissociation constant

PKA  protein kinase A

PML  promyelocytic leukemia bodies

poly A  poly(adenylic acid)

poly N  poly([random base] ribonucleic acid)

poly U  poly(uridylic acid)

RNA  ribonucleic acid

s  seconds

SAICAR  5-aminoimidazole-4-(N-succinocarboxamide) ribonucleotide

TAMRA  5-carboxytetramethylrhodamine

THF  tetrahydrofolic acid

TMAO  trimethylamine N-oxide

TMB  3,3’,5,5’ tetramethylbenzidine

U  units (enzyme activity)

$V_{\text{max}}$  maximum reaction velocity
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For my parents
Chapter 1

Introduction


The author of this dissertation wrote sections 1.1, 1.3-1.5, 1.8, and portions of 1.2, 1.6 and 1.7. Bradley Davis wrote the remaining portions of 1.2, 1.6 and 1.7.

1.1 Biological Cell Complexity and Motivation for Cellular Mimics

Biological cells are incredibly complex, yet highly organized. Reactions are confined in subcellular compartments, which provide a physical location segregated from the rest of the cell. Some compartments are membrane-bound, while others are not. These compartments are composed of a myriad of molecules, which typically include proteins and nucleic acids. Enzymes must operate in this complex environment, and their activity is controlled by such means as spatial localization to certain compartments, post-translation modifications, or associations with other enzymes/proteins. While no single macromolecule is present at a high concentration, the collective concentration of all biomolecules creates an environment that contains ~30% by weight macromolecules. In *vitro*, solutions of one or more polymers at sufficient concentration can result in the formation of aqueous phases, which provides coexisting environments with different physical properties and local concentrations of molecules. In fact, recent work has suggested that some biological compartments may be liquid in nature. It is of interest to
create aqueous phase model systems in order to study the physicochemical aspects of the cell. Additionally, it is advantageous to mimic cellular processes on a simplified level in order to have precise control over specific experimental variables. The subject of this dissertation is the use of aqueous phase model systems for the purpose of studying phase separation, enzyme activity, and compartmentalization of biomolecules.

### 1.2 Cellular Compartmentalization

Some cellular compartments are membrane bound, one of the most recognized being the mitochondrion (Figure 1-1). It is often described as the powerhouse of the cell because it produces the cellular currency adenosine triphosphate (ATP) from a series of electron transfer reactions in a process known as oxidative phosphorylation.\(^4\)\(^,\)\(^5\) The enzymes of the citric acid cycle are organized within the mitochondrial matrix, many of which are bound to the inner mitochondrial membrane (IM in Figure 1-1).\(^6\)\(^,\)\(^7\) The citric acid cycle generates energy necessary for oxidative phosphorylation from the oxidation of biofuels in the form of acetyl coenzyme A.\(^4\) It also produces molecular precursors for other cellular processes, such as amino acid synthesis.\(^4\) Organization of these enzymes provides an additional level of compartmentalization within the mitochondrion itself. These enzymes have recently been shown to form a multienzyme complex in which the enzyme substrates are channeled; the negatively charged substrates are transferred along the positively charged protein-protein interfaces.\(^8\) In addition to mitochondria, lysosomes are another membrane-bound organelle where cellular components are broken down by a number of hydrolytic enzymes.\(^9\) Lysosomes have an internal pH of ~4.8 which is in stark
contrast to the cytoplasmic pH = 7.0-7.3.\textsuperscript{9} This demonstrates that subcellular compartments can display environmental differences from other cellular compartments coexisting within the same cell.

**Figure 1-1.** High resolution scanning electron microscopy image of the surface (left) and interior structure (right) of mitochondrion (M) from a rat pancreas exocrine cell. Other labeled features are outer mitochondrial membrane (OM), inner mitochondrial membrane (IM), cristae (C), tubular cristae (tC), rough endoplasmic reticulum, (rER) and ribosome (r). Reproduced from reference 2 with permission from Elsevier.

Other cellular compartments are non-membrane bound; the nucleus harbors a number of such compartments. The most prominent nuclear compartment is the nucleolus which is composed of RNA, proteins, and the enzymes required for ribosome synthesis, which is carried out there.\textsuperscript{10} Cajal bodies and nuclear speckles are closely related nuclear compartments shown in Figure 1-2. Cajal bodies are thought to be involved in post-transcriptional RNA processing and have been implicated in the biogenesis of pre-mRNA
splicing factors and the assembly of small nuclear ribonucleic proteins.\textsuperscript{11, 12} Once processed, these splicing factors require further assembly and modifications within nuclear speckles, which are often found to be directly associated with Cajal bodies and are thought to serve as a storage compartment for the spliceosomal components.\textsuperscript{12, 13} This colocalization of splicing factor processing and assembly with subsequent storage near active transcription sites shows a highly organized compartmentalization within the nucleoplasm. Ribosomes are another example of a non-membrane bound organelle found in the cytoplasm. They are composed of RNA and protein and serve as the location for protein biosynthesis.\textsuperscript{9} It is evident from these examples and others that compartmentalization of reactions is a hallmark of biological cells.

\textbf{Figure 1-2.} Prominent nuclear bodies visualized by optical microscopy. (A) Labeled nucleoli (light blue) and Cajal bodies (yellow). (B) Labeled splicing factors in nuclear speckles (pink). Reproduced from reference 10. Reprinted with permission from AAAS.

Recent observations have demonstrated the liquid-like nature of some nonmembranous compartments in both the cytoplasm and nucleoplasm. P granules are protein/RNA-rich compartments found in the germ cell cytoplasm of the nematode worm
Caenorhabditis elegans.\textsuperscript{3} Initially, P granules are uniformly distributed throughout the cell, but eventually the cell polarizes and begins to differentiate into two specialized cells upon the first cell division. Subsequently, P granules are localized to only one of the cells. Brangwynne and coworkers found that the P granules underwent fusion, dripping, and wetting (Figure 1-3A) and are capable of rapidly forming and dissolving, which strongly suggests that these compartments are liquid droplets.\textsuperscript{14} Recent work has also suggested that P granule formation/dissolution is under control of a kinase and phosphatase pair that operates on intrinsically disordered proteins found in the P granules.\textsuperscript{15} Brangwynne and coworkers also demonstrated that the nucleoli of Xenopus laevis oocytes behave as liquid droplets through fusion and fission events (Figure 1.3-B,C).\textsuperscript{16} When the nucleoli fuse, the volume is conserved and a spherical shape is maintained, in the same way liquid droplets coalesce. The fluid-like behavior of nucleoli permits the coalescence of two nucleoli while a third displays a characteristic “pinch-off” behavior of viscous liquids as the nucleoli separate. The spherical nature was attributed to an apparent surface tension that acts to minimize the surface area of the nucleolus. This surface tension is approximately 10 mN/m and is roughly an order of magnitude higher than the surface tension of the C. elegens P granules.\textsuperscript{14, 16} Both of these nuclear and cytoplasmic RNA-protein compartments display surface tensions that fall in the expected regime for macromolecular liquids. From these surface tension estimates, the authors found that the oocyte nucleoli display an apparent viscosity of approximately $10^6$ cP, similar to honey, and it is 1000 times more viscous than that of P granules.\textsuperscript{14, 16} These observations indicate that the RNA/protein compartments display characteristics of liquid aqueous phases.
Figure 1-3. In vivo liquid-like compartments. (A) P granules in *C. elegans* dripping around a nucleus (N), fusing and coalescing. (B) Nucleoli from *Xenopus laevis* oocytes are fusing and pinching off (within dashed line). (C) The pinching off behavior is highlighted. Panel A from reference 14. Reproduced with permission from AAAS. Panels B and C from reference 16. Reproduced with permission from The National Academy of Sciences, USA.

1.3 Macromolecule Solution Chemistry

These observations beg the question: What is the biological activity of the RNA, proteins and other molecules inside these compartments? One might anticipate that the presence of so many “background” macromolecules could alter the activity of a biomolecule compared to buffer solution. To simplify interpretation and to avoid wasting
precious biomacromolecular reagents, biochemicals and their reactions have typically been studied in idealized, dilute buffer solution conditions (typically less than 1 mg/mL of nucleic acids, proteins, small molecules, etc.). This approach has been productive, and is responsible for the major part of our understanding of biomolecule structure, function, and associations. In recent years, however, it has become clear that the crowded intracellular environment in which these macromolecules typically act can both quantitatively and qualitatively change the outcome of important biochemical reactions ranging from conformational states to association equilibria to enzymatic activities. The importance of performing studies under more realistic solution conditions is increasingly appreciated, particularly with regard to mimicking the environment of the cytoplasm or nucleoplasm. The presence of macromolecules at relatively high concentrations, termed “macromolecular crowding,” leads to a considerable excluded volume as well as chemically attractive and repulsive forces between solutes. Consequently, such solutions are not thermodynamically ideal, and solute chemical activities can differ greatly depending on the solute concentration.

1.3.1 Thermodynamic Nonideality

The assumption of thermodynamic ideality, while generally reasonable for dilute solutions, is invalid for macromolecularly crowded media such as the intracellular environment. Ideal solutions are defined to have an enthalpy of mixing equal to zero. In addition, it is assumed that the total volume does not change upon mixing. This is analogous to the assumptions made when dealing with ideal gases (i.e., the volume of the
individual gas molecules can be ignored and interactions between the molecules are negligible). Consider the chemical potential $\mu_A$ of a solute, A, in a solution. It can be expressed using the thermodynamic activity $a_A$ in the following equation:

$$\mu_A = \mu_A^0 + RT \ln a_A \quad \text{(Equation 1-1)}$$

where $\mu_A^0$ is the standard state chemical potential, $R$ is the gas constant, and $T$ is the temperature. The chemical activity of a solute is related to its concentration by the activity coefficient,

$$a_A = \gamma_A c_A \quad \text{(Equation 1-2)}$$

where, $\gamma_A$ is the activity coefficient of solute A and $c_A$ is the concentration of the solute. The activity coefficient can be further defined as

$$\gamma_A = \exp \left( \frac{\langle f_A \rangle}{k_B T} \right) \quad \text{(Equation 1-3)}$$

where $\langle f_A \rangle$ is the free energy change associated with the equilibrium free energy of interaction between a molecule A and all other solutes, $k_B$ is the Boltzmann constant, and $T$ is the temperature.$^{1,22,23}$

In ideal solutions, the activity or effective concentration is assumed to be equal to the concentration of solute A ($a_A = c_A$ and $\gamma_A = 1$). Chemical interactions between all species in solution are identical. This assumption can introduce large errors when dealing with concentrated mixtures of macromolecules. Enthalpy of mixing is not negligible when interactions between different pairs of molecules (solute A–solute A, solute B–solute B, A–B, A–solvent, B–solvent) are substantially different because of differences in intermolecular forces between them from van der Waals, electrostatic, and hydrogen bonding. In solutions of macromolecules, the volume occupied by solutes can also be a
large fraction of the total volume of solution (e.g., up to 30%). Some simple predictions can be made: if interactions are repulsive, and/or volume exclusion dominates, \( \gamma_A \) will be greater than 1, resulting in increased chemical activity for solute A; attractive interactions can result in \( \gamma_A \) being greater or less than 1, depending on the magnitude of excluded volume effects.\(^{23}\)

### 1.3.2 Macromolecular Crowding

Macromolecular crowding refers to the effects of adding macromolecules to a solution, as compared to a solution containing no macromolecules. Substantial differences are observed for a wide range of biomacromolecular interactions and activities in dilute vs. crowded solutions.\(^{20}\) This is interpreted as the combined outcome of both general types of interactions introduced earlier: (1) excluded volume, which is a result of inaccessibility of molecules to occupy space because of the presence of background macromolecules, and (2) chemical effects, which are caused by the attraction and repulsion between molecules. It is not necessary that any one solute should be present in high concentrations for the solution to be crowded. No single protein, nucleic acid sequence, or other polymer is necessarily present in high concentration in a particular intracellular environment; rather, it is the cumulative effect of all macromolecules that renders it crowded.

A significant consequence of volume exclusion is that the thermodynamic activity, \( a \), of a biological macromolecule of interest in crowded conditions can increase orders of magnitude over its value in the absence of crowding agents as illustrated in
An early example of this was the case of hemoglobin. At a concentration of 200 g/L in solution, the activity coefficient, $\gamma$, is about 10. At 300 g/L (similar to the concentration in a normal red blood cell), $\gamma$ rises to 100. At this hemoglobin concentration, a considerable fraction of the total volume is occupied by hemoglobin (for comparison, at 350 g/L hemoglobin, excluded volume is 30% of the total volume). A simple geometrical model using a hard quasi-sphere that factors in the available volume was developed to account for this dependence, and it agreed well with the crystal structure of hemoglobin. Since then, numerous studies have found substantial effects caused by crowding, and in many cases models based on volume exclusion have provided satisfactory qualitative explanations. Crowding agents and reactants are often modeled as hard spheres, which is useful for identifying trends. More elaborate models such as the direct simulation coarse-grained model by Cheung et al. and atomistic simulations by Feig and Sugita have been developed, and future models will offer more insight into the effects by quantitatively describing chemical interactions.
Figure 1-4. The effect of volume exclusion on chemical activity can be visualized within the volume encompassed by a square containing macromolecules (black spheres). Another molecule can be added only if the core is located in the accessible volume (blue). Panel (A) represents the volume available to a molecule of small size. Panel (B) is the volume available to a molecule of comparable size. Reprinted from reference 17 with permission from the American Society for Biochemistry and Molecular Biology.

Predictions for crowding effects caused by volume exclusion alone favor more condensed structures over less condensed forms. For example, equilibria for folding, association, polymerization, and aggregation reactions shift toward the multimeric forms from the monomeric forms. However, the chemical effects of crowding can either enhance stabilization of native, folded states or actually lead to unfolded states. Nonspecific repulsive interactions that occur as a result of minimizing surface exposure (such as during protein folding and association) lead to a stabilizing effect and these can enhance the effects of volume exclusion. However, nonspecific attractive interactions of a protein with the surroundings can have quite the opposite effect and result in destabilization, which can completely negate the stabilizing effects of volume
Miklos and coworkers found that macromolecular crowding agents lysozyme and bovine serum albumin destabilized the structure of the enzyme barley chymotrypsin inhibitor 2, the opposite of what would be expected as a result of volume exclusion alone. They interpret this as a result of nonspecific interactions with the protein crowding agents. Record and coworkers examined the effects of both chemical interactions and excluded volume using polyethylene glycol (PEG) of varying chain lengths (i.e., a single ethylene glycol unit up to MW 20,000) and quantitatively describing the effects of each on duplex and hairpin formation within DNA. Oligoethylene glycols, up to 200 Da, had a destabilizing effect on both hairpin and duplex formation, because of favorable interactions between the PEG and DNA bases. Increasing the molecular weight of the PEG (and therefore increasing the effect of excluded volume) caused stabilization of the duplex and less destabilization of the hairpin. Stabilization was a result of inaccessibility of the DNA to the interior PEG subunits of the PEG polymer at higher molecular weight and a decrease in volume fraction to favor DNA duplex and hairpin formations.

With respect to enzyme activity, macromolecular crowding can increase, decrease, or have no effect on enzyme activity. Changes in enzyme activity in crowded solutions compared to a buffer solution may be explained by changes in the structure of the enzyme, which in turn, affects substrate binding to the active site. Multicopper oxidase had lower activity in a crowded solution compared to buffer, possibly due to the enzyme adopting a more rigid conformation, which, in turn, restricted the substrate’s access to the active site. Other factors, such as reduced diffusion of enzymes and substrates alter enzyme activity. One type of interaction that has been
overlooked with respect to macromolecular crowding and enzyme activity is the interactions of enzyme substrates and crowding agents. This will be explored in Chapter 4, where the crowding effects of a single enzyme with two different substrates are studied.

Because of the complex aspects of macromolecular crowding it is not yet possible to make quantitative predictions for the impact of crowding on arbitrary reactions of interest a priori. It is, nonetheless, important to bear these effects in mind when considering the cellular milieu because the same types of phenomena at play in macromolecular crowded solutions in vitro are expected to be important.

1.4 Aqueous Phase Separation

In addition to the effects described earlier, another consequence of macromolecular crowding can be the formation of distinct coexisting aqueous phases. Solutions that contain one or more polymers at sufficient concentrations commonly phase-separate to form two or more coexisting aqueous phases. This is a relatively general phenomenon that occurs for a wide range of different polymeric solutes, including proteins and nucleic acids. Several types of phase separations are possible, and they can be classified based on the polymers being polyelectrolytes or otherwise, a salt being present in high concentration, and the number of phases being observed. Since the coexisting phases have different properties, biomolecule partitioning among these phases is possible and could provide a means of compartmentalization.
Nucleic acids, as polyanions, can be expected to undergo condensation and, potentially, phase separation in the presence of polycationic cosolutes. Phase separation in concentrated protein solutions is a well-known phenomenon. Proteins in the cell carry a variety of surface charges but generally a lower charge density than nucleic acids. Major classes of phase separation in aqueous polymer solutions that are most relevant for the intracellular environment are considered here. They are necessarily simpler than anything one might anticipate in the cell but provide a framework for understanding the types of demixing in polymer solutions. Here, I will discuss three types of phase-separated systems that appear in later chapters: coacervation, nonionic polymer-polymer phase separation, and polymer-salt phase separation. I refer the interested reader to more comprehensive sources for additional information on other types of phase separation not described here, including polymer-surfactant and polymer-alcohol systems.

1.4.1 Coacervation

Solutions in which one or more polyelectrolytes are present can, under the right conditions, phase-separate to form polyelectrolyte-rich phases, referred to as coacervates. The coacervate phase is a relatively small, dense, and polymer-rich liquid phase, and is accompanied by a dilute phase of much larger volume, which is referred to as the equilibrium or supernatant phase. The coacervate droplets can be differentiated from precipitates or aggregates that form in solutions of similar composition, because the latter are solids rather than liquids and hence will not exhibit spherical shapes, flow, etc.
Simple coacervation refers to systems in which a single polyelectrolyte species phase-separates in the presence of a small molecule or salt. For example, type-B gelatin (a polyampholyte obtained from denatured collagen) forms a simple coacervate when alcohols are added, because of decreased solvation. The phase behavior of the gelatin-alcohol system depends strongly on ionic strength and pH, with a single-phase, liquid–liquid coexistence, and aggregation all possible outcomes depending on the composition of the solution. This is typical for many different polyelectrolytes of synthetic or biological origin. Biologically important polyanions such as DNA or RNA can also form aggregates in the presence of condensing agents such as polyamines (spermine, spermidine), cobalt hexamine, other cationic polymers such as polylysine, highly basic proteins (histones), multivalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)), neutral polymers, and alcohols. These agents generally work by inducing precipitation by a few means: decreasing DNA repulsions by screening the charge of the phosphate, reordering water structure, or making DNA–solvent interactions less favorable. Ethanol-induced precipitation of DNA is not unlike the alcohol-induced coacervation and, eventually, the aggregation observed for simple coacervates.

Complex coacervation involves one macromolecule and a small molecule, or two or more macromolecules that are oppositely charged. The charge density of the macromolecule(s) must be large enough for electrostatic interactions to keep the droplets from dissolving into the equilibrium phase to form a homogenous solution, but not large enough to induce precipitation of solid aggregates. Bulk phase images and microscopy images of complex coacervates with poly(udidylic acid) RNA and a cationic peptide
(sequence RRASLRRASL) as the phase forming components are presented in Figure 1-5.

This phase system is the subject of Chapter 2.

![Figure 1-5](image)

**Figure 1-5.** Complex coacervates of poly U RNA and an arginine-rich peptide (RRASLRRASL). Labeled peptide has been added to aid in visualization. (A) A turbid coacervation solution. (B) A completely coalesced coacervate phase shown at bottom of tube. (C) A transmitted light with differential interference contrast (DIC) image of complex coacervates. (D) fluorescence image of complex coacervates that have been false-colored.

The driving force for complex coacervate formation has been described as predominantly entropically driven. As the oppositely charged molecules interact, counter ions that were once associated with the macromolecule(s) are released. Reports also suggest that it is partially enthalpically driven, because of the electrostatic free energy of the system. The phase behavior of complex coacervate systems has been studied since their original discovery, with a few key general principles that apply to coacervate systems. Mainly, the complex phase as a whole is nearly electrically neutral, yet highly polarizable. For example, Burgess and Carless used microelectrophoretic measurements on complex coacervates of gelatin and acacia and found that the optimum pH for coacervation (that which leads to the greatest volume of the coacervate phase) was equal to the electrical equivalence pH (net charge = 0). Other studies also suggest that there is
a fixed stoichiometry determined by the charge density of the phase-forming components at a specific pH.\textsuperscript{48} Salt can also affect the phase formation; for every coacervate, there exists a critical salt concentration at which the two-phase system will become a homogenous solution as a result of charge screening. This value is experimentally determined and varies with the polyanion/polycation pair increasing as the polymer chain lengths and charge densities are increased.\textsuperscript{56} For example, for a charge-matched poly(L-lysine)/poly(L-glutamic acid) mixture, the critical salt concentration increased from 300 mM NaCl for a chain length $N = 30$ to $\sim 1000$ mM NaCl for $N = 400$.\textsuperscript{56}

Given the importance of polyelectrolytes such as RNA, DNA, and cationic polypeptides/proteins in the cell, it is reasonable to suspect that aqueous phase separation based on macromolecular charge may be a factor in forming RNA/protein-rich compartments described earlier.

### 1.4.2 Nonionic Polymer/polymer Phase Separation

In addition to complex coacervation, aqueous phase separation can occur in a solution of two or more neutral polymers, which results in two or more separate crowded phases, each enriched in one of the polymers.\textsuperscript{38} The process involves enthalpic interactions between polymers, changes in hydration, and entropic contributions that can dominate because of macromolecular crowding. Developing models that can predict phase behavior and solute partitioning has been an active area of research, because of interest in using these systems for bioseparations and the empirical manner in which separation protocols are generally determined.\textsuperscript{57-63} One of the most widely studied
aqueous phase systems of two polymers is that formed from PEG and dextran. It undergoes phase separation above a few weight percent of each polymer. An aqueous two-phase system (ATPS) of 10% (w/w) PEG 8 kDa and 10% (w/w) dextran 10 kDa is shown in Fig. 1-6. The upper phase, stained blue, is the PEG-rich phase, and the lower colorless phase is the dextran-rich phase. Mechanical agitation of the ATPS disperses droplets of one phase within the other (in this case, PEG-rich phase droplets are suspended in a continuous dextran-rich phase). Other phase systems composed of synthetic polymers, polysaccharides, or proteins have also been developed and studied, and have found uses in biotechnology for biomolecule separation.\textsuperscript{46, 47} The process is not limited to biphasic systems; multiphase systems can be generated by addition of more phase-forming components,\textsuperscript{38} and it should be noted that multiple types of phase separation can occur in the same solution (e.g., coacervates and nonionic polymer-rich).

A PEG/dextran ATPS will be used as the phase system in Chapter 5.

\textbf{Figure 1-6.} An aqueous two-phase system of polyethylene glycol and dextran with labeled PEG within a cuvette (left) and confocal microscope image after agitation (right). Reprinted with permission from Elsevier.
1.4.3 Polymer/salt Phase Separation

Phase separation can also occur with a single uncharged polymer when a high concentration of salt is present. The result is a polymer-rich, salt-poor, top phase and a salt-rich, polymer-poor bottom phase.\textsuperscript{46} Virtually all polymer-salt aqueous phase systems created to date are composed of PEG and a salt with a multivalent anion (sulfate, phosphate, or citrate) and metal cation(s).\textsuperscript{64} The mechanism for phase separation in these systems is less understood in comparison to polymer-polymer systems. It is has been suggested that PEG ether oxygen-cation interactions can dominate over water-cation hydration interactions, and as a result, the charge dense anions are excluded from interacting with the PEG.\textsuperscript{46, 64} Changes in water structure may also play a role. These systems have typically been used for purifying valuable biomolecules in industry as a less expensive alternative to the PEG/dextran system because of the low cost of salt compared to dextran.\textsuperscript{46} However, as demonstrated in Chapter 3, a PEG/citrate system can be used as a cellular mimic to study enzyme and substrate localization.

1.5 Compartmentalization Induced By Aqueous Phase Separation

The coexistence of multiple phases with differing compositions, viscosities, densities, and so on means that solutes may accumulate more in one of the phases over the others, leading to differences in local concentration between the phases. The equilibrium distribution of a solute in an ATPS is described in terms of the partitioning coefficient, $K$, which is the concentration of the solute in the top phase ($C_{top}$) relative to that in the bottom phase ($C_{bottom}$):
Many factors influence how a solute partitions in an ATPS, including the properties of the polymer phases such as polymer identities, structures, molecular weights, concentrations, pH, ionic strength as well as the properties of the solute including the size, hydrophilicity/hydrophobicity, and any interactions with the ATPS components.\textsuperscript{38, 65} Protein partitioning studies in the PEG/dextran ATPS have demonstrated that native proteins usually partition to the more hydrophilic dextran phase, while denatured proteins partition to the hydrophobic PEG-rich phase. In the denatured state, more internal hydrophobic amino acids of the protein are exposed.\textsuperscript{66} Charge and size play a role in partitioning, as charged species are likely to interact with any charged components of the ATPS, and large molecules have larger areas of interaction with the phase-forming components.\textsuperscript{38, 67} For example, RNA partitioning in a PEG/dextran ATPS is strongly length dependent, with \(1/K = \sim 40\) and \(\sim 3000\) for 15 and 159 nucleotide RNAs, respectively.\textsuperscript{68} Coacervates composed of poly(diallyldimethylammonium) chloride (PDDA) and ATP were also able to sequester globular proteins such as green fluorescent protein (GFP) at an 86-fold higher concentration within the coacervate phase droplet as compared to the surrounding phase.\textsuperscript{69}

Considering the possibility of coexisting phases in the cell, we can anticipate that a solute’s partitioning behavior would be dominated by binding interactions with molecules already localized in the phase. These binding partners could be the protein and nucleic acids responsible for its formation, or another solute that partitioned strongly to
the phase. The number of binding sites and strength of the interactions would lead to different degrees of localization to the phase compartment and residence times within it.

1.6 Aqueous Phase Separation and Compartmentalization for Localizing Enzyme Activity

Localization by partitioning into different aqueous phase compartments offers a mechanism for maintaining distinct molecular composition in the different types of compartments. It has been hypothesized that colocalization of enzymatic activity may serve as a general means of metabolic regulation.\textsuperscript{70-72} Colocalization of enzyme activity reduces intermediate transient time between sequential processing events while increasing local intermediate concentrations to enhance catalytic efficiency.\textsuperscript{73, 74} These theories have thus far been focused more on applications within the cytoplasmic compartments/organelles; however, such principles are equally valid within the nucleoplasm because of the comparable environments.

Several studies have shown enhanced enzymatic activity through compartmentalization. Strulson and coworkers utilized a PEG/dextran ATPS to enhance RNA cleavage via compartmentalization (Figure 1-7).\textsuperscript{68} Variants of the hammerhead ribozyme were composed of a separate enzyme and substrate strand that induced the ribozyme to self-cleave upon association. As a result of partitioning, the RNA was localized into dextran-rich phase droplets by as much as 3000-fold over its concentration in the PEG-rich continuous phase. With a constant total volume and amount of RNA added, the local concentration of RNA depended on the volume ratio for the two phases, with smaller volumes of the dextran-rich phase leading to higher local RNA
concentrations and, consequently, increasing the cleavage rate. Rate enhancements as high as 66-fold were observed, with increases tunable by the phase volume ratio. This work points to a possible functional significance of the total volume of any given phase as a means of tuning local concentration and, hence, reaction rates without varying the partitioning preference of the molecule, which could be relevant to nuclear body sizes/numbers.

**Figure 1-7.** RNA localization and enhanced rates of reaction in dextran-rich phase droplets. Confocal images show (A) DIC, (B) labeled dextran (red), and (C) labeled RNA (green). By varying the dextran:PEG volume ratio in 10 mM Mg$^{2+}$ (D) and 0.5 mM Mg$^{2+}$ (E), RNA is increasingly localized to the dextran-rich phase and reaction rate is increased. The volume ratios tested were 1:0 (black circles), 1:5 (blue squares), 1:12.5 (red diamonds), 1:50 (light blue triangles), and 1:100 (green inverted triangles). Reprinted from reference 68 with permission from Macmillan Publishers Ltd: Nature Chemistry.
Increased rates for protein enzymes have also been observed upon compartmentalization in aqueous phases. Koga et al. observed a two-fold rate increase for an ATP-dependent reaction involving hexokinase when the enzyme was encapsulated within poly(L-lysine)/ATP coacervate droplets as compared to bulk solution.\textsuperscript{75} Colocalization of sequential enzymes was accomplished by incorporating the minimal complex of actinorhodin polyketide synthase (PKS), which contains three enzymatic functions, into the PDDA/ATP coacervate system. The minimal PKS complex was sequestered into the coacervate droplets, as was its substrate malonyl-CoA, leading to an 18-fold increase in product yield as compared to control assays with similar concentrations of either PDDA or ATP present.\textsuperscript{76} One might expect that increased concentrations of enzymes will always lead to an enhanced rate of reaction, however, that is not always observed. In Chapter 5, we show that enzymes of the de novo purine biosynthesis pathway do not experience a large kinetic effect from colocalization despite increased local concentrations.

Another possible advantage of compartmentalization is control over the location of a reaction. Cacace and Keating demonstrated that even a weakly-partitioned enzyme ($K = 0.12$) could lead to nearly quantitative product formation only in the dextran-rich phase of a PEG/dextran ATPS.\textsuperscript{77} This work used urease to catalyze formation of the mineral calcium carbonate by locally producing carbonate anion in the dextran-rich phase. Under optimized conditions of enzyme concentration and volume ratio, $<1$ mol\% mineral could be detected in the PEG-rich phase despite the only 8.3-fold higher concentration of urease in the dextran-rich phase and the fact that $\text{Ca}^{2+}$ ($aq$) was initially present in equal concentrations in both phases. For the cell, the ability to restrict reactions
to nonmembranous compartments is as important as increasing local reaction rates. This idea will be explored in relation to the sequential enzyme activity of glucose oxidase and horseradish peroxidase in Chapter 3 and with the de novo purine biosynthesis enzymes in Chapter 5.

1.7 Dissertation Overview

It is apparent that the cell is an intricate matrix, as it is composed of many coexisting environments carrying out cellular functions. The chapters that follow describe the use of aqueous phase model systems in order to understand biological compartmentalization and its significance, as well as enzyme activity in heterogeneous crowded media.

Chapter 2 is concerned with using complex coacervates of poly U RNA and a peptide (RRASLRRASL) for compartmentalization of biomolecules. I demonstrate that a protein phosphatase can remove phosphate groups from the peptide, which makes the peptide more positively charged, and subsequently results in phase separation. This process was reversible; already formed RNA/peptide coacervates are dissolved by addition of a protein kinase. The kinase adds phosphate groups to the cationic peptide and phase separation no longer occurs. This work is significant because it demonstrates that phosphorylation can be a viable means for compartment formation in cells, and that RNA/peptide-rich compartments are capable of increasing concentration of solutes over 1000-fold.
Chapter 3 focuses on the use of a PEG/citrate ATPS as heterogeneous reaction media to study the sequential enzymes glucose oxidase (GOX) and horseradish peroxidase (HRP). We find that the enzyme activity is highly dependent on the solution composition, especially for the HRP reaction. The HRP activity is substantially decreased in the PEG-rich phase compared to the citrate-rich phase. The enzymes partition to the citrate-rich phase, but one of the required substrates strongly partitions to the PEG-rich phase, which has a substantial impact on enzyme activity. A mathematical model of the enzyme kinetics in the biphasic system is described that accurately fits the experimental data. The model is able to predict the enzyme kinetics under a different set of reaction conditions, which demonstrates its usefulness for other enzymes and biphasic systems, as it is also used in Chapter 5. The sequential reaction is dependent on the presence of the interface, which suggests that sequestration of substrates to a compartment separate from the active enzymes can serve as means of metabolic regulation.

Based on the observations in Chapter 3, I take a more detailed look at HRP enzyme activity in Chapter 4. I measure HRP kinetics in the presence of different macromolecular crowding agents (PEG 8k, dextran 10k) and cosolutes (PEG 400 and glucose) with two different substrates: o-phenylenediamine (OPD) and 3, 3’, 5, 5’ tetramethylbenzidine (TMB). The activity with the more hydrophobic substrate TMB is substantially decreased compared to the activity with OPD, especially in the higher weight percent PEG solutions. Pulsed-field gradient nuclear magnetic resonance (PFG-NMR) is used to measure the diffusion of the substrates in the different media. The diffusivity of the TMB is slowed much more in PEG than that of OPD, due to hydrophobic PEG-TMB interactions. This work is significant because it examines
macromolecular crowding effects with respect to a small molecule substrate, which has not been explicitly investigated previously.

In Chapter 5, we use a PEG/dextran ATPS as heterogeneous reaction media to investigate the kinetics of sequential enzymes from the de novo purine biosynthesis pathway. The enzymes and substrates partition to the dextran-rich phase. By decreasing the PEG:dextran volume ratio, we increase the local concentration of enzymes in the dextran-rich phase. However, somewhat surprisingly, we observe that the enzyme activity is not substantially increased because there is decrease in total number of moles of enzyme in the dextran-rich phase as the volume ratio increased. The mathematical model from Chapter 3 is adapted to this system, and it allows us to probe reaction conditions that are difficult to achieve experimentally (e.g. changing enzyme partitioning). The results suggest that significant advantages from enzyme compartmentalization can be achieved by localizing essentially all copies of active enzyme to a compartment.

Finally, Chapter 6 discusses some general conclusions and future directions.

1.8 References


Chapter 2

Phosphorylation Mediated RNA/Peptide Complex Coacervation: A Model for Intracellular Liquid Organelles

This chapter has been submitted for publication to a high-impact chemistry journal.

2.1 Abstract

Biological cells are highly organized with numerous subcellular compartments. Phosphorylation has been hypothesized as a means to control assembly/disassembly of liquid-like RNA- and protein-rich intracellular bodies that lack delimiting membranes. Here, we demonstrate that charge-mediated interaction, or complex coacervation, of RNA with a ten-residue peptide can generate simple model liquid organelles capable of reversibly compartmentalizing biomolecules. Formation and dissolution of these liquid bodies was controlled by changes in peptide phosphorylation state using a kinase/phosphatase enzyme pair. The droplet-generating phase transition responded to modification of even a single serine residue. Electrostatic interactions between the short cationic peptide (with phosphorylation-dependent net charge of approximately +4, +2 or 0) and the much longer polyanionic RNA drove phase separation. Coacervates were also formed on silica beads, a primitive model for localization at specific intracellular sites. This work supports phosphoregulation of complex coacervation as a viable mechanism for dynamic intracellular compartmentalization in membraneless organelles.
2.2 Introduction

Compartmentalization is characteristic of all living cells. In eukaryotes, both membrane-bounded and membraneless organelles are important in spatiotemporal organization of subcellular processes. Certain membraneless compartments composed of RNA and protein are now appreciated to have liquid-like characteristics and thought to form by phase separation.\(^1\)\(^-\)\(^6\) For example, cytoplasmic P granules in \textit{C. elegans} were demonstrated to have liquid properties such as rapid condensation and dissolution, fusion, and wetting.\(^1\) The nucleus contains a host of dynamic, non-membrane bound bodies including nucleoli, Cajal bodies, nuclear speckles and others that vary in size, number and/or composition over time and can appear and disappear during mitosis.\(^7\),\(^8\) Mechanisms for formation/dissolution of these “liquid organelles” and for targeting molecules to them remain incompletely understood. Several studies point to the importance of multivalent interactions and post-translational modifications in modulating both formation and molecular occupancy.\(^4\),\(^7\)-\(^13\) Phosphorylation is a ubiquitous post-translational modification that regulates cellular processes and signaling pathways by controlling protein structure, activity, interactions, and localization.\(^14\),\(^15\) Given that so many cellular processes are controlled by protein phosphorylation states and the large number of kinases and phosphatases, it is reasonable to anticipate that the phosphorylation state of cytoplasmic and nuclear body components may be responsible wholly or in part for their formation and disassociation. Indeed, kinase and phosphatase activity have been implicated in dissolution and formation, respectively, of two kinds of liquid-like RNA granules.\(^16\),\(^17\) In vitro evidence also supports a possible role for
phosphorylation in liquid-liquid phase separation: Rosen and coworkers demonstrated that a phosphorylated peptide modeled after the transmembrane protein nephrin tail sequence facilitated its participation in phase separation.\textsuperscript{13}

There are several ways phosphorylation could regulate formation and occupancy of liquid-like intracellular bodies such as P granules: specific biorecognition, conformational changes, and/or electrostatic interactions.\textsuperscript{15, 18, 19} The simplest of these is electrostatics. Changes in charge-charge interactions upon altering addition or removal of a phosphate moiety could regulate intracellular droplet formation by complex coacervation, a type of phase separation that occurs due to electrostatic attraction between oppositely charged macromolecules. Complex coacervation leads to polyelectrolyte-rich droplets and is a possible mechanism of liquid-liquid phase separation in vivo.\textsuperscript{20-23} Biomolecule compartmentalization and enzymatic reactions have been studied in coacervates and other aqueous biphasic systems in vitro,\textsuperscript{20, 21, 24-29} but generally the phase-forming components are not themselves undergoing reaction. Recently, Huck and coworkers showed that polypeptide coacervates could be disassembled by protease activity, illustrating a unidirectional enzymatic action on phase-forming polymers to direct a phase change.\textsuperscript{30} Here, we report that enzymatic phosphorylation/dephosphorylation of a short peptide reversibly controls droplet formation in an RNA/peptide system.

Our minimal synthetic membraneless organelles are complex coacervates composed of poly(uridylic acid), and a cationic peptide with two phosphorylatable serines. We demonstrate control over droplet formation by changing the peptide phosphorylation state (and therefore, its charge density). The enzyme lambda protein
phosphatase (λ-PP) dephosphorylates the peptide, creating a positively charged peptide that subsequently results in coacervation. We show that this process can be carried out in the other direction by phosphorylating the peptide using protein kinase A (PKA), causing coacervation to no longer occur. By modulating the activity of the kinase and the phosphatase, this process was reversible. Fluorescently-tagged peptides, enzymes and short model RNA sequences partition into these model liquid organelles, increasing local solute concentrations by up to 1150×.

2.3 Results

2.3.1 Poly U/RRASLRRASL Coacervates

We chose polyuridylic acid RNA (poly U, MW 600k-1000k) to serve as a model polyanion because of its random coil structure. The peptide sequence RRASLRRASL was designed to serve as the polycation. The sequence was inspired by the peptide LRRASLG or “Kemptide” which is a model synthetic substrate for PKA (see below). Addition of RRASLRRASL to a buffered solution of 0.05 % (w/w) (~0.06 mg/mL) poly U resulted in complex coacervation (Figure 2-1A). Turbidity measurements in which the concentration of RRASLRRASL was varied from 0-1000 μM (0-1.9 mg/mL) with a fixed concentration of 0.05 % poly U indicated that the critical coacervate concentration (CCC) was 250 μM RRASLRRASL (Figure 2-1B). Turbidity indicated phase separation (Figure 2-1C); centrifugation resulted in a coalescence of the coacervate droplets to form one drop at the bottom of the tube (Figure 2-1D). The coacervate
droplets were generally less than 5 µm in diameter before coalescence (Figure 2-1E, F). At 1000 µM total peptide concentration, the supernatant phase contained 570 ± 20 µM peptide, hence approximately 43% of the peptide is concentrated into the < 2 µL volume coacervate phase. The suspended droplets were slightly negatively charged (zeta potential = -12 ± 5 mV), in good agreement with estimation of charges based only on the RNA and peptide. For the poly U, we assume that there is 1 negative charge from the phosphate backbone of each monomer and use a molecular weight of uridine monophosphate and calculated that there is ~1.7 mM charge. The concentration of positive charges from the peptide is ~1.6 mM, which predicts that there would be a slight net negatively charged coacervate phase. Charge screening by additional NaCl decreased the turbidity to ~35% at 50 mM NaCl, and coacervates dissolved at 100 mM NaCl (Figure 2-2). We conclude that coacervation in this system is largely driven by electrostatic attraction between the negatively-charged RNA and the positively-charged peptide.
Figure 2-1. Illustrative representation and characterization of the poly U/RRASLRRASL coacervate system. Fluorescent peptide (TAMRA-RRASLRRASL) has been added to the samples to aid in visualization. (A) Schematic illustration of poly U RNA added to a solution of RRASLRRASL, which results in complex coacervation. (B) A phase diagram where the concentration of RRASLRRASL is varied. (C) Photograph of bulk phase coacervates illustrating turbidity. (D) Coacervates after centrifugation showing the coalesced coacervate phase at the bottom of the tube. (E) A transmitted light differential interference contrast (DIC) image of coacervate phase droplets. (F) Fluorescence image of the same coacervate phase droplets. TAMRA-RRASLRRASL fluorescence has been false-colored red for visualization. The buffer used was 50 mM HEPES, pH 7.4, 4 mM MgCl₂, 2.25 mM ATP, 1.6 mM DTT at 37 °C.
Phosphorylation of RRASLRRASL decreases its net positive charge, and consequently, we hypothesized that complex coacervation would be inhibited. Each Arg contributes a positive charge, for a total of +4. A typical N terminal amino group pK<sub>a</sub> is 8.0, which means ~80% of the N-termini are positively charged. The C terminal carboxyl group is deprotonated (pK<sub>a</sub> = 3.0), therefore, there is a net partial negative charge contribution from the ionizable groups at the peptide termini. Single phosphorylation of RRASLRRASL results in two possible structural isomers, RRApSLRRASL and RRASLRRApSL. At pH 7.4, the dibasic phosphate group adds a -2 charge and lowers the pI of the peptide to 11.5. If both sites are phosphorylated, the peptide becomes RRApSLRRApSL, with a nearly neutral charge (pI = 7.6). Addition of one phosphate modestly lowers the peptide pI, but addition of both phosphate groups significantly lowers the peptide pI. As anticipated, addition of 1.5 mM of RRApSLRRApSL (~0.3 mM net negative charge) did not result in complex coacervation (3 ± 1 % turbidity).
Single phosphorylation of RRASLRRASL results in two possible structural isomers, RRApSLRRASL and RRASLRRApSL. Addition of up to 1.5 mM of either isomer (~2.7 mM net positive charge) to 0.05 % poly U also did not result in complex coacervate formation (2 ± 2 % and 0.3 ± 0.2 % turbidity, respectively). The positive charge density of these singly-phosphorylated peptides was not sufficient to induce phase separation. These results indicated that the complex coacervation was dependent on charge density, which can be controlled by phosphorylation state, and that even a single phosphorylation event is sufficient to prevent complex coacervation in this system.

2.3.2 Dephosphorylation of RRApSLRRApSL by λ-PP Drives Coacervation

We next explored if a phosphatase could be used to cleave the phosphate groups of RRApSLRRApSL in a solution with poly U and result in phase separation. Bacteriophage lambda protein phosphatase (λ-PP) is a well-characterized Ser/Thr protein phosphatase that requires Mn$^{2+}$ for activity.$^{35-37}$ λ-PP has broad specificity,$^{35}$ and it is commonly used to dephosphorylate peptides and proteins.$^{35, 38, 39}$ Addition of 0.001 mg/mL λ-PP (800 U/mL, 0.040 µM) to a solution containing poly U and RRApSLRRApSL, resulted in a turbid solution after 10 minutes (Figure 2-3 A & B, Figure 2-4). Without λ-PP, the solution does not become turbid (red traces in Figures 2-3B and 2-4).
RRapSLRRAPSL
pI = 7.6

RRapSLRRASL
pI = 11.5

RRASLRRApSL
pI = 12.5
Required for phase formation

b

Turbidity (100 % T)

Time (min)

2.0 min 12.3 min 19.6 min 32.0 min

silica beads

10 µm

c

Absorbance (mAU)

Time (min)

15.0 14.0 13.0 12.0

2.0 min 12.3 min 19.6 min 32.0 min

RRapSLRRASL
RRapSLRRASL
RRapSLRRASL
RRapSLRRASL
RRapSLRRASL

d

Concentration (µM)

Time (min)

150 100 50 0

100 80 60 40 20 0

18.00 min 18.08 min 18.17 min 18.33 min

10 µm

18.00 min 18.08 min 18.17 min 18.33 min

RRapSLRRASL
RRapSLRRASL
RRapSLRRASL
RRapSLRRASL
**Figure 2-3.** Activity of λ-PP upon the peptide and resulting phase separation. (A) Schematic representation of enzyme activity upon the phosphorylated analogs of RRASLRRASL. (B) Plot of solution turbidity as function of time with enzyme (black trace) and without enzyme (red trace). (C) HPLC chromatogram showing separation of the RRASLRRASL and its phosphorylated analogs. (D) Phosphatase reaction progress reported as concentration of each of the peptides as a function of time. Error bars represent standard deviation of 3 trials. (E) Formation of coacervate droplets during the phosphatase reaction. Top row is transmitted light images and bottom row is TAMRA-labeled peptide fluorescence at the same time points. Peptide fluorescence can be seen around the beads prior to extensive droplet formation in solution. Coacervate droplets form in solution and on the silica bead surface. Droplets in focus are suspended in solution, while droplets that have fallen to the surface are out of the focal plane. (F) Time course of a coacervate droplet wetting the surface of a silica bead. The buffer contained 50 mM HEPES, pH 7.4, 4 mM MgCl₂, 1 mM MnCl₂, 1.6 mM DTT at 37 °C.

**Figure 2-4.** Additional phosphatase turbidity trials. Black traces are the samples with phosphatase added. Red traces are the samples without phosphatase.

We monitored the reaction by measuring the concentration of each peptide species as a function of time using HPLC (Figure 2-3C). The reaction proceeds fast initially; ~ 200 µM of RRApSLRRApSL was consumed within the first few seconds, and the concentration was below 100 µM by 20 minutes (Figure 2-3D). Dephosphorylation at the second Ser residue was more rapid than the first; at 5 mins, more RRApSLRRASL was generated (360 ± 10 µM) than RRASLRRApSL (190 ± 20 µM). To make the final
product RRASLRRASL, both of the singly phosphorylated peptides must be acted upon again by $\lambda$-PP, indicating competition among the peptide substrates for the active site. RRASLRRASL concentration increased steadily for the first 20 minutes, and then increased more slowly for the remainder of the reaction. This is most likely due to accumulation of the inorganic phosphate byproduct, a competitive inhibitor for $\lambda$-PP ($K_i = 710 \mu M$). Between ten and fifteen minutes, RRASLRRASL concentration increased from 240 ± 80 $\mu M$ to 370 ± 60 $\mu M$. Because the solution composition was different than the poly U/RRASLRRASL coacervates described above, we measured the CCC in the presence of the approximate maximum concentrations of the singly phosphorylated peptides that was reached during the reaction. The CCC falls to 200 $\mu M$ (Figure 2-5), within the concentration range difference for RRASLRRASL from 10 to 15 minutes. This is consistent with our observed time of coacervate formation triggered by $\lambda$-PP activity. We carried out the reaction without poly U, disallowing the possibility of coacervate formation to compare how the enzyme activity was changed in the presence of the coacervates to a buffer solution. The reaction activity was decreased slightly compared to the coacervate samples (Figure 2-6), but the same trends were observed. These results show that coacervate formation was dependent on the presence of RRASLRRASL. Dephosphorylation of RRApSLRRApSL by $\lambda$-PP was able to produce RRASLRRASL and subsequently resulted in complex coacervate formation.
**Figure 2-5.** Phase diagram of poly U/RRASLRRASL in phosphatase buffer conditions with 375 µM RRApSLRRASL and 190 µM RRASLRRApSL. The CCC shifts to 200 µM RRASLRRASL.

**Figure 2-6.** Phosphatase reaction trace in buffer. No poly U has been added, disallowing the possibility of coacervation.

We also monitored coacervate formation by confocal microscopy. Figure 2-3E shows DIC and fluorescence images throughout the course of the reaction. Silica beads were added to the sample in order to understand how the coacervate phase interacts with the bead surface and to focus the sample because the solution was initially transparent. At 12.3 minutes, the fluorescent peptide has coated the silica beads, about the same time that coacervates start to form in solution and begin to fall to the coverslip surface. Figure 2-7
highlights nucleation on beads. At 32 minutes, the coverslip is coated in the coacervate phase, as well as the silica beads. In another reaction sequence, a droplet fuses with the existing coating of coacervate phase around a bead (Figure 2-3F).

Figure 2-7. Reaction sequence images highlighting coacervation around the silica beads. The reaction buffer was the same, but the concentration of phosphatase was reduced by one-half and the concentration of RRAPSLRRAPSL was reduced to 750 µM. Under these conditions, it was possible to favor formation of coacervate droplets on the negatively-charged silica surface of the beads (and the underlying silica coverslip) over solution-phase formation.

2.3.3 Phosphorylation by PKA Results in Dissolution

We next investigated if the system could proceed the other direction in which a kinase would act upon RRALSRRASL in the poly U/RRASLRRASL system and cause dissolution of the coacervate droplets to form a single-phase solution. We used PKA to carry out the phosphorylation of RRASLRRASL (Figure 2-8A). Like λ-PP, PKA is a well-characterized enzyme, and it is often considered a prototypical kinase. It has a
consensus phosphorylation sequence of R-R-X-S/T-Φ, where X is any amino acid and Φ is a hydrophobic amino acid. The sequence RRASL has been used as a substrate for PKA, so we anticipated that PKA could act upon RRASLRRASL. Poly U/RRASLRRASL coacervates were prepared in the same buffer, except that MnCl$_2$ was replaced with ATP, the phosphate source for kinase activity. We found that kinase activity was significantly diminished in the presence of Mn$^{2+}$, as previously observed. Addition of protein kinase A at a final concentration of 0.01 mg/mL (86 units/mL, 0.24 µM) resulted in a transparent solution after about 6 minutes. (Figure 2-8, Figure 2-9). Dissolution was rapid; the sample went from turbid to transparent in about 2 minutes.
Figure 2-8. Activity of PKA and dissolution of the coacervate phase. (A), Schematic representation of PKA activity upon RRASLRRASL. (B) Plot of solution turbidity as function of time with enzyme (black trace) and without enzyme (red trace). (C) Kinase reaction progress reported as concentration of each of the peptides as a function of time. Error bars represent standard deviation of 3 trials. (D) Poly U/RRASLRRASL coacervate dissolution by PKA activity. The top row is transmitted light optical microscope images and the bottom row is TAMRA-labeled peptide fluorescence at the same time points. The droplets begin to disappear around 12 minutes and are almost entirely dissolved by 17 minutes. The buffer used was 50 mM HEPES, pH 7.4, 4 mM MgCl$_2$ 2.25 mM ATP, 1.6 mM DTT at 37 °C.
Figure 2-9. Additional kinase turbidity trials. Black traces are the samples with kinase added. Red traces are the samples without kinase.

Reaction progress for PKA is given in Figure 2-8B. RRASLRRASL reacted quickly, depleting to 320 ± 40 µM by 5 minutes and 140 ± 40 µM by 10 minutes. The CCC of RRASLRRASL in the presence of the singly phosphorylated peptides was unchanged from 250 µM (Figure 2-10). The CCC is within the time scale of RRASLRRASL concentration change in which turbidity decreased. The second Ser was more easily phosphorylated than the first; RRASLRRAPSL reached a concentration of 460 ± 40 µM at 5 minutes while RRApSLRRASL only reached 140 ± 10 µM at the same time. Different phosphorylation rates have been observed with different flanking sequences to RRASL, as we observe here. RRApSLRRAPSL steadily increased during the reaction, reaching a concentration of 970 ± 30 µM at 60 minutes. We measured kinase activity without poly U and saw a very similar reaction trace, indicating that enzyme activity was not significantly changed by the presence of coacervates (Figure 2-11). Turbidity persisted in controls lacking kinase (red traces, Figure 2-8B and 2-9).
Figure 2-10. Phase diagram of poly U/RRASLRRASL coacervates in kinase buffer conditions with 160 µM RRAPSLRRASL and 460 µM RRASLRRAPSL. The CCC remains at 250 µM RRASLRRASL.

Figure 2-11. Kinase reaction trace in buffer. No poly U has been added, disallowing the possibility of coacervation.

Droplet dissolution was monitored using confocal microscopy (Figure 2-8D). Here, kinase concentration was reduced by one-half (0.005 mg/mL) to allow the droplets to persist for a longer period of time to aid in visualization. Initially, the droplets fell to the surface. At 12.5 minutes, the droplets that had been at the coverslip were significantly smaller. By 17 minutes, nearly all the coacervates were dissolved and a one-phase solution remained. These droplets remained more spherical when they are in contact with the coverslip surface and did not coalesce as much as the λ-PP generated coacervates did.
Since the concentration of RRASLRRASL was decreasing, the coacervate droplets were shrinking and remained in contact with themselves rather than with the glass surface. Also, it is likely that the kinase in the dilute phase was acting on RRASLRRASL at the interface of the coacervate phase and the dilute phase.

### 2.3.4 Compartmentalization of Biomolecules within Coacervate Droplets

We measured the concentrations of fluorescently-labeled peptides, phosphatase, kinase and 15 base RNA strands within the two coacervate systems: poly U/RRASLRRASL coacervates and the mixed phosphorylation state coacervates generated by the λ-PP reaction. The volume of the coacervate was very small (< 1 µL in a 125 µL total volume), so we used confocal fluorescence microscopy to image (Figure 2-12, Figure 2-13) and measure the concentrations of the solutes in the coacervate phase (Table 2-1). Because the peptides and RNA partitioned very strongly, their concentration in the supernatant phase was below the limit of quantification. RRASLRRASL was quite concentrated inside the droplets in both systems; when 1 µM labeled peptide was added its concentration in the droplets was ~800 µM. This exceeds our estimate of ~50× based on unlabeled peptide concentration in the dilute phase; the difference may be due to the TAMRA label, which adds mass and impacts charge density. Labeled PKA and λ-PP also accumulated somewhat in the droplets, however a significant fraction of both enzymes remained in the supernatant.
**Figure 2-12.** Confocal microscopy of poly U/RRASLRRASL coacervates with fluorescent solutes. (A) TAMRA-RRASLRRASL, (B) Alexa 488 PKA, (C) Alexa 647 poly N15, (D) Alexa 647 poly A15. Transmitted (DIC) images are shown in left-hand panels and fluorescence in right-hand panels. Fluorescence images have been false-colored for visualization, with colors chosen to be unique for each dye.

**Figure 2-13** Additional biomolecule localization within coacervates images. (A) TAMRA-RRASLRRASL in λ-PP generated coacervates. (B) Alexa 488 labeled λ-PP in λ-PP generated coacervates. (C) Alexa 647 poly N 15 in λ-PP generated coacervates. (D) Alexa 647 poly A 15 in λ-PP generated coacervates. (E) TAMRA-LRRASLG in poly U/RRASLRRASL coacervates. (F) TAMRA-LRRApSLG in poly U/RRASLRRASL coacervates.
Table 2-1. Concentrations of fluorescent solutes inside the coacervate systems. The error with each measurement represents the standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Concentration added (µM)</th>
<th>Coacervate phase concentration (µM)</th>
<th>Supernatant phase concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly U/RRASLRRASL coacervates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAMRA-RRASLRRASL</td>
<td>1.0</td>
<td>800 ± 200</td>
<td>-</td>
</tr>
<tr>
<td>Alexa 488 PKA</td>
<td>0.24</td>
<td>1.8 ± 0.6</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Alexa 647 poly N15</td>
<td>0.020</td>
<td>16 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>Alexa 647 poly A15</td>
<td>0.020</td>
<td>23 ± 6</td>
<td>-</td>
</tr>
<tr>
<td>TAMRA-LRRASLG</td>
<td>1.0</td>
<td>240 ± 40</td>
<td>-</td>
</tr>
<tr>
<td>TAMRA-LRRApSLG</td>
<td>1.0</td>
<td>170 ± 20</td>
<td>-</td>
</tr>
<tr>
<td>Poly U/mixed phosphorylation RRASLRRASL coacervates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAMRA-RRASLRRASL</td>
<td>1.0</td>
<td>800 ± 200</td>
<td>-</td>
</tr>
<tr>
<td>Alexa 488 λ-PP</td>
<td>0.25</td>
<td>2.5 ± 0.7</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>Alexa 647 poly N15</td>
<td>0.020</td>
<td>12 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>Alexa 647 poly A15</td>
<td>0.020</td>
<td>13 ± 3</td>
<td>-</td>
</tr>
</tbody>
</table>

To evaluate the potential for phosphorylation state to control partitioning into droplets, we measured the concentration of fluorescently-labeled Kemptide and phosphorylated Kemptide (LRRApSLG). The more positively charged Kemptide (pI = 12.0\textsuperscript{45}) partitioned stronger than pKemptide (pI = 7.4\textsuperscript{45}), indicating that this post-translational modification could be used to modulate local peptide concentrations. We also examined the partitioning of two short strands of fluorescent RNA, a poly N15 (where N= any base) and a poly A15. The poly A15 can base pair with poly U to a greater extent than poly N15. We did not observe a strong difference in partitioning between these sequences, however both accumulated to a greater extent in the poly U/RRASLRRASL coacervates than the mixed phosphorylation state coacervates. A
possible explanation is that the poly U/RRASLRRASL coacervates, with their more positive peptide, provide less electrostatic repulsion of the labeled RNAs compared to the mixed phosphorylation state.

### 2.3.5 Reversible Coacervation by Controlling Phosphatase and Kinase Activities.

Coacervates could be reversibly formed and dissolved in the same solution by controlling the phosphatase and kinase activity by addition of reagents. The turbidity and kinetic plots are given in Figure 2-14 and Figure 2-15. For the first 18 minutes, λ-PP acted upon RRApSLRRApSL and mixed phosphorylation state coacervates were generated. RRASLRRASL concentration reached 510 ± 40 µM, well above the CCC range of 200-250 µM. At 20 minutes, EDTA, ATP and PKA were added. EDTA was added to complex Mn$^{2+}$ because kinase activity was significantly lowered in the presence of Mn$^{2+}$. EDTA preferentially complexed Mn$^{2+}$ over the other metal cations present.$^{46}$ The coacervates began to dissolve and the solution became transparent. RRASLRRASL concentration was 120 ± 50 µM at 38 minutes, below the CCC range. At 40 minutes, more Mn$^{2+}$ was added to reactivate the λ-PP already present in solution. The solution became turbid again, at a slower rate than the first cycle due to phosphate inhibition from the first cycle. At 78 minutes, RRASLRRASL reached 361 ± 4 µM, above the CCC range. Finally, we added more EDTA and ATP to the kinase already in solution, and the coacervates dissolved again (RRASLRRASL = 110 ± 50 µM at 98 minutes). We confirmed that coacervate formation/dissolution was the result of RRASLRRASL
concentration rather than changes in solution ionic strength/composition from adding the reagents (Figure 2-16).

Figure 2-14. Reversible coacervation modulated by changes in enzyme activity. (A) A schematic illustration of modulation of the enzyme activities during the reversibility reaction. (B) A turbidity plot of the reaction as a function of time. (C) Reaction activity of the peptide species as a function of time. Error bars represent standard deviation of 3 trials.
Figure 2-15. Additional reversibility trials. These were performed under identical conditions to those in Figure 2-14.

Figure 2-16. Turbidity control experiments. (A) At 20 minutes, EDTA and ATP were added, but the kinase was not. The sample remained turbid. (B) At 20 minutes, EDTA, ATP and kinase were added as normal. At 40 minutes, Mg\(^{2+}\) was added instead of Mn\(^{2+}\). The sample does not become turbid. (C) At 20, the EDTA, ATP, and kinase were added as normal and at 40 minutes the Mn\(^{2+}\) was added as normal. At 80 minutes, ADP and EDTA were added. The turbidity decreased, but not nearly as much as from the active kinase with ATP.
2.4 Discussion

The RNA/peptide system described here provides a minimal synthetic model for regulation of intracellular droplet formation by post-translational modifications. Complex coacervation of relatively long poly U RNA polyanion with a short cationic peptide led to formation of liquid droplets enriched in macromolecules. Enzymatic addition or removal of a phosphate moiety at even one of two sites on the peptide was sufficient to cause assembly/disassembly by altering the electrostatic interactions between the peptide and RNA. Peptide phosphorylation state and RNA sequence also influenced solute partitioning into the droplets. Our results in this minimal system therefore support a role for phosphatase and kinase activity in regulating the reversible formation and occupancy of liquid-like intracellular compartments.

Our synthetic “liquid organelle” system lacks specific biorecognition between components, and as such is presumably much simpler than its in vivo counterparts. Here, interactions between the poly U RNA and the peptide were designed to be largely electrostatic in nature. Although both the RNA and the peptide also contain hydrophobic moieties (i.e., uridine bases on the RNA and hydrophobic amino acid residues on the peptide), a simple charge-based view of the interactions was both necessary and sufficient to predict when association to form liquid droplets would occur and when it would not. Indeed, we found that the negatively-charged surface of silica beads acted as a nucleation site for coacervate droplets, effectively serving as an even better polyanion than poly U. The ability to grow liquid droplets around pre-existing structures is reminiscent of intracellular liquid bodies and could serve as simple models for structures
such as nucleolar organizing regions, which are clusters of ribosomal RNA around which nucleoli form.\(^9\)

The RRASLRRASL/poly U system was extremely sensitive to peptide charge, switching on/off its ability to form droplets with the removal/addition of a single phosphate. This sensitivity is expected to be beneficial in vivo, and could presumably be tuned to suit the physiological requirements of the cell by taking advantage of the greater complexity in molecular composition found intracellularly. For instance, liquid-liquid phase separation in biology often involves stretches of intrinsically disordered proteins that contain many repeats of interacting domains;\(^{47-49}\) this increased multivalency as compared to the short peptide used here would provide greater interaction strength and stabilize coacervation at higher (i.e. physiological) salt concentrations.

2.5 Experimental Section

2.5.1 Materials

Poly(uridylic acid) potassium salt (MW 600 – 1,000 kDa), protein kinase A catalytic subunit from bovine heart, HEPES, HEPES sodium salt, dithiothreitol, magnesium chloride, manganese chloride, and adenosine 5’-triphosphate disodium salt hydrate were purchased from Sigma-Aldrich (St Louis, MO). Lambda protein phosphatase was obtained from New England Biolabs (Ipswich, MA). RRASLRRASL, RRApSLRRASL, RRApSLRRApSL, RRAPSLRRApSL, and N terminus labeled tetramethylrhodamine (TAMRA) RRASLRRASL, LRRASLG, and LRRApSLG were
custom synthesized as the trifluoroacetic acid salts by GenScript (Piscataway, NJ). An amino acid analysis report was requested with the peptides to determine the net peptide content for calculation of peptide concentrations. Table 5-2 gives the pI and molecular weight of the peptides and enzymes. The Alexa Fluor 488 Microscale Protein Labeling Kit was purchased from Life Technologies (Carlsbad, CA) to label the enzymes according to Life Technologies instructions. The RNA oligos poly A15 and poly N15 (random sequence), each with a 5’ Alexa Flour 647 label, were synthesized by Integrated DNA Technologies (Coralville, IA) All components were dissolved in nuclease-free water from Amresco (Solon, OH) or deionized water from a Barnstead Nanopure System with a resistivity of at least 18.2 MΩ·cm.

Table 2-2. Molecular weight and isoelectric point (pI) of solutes in this work.

<table>
<thead>
<tr>
<th>System component</th>
<th>Molecular weight (g/mol)</th>
<th>Isoelectric point (pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly U</td>
<td>600k-1000k</td>
<td>-</td>
</tr>
<tr>
<td>RRASLRRASL</td>
<td>1189</td>
<td>12.5(^a)</td>
</tr>
<tr>
<td>RRApSLRRASL</td>
<td>1267</td>
<td>11.5(^a)</td>
</tr>
<tr>
<td>RRASLRRAlpSL</td>
<td>1267</td>
<td>11.5(^a)</td>
</tr>
<tr>
<td>RRApSRRAPSL</td>
<td>1345</td>
<td>7.6(^a)</td>
</tr>
<tr>
<td>TAMRA- RRASLRRASL</td>
<td>1598</td>
<td>-</td>
</tr>
<tr>
<td>TAMRA-LRRASLG</td>
<td>1184</td>
<td>-</td>
</tr>
<tr>
<td>TAMRA-LRRAPSLG</td>
<td>1264</td>
<td>-</td>
</tr>
<tr>
<td>Lambda Protein</td>
<td>25,219</td>
<td>5.5(^a)</td>
</tr>
<tr>
<td>Phosphatase ((\lambda)-PP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein Kinase A (PKA)</td>
<td>40,862</td>
<td>7.0-7.8(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Calculated using Scansite\(^45\)
\(^b\) A range of 3 isozymes\(^50\)
2.5.2 Coacervate Sample Preparation

Samples were prepared by adding all of the components from stock solutions. Peptide stock solutions were made in deionized water. For samples without enzymes, the poly U was the last component added. When enzymes were added, the enzyme was the last component. The samples were thoroughly mixed by pipetting up and down. Zeta potential measurements were collected on a Malvern Zetasizer Nano ZS using a capillary cell. For enzyme activity measurements, the enzyme was added and mixed by pipette and the reaction proceeded without mixing on a heat block at 37 °C. Aliquots were removed and added to 7× the volume of a solution of acidic salt (250 mM NaCl, 150 mM H₃PO₄, pH ~1) in order to stop the kinase or phosphatase and dissolve samples containing coacervates for HPLC analysis.

2.5.3 HPLC of Peptides and Phosphopeptides

The peptides were separated and quantified using a modified procedure for separating Kemptide and phospho-Kemptide. HPLC analysis was done on an Agilent 1260 Infinity HPLC system with a 1260 Infinity Quaternary Pump, Thermostatted Column Compartment, Diode Array Detector, Autosampler and Agilent Chemstation software using an Agilent ZORBAX Eclipse Plus C18 Rapid Resolution column (4.6 x 100 mm, 3.5 µm) and a ZORBAX Eclipse Plus C18 Analytical Guard column (4.5 x 12.5 mm, 5 µm). The method used a gradient of HPLC grade water with 0.1% (v/v) trifluoroacetic acid (A) and acetonitrile/HPLC water (75:25) with 0.1% (v/v) trifluoroacetic acid (B). A linear gradient of 0-42% of B for the first 20 minutes and 42%
to 100% B from 20 to 25 minutes was used. A flow rate was 1 mL/min using an analysis wavelength of 220 nm and a 25 °C column compartment temperature.

2.5.4 Turbidity Measurements

Turbidity is a method used to determine if phase separation has occurred in solution. The turbidity varies between 0% (100% light transmittance) and 100% (no light transmittance). Turbidity measurements were collected using an Agilent 8453 diode-array UV-visible spectrophotometer with Agilent ChemStation software and an Agilent 89090A Peltier temperature controller at a wavelength of 500 nm.

2.5.5 Confocal Microscopy

Confocal images were acquired using a Leica TCS SP5 laser scanning confocal inverted microscope (LSCM) with a 63x oil objective and an Instec TSA021 temperature controlled stage held at 37 °C. Samples were prepared on no. 1.5 microscope coverslips with a silicone spacer. Excitation/Emission wavelengths used were: 488 nm/509-529 nm for Alexa 488 labeled enzymes, 543 nm/569-589 nm for TAMRA-peptides, and 633 nm/655-685 nm for Alexa 647 labeled RNAs. TAMRA peptide samples were imaged separately from the other components to prevent signal overlap. Solute concentrations were calculated by measuring the pixel intensity of the fluorescent images and comparing to calibration curves of known concentrations of solutes prepared in buffer imaged under identical conditions.
2.5.6 Reversibility Reaction Conditions

The following reaction conditions were used for the reversibility reaction: At 0 minutes: 50 mM HEPES buffer, pH 7.4, 1 mM RRApSLRRApSL, 0.05 wt. % poly U, 4 mM MgCl₂, 0.2 mM MnCl₂, 1.6 mM DTT, and 0.002 mg/mL λ-PP in a total volume of 250 µL. At 18 minutes, a 10 µL aliquot was removed for analysis, followed by addition of reagents to final concentrations of 0.6 mM EDTA, 1.25 mM ATP, and 0.0025 mg/mL PKA at 20 minutes to replace the 10 µL. At 38 minutes, a 10 µL aliquot was removed. MnCl₂ was added at a concentration of 0.8 mM (1 mM total) at 40 minutes. At 78 minutes, another aliquot was removed, followed by addition of 0.6 mM EDTA (1.2 mM total) and 1.5 mM ATP (2.75 mM total) at 80 minutes.

2.6 References


Chapter 3

Coupled Enzyme Reactions Performed in Heterogeneous Reaction Media:
Experiments and Modeling for Glucose Oxidase and Horseradish Peroxidase
in a PEG/Citrate Aqueous Two-Phase System


The author of this dissertation and Bradley Davis contributed equally. The author focused on conceiving and designing experiments and optimizing the experimental conditions. Bradley Davis focused on data management and communicated the experimental data to the chemical engineers. The author and Bradley Davis both collected and analyzed the data and wrote the manuscript. Negar Hashemian developed the mathematical model and wrote the mathematical modeling portion of the manuscript.

3.1 Abstract

The intracellular environment in which biological reactions occur is crowded with macromolecules and subdivided into microenvironments that differ in both physical properties and chemical composition. The work described here combines experimental and computational model systems to help understand the consequences of this heterogeneous reaction media on the outcome of coupled enzyme reactions. Our experimental model system for solution heterogeneity is a biphasic polyethylene glycol (PEG)/sodium citrate aqueous mixture that provides coexisting PEG-rich and citrate-rich phases. Reaction kinetics for the coupled enzyme reaction between glucose oxidase (GOX) and horseradish peroxidase (HRP) were measured in the PEG/citrate aqueous
two-phase system (ATPS). Enzyme kinetics differed between the two phases, particularly for the HRP. Both enzymes, as well as the substrates glucose and H$_2$O$_2$, partitioned to the citrate-rich phase; however, the Amplex Red substrate necessary to complete the sequential reaction partitioned strongly to the PEG-rich phase. Reactions in ATPS were quantitatively described by a mathematical model that incorporated measured partitioning and kinetic parameters. The model was then extended to new reaction conditions, i.e., higher enzyme concentration. Both experimental and computational results suggest mass transfer across the interface is vital to maintain the observed rate of product formation, which may be a means of metabolic regulation in vivo. Although outcomes for a specific system will depend on the particulars of the enzyme reactions and the microenvironments, this work demonstrates how coupled enzymatic reactions in complex, heterogeneous media can be understood in terms of a mathematical model.

3.2 Introduction

Important differences between the dilute buffers typically used for biochemical studies and the intracellular environments in which biomolecules such as enzymes actually operate are increasingly realized.\textsuperscript{1-7} These can include the following: (1) excluded volume effects due to high concentrations of other background molecules, (2) attractive and repulsive interactions between molecules of interest and other solutes or solvent molecules, and (3) physical and chemical heterogeneity in the reaction medium. The first two differences can be approximated by including macromolecular crowding agents either alone or in concert with small molecules that interact with
biomacromolecules of interest. In this manuscript, we focus on heterogeneity, which has received considerably less attention compared to crowding and chemical effects. The existence of microenvironments within the cell could impact local and overall reaction kinetics due to variations in local reactant, enzyme, or inhibitor concentrations, chemical interactions, excluded volume, and/or local viscosities. Here, we achieve chemical and physical heterogeneity by using a polyethylene glycol (PEG)/citrate aqueous two-phase system (ATPS). This ATPS has PEG-rich and citrate-rich phases that differ substantially in viscosity, macromolecular crowding, and salt concentration. Thus, although its components are not those of the intracellular environment, it offers a test system for evaluating the impact of heterogeneous media on a coupled biochemical reaction.

Experimental and modeling studies have demonstrated the impact of macromolecular crowding agents such as polyethylene glycol (PEG), dextran, or Ficoll on the structure, association, and activity of various biomacromolecules. A major aspect of the macromolecular crowding effect is due to excluded volume from intracellular polymers (proteins, nucleic acids, polysaccharides) that combined can make up ~30% weight percent in cytoplasm. Additionally, chemical effects due to attractive and repulsive interactions between molecules (solutes and/or solvent molecules) can alter outcomes as compared to dilute solution. These chemical effects are observed even for small molecule cosolutes that do not exclude appreciable volume (e.g., ethylene glycol, trimethylamine $N$-oxide (TMAO)). For example, Record and co-workers found that DNA duplexes and hairpins were destabilized by small molecular weight PEG due to favorable interactions with the PEG monomers. Such efforts to better mimic the
crowded environments in which biomacromolecules function are very important to our understanding of macromolecular crowding in vivo, but because they are performed in homogeneous media, they do not capture all aspects of the intracellular environment.

The intracellular milieu is heterogeneous in addition to being crowded. Different concentrations of various small molecules, ions, proteins, and nucleic acids are found in different regions within the cell and its compartments. The concentration of biomolecules into subcellular compartments could offer a means of increasing reaction rates and controlling the site of a reaction, or regulating a pathway based on the formation and dissolution of a compartment. Reaction compartmentalization is thought to be crucial for a variety of cellular functions including metabolism, transcription and translation, and cell division. For example, the citric acid cycle is confined to the mitochondrial membrane, and lysosomes perform their catabolic functions separate from the rest of the cell. In addition to the membrane-bounded organelles, numerous other subcellular and subnuclear compartments have been identified that lack membranous boundaries. Some structures are transient, such as the purinosome, with formation/dissolution thought to correspond to biological activity. Two nonmembrane bounded compartments, the nucleolus and P-granules, have recently been demonstrated to behave as liquids, suggesting that these subcellular structures are the result of aqueous phase separation.

Single enzymatic reactions have been performed in polymer/salt ATPS and aqueous/organic biphasic media; these systems are attractive for bioconversion reactions for which the substrate and enzyme partition to the same phase (generally the bottom, salt-rich, or aqueous phase), while the product partitions to the other phase (generally the
top, polymer-rich, or organic phase), where it is prevented from inhibiting the reaction and can be continuously removed if desired.\textsuperscript{31-33} These reactions are often performed with bulk phases (macroenvironments) that have a well-defined interfacial area rather than with media in which one phase exists as droplets dispersed in the other (microenvironments); this facilitates continuous product removal. A sequential reaction of lipase and lipoxygenase has been performed in a macroheterogeneous octane/aqueous buffer system of carefully controlled interfacial area, where the substrates partitioned to the octane phase and the enzymes to the aqueous phase. Experiments and simulations showed that the rate of the second reaction was determined by the first reaction and also by mass transfer in this system.\textsuperscript{34}

Few studies have attempted to mathematically model enzymatic reactions occurring within heterogeneous media.\textsuperscript{34} Instead, most have focused on predicting partitioning coefficients in the equilibrium state\textsuperscript{35, 36} or the phase behavior of the ATPS.\textsuperscript{14,37} To accomplish this goal, they employed thermodynamic models based on Gibbs excess (G\textsuperscript{E}-models). On the other hand, a few papers have exploited models describing the behavior of heterogeneous liquid–liquid (organic/aqueous) systems to find the concentration profile in time.\textsuperscript{38, 39} Quadros et al. used linear regression to derive a statistical model to estimate the product concentration,\textsuperscript{38} while van Woezik and Westerterp used conservation equations to derive a mechanistic model to study the reaction rates in a semi batch reactor.\textsuperscript{39} Additionally, a continuous flow of ATPS in which there is no chemical reaction has been modeled to understand the steady state and transient behavior of the system.\textsuperscript{40} However, to the knowledge of the authors, there is no mathematical model presented in the open literature to predict the dynamic behavior of
partitioned species in an ATPS, in which both mass transfer and chemical reactions have to be accounted for simultaneously. Moreover, previous modeling efforts did not consider the microscale geometry of the model and as a result investigated only changes of average concentrations of the species with time. In this work, we take a mechanistic modeling approach and develop a complex model that also includes an interesting interface geometry.

Here, the well-studied enzymes glucose oxidase (GOX) and horseradish peroxidase (HRP) were used to perform a sequential reaction in a PEG/citrate ATPS that was mixed to generate droplets during the reaction (Scheme 3-1). The PEG/citrate ATPS was selected for this study because its two aqueous phases differ greatly in composition: the top, polymer-rich phase is crowded and viscous, while the bottom, citrate-rich phase is quite salty. The phases impact enzyme kinetics differently, more so than would be expected from typical polymer/polymer ATPS such as the PEG/dextran system where both phases are more similar in crowding and salt concentration. Additionally, partitioning leads to differences in local concentrations for the enzymes and some of the small molecules. A computational model that takes into account measured enzyme kinetics for each phase as well as enzyme and substrate partitioning was then derived and informed on the basis of experimental results for the two-phase system. Through formulating the governing mass transfer equations for this system, we obtained a system of coupled PDEs. Solving these equations simultaneously using finite element methods in COMSOL provided us with temporal as well as spatial concentration distributions of the species in both phases. The kinetics for the sequential reaction were well-described by
this model, which was then used to predict reaction kinetics at higher enzyme concentrations.

Scheme 3-1. (A) The Sequential Enzyme System of Glucose Oxidase (GOX) and Horseradish Peroxidase (HRP) with the Substrates, Intermediates, and Products of Interest Shown;\(^a\) (B) Illustration Depicting How the Enzymes, Substrates, and Products Partition within a PEG:Citrate ATPS

\(^a\) The reaction was monitored by the fluorescent product resorufin.

This work demonstrates how, despite substantial and nontrivial media effects for the different phases, by experimentally determining key parameters (partitioning coefficients, \(K_M\), \(k_{cat}\) in each phase), a sequential reaction within a heterogeneous reaction
medium can be understood in terms of simple kinetic and partitioning experiments with the aid of mathematical modeling.

3.3 Results and Discussion

To understand the sequential enzyme reaction of Scheme 3-1 in the ATPS, and to generate an accurate mathematical model for this reaction, it was first necessary to characterize the content and kinetic effects of the individual phases. Enzymatic reactions in the full ATPS were then performed and a mathematical model derived to describe the kinetics in this system. The model was then used to predict the enzyme activity at a higher concentration of HRP.

3.3.1 Phase Composition and Properties

The PEG:citrate ATPS had an overall composition of 13.3 w/w % PEG 8 kDa and 10.0 w/w % citrate prepared in a 50 mM sodium phosphate buffer pH 7.4 with 1 mM EDTA. This ATPS has roughly equal volumes of a PEG-rich top phase and a citrate-rich bottom phase, which have very different chemical and physical properties that impact the enzymatic reactions (Table 3-1). The PEG-rich phase contained most of the polymer, making it much more macromolecularly crowded (24 vs <1 w/w % PEG) and ~17× more viscous than the citrate-rich phase, while the citrate-rich phase was considerably saltier (1.1 M citrate vs ~100 mM). A phase diagram for the PEG 8 kDa/citrate system, with the location of the composition highlighted, is included as Figure 3-1.
Table 3-1. Physical Properties of a 13.3% PEG 8k and 10% citrate ATPS

<table>
<thead>
<tr>
<th>Phase</th>
<th>wt% PEG</th>
<th>*Molal PEG</th>
<th>wt% citrate</th>
<th>*Molal citrate</th>
<th>Viscosity (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-rich</td>
<td>23.79 ± 0.09</td>
<td>0.04</td>
<td>3.53 ± 0.09</td>
<td>0.1</td>
<td>38.8 ± 0.3</td>
</tr>
<tr>
<td>Citrate-rich</td>
<td>0.6 ± 0.1</td>
<td>0.0008</td>
<td>14.9 ± 0.3</td>
<td>0.6</td>
<td>2.25 ± 0.01</td>
</tr>
</tbody>
</table>

*Solutions were prepared based on mass of solute and mass of solvent. Molality approximated based on experimentally determined weight percents for the phases, see Experimental Section.

Figure 3-1. Phase diagram of PEG-8k and citrate. Samples prepared above the coexistence curve (red) phase separate and were visually turbid whereas those below exist as a single phase (blue). The 13.3% PEG-8k and 10% citrate composition used in this study is marked with a black diamond.

3.3.2 Enzyme Kinetics in the Individual Phases

On the basis of their different compositions, we anticipated that enzyme kinetics would be different in the two phases of the ATPS. Results from Michaelis–Menten
assays performed in each of the individual phases are shown in Figure 3-2 and Table 3-2. GOX kinetics were similar, but not identical, between the two phases, while differences in HRP kinetics were substantial. The $K_M$ value for $H_2O_2$ was $\sim 30 \times$ lower in the PEG-rich phase than in the citrate-rich phase, and $k_{cat}$ was nearly 2 orders of magnitude lower in the PEG-rich phase. $K_M$ for the other substrate of HRP, Amplex Red, could not even be measured in the PEG-rich phase as the rate continued to increase with increasing Amplex Red concentration even at high concentrations (up to 1 mM). Possible explanations for these large effects on the HRP reaction in the PEG-rich phase include changes in enzyme conformation or the increased solubility of the hydrophobic substrate Amplex Red in the PEG-rich phase. PEG has been reported to interact with hydrophobic amino acids in proteins and to increase the solubility of hydrophobic solutes in aqueous solution; we reason it may be competing with the enzyme for Amplex Red.

Figure 3-2. Michaelis–Menten assays for GOX and HRP in the PEG-rich phase (open circles) and citrate-rich phase (closed circles). (A) Effect of glucose concentration on GOX rate, measured at 0.05 U/mL GOX (2.1 nM). Effect of substrate concentration on HRP rate for (B) peroxide and (C) Amplex Red. HRP concentrations were 0.005 U/mL (0.45 nM) for PEG-rich phase experiments and 0.0005 U/mL (0.045 nM) for the citrate-rich phase.
Table 3-2. Michaelis–Menten Constants of GOX and HRP within PEG:Citrate ATPS

<table>
<thead>
<tr>
<th></th>
<th>$K_M$ (µM)</th>
<th>$V_{max}$ (µmol/min/mg)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GOX (glucose)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-rich phase</td>
<td>3400 ± 400</td>
<td>73 ± 2$^a$</td>
<td>194 ± 5</td>
</tr>
<tr>
<td>citrate-rich phase</td>
<td>5400 ± 200</td>
<td>66.9 ± 0.6$^a$</td>
<td>178 ± 2</td>
</tr>
<tr>
<td><strong>HRP (H$_2$O$_2$)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-rich phase</td>
<td>5 ± 1</td>
<td>28 ± 2$^b$</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>citrate-rich phase</td>
<td>150 ± 20</td>
<td>2600 ± 200$^c$</td>
<td>1900 ± 100</td>
</tr>
<tr>
<td><strong>HRP (Amplex Red)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-rich phase</td>
<td>n.a.$^d$</td>
<td>n.a.$^b$</td>
<td>n.a.</td>
</tr>
<tr>
<td>citrate-rich phase</td>
<td>60 ± 10</td>
<td>2300 ± 100$^c$</td>
<td>1700 ± 100</td>
</tr>
</tbody>
</table>

$^a$V$_{max}$ for enzyme concentrations of 0.05 U/mL. $^b$V$_{max}$ for enzyme concentrations of 0.005 U/mL. $^c$V$_{max}$ for enzyme concentrations of 0.0005 U/mL. $^d$Not applicable. Rate increased linearly to the limit of substrate solubility.

We also performed the sequential reaction in the individual phases. Reactions contained 0.05 U/mL GOX, 0.005 U/mL HRP, 1 mM glucose, and 50 µM Amplex Red. An initial lag period was observed for the first ~3 min in the citrate-rich phase as the concentration of peroxide generated by GOX increased (Figure 3-3). After 10 min, 15.4 ± 1.6 µM resorufin had been formed in the citrate-rich phase as compared with only 0.69 ± 0.05 µM in the PEG-rich phase, an approximately 22-fold difference. These results, along with the individual assays described above, suggested that PEG had a detrimental effect on HRP activity, particularly with respect to Amplex Red. This in turn made the sequential reaction much slower in the PEG-rich phase than the citrate-rich phase.
Figure 3-3. Product formation of the sequential GOX and HRP reaction in the citrate-rich phase (inverted orange triangles) and the PEG-rich phase (blue triangles).

3.3.3 Partitioning

All of the reactions described above were performed in single phases. When both phases of the ATPS are present, the enzyme and substrate concentrations may differ between the phases; this partitioning can impact the sequential kinetics. Solute partitioning is quantified as the partitioning coefficient, \( K = \frac{C_P}{C_C} \), where \( C_P \) and \( C_C \) are the solute’s concentration in the PEG-rich and citrate-rich phases, respectively. Except where noted, a 1:1 volume ratio of PEG-rich to citrate-rich phases was used for these measurements. Table 3-3 reports partitioning values for enzymes and small molecules of interest in the sequential reaction. Enzymes were fluorescently labeled with different dyes for these measurements and were tested simultaneously, since any potential protein–protein interactions would affect their partitioning coefficient.\(^{41}\) Both enzymes were more concentrated in the citrate-rich phase. \( K_{\text{GOX}} = 0.036 \) and \( K_{\text{HRP}} = 0.63 \), indicating a 28-fold and 1.6-fold concentration excess for GOX and HRP, respectively, in
this phase. Glucose and peroxide also partitioned somewhat to the citrate-rich phase ($K_g = 0.53$ and $K_p = 0.6$, respectively), while the hydrophobic substrate and product strongly partitioned to the PEG-rich phase with $K_a = 60$ for Amplex Red and $K_r = 23$ for resorufin.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Partitioning Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4:1</td>
</tr>
<tr>
<td>GOX</td>
<td>0.023 ± 0.006</td>
</tr>
<tr>
<td>HRP</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>glucose</td>
<td>n.a.</td>
</tr>
<tr>
<td>hydrogen peroxide</td>
<td>n.a.</td>
</tr>
<tr>
<td>Amplex Red</td>
<td>n.a.</td>
</tr>
<tr>
<td>resorufin</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

GOX partitioning was determined at the concentration used in the enzyme assays (2.1 nM). HRP was measured at 4.5 nM HRP because at 0.45 nM, which was used in the sequential assays, the fluorescence was too low to quantify. Not applicable. Partitioning coefficients of small molecules were only measured at a 1:1 PEG:citrate volume ratio.

The partitioning coefficient is a thermodynamic constant and should not normally change with volume ratio or solute concentration; however, exceptions are well-known for proteins in PEG:salt ATPS because the salt-rich phase may “salt out” the protein into the PEG-rich phase or it may precipitate to the interface.\(^\text{45-47}\) The increased apparent hydrophobicity of proteins in high salt solutions can lead to changes in partitioning, in particular an increased preference for the more hydrophobic PEG-rich phase, and/or multimerization or aggregation of the protein. Additionally, at distances far from the critical point of the phase diagram, small deviations from the tie line, caused by minor dilution from adding the enzymes/substrates to the ATPS, can change partitioning even
in the absence of salting out effects. Therefore, we measured the partitioning coefficients of GOX and HRP at all the volume ratios and diluted the samples by the same amount, as will be done with the assays below. We did not see evidence of protein precipitation in our system (see below); however, differences in partitioning with volume ratio were observed for both proteins (Scheme 3-2, Table 3-3). These differences in enzyme partitioning with volume ratio, while underscoring the importance of careful analysis of the experimental system, also enabled us to examine the effect of such changes on the overall reaction kinetics. As the volume of the citrate-rich phase increased, both enzymes partitioned less strongly. GOX remained partitioned in the citrate-rich phase; however, for HRP, which partitioned only slightly to the citrate-rich phase at a volume ratio of 1:1, a switch in partitioning preference to the PEG-rich phase was observed at a 1:4 PEG-rich to citrate-rich volume ratio. We also measured the effect of enzyme concentration on partitioning at the three volume ratios (Figure 3-4). $K_{\text{GOX}}$ was sensitive to both concentration and volume ratio. $K_{\text{HRP}}$ however was insensitive to the concentration of HRP over the range tested (4.5–45 nM). We assume $K_{\text{HRP}}$ measured at 4.5 nM HRP is valid at 0.45 nM, the concentration used in Figure 3-3, which was below our quantification limits for $K_{\text{HRP}}$ determination.
Scheme 3-2. After an ATPS is prepared, the phases can be separated and later reconstituted to form PEG:citrate volume ratios. From left to right: 4:1, 1:1, and 1:4.

Figure 3-4. The partitioning coefficient of GOX (top) and HRP (bottom) was measured while varying enzyme concentration in the experimental volume ratios: 4:1 (squares), 1:1 (diamonds), and 1:4 (circles). GOX partitioning is volume ratio and enzyme concentration dependent, whereas HRP partitioning is volume ratio dependent.
Due to the known salting out behavior in these systems as discussed above, confocal microscopy was used to determine if any aggregation of protein at the interface could be observed. Fluorescently labeled enzymes were added at 21 and 45 nM of GOX and HRP, respectively (10× GOX, and 10× or 100× used for HRP as compared to the assays). The sample was vortexed and quickly placed on a coverslip for imaging (Figure 3-5). The observed partitioning of the enzymes was consistent with the bulk partitioning measurements and no obvious aggregation or precipitation to the interface was observed, although any multimeric complexes that remained in suspension may be too small to be seen. Ideally, we would have measured resorufin production via confocal microscopy as well; however, laser illumination has been shown to induce resorufin production in the presence of HRP, even without the peroxide substrate.48 Unfortunately, the rate of this undesirable reaction was too rapid to be ignored in our system (Figure 3-6); hence, we were unable to experimentally observe the spatial distribution of resorufin production at the microscale.
Figure 3-5. GOX and HRP partitioned in 4:1, 1:1, and 1:4 PEG:citrate volume ratios. Transmitted light (DIC) images are on the left. Confocal fluorescence images have been false-colored: blue is PEG 5000-Alexa 647, to indicate the location of the PEG-rich phase while green and red channels indicate fluorescence from Alexa 488-labeled GOX and Alexa 546-labeled HRP, respectively. Labeled GOX and HRP were included at 0.5 U/mL. The drop sizes in the figure are not meant to be representative of the entire sample, as the coalescence rate of this ATPS was very high and the true droplet diameter while mixing certainly resulted in a smaller drop size distribution; rather it is meant to show the enzyme localization. Notably, the HRP appears to be partitioned relatively equally in the 1:4 system, partitioned slightly in the 1:1, and partitioned the strongest in the 4:1. The GOX is partitioned to the citrate-rich phase for each ratio. Scale bar = 250 µm.
Figure 3-6. Assays could not be quantified in real time by confocal microscopy due to background resorufin production. (A) Assay conducted in a 1:4 PEG:citrate volume ratio. (B) Control without glucose. In both (A) and (B), transmitted light (DIC) images are on the left while blue and red channels correspond to fluorescently-labeled PEG to indicate the PEG-rich phase droplets, and produced resorufin, respectively. It is evident that resorufin is being formed through photooxidation as described previously.\textsuperscript{48} Scale bar = 250 µm.

3.3.4 Enzyme assays in the ATPS

We used the same conditions as the individual phases; continuously mixing the system induced the formation of phase droplets, increasing the surface area for exchange of enzymes and substrates between the phases. The rate of formation of resorufin was significantly different among the volume ratios with the trend (PEG-rich: citrate-rich) 4:1
<1:1 < 1:4 (Figure 3-7). Interpretation of these data is nontrivial due to the differences in reaction rates in the two media, the partitioning of small molecules and enzymes, and the variation in enzyme partitioning with volume ratio. In an effort to better understand the reactions in ATPS, we also conducted assays in which the enzymes and substrates were partitioned, but there was no interface available. This was achieved by adding all of the reaction components and physically separating the two phases, thereby allowing the reactions to proceed with mixing in separate containers. We observed that, for all of the prepartitioned phase controls, resorufin was produced quickly in the citrate-rich phase and leveled off at the prepartitioned amount of Amplex Red that was in that phase. The PEG-rich phase controls proceeded linearly throughout at each ratio at a much slower rate (Figure 3-7).

Figure 3-7. PEG:citrate volume ratios (A) 4:1, (B) 1:1, and (C) 1:4. The assay conditions were 2.1 nM GOX, 0.45 nM HRP, 1 mM glucose, and 50 µM Amplex Red. The points represent the experimental data. Black traces represent the model predictions to experimental ATPS volume ratios. Model parameters are obtained from prepartitioned assays in separated PEG-rich phase (blue triangles) and citrate-rich phase (inverted orange triangles) and single phase control assays (Figure 3-2). Insets highlight the phase-separated controls.
3.3.5 Mathematical Modeling

To describe the reaction within the two-phase system, we developed a mathematical model to describe the species concentration as a function of space and time that took into account the partitioning coefficients of the species as well as the reaction rates in each phase.

3.3.5.1 Computational Domain

We assumed the ATPS consisted of droplets of the first phase (the one in the least amount) in a second phase medium. Thus, on the basis of the PEG:citrate volume ratio, the droplets created contained either a PEG-rich phase or a citrate-rich phase surrounded by the opposite media. In the case of 1:1 ratio, we performed simulations for both PEG and citrate droplets; there was no significant difference in the simulation predictions. Assuming that the droplets are distributed uniformly within the solution, we limited our attention to the interactions between one droplet and its immediate surroundings, including other droplets (shown in Figure 3-8A). Taking advantage of the symmetry of the problem to further reduce the computational demands, one-eighth of the domain was simulated in COMSOL shown in Figure 3-8B. The droplets were approximated as spheres of radius $R = 50 \, \mu m$, based on the approximate droplet sizes imaged right after mixing, before extensive coalescence. The edge length of the cube, $d$, was calculated on the basis of the volume ratio of the phases.
Figure 3-8. Illustration of the geometry used in modeling. (A) For a 1:4 PEG:citrate volume ratio, PEG-rich phase droplets (blue) are within the continuous citrate-rich phase (orange). (B) The computational domain of the mathematical model is a subsection of part A.

3.3.5.2 Mass Conservation Equations

Modeling the mentioned two-phase system involved the coupling of two phenomena, i.e., mass diffusion and chemical reaction. Under the assumption that the diffusion coefficients are constant and that convective phenomena can be neglected for the considered simulation volume (i.e., the velocity variation of the fluid within the computational domain is negligible), the material conservation equations obtain the following partial differential equation (PDE) expression:

$$\frac{\partial c_{i,j}}{\partial t} - D_{i,j} \nabla^2 c_{i,j} = r_{i,j}$$

(Equation 3-1)

Here, $i$ denotes the species, i.e., $i = \{g, p, a, r\}$ which represents glucose, peroxide, Amplex Red, and resorufin, respectively; $j = \{P, C\}$ denotes the corresponding PEG-rich or citrate-rich phase, respectively; and $c_{i,j}$ and $D_{i,j}$ are the corresponding concentration and diffusion coefficient of species $i$ in phase $j$, respectively. The diffusion coefficients were calculated from the Stokes–Einstein equation, using the viscosities of the phases listed in
Table 3-1. The net rate of the reactions that involve species $i$ in phase $j$ are represented by $r_{i,j}$. To derive expressions that are consistent with the geometry and the boundary conditions of the problem, we employed spherical coordinates within the droplet domain and Cartesian coordinates within the surrounding cubic domain. The Laplace operator (of appropriate form depending on the coordinate system) is denoted by $\nabla^2$. For each species $i$, at the interface between the two phases of the droplet, the fluxes are continuous (interfacial mass conservation), and concentrations are related by the partitioning coefficient, $K_i$ (interfacial chemical potential equilibrium), presenting us with the boundary conditions:

$$
\left. c_{i,p}(r,t) \right|_{r=R} = K_i \left. c_{i,\infty}(r,t) \right|_{r=R}, 
- \left. D_{i,p} \nabla c_{i,p} \right|_{r=R} = - \left. D_{i,\infty} \nabla c_{i,\infty} \right|_{r=R} \quad \text{(Equation 3-2)}
$$

where $\nabla$ denotes the gradient operator of appropriate form depending on the coordinate system. In case one species is consumed or produced in one phase, these boundary conditions by transporting mass from one phase to the other guarantee that the partitioning condition is still satisfied and the species is always in a thermodynamic equilibrium at the interface.

Also, due to the symmetric nature of the model, periodic boundary conditions are applied at opposite faces of the cube:

$$
c_i |_a = c_i |_b, -D_{i,p} \nabla c_{i,p} |_a = D_{i,\infty} \nabla c_{i,\infty} |_b \quad \text{(Equation 3-3)}
$$

where $a$ and $b$ denote two opposite faces of the cube and $F_{i,j}|l = -D_{i,j} \nabla c_{i,j}|l$ represents the inward flux to the phase $j$ of the $i$ component at face $l$. To solve the presented system, the reaction expressions need be identified.
3.3.5.3 Reaction Rate Expressions

We assume oxygen is in excess in the considered experiments; therefore, the GOX reaction can be modeled by the Michaelis–Menten equation. However, in the second reaction, two substrates both influence the reaction rate. Consequently, the describing reaction rate requires a more complex expression. In this work, the Dalziel expression was utilized to model this enzymatic reaction rate.\(^{49, 50}\) Note that the species are sufficiently dilute that we may assume the product inhibitory effect is insignificant\(^{51}\) and we experimentally observed no rate decrease throughout the reaction.

The rate of glucose consumption is equal to the peroxide production rate, and it is dependent only on glucose and GOX concentrations. On the other hand, peroxide and Amplex Red are consumed in the second reaction and produce resorufin at the same rate. As a result, the net production rate of the various species for both phases can be expressed in the following form:

\[
r_{g,j} = -\frac{k_{\text{cat},1,j}c_{\text{GOX},j}c_{g,j}}{K_{M,j} + c_{g,j}} \\

r_{p,j} = \frac{k_{\text{cat},1,j}c_{\text{GOX},j}c_{g,j}}{K_{M,j} + c_{g,j}} - \frac{k_{\text{cat},2,j}c_{\text{HRP},j}c_{p,j}c_{a,j}}{k_{12,j} + k_{2,j}c_{a,j} + k_{1,j}c_{p,j} + k_{0,j}c_{p,j}c_{a,j}} \\

r_{a,j} = -\frac{k_{\text{cat},2,j}c_{\text{HRP},j}c_{p,j}c_{a,j}}{k_{12,j} + k_{2,j}c_{a,j} + k_{1,j}c_{p,j} + k_{0,j}c_{p,j}c_{a,j}} \\

r_{r,j} = \frac{k_{\text{cat},2,j}c_{\text{HRP},j}c_{p,j}c_{a,j}}{k_{12,j} + k_{2,j}c_{a,j} + k_{1,j}c_{p,j} + k_{0,j}c_{p,j}c_{a,j}}
\]

(Equations 3-4, 3-5, 3-6, 3-7)

The parameters \(k_{\text{cat},2,j}, k_{12,j}, k_{2,j}, k_{1,j},\) and \(k_{0,j}\) in Dalziel’s expression were unknown. Therefore, the experimental data of the prepartitioned phase controls and the
nonpartitioned controls in the PEG-rich phase and the citrate-rich phase, described earlier, were used to calculate the Dalziel parameters for HRP (Table 3-4).

Table 3-4. Optimized Parameters from Prepartitioned and Phase Controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>PEG-rich phase</th>
<th>Citrate-rich phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{k_{cat,2}}{k_0} )</td>
<td>([\mu M \cdot s]^{-1})</td>
<td>20.41</td>
<td>150.03</td>
</tr>
<tr>
<td>( \frac{k_{12}}{k_0} )</td>
<td>([\mu M]^2)</td>
<td>5.69</td>
<td>10.21</td>
</tr>
<tr>
<td>( \frac{k_1}{k_0} )</td>
<td>([\mu M])</td>
<td>0.03</td>
<td>7.86</td>
</tr>
<tr>
<td>( \frac{k_2}{k_0} )</td>
<td>([\mu M])</td>
<td>0.02</td>
<td>2.16</td>
</tr>
</tbody>
</table>

To identify the unknown parameters, a least-squares problem was formulated. To simplify the problem at hand, we assumed that the enzyme activities are constant during the experiment. Moreover, since during the control experiments the samples were being mixed, we assumed the concentration of all species in each phase is uniform (well mixed system assumption). Therefore, the original governing material conservation equations of Equation 3-1 simplified to a system of ordinary differential equations (ODEs) for the control experiments

\[
\frac{\partial c_{i,j}}{\partial t} = r_{i,j}(c_{n,j})
\]  

(Equation 3-8)
where $c_{n,j}$ represents a vector of all the species concentrations at phase $j$, respectively. Note that the ordinary differential equation system was employed only to calculate the reaction rate constants for the control experiments.

### 3.3.5.4 PDE Model

As expressed in Equations 3-1 – 3-3, the species concentrations versus time are obtained through the solution of a system of partial differential equations. To aid the stability of the simulation, it is convenient to nondimensionalize the equations:

$$ \frac{\partial C_{i,j}}{\partial T} - \alpha_{i,j} \nabla^2 C_{i,j} = R_{i,j} $$  \hspace{1cm} (Equation 3-9)

$$ R_{g,j} = -\frac{\beta_j C_{g,j}}{\gamma_j + C_{g,j}} $$  \hspace{1cm} (Equation 3-10)

$$ R_{p,j} = \frac{\beta_j C_{g,j}}{\gamma_j + C_{g,j}} - \frac{\phi_{0,j} C_{a,j} + \phi_{1,j} C_{p,j} + C_{p,j} C_{a,j}}{\phi_{12,j} + \phi_{2,j} C_{a,j} + \phi_{1,j} C_{p,j} + C_{p,j} C_{a,j}} $$  \hspace{1cm} (Equation 3-11)

$$ R_{a,j} = -\frac{\phi_{0,j} C_{p,j} C_{a,j}}{\phi_{12,j} + \phi_{2,j} C_{a,j} + \phi_{1,j} C_{p,j} + C_{p,j} C_{a,j}} $$  \hspace{1cm} (Equation 3-12)

$$ R_{r,j} = \frac{\phi_{0,j} C_{p,j} C_{a,j}}{\phi_{12,j} + \phi_{2,j} C_{a,j} + \phi_{1,j} C_{p,j} + C_{p,j} C_{a,j}} $$  \hspace{1cm} (Equation 3-13)

The dimensionless parameters are defined in Table 3-5. Furthermore, $c_{g,0}$ is the initial glucose concentration, and is equal to 1 mM in all experiments. The time length of the experiments is denoted by $\tau$ and is 10 min. The partial differential equation model was used to obtain species concentrations for the ATPS and draw conclusions.
Table 3-5. Definitions of the Dimensionless Parameters used in Equations 3-9 –3-13

<table>
<thead>
<tr>
<th>Dimensionless Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_i$</td>
<td>$\frac{c_i}{c_{g,0}}$</td>
</tr>
<tr>
<td>$T$</td>
<td>$\frac{t}{\tau}$</td>
</tr>
<tr>
<td>$\alpha_i$</td>
<td>$\frac{D_\tau}{d^2}$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$k_{cat,1}c_{GOX}\tau$</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>$\frac{K_M}{c_{g,0}}$</td>
</tr>
<tr>
<td>$\phi_0$</td>
<td>$\frac{k_{cat,2}c_{HRP}\tau}{k_0}$</td>
</tr>
<tr>
<td>$\phi_{12}$</td>
<td>$\frac{k_{12}}{c_{g,0}k_0}$</td>
</tr>
<tr>
<td>$\phi_2$</td>
<td>$\frac{k_2}{c_{g,0}k_0}$</td>
</tr>
<tr>
<td>$\phi_1$</td>
<td>$\frac{k_1}{c_{g,0}k_0}$</td>
</tr>
</tbody>
</table>

3.3.6 Simulation Results

Upon computing the reaction parameters, we employed the PDE model of Equations 3-9 –3-13 to simulate the system (using COMSOL). In Figure 3-9, we present the spatial distribution of the reactant and product species at a time of 10 min for the 1:4 volume ratio case. We observe that the three reactant concentrations are relatively
uniform in each phase. That would allow us to consider a well-mixed system assumption for each phase, simplifying the model description to the ODE form of Equation 3-14.

\[
\frac{\partial C_{i,c}}{\partial T}(1 + \zeta K_i) = R_{i,c} \left(C_{n,c}\right) + R_{i,P} \left(K_i C_{n,c}\right) \zeta \quad \text{(Equation 3-14)}
\]

Mathematically, a possible explanation is that the \( \alpha_i \) parameters in Equation 3-9 are large enough compared to the other terms’ coefficients and hence the concentration gradients are approximately zero. Note that the resorufin concentration varies significantly as a function of space; however, as it does not enter the reaction rate expressions and we only employ the total amount of resorufin produced when calculating the reaction rate constants, it does not affect the least-squares solution accuracy. This observation remained valid for the other investigated cases also (i.e., different volume ratios, drop sizes, and diffusivity) (Figures 3-10 – 3-13). That simplification would however cause some minor errors in prediction, which would become pronounced when the reactions become faster at higher enzyme concentrations (Figure 3-14). To prevent the onset of such prediction errors, we proceeded with the PDE model predictions.
Figure 3-9. Concentration profiles of all species in a 1:4 PEG:citrate volume ratio, depicted from the center of a phase droplet outward at the end of the assay. (A) glucose, (B) peroxide, (C) Amplex Red, and (D) resorufin show that there is a uniform distribution within each phase. The dotted line represents the 50 µm radius of the phase droplet.

Figure 3-10. Concentration profiles at the end of the assay (t = 10 min) within a 1:1 PEG:citrate volume ratio, depicted from the center of a phase droplet outwards. Panels show the various substrates and products: (A) glucose, (B) peroxide, (C) Amplex Red, and (D) resorufin profiles display that the concentration throughout the droplet is uniform, except in proximity of the interface. The dotted line represents the 50 µm radius of the phase droplet.
Figure 3-11. Concentration profiles within a 1:4 PEG:citrate volume ratio with 100× less diffusivity, depicted from the center of a phase droplet outwards at the end of the assay (t = 10 min). (A) Glucose, (B) peroxide, (C) Amplex Red, and (D) resorufin profiles illustrate that the concentration remains uniform with decreased diffusion. The dotted line represents the 50 µm radius of the phase droplet.

Figure 3-12. Concentration profiles of a 25 µm radius phase droplet, within a 1:4 PEG:citrate volume ratio, depicted from the center of a phase droplet outwards at the end of the assay (t = 10 min). (A) Glucose, (B) peroxide, (C) Amplex Red, and (D) resorufin profiles illustrate that the size of the droplet does not significantly alter the uniform distributions. The dotted line represents the 25 µm radius of the phase droplet.
Figure 3-13. Concentration profiles of a 75 µm radius phase droplet, within a 1:4 PEG:citrate volume ratio, depicted from the center of a phase droplet outwards at the end of the assay (t = 10 min). (A) Glucose, (B) peroxide, (C) Amplex Red, and (D) resorufin profiles suggest that the assumed 50 µm radius for our experimental data did not affect our predictions. The dotted line represents the 75 µm radius of the phase droplet.

Figure 3-14. PEG:citrate volume ratios (A-1,2) 4:1, (B-1,2) 1:1, (C-1,2) 1:4. The assay conditions were 2.1 nM GOX, 1 mM glucose, 50 µM Amplex Red, 0.45 nM or 4.5 nM HRP in (A1, B1, C1) and (A2, B2, C2) correspondingly. The black markers represent the experimental data. Model predictions to experimental ATPS volume ratios with (PDE model of Equation 3-9) and without considering diffusion (ODE model of Equation 3-14) are presented with black and red traces respectively.
Looking back at Figure 3-7, we can compare the experimental data points with the solid lines from the performed simulations in the ATPS at three different volume ratios that represent the model of Equation 3-9 – 3-13 prediction using optimized parameters. The reaction dynamics were obtained on the basis of Figure 3-3 and the individual phases of Figure 3-7. Despite the complexity of the experimental system due to partitioning and different media effects in the PEG-rich and citrate-rich phases, we observed good agreement between the predictions and the experiment in ATPS for volume ratios 4:1 and 1:1. At a volume ratio of 1:4, where the citrate-rich phase is largest, the model somewhat underpredicts the experimental data. The nonpartitioned citrate-rich phase control is not as well fit by the model as the nonpartitioned PEG-rich phase (Figure 3-15). This suggests additional effects in the citrate-rich phase that differ between the citrate-rich phase control and the prepartitioned citrate-rich controls, and may also be responsible for the underprediction of resorufin production in the 1:4 ATPS (Figure 3-7). Changes in enzyme specific activity with enzyme concentration due to the high salt of this phase is a possible explanation, any salting-out effects (e.g., changes in hydration leading to possible conformational changes or multimerization) are expected to be less apparent at lower protein concentrations. The citrate-rich phase control had a lower enzyme concentration than the corresponding phase of the ATPS samples or prepartitioned controls. Nonetheless, the PDE model predicts the ATPS reaction well, especially for systems in which the citrate-rich phase is of equal or smaller volume as compared to the PEG-rich phase.
3.3.7 Model Predictions at Different Enzyme Concentration.

Initially, we optimized the kinetic reaction parameters from single-phase assay control experiments in which, since they were uniform, diffusion phenomena could be neglected. We then employed the governing mass conservation equations of Equations 3-9 – 3-13 to describe the ATPS. In order to ensure the mathematical model properly captured the importance of enzyme and substrate spatial localization, we conducted the assay under the same conditions as previously described except that a 10× higher concentration of HRP was used (0.05 U/ mL).

Initially, predictions for each of the prepartitioned individual phases at the three volume ratios were carried out employing the ODE model of Equation 3-8 (Figure 3-16; insets). Additionally, the nonpartitioned controls were conducted (Figure 3-17) and compared to the ODE model predictions. We found that, for the nonpartitioned controls, the ODE mathematical model of Equation 3-8 overpredicts the resorufin formation in the
citrate-rich phase. This is a larger effect than that seen at the lower enzyme concentrations (Figure 3-15), and as discussed above may have been the result of some enzyme activity loss due to salting-out effects described by Huddleston et al. and others.\textsuperscript{45, 46, 52} For the prepartitioned PEG-rich phase separated reactions, the ODE model of Equation 3-8 showed good agreement, except for the 1:1 PEG-rich phase which we attribute to experimental error; this particular set of samples had greater variability than the others, most likely caused by errors in separating the two phases from each other.

\textbf{Figure 3-16.} PEG:citrate volume ratios (A) 4:1, (B) 1:1, and (C) 1:4 with 10× more HRP. Model predictions were applied to experimental ATPS volume ratios (black traces) and prepartitioned assays in separated PEG-rich phase (blue triangles) and citrate-rich phase (orange triangles). Insets highlight the phase-separated controls.
Figure 3-17. Sequential assays were performed with a higher HRP concentration of 0.05 U/mL. Controls in PEG-rich phase (blue triangles) and citrate-rich phase (orange triangles) are displayed with predictions from the ODE mathematical model (solid lines).

For the ATPS reactions, we see good agreement at the 4:1 and 1:1 volume ratios and a small underprediction by the PDE model of Equation 3-9 for the 1:4 case. The significance of diffusion can be illustrated here, since if we employ the ODE model of Equation 3-14 predictions (assuming well-mixed individual phases and instantaneous interfacial transport), we see good agreement at the 4:1 volume ratio, a small overprediction by the model at 1:1, and a large overprediction for the 1:4 case (Figure 3-14). The PDE model of Equation 3-9 can thus be reasonably expected to predict the activity if the enzyme concentration changes, but if the partitioning coefficient or enzyme activity were to change unexpectedly, then the model would not be able to predict the reaction kinetics without further information (i.e., the new $K_{enzymes}$ and activities). The behavior of the system at other volume ratios may also be directly predicted, but the accuracy of the predictions will similarly depend on the accuracy of the enzyme
partitioning coefficients and activities, which for these complex systems cannot always be extended to new conditions without experimental verification.

### 3.3.8 The Role of Diffusion and Interface

To further understand the role of the interface and the diffusion in the system, we assayed the enzymes in a cuvette in the bulk where the reaction was unmixed (Figure 3-18). We observed resorufin formation in the citrate-rich phase as early as 1.5 min. After 10 min, we saw the pink product resorufin was being formed at the interface. Over the next several minutes, the interface remained bright pink as the resorufin diffused throughout the PEG-rich phase. At 180 min, the PEG-rich phase was nearly uniformly pink. These results showed that, because the substrate was strongly partitioned to the PEG-rich phase, diffusion across the interface was critical for product formation. These observations were consistent with the concentration profile data in Figure 3-9, where the resorufin concentration shows significant variations in space, especially at the interface. The accumulation of resorufin in the PEG-rich phase was due to partitioning, although it was preferentially produced in the citrate phase due to the higher concentration of the enzymes there, and hence initial resorufin concentrations were highest in the interfacial region.
Figure 3-18. A 1:1 PEG:citrate volume ratio assay was conducted in a cuvette without mixing. The production of resorufin is clearly visible at the interface of the phases. Eventually, resorufin was homogeneously distributed in the PEG-rich phase.

Additionally, we ran the volume ratio assays without mixing and quantified the amount of resorufin formed. The enzymes and substrates were added to an ATPS and briefly vortexed to make a homogeneously mixed sample, and the reaction was immediately aliquotted into individual containers and centrifuged to reform the distinct phase-separated system. Interfacial area was therefore substantially decreased in this assay as compared to the mixed sample that produced small phase droplets. We found there was a significant decrease in the concentration of resorufin formed at 10 min for all of the volume ratios at 0.05 U/mL of both enzymes (Figure 3-19). The largest difference was for the 4:1 ratio, where only 2.9 ± 0.5 µM resorufin was made at 10 min compared to 18.2 ± 2.6 µM for the mixed assays. During the volume ratio assays that were continuously mixed, there was sufficient interfacial area for the substrate to diffuse across the interface to permit product formation and we observed increased resorufin production. This suggested that the interfacial area and ability of the substrates,
particularly Amplex Red, to diffuse into the citrate-rich phase where the majority of the enzymes were localized was necessary for maximal resorufin production.

![Graph](image)

**Figure 3-19.** Product formation within various samples that after initial vortexing to provide a homogenous mixture were centrifuged so the reaction would have to proceed in the two separated phases, thus providing one interface with substantially less surface area. Several unmixed PEG:citrate volume ratios were used: 4:1 (squares), 1:1 (diamonds), and 1:4 (circles).

The effect of diffusivity and partitioning coefficient in the behavior of the system would be even more pronounced if resorufin was a reaction intermediate due to its predicted and observed formation primarily at the interface. In the present work, we employed a mathematical model to observe the spatiotemporal profile of species (Figures 3-9 – 3-13). It is important to consider interfacial phenomena especially when the production of a species happens at a compartment where the species has low solubility and much higher solubility in a different compartment in contact with it. This phenomenon becomes especially important when this happens to a reaction intermediate.
For the rest of the species, the effect of interfacial diffusion is significantly less pronounced than that for resorufin.

3.4 Conclusion

We described a sequential reaction within heterogeneous biphasic media consisting of two distinct phases with very different chemical and physical properties. A well-studied sequential enzyme pair was used to investigate complex cellular metabolism where local concentrations of metabolites are ever-changing due to partitioning within the biphasic system. Even with the complex behavior of the system, a mathematical model was developed that could reasonably approximate the sequential reaction at a different enzyme concentration using enzyme and substrate partitioning coefficients in addition to the rates in individual phases. To design this mathematical model, we needed to know the corresponding reaction rate parameters. The GOX reaction parameters were obtained experimentally using Michaelis–Menten expressions; however, the HRP rate reaction did not follow Michaelis–Menten kinetics. As a result, we first optimized the unknown Dalziel parameters using the least-squares method to find the best fitting curve which describes the total average resorufin concentration in time. We then validated the model by predicting produced resorufin in an ATPS in different volume ratios. Finally, we showed the obtained parameters could even be employed to predict the product concentration in a higher level of HRP concentration.

This general combined experimental and computational approach should be applicable to other synthetic or biological phase separated media, where local
environments differ in enzyme concentrations or activities, physical properties such as viscosity, ionic strength, or crowding effects, and partitioning of reaction substrates, intermediates, and products. Although the rate behavior will vary with the specific system under evaluation, by knowing the reaction parameters in each phase, product formation in the complex media can be predicted. This work complements studies in the literature that have focused on the effects of macromolecular crowding in terms of excluded volume and chemical attractive/repulsive effects. The findings are also relevant for biotechnological applications, where PEG/salt ATPS are used primarily to increase an enzymatic product yield. Careful understanding of enzyme rates in addition to enzyme and substrate partitioning coefficients in those cases may lead to a more efficient output.

3.5 Experimental Section

3.5.1 Materials

Poly(ethylene glycol) 8 kDa, sodium citrate tribasic dihydrate, D-(+)-glucose, 30% hydrogen peroxide solution, o-dianisidine hydrochloride tablets, glucose oxidase from Aspergillus niger type X-S, sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, and Amicon 0.5 mL filters (MWCO 3000) were purchased from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase EIA grade, Amplex Red reagent, Amplex Red/Amplex Ultra Red Stop Reagent, Alexa Fluor 488, Alexa Fluor 546, and Alexa Fluor 647 labeling kits, and 13 mm SecureSeal Spacers were purchased from Life Technologies (Carlsbad, CA). mPEG- NH2 MW 5000 was purchased from Shearwater
Polymers. Dimethylsulfoxide was purchased from Alfa Aesar. Ethylenediaminetetraacetic acid (EDTA) was purchased from IBI Scientific (Peosta, IA). Deionized water with a resistivity of 18.2 MΩ·cm from a Barnstead NANOpure Diamond water purification system (Van Nuys, CA) was used for all experiments. Buffers were filtered using 0.45 µm pore size Nalgene filter units. All reagents were used as received without further purification.

3.5.2 Instrumentation

Fluorescently labeled enzyme concentrations and resorufin concentrations were measured using a Horiba Jobin Yvon Fluorolog 3-21 fluorimeter with Fluor-Essence software. The citrate composition of the ATPS and glucose partitioning within the ATPS were determined using an Agilent 1260 HPLC system with a 1260 Infinity Quaternary Pump, 1260 Infinity Thermostatted Column Compartment, 1260 Infinity Diode Array Detector, a 1260 Infinity Manual Injector, and Agilent ChemStation software. The GOX activity and degree of enzyme labeling were determined using an Agilent 8453 diode-array UV–visible spectrometer with Agilent ChemStation Software. Confocal images were acquired using a Leica TCS SP5 laser scanning confocal inverted microscope (LSCM) with a 20× air objective. Refractive index measurements of the aqueous two-phase system were made using a Leica Abbe Auto Refractometer. Viscosity measurements were made using an Ostwald viscometer.
3.5.3 ATPS Preparation

A phase diagram was created to determine which weight percents of PEG and citrate would form an ATPS. Several citrate weight percents were chosen, and the weight percent of PEG was varied close to the expected weight percents that would cause phase separation. Samples were vortexed, and phase separation was observed when a turbid solution formed, indicating phase separation. A 50.00 g ATPS was prepared by addition of 6.67 g of PEG 8 kDa and 5.00 g of sodium citrate tribasic dihydrate in 38.33 g of 50 mM sodium phosphate buffer pH 7.4 with 1 mM EDTA. EDTA was added to complex any trace metal ions present from the sodium citrate. After the PEG and citrate had dissolved, it was added to a separatory funnel and allowed to phase separate overnight. At these weight percents, the ATPS as prepared was approximately a 1:1 PEG-rich phase:citrate-rich phase. After separation, each phase was collected in separate containers so that they could be recombined at the desired volume ratios (PEG-rich:citrate-rich, 1:4, 1:1, 4:1).

3.5.4 Phase Composition Determination

The composition of each phase was determined using a combination of refractometry and HPLC. The weight percent of citrate in each phase was determined by HPLC using standards of known weight percents of citrate. The citrate was isocratically separated at 0.3 mL/min with 0.013 N H₂SO₄ as a mobile phase on an Aminex HPX 87H cation exchange column (300 × 7.8 mm i.d.) with a Micro-Guard IG Cation H precolumn from Bio-Rad at 25 °C for 35 min, using an analysis wavelength of 210 nm. The weight
percent of PEG 8 kDa was determined through refractometry. The refractive index of each phase was measured. Calibration curves of known weight percents of PEG 8 kDa and citrate were created. Due to the additive nature of refractive indices, the known contribution of citrate was subtracted from the refractive index of each phase. The remaining refractive index contribution was attributed to PEG.

3.5.5 Protein Labeling

Glucose oxidase and horseradish peroxidase were labeled according to the manufacturer’s instructions with Alexa Fluor 488 and Alexa Fluor 546, respectively. mPEG-NH2 MW 5000 was labeled with Alexa Fluor 647. Free dye was removed from the labeled polymer using an Amicon 3000 MWCO filter.

3.5.6 Partitioning

Fluorescently labeled GOX and HRP were measured by fluorimetry. The enzymes were added at a final concentration of 0.05, 0.25, or 0.5 U/mL each in a total volume of 1050 µL (1000 µL of each volume ratio and 50 µL of enzymes in buffer.) The enzymes were briefly vortexed and settled for 1 h and centrifuged to reform the distinct phases. An aliquot from each phase was taken, and the fluorescence was measured. The concentration of enzyme in each phase was determined using calibration curves of a known amount of enzyme in each phase. Resorufin partitioning was measured by fluorimetry. Resorufin was added to a 1:1 ATPS (500 µL of PEG-rich phase, 500 µL of
citrate-rich phase, 50 µL of buffer/sample), and after mixing, the samples were centrifuged to form distinct phases. Amplex Red partitioning was determined by addition of Amplex Red to a 1:1 ATPS at a final concentration of 50 µM. The solution was vortexed and phase separated by centrifugation. An aliquot from each phase was taken and placed in separate centrifuge tubes. A small excess of hydrogen peroxide was added with 0.5 U/mL of HRP in order to convert all of the Amplex Red to resorufin. The reaction proceeded to completion, and the resorufin fluorescence was measured in each phase. A similar approach was used to measure the hydrogen peroxide concentration in each phase using excess Amplex Red. Glucose partitioning was measured by HPLC. Glucose was partitioned in a 1:1 ATPS, and samples were mixed by inversion for 10 min and phase separated by centrifugation. An aliquot from each phase was diluted 10× before injection on the HPLC. The phase samples were isocratically separated at 0.2 mL/min with a mobile phase of 0.005 N H₃PO₄ on an Aminex HPX 87H cation exchange column (300 × 7.8 mm i.d.) with a Micro-Guard IG Cation H precolumn from Bio-Rad at 80 °C for 35 min at an analysis wavelength of 190 nm.54, 55

3.5.7 Enzyme Assays. Michaelis–Menten Parameters in Individual Phases

All enzyme assays were repeated three times. GOX was assayed using a modified procedure provided by Sigma-Aldrich.56 The enzyme activity was measured in each phase individually at a final concentration of 0.05 U/mL of glucose oxidase, 0.160 mM o-dianiside dihydrochloride, and 6 U/mL of HRP while varying the glucose concentration from 0 to 75 mM. The activity was measured for 3 min, and an extinction coefficient of
oxidized o-dianisidine (7.5 mM$^{-1}$ cm$^{-1}$) at 500 nm was used to calculate the product formation. The standard Michaelis–Menten equation (Equation 3-15) was used to fit the data in order to determine $K_M$ and $V_{\text{max}}$ using Igor CarbonPro nonlinear regression analysis. For HRP in the citrate-rich phase, a concentration of 0.0005 U/mL was used. To determine the $V_{\text{max}}$ and $K_M$ of HRP with respect to peroxide, the peroxide concentration was varied from 0 to 300 µM, while the Amplex Red concentration was fixed at 400 µM. Exposure to light was avoided for all Amplex Red assays due to the known photo-oxidation of Amplex Red to resorufin.$^4$ The $K_M$ with respect to Amplex Red was determined by varying the Amplex Red concentration from 0 to 500 µM, while hydrogen peroxide was fixed at 1 mM. For HRP in the PEG-rich phase, a concentration of 0.005 U/mL was used. Hydrogen peroxide was varied from 0 to 400 µM with a fixed concentration of Amplex Red at 400 µM. All reactions proceeded for 5 min. Time points were taken by removing an aliquot during the assay. The reaction was stopped with Amplex Red Stop Reagent, and the concentration of resorufin was measured at each point. The activity was calculated by the slope of the resulting linear plot of concentration vs time.

$$V_0 = \frac{V_{\text{max}} [S]}{K_M + [S]}$$  \hspace{1cm} (Equation 3-15)

### 3.5.8 Enzyme Assays. Single Phase Controls

The single-phase controls consisted of 0.05 U/mL GOX and 0.05 or 0.005 U/mL HRP. The final concentrations of substrates were 1 mM glucose and 50 µM Amplex Red
using 1 mL of either PEG-rich or citrate-rich phase in addition to the 50 µL of enzymes, substrates, and buffer. For comparison, the assay was conducted in buffer and a control was done in buffer without the addition of glucose to ensure Amplex Red was not being converted to resorufin due to its known oxidation by light.\textsuperscript{48}

### 3.5.9 Mixed Volume Ratios

The ATPS samples were prepared by addition of the appropriate amounts of each phase to reach a final concentration of 1 mL (e.g., a 1:4 PEG:citrate volume ratio would contain 200 µL of PEG-rich phase and 800 µL of citrate-rich phase). The final concentrations were 0.05 U/mL GOX, 0.05 or 0.005 U/mL HRP, 1 mM glucose, and 50 µM Amplex Red. The volume of added enzymes, substrates, and excess buffer was maintained at 50 µL throughout all assays to ensure only minor dilution of the ATPS. The enzymes and the Amplex Red were vortexed to uniformly mix the sample. A 100 µL aliquot was taken to serve as the zero time point and added to 200 µL of Amplex Red Stop Reagent. The reaction was then initiated with addition of glucose, vortexed again, and placed on a rotisserie that mixed at \( \sim 18 \) rpm. For each time point (1, 2, 3, 5, 7.5, 10 min), a homogeneous 100 µL aliquot was removed from the reaction and immediately added to 200 µL of stop reagent. This not only stopped the HRP reaction but also diluted the sample to one phase so the resorufin concentration could be measured by fluorimetry.
3.5.10 Physically Separated Phases

Prepartitioned phase separated control samples were prepared by adding enzymes and Amplex Red to the experimental volume ratios. After vortexing, the sample was centrifuged and the distinct phases were reformed. The phases were physically separated and transferred to separate reaction containers. To initiate the reaction, the calculated partitioned amount of glucose was added to each phase. Aliquots were taken at the necessary time points and were diluted with Stop Reagent.

3.5.11 Unmixed Volume Ratios

Samples were prepared in the same manner as the volume ratio assays by addition of the enzymes and Amplex Red to each volume ratio. Glucose was added, but after vortexing, the samples were promptly aliquoted into individual containers and centrifuged to reform the two phases. Each aliquot was then stopped at the desired time point with 200 µL of Stop Reagent, and the resorufin concentration was measured. To visualize this further, a 1:1 volume ratio assay was transferred to a quartz cuvette. After initial sample preparation, the sample was vortexed and subsequently centrifuged to induce phase separation. The phases were separated and carefully reconstituted in the cuvette. Photographs were taken with a Kodak EasyShare camera to monitor product formation.
3.5.12 Confocal Microscopy

To visualize enzyme partitioning, images were collected on a Leica TCS SP5 confocal microscope with excitation at 488, 543, and 647 nm for Alexa Fluor 488, Alexa Fluor 546, and Alexa Fluor 647, respectively. GOX and HRP were added to the experimental volume ratios at a final concentration of 0.5 U/mL. Samples were thoroughly vortexed prior to imaging.

3.5.13 Simulation Method

To simulate the developed model with partial differential equations as governing equations, we used COMSOL 4.3a. The maximum element size of the created mesh in the simulation was 0.05. Additionally, the dimensionless time element during the study was set to $10^{-2}$. In order to address the concentration discontinuity present at the interface, we employed a change of variables to have continuous values in the equations. Then, we related the corresponding local concentrations of each phase through using Equation 3-2. The rest of the computations that are discussed in the Results and Discussion section were performed using MATLAB R2009. To find the unknown Dalziel’s parameters, the resorufin concentration was predicted in time in single-phase control consisting of 0.05 U/mL GOX and 0.005 U/mL HRP for both PEG and citrate. Then, using a genetic algorithm in MATLAB, the relative prediction error of the mathematical model for each phase using a least-squares error formulation was minimized.
3.6 References


36. Großmann, C.; Tintinger, R.; Zhu, J.; Maurer, G. Partitioning of Some Amino Acids and Low Molecular Peptides in Aqueous Two-Phase Systems of


Chapter 4

Interactions of Macromolecular Crowding Agents and Cosolutes with Small-Molecule Substrates: Effect on Horseradish Peroxidase Activity with Two Different Substrates


The author of this dissertation conceived, designed, and performed the experiments, analyzed the data, and wrote the manuscript. Bradley Davis assisted in enzyme activity data collection and analysis. Emmanuel Hatzakis assisted in NMR data collection and analysis.

4.1 Abstract

The importance of solution composition on enzymatic reactions is increasingly appreciated, particularly with respect to macromolecular cosolutes. Macromolecular crowding and its effect on enzymatic reactions has been studied for several enzymes and is often understood in terms of changes to enzyme conformation. Comparatively little attention has been paid to the chemical properties of small-molecule substrates for enzyme reactions in crowded solution. In this article, we studied the reaction of horseradish peroxidase (HRP) with two small-molecule substrates that differ in their hydrophobicity. Crowding agents and cosolutes had quite different effects on HRP activity when the substrate used was 3,3′,5,5′-tetramethylbenzidine (TMB, which is hydrophobic) as compared to o-phenylenediamine (OPD, which is more hydrophilic).
Reaction rates with TMB were much more sensitive to the presence of crowding agents and cosolutes than OPD, suggesting that the small-molecule substrates may themselves be interacting with crowders and cosolutes. At high polyethylene glycol (PEG) concentrations (25–30 wt/wt %), no reaction was observed for TMB. Even at lower concentrations, Michaelis constants ($K_M$) for HRP with the more hydrophobic substrate increased in the presence of crowding agents and cosolutes, particularly with PEG. Diffusion of TMB and OPD in the PEG and dextran reaction media was evaluated using pulsed field gradient nuclear magnetic resonance (PFG-NMR). The diffusivity of the TMB decreased 3.9× in 10% PEG 8k compared to that in buffer and decreased only 1.7× for OPD. Together, these data suggest that weak attractive interactions between small molecule substrates and crowders or cosolutes can reduce substrate chemical activity and consequently decrease enzyme activity and that these effects vary with the identity of the molecules involved. Because many enzymes can act on multiple substrates, it is important to consider substrate chemistry in understanding enzymatic reactions in complex media such as biological fluids.

4.2 Introduction

The cytoplasm of biological cells is composed of approximately 30% by volume biomacromolecules such as proteins, nucleic acids, and polysaccharides.1, 2 This environment differs from the idealized dilute buffer solutions in which enzymes are often studied.2–6 The effects of macromolecular crowding on enzymatic reactions are becoming increasingly realized with reports of enhanced rates of reaction,2, 7-12 some with loss of
activity,\textsuperscript{12-15} or little to no changes.\textsuperscript{16} Substrate-dependent effects of crowding agents and cosolutes on enzyme activity are generally not considered. The possibility of substrate-dependent differences in the effects of crowding on enzyme activity warrants investigation because many enzymes act on multiple substrates in vivo,\textsuperscript{17} and for ease of analysis, many enzymes are routinely assayed using non-native substrates.\textsuperscript{18, 19} In this article, the activity of horseradish peroxidase (HRP) with respect to two different commonly used substrates is investigated in the presence of background macromolecular crowding agents and cosolutes.

The volume exclusion aspect of macromolecular crowding has been widely studied for many proteins and nucleic acids.\textsuperscript{2} For associative interactions of proteins and nucleic acids, the effects of excluded volume alone will favor more compact structures.\textsuperscript{2, 20} Changes in enzyme activity in the presence of crowding agents are often ascribed to changes in enzyme conformation.\textsuperscript{21} For example, Pozdnyakova and Wittung-Stafshede report that for the enzyme multicopper oxidase, a weaker binding of the substrate was observed and may be explained by the active site adopting a more rigid conformation, restricting substrate binding.\textsuperscript{14} At higher concentration of crowding agent, a decrease in the Michaelis constant, $K_M$, was observed, possibly due to an increase in the effective concentration of the enzyme and substrate. In another example, a conformational change in the enzyme isochorismate synthase (EntC) was observed in Ficoll solution, which resulted in a decrease in the $K_M$ of the substrate.\textsuperscript{22} There are other factors that may affect the kinetics of an enzyme in crowded solution, such as reduced and/or anomalous diffusion\textsuperscript{23, 24} and the activity of water.\textsuperscript{25, 26} Therefore, it is difficult to predict a priori for
a particular enzymatic reaction of interest the kinetic effects of macromolecular crowding.

The chemical effects of macromolecular crowding have only begun to be realized in the past decade. Chemical interactions between a solute and the other species in solution can be repulsive, resulting in increased chemical activity for the solute or attractive, which decreases its chemical activity. For macromolecules such as proteins or DNA, repulsive interactions with background molecules will enhance the effects of volume exclusion. Attractive interactions, however, often lead to a more destabilized structure and can promote unfolding; the protein chymotrypsin inhibitor 2 was recently shown to be destabilized in reconstituted E. coli cytosol despite the excluded volume ($\geq 100\text{g/L macromolecules}$). Typically, chemical effects of macromolecular crowding are only investigated with respect to the effect on other macromolecules, but small-molecule substrates could potentially experience attractive or repulsive interactions with the background macromolecules. The phenomenon known as substrate channeling occurs when an intermediate is transferred from one enzyme active site to another without diffusing in the bulk solvent. While some examples of this are more due to steric constraints like tryptophan synthase (where the hydrophobic substrate indole is transferred by means of a hydrophobic tunnel), others like dihydrofolate reductase-thymidylate synthase transfer the negatively charged substrate dihydrofolate by means of electrostatic interaction with positively charged residues in the enzymes. Record and co-workers separated the chemical effects from the volume exclusion effects of PEG on DNA structure. They showed that attractive interactions between DNA and small molecular weight PEGs led to destabilization of DNA hairpin and duplex formation.
Often, researchers will use a particular small-molecule substrate so that the effects of macromolecular crowding (more specifically, excluded volume) on the substrate can be ignored because it does not occupy much volume compared to the macromolecules,\textsuperscript{13, 15} however, this neglects any chemical interactions that may be present between the substrates and crowders.

In addition to crowded solutions of large macromolecular solutes, small-molecule cosolutes/osmolytes can alter enzyme kinetics. In general, compatible osmolytes have a stabilizing effect on protein structure because of their exclusion from the protein–water interface, which promotes a more compact, folded structure;\textsuperscript{35-37} osmolytes may enhance or decrease enzyme activity.\textsuperscript{38} For example, hexokinase activity was decreased in the presence of glycerol, was increased in the presence of betaine and urea solution, and was not significantly affected in trimethylamine N-oxide (TMAO).\textsuperscript{38} Although the osmolytes with the exception of urea are expected to be excluded from the enzyme surface, the authors suggest that some type of interaction with the hexokinase caused the activity to change. Recent work by Howell and co-workers indicates the possibility of osmolytes interacting with small-molecule substrates through weak binding interactions. They report preferential interactions of folate (a model compound for dihydrofolate reductase, substrate dihydrofolate) with osmolytes betaine and dimethyl sulfoxide (DMSO).\textsuperscript{39} Like macromolecular crowders, the effects of cosolute interactions also cannot easily be predicted.

We report the significant effects of various macromolecular crowding agents (polyethylene glycol (PEG) 8k, dextran 10k) and cosolutes (PEG 400 and glucose) on the reaction of HRP. We analyzed the effect of the different crowding agents and cosolutes
on the $K_M$ and the maximal velocity ($V_{\text{max}}$) of two substrates, o-phenylenediamine (OPD) and 3,3′,5,5′-tetramethylbenzidine (TMB). These substrates were chosen due to their different hydrophobic properties. We reasoned that due to the relative hydrophilic/hydrophobic nature of the background molecules, we would observe various effects on enzymatic activity due to interactions between the background macromolecules and the substrates. We found that the effect on the kinetics was modest for the OPD substrate but substantial for the TMB reaction, especially with respect to the relatively more hydrophobic crowder, PEG 8k. We also measured the diffusion coefficients of the OPD and TMB in buffer, 10% PEG 8k and 10% dextran 10k solutions using pulsed field gradient nuclear magnetic resonance (PFG-NMR). The presence of PEG decreased the diffusion coefficient for TMB disproportionately as compared with OPD. This suggests that weak attractive interactions between the more hydrophobic TMB and the PEG are responsible for the different impact of this crowder on HRP reaction rates with the two substrates.

4.3 Results and Discussion

HRP activity is commonly used as a reporter in an enzyme-linked immunosorbent assay (ELISA) and other types of bioassays due to its stability and the ease of detecting its products. HRP can oxidize many different aromatic substrates, which made it possible for us to select two common substrates for comparison in this investigation. We had previously measured HRP kinetics as part of a coupled reaction in a PEG/sodium citrate aqueous two-phase system (ATPS). HRP activity toward its substrate Amplex
Red in the more hydrophobic PEG-rich phase was decreased substantially as compared to the citrate-rich phase. Indeed, kinetics in the PEG-rich phase could not be fit with the standard Michaelis–Menten equation because the rate increased linearly up to the point of substrate solubility. This observation, coupled with Amplex Red’s strong partitioning preference for the PEG-rich phase of the ATPS, suggested to us that there could be an attractive interaction between the substrate and the PEG leading to decreased availability of the Amplex Red.

For the present study, we chose two chemically distinct substrates, OPD and TMB, to evaluate whether substrate–crowder interactions could be important. The value log D is a measure of the partitioning of a compound with ionizable functional groups in a water–octanol system, and it serves to gauge the relative lipophilicity (hydrophilicity/hydrophobicity) of a small molecule. Compounds with log D > 0 partition to the octanol phase of a water–octanol system and are more lipophilic. OPD is highly water-soluble and one of the least hydrophobic HRP substrates (log D at pH 7 = 0.24); TMB is sparingly water-soluble and one of the most hydrophobic substrates (log D at pH 7 = 2.67) (Chart 4-1). The OPD reaction was monitored by the absorbance of the product 2,3-diaminophenazine (DAP). The TMB reaction was monitored using the diamine/diimine charge-transfer complex. We verified that the established extinction coefficients for these products were valid in the complex media used here (see the Experimental Section). PEG was chosen as a crowding agent and as a small-molecule cosolute in these experiments because it has been widely used as a crowding agent and is known to have interactions with hydrophobic molecules and with hydrophobic portions of proteins. Dextran 10k was chosen because it is a commonly used crowding agent. The
buffer used in this work was 50 mM sodium phosphate, pH 7.4, with 1 mM EDTA added to complex any trace metal ions that may cause background oxidation of the small-molecule substrates.

**Chart 4-1. Substrates Used in This Work.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chemical Structure</th>
<th>Log D</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-phenylenediamine (OPD)</td>
<td><img src="image.png" alt="OPD" /></td>
<td>0.24</td>
</tr>
<tr>
<td>3,3',5,5'-tetramethylbenzidine (TMB)</td>
<td><img src="image.png" alt="TMB" /></td>
<td>2.67</td>
</tr>
</tbody>
</table>

**4.3.1 HRP Activity, $K_M$ of Substrates in Macromolecular Crowding Agents**

We first measured the effect of crowding on HRP reaction kinetics for the OPD and TMB substrates in solutions of the macromolecular crowding agents, PEG 8k and dextran 10k, (Figure 4-1 and Figure 4-2). Data were fit with the Michaelis–Menten equation; Table 4-1 summarizes $K_M$ and $V_{max}$ values for each set of conditions. In all cases, $K_M$ increased as the weight percent of the crowding agent increased; however, the effects were different both between crowders for the same substrate and between substrates for the same crowder. For TMB, $K_M$ increased more than 2-fold in just 5% and more than 10-fold in 20% PEG 8k. Above 25% PEG 8k, the reaction of HRP with TMB was essentially shut down, and data could not be fit with Michaelis–Menten kinetics. PEG 8k also increased the $K_M$ for OPD but only at higher concentrations of crowding agent. At 10% PEG 8k, $K_M$ for OPD was the same as that without crowder, while by 30%
PEG, it had increased more than 10-fold. Dextran also increased $K_M$ for both substrates but to a lesser degree than PEG. At 30% dextran, the $K_M$ for TMB increased 6-fold, while the $K_M$ for OPD increased nearly 3-fold.

Figure 4-1. Michaelis–Menten plots of the reaction of HRP with TMB and OPD in different crowding agents. (A) TMB in PEG 8k; (B) OPD in PEG 8k; (C) TMB in dextran 10k; (D) OPD in dextran 10k; The HRP concentration was 0.005 U/mL (0.45 nM) for all assays. The data points are the average of three measurements with standard deviation error bars. The traces are the fit to the standard Michaelis–Menten equation. TMB data for 30% PEG 8k could not be fit to the equation.
Figure 4-2. Additional kinetic traces for TMB (A) PEG 8k, (B) dextran 10k, (C) PEG 400 and (D) glucose at 15% and 25%.
Table 4-1. Michaelis–Menten Reaction Parameters for TMB and OPD in the Various Macromolecular Crowding Agents

<table>
<thead>
<tr>
<th>Media</th>
<th>Wt. %</th>
<th>$K_M$ (µM)</th>
<th>$V_{max}$ (µM/min)</th>
<th>$K_M$ (µM)</th>
<th>$V_{max}$ (µM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffer</td>
<td>0</td>
<td>100 ± 10</td>
<td>6.6 ± 0.3</td>
<td>25 ± 4</td>
<td>1.86 ± 0.06</td>
</tr>
<tr>
<td>PEG 8k</td>
<td>5</td>
<td>240 ± 40</td>
<td>5.1 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>500 ± 100</td>
<td>3.5 ± 0.4</td>
<td>26 ± 3</td>
<td>1.55 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1100 ± 500</td>
<td>2.5 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1100 ± 800</td>
<td>1.0 ± 0.5</td>
<td>45 ± 9</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>n.a.</td>
<td>n.a.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>n.a.</td>
<td>n.a.</td>
<td>280 ± 50</td>
<td>1.28 ± 0.10</td>
</tr>
<tr>
<td>dextran 10k</td>
<td>5</td>
<td>110 ± 10</td>
<td>6.7 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>190 ± 40</td>
<td>6.3 ± 0.5</td>
<td>38 ± 3</td>
<td>1.90 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>210 ± 70</td>
<td>5.3 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>300 ± 100</td>
<td>4.0 ± 0.6</td>
<td>36 ± 3</td>
<td>1.68 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>600 ± 200</td>
<td>5 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>600 ± 300</td>
<td>4 ± 1</td>
<td>70 ± 10</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

n.a. Not applicable. That data could not be fit with Michaelis-Menten kinetics

An increase in $K_M$ can be attributed to changes in the active site of an enzyme.\textsuperscript{13}

We cannot rule out this contribution to the increase in $K_M$ as substrate access can be hindered by changes in the local protein environment of HRP.\textsuperscript{46} However, we expect that the local protein environment in the crowding agents would be the same regardless of the substrate used. Another study reported that dextran had little effect on HRP $K_M$ using the substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), observing a
small decrease that was not statistically different compared to the $K_M$ in buffer.\textsuperscript{47} ABTS is more hydrophilic than OPD, with log D = $-3.59$ at pH 7;\textsuperscript{48} therefore, it is not surprising that the $K_M$ was not changed in dextran. In our previous work using Amplex Red, with log D = 1.94,\textsuperscript{49} HRP activity could not be fit to Michaelis–Menten kinetics,\textsuperscript{41} which is also consistent with this hydrophobic substrate interacting with the PEG. An increase in $K_M$ may also be caused by increased diffusion resistance within the sample.\textsuperscript{13,50} While an increase in viscosity due to the crowding agents could account for some of the observed decrease in enzyme kinetics, it cannot account for the much larger changes that we observed in $K_M$ and $V_{\text{max}}$ for the TMB substrate as compared with the OPD substrate. These substrates have similar molecular weights (OPD $M_w$ = 108.14 Da; TMB $M_w$ = 240.34 Da); therefore, we would expect the diffusivity of both substrates to decrease similarly if viscosity was the only factor affecting the diffusion; that was not observed here. Experimentally determined viscosities for weight percents of crowding agent and cosolute at 17 °C (lab temperature used in enzyme assays) and 25 °C (temperature of NMR experiments) are compiled in Table 4-2.
Table 4-2. Viscosity of 10%, 20%, and 30% Solutions at Lab Temperature (17 °C) and NMR Experiment Temperature (25 °C).

<table>
<thead>
<tr>
<th>media</th>
<th>Wt. %</th>
<th>Viscosity (cP) at 17 °C</th>
<th>Viscosity (cP) at 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer</td>
<td>0</td>
<td>1.090 ± 0.003</td>
<td>0.931 ± 0.008</td>
</tr>
<tr>
<td>PEG 8k</td>
<td>10</td>
<td>6.37 ± 0.04</td>
<td>5.12 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>21.5 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>58.6 ± 0.4</td>
<td>-</td>
</tr>
<tr>
<td>dextran 10k</td>
<td>10</td>
<td>3.08 ± 0.06</td>
<td>2.55 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8.25 ± 0.08</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>23.1 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>PEG 400</td>
<td>10</td>
<td>1.709 ± 0.009</td>
<td>1.46 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.68 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.38 ± 0.08</td>
<td>-</td>
</tr>
<tr>
<td>glucose</td>
<td>10</td>
<td>1.53 ± 0.01</td>
<td>1.29 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.27 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.39 ± 0.06</td>
<td>-</td>
</tr>
</tbody>
</table>

4.3.2 $V_{\text{max}}$ Analysis in Macromolecular Crowding Agents

The $V_{\text{max}}$ of the substrates was either statistically unchanged or decreased with respect to increasing weight percent of the crowding agents. For OPD, $V_{\text{max}}$ decreased slightly in the PEG 8k and was essentially unchanged with respect to buffer in dextran 10 kDa. For TMB, $V_{\text{max}}$ decreased steadily in PEG 8k until there was no reaction and decreased slightly in dextran 10k.

A decrease in $V_{\text{max}}$ has usually been attributed to a change in the active site of the enzyme by the environmental surroundings. Other studies report crowding effects on HRP activity. Altikatoglu and Basaran studied the reaction with respect to the substrate $o$-
dianisidine in various molecular weights of dextran and found that the activity was decreased in dextran 17.5k compared to that in buffer, curiously was increased \( \sim 2 \)-fold in dextran 75k, and then decreased as the molecular weight of the dextran increased.\(^{52}\) Pitulice et al. measured the rate with respect to ABTS and also found that the \( V_{\text{max}} \) was decreased,\(^{47}\) as we observed here. These studies and our work point to a general volume exclusion effect that is changing the enzyme active site. Aromatic substrates bind to HRP by a solvent-exposed heme edge that has an active site composed of flexible amino acids; it can accommodate many different small aromatic molecules.\(^{53}\) Volume exclusion could cause a conformation change that is sterically restricting access to the active site. Polymer–protein interactions\(^{27, 54, 55}\) can also be restricting substrate access. As described above, this may contribute to the macromolecular crowding effect that we and others observe but cannot adequately explain that the \( V_{\text{max}} \) decreased to zero for the TMB substrate and only slightly decreased or stayed the same for the OPD substrate under the same HRP crowding conditions.

### 4.3.3 HRP Activity in the Presence of Small Molecule Cosolutes

To further investigate potential chemical interactions with the media without the excluded volume effects of the polymers, we used PEG 400 and glucose as cosolutes and conducted each of the reactions in solution up to 30 wt % (Figure 4-2, Figure 4-3, Table 4.3). HRP kinetics were not substantially changed with respect to the OPD substrate in either cosolute. For the TMB substrate, however, \( K_M \) was increased substantially. This is consistent with a chemical interaction between the TMB substrate and the PEG 400, as
was observed with the PEG 8k. With TMB, the $K_M$ in glucose also increased but increased to a much smaller extent than that for PEG 400, similar to the larger effect of PEG 8k than dextran in Figure 4-1. Together, these data for small molecules suggest that chemical interactions are important for understanding the differences between the two small-molecule substrates. The larger effects seen for macromolecular cosolutes also indicate that excluded volume effects account for some of the observed changes in HRP kinetics as compared with reactions performed in buffer.
Figure 4-3. Michaelis–Menten plots of the reaction of HRP with OPD and TMB in different cosolutes. (A) TMB in PEG 400; (B) OPD in PEG 400; (C) TMB in glucose; (D) OPD in glucose. The HRP concentration was 0.005 U/mL (0.45 nM) for all assays. The data points are the average of three measurements with standard deviation error bars. The traces are the fit to the standard Michaelis–Menten equation.
Table 4-3. Michaelis-Menten Reaction Parameters for TMB and OPD in the Cosolutes.

<table>
<thead>
<tr>
<th>media</th>
<th>wt. %</th>
<th>$K_M$ (µM)</th>
<th>$V_{max}$ (µM/min)</th>
<th>$K_M$ (µM)</th>
<th>$V_{max}$ (µM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer</td>
<td>0</td>
<td>100 ± 10</td>
<td>6.6 ± 0.3</td>
<td>25 ± 4</td>
<td>1.86 ± 0.06</td>
</tr>
<tr>
<td>PEG 400</td>
<td>5</td>
<td>180 ± 30</td>
<td>8.0 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>220 ± 50</td>
<td>7.4 ± 0.7</td>
<td>18 ± 2</td>
<td>1.74 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>300 ± 60</td>
<td>7.6 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>500 ± 100</td>
<td>7.2 ± 0.8</td>
<td>28 ± 4</td>
<td>1.49 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>700 ± 200</td>
<td>6.5 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2400 ± 1700</td>
<td>10 ± 5</td>
<td>50 ± 12</td>
<td>1.65 ± 0.10</td>
</tr>
<tr>
<td>glucose</td>
<td>5</td>
<td>150 ± 30</td>
<td>7.7 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>130 ± 30</td>
<td>6.2 ± 0.4</td>
<td>32 ± 3</td>
<td>1.90 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>150 ± 30</td>
<td>6.3 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>150 ± 30</td>
<td>6.1 ± 0.5</td>
<td>40 ± 5</td>
<td>1.74 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>180 ± 20</td>
<td>5.9 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>210 ± 50</td>
<td>5.1 ± 0.5</td>
<td>110 ± 30</td>
<td>1.79 ± 0.09</td>
</tr>
</tbody>
</table>

4.3.4 Diffusion Coefficients of OPD and TMB in Different Media

PFG-NMR was used to measure the impact of the PEG 8 kDa and dextran 10 kDa crowders on OPD and TMB diffusion coefficients. When translational diffusion of small molecules is reduced due to interactions with polymers in solution, this can be measured using PFG-NMR.56-59 We measured the diffusion coefficient, $D$, of OPD and TMB together in buffer alone, 10% PEG 8k, and 10% dextran 10k. This crowder concentration was chosen because a difference in enzyme activity was observed at 10%, and higher
polymer concentrations are more challenging for NMR diffusion experiments. A schematic way to view the diffusion data is the diffusion ordered NMR (DOSY) representation where the signal attenuation at each chemical shift is inverted by an approximate inverse Laplace transformation (ILT). The DOSY representation of the data is presented as a 2D spectrum with chemical shifts on the horizontal axis and the distribution of the diffusion coefficients on the vertical axis shown in Figure 4-4. The individual 1D $^1$H spectra are also included for buffer, 10% PEG, and 10% dextran.
Figure 4-4. $^1$H DOSY plot of OPD and TMB in buffer (black), 10% PEG 8k (red), and 10% dextran 10k (blue) with the corresponding 1D spectra above. Chemical shifts are OPD: $\delta = 6.7$ and TMB: $\delta = 2.2$ and 7.2. HDO appears in the 10% PEG at $\delta = 4.7$.

Diffusion coefficients for each substrate in buffer, 10% PEG 8k, and 10% dextran 10k are given in Table 4-4. The coefficients were calculated using Equation 2 (see the Experimental Section). $D_{\text{OPD,PEG}}$ decreased by a factor of 1.7 compared to $D_{\text{OPD,buffer}}$, and the $D_{\text{TMB,PEG}}$ decreased by a factor of 3.9. For dextran, the diffusion coefficients
decreased by a similar factor. $D_{\text{OPD,dex}}$ decreased 1.6× compared to buffer, while $D_{\text{TMB,dex}}$ decreased 2.3× compared to buffer.

**Table 4-4.** Summary of Measured Diffusion Coefficients of OPD and TMB in Different Media.

<table>
<thead>
<tr>
<th>substrate</th>
<th>diffusion coefficient $D$ (10^{-10} m^2/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>buffer</td>
</tr>
<tr>
<td>OPD</td>
<td>11.8 ± 0.2</td>
</tr>
<tr>
<td>TMB</td>
<td>7.18 ± 0.07</td>
</tr>
</tbody>
</table>

We expect that the diffusion coefficients would decrease due to the increased viscosity of these solutions, as anticipated by the Stokes–Einstein equation, which can provide a first-order estimate of a diffusion coefficient.\(^6^0\) Diffusion coefficients do not always scale proportionately with viscosity in liquids because diffusion reflects short-range interactions while viscosity often depends on longer-range interactions in the solution.\(^6^1\) The larger decrease in the diffusion coefficient of TMB in PEG versus OPD in PEG suggests that there is an interaction between the TMB and PEG that is either smaller or not present between OPD and PEG. The fold-decrease for the OPD and TMB in dextran was not identical but was more similar in magnitude than that for OPD and TMB in PEG. The change in diffusion that we observe for OPD and TMB can explain the large difference in activity that we observed for the two substrates; the effective concentration of the TMB was decreased in the increasing concentrations of PEG due to attractive interactions with the PEG. The nature of the TMB–PEG interaction is not a strong binding because we did not observe a large chemical shift of the proton signal or a complete loss of signal. Rather, we interpret these data as indicative of a weakly
attractive and dynamic association between TMB and PEG, such as has been observed with other hydrophobic molecules\textsuperscript{62} and hydrophobic portions of proteins.\textsuperscript{2} It is also possible that the TMB interacts with different microenvironments in the solution as nanostructuring has been suggested to occur in a polymer solution.\textsuperscript{24} Nanostructuring can lead to subdiffusion of solutes; subdiffusion has been described previously in model crowding conditions\textsuperscript{63,64} and in cellular environments.\textsuperscript{65,66}

4.4 Conclusion

This study demonstrates substrate-specific crowding effects for the same enzyme in the same crowder, which appear to arise due to differences in weak chemical interactions between the polymeric crowders and the small-molecule enzyme substrates. It underscores the multiple types of interactions that can occur as the complexity of biological (or biomimetic) media is increased. The reaction of HRP with the more hydrophobic substrate, TMB, is substantially more sensitive to the presence of crowders and cosolutes than the reaction with OPD. This is consistent with weakly attractive interactions between the substrates and the background molecules resulting in decreased chemical activity of the small-molecule substrate.

Although the substrates and crowders investigated here are not themselves biologically relevant in vivo, biological metabolites almost certainly experience interactions with the components of the cell. Amino acids with hydrophobic side chains (tyrosine, phenylalanine, and tryptophan) and other metabolites with aromatic ring structures (e.g., dimethylbenzimidazole, riboflavin) could experience attractive
interactions with hydrophobic areas inside of cells, such as cell membranes and hydrophobic parts of proteins. The abundance of uncharged, hydrophobic metabolites in cells is low compared to charged hydrophilic species; it has been suggested that this is due to the possibility of hydrophobic species having high membrane permeability, which could lead to metabolite leakage or membrane accumulation. These types of interactions in cells need not be only hydrophobic; electrostatic, van der Waals attractions, and hydrogen bonding could affect the chemical activity of a small-molecule metabolite in the nonideal cell environment.

The results presented here indicate that possible chemical effects of biological and biomimetic media must be examined not only for biomacromolecules but also for biologically important small molecules such as enzyme substrates. This will be important for understanding not only enzymatic activity in complex media such as the cytoplasm or nucleoplasm but also for any process that relies on the chemical activity of a small molecule (e.g., binding to nucleic acids, proteins, or polysaccharides or insertion into membranes).

4.5 Experimental Section

4.5.1 Materials

PEG 8 kDa, dextran 10 kDa from *Leuconostoc mesenteroides*, PEG 400, D-(+)-glucose, 30% hydrogen peroxide solution, OPD, 3,3′,5,5′-tetramethylbenzidene, sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, deuterium oxide,
DMSO-$d_6$, and Amicon 0.5 mL filters (MWCO 3000) were purchased from Sigma-Aldrich (St. Louis, MO). HRP EIA grade was purchased from Life Technologies (Carlsbad, CA). DMSO was purchased from Alfa Aesar, and ethylenediaminetetraacetic acid (EDTA) was purchased from IBI Scientific (Peosta, IA). Deionized water with a resistivity of 18.2 MΩ·cm from a Barnstead NANOpure Diamond water purification system (Van Nuys, CA) was used for all experiments. Buffers were filtered using a 0.45 µm pore size Nalgene filter units. All reagents were used as received without further purification. Viscosity measurements were made using an Ostwald viscometer.

### 4.5.2 Enzyme Assays

The reaction progress of HRP was followed using an Agilent 8453 diode array UV–visible spectrometer with Agilent ChemStation software. All assays were repeated three times. The final concentration of enzyme for both substrates was 0.005 U/mL (0.45 nM) HRP, with hydrogen peroxide held in excess at 8.8 mM. 68 OPD and TMB concentrations were varied from 0 to 1000 µM in the various weight percents of cosolutes and crowding agents dissolved in 50 mM sodium phosphate buffer, pH 7.4, with 1 mM EDTA. OPD stock solutions were made by dissolving the OPD tablet in the sodium phosphate buffer and used immediately. New solutions were made if any color in the stock solution was observed. TMB stock solutions were made by dissolving the TMB solid in DMSO. The activity of the enzyme for the OPD substrate with respect to both substrates was measured for 2 min, and the activity was calculated using an extinction coefficient of 16,700 M$^{-1}$ cm$^{-1}$ for the product (2,3-diaminophenazine) at 417 nm. 69 For
the TMB substrate, the activity was calculated using the extinction coefficient of 39,000 M$^{-1}$ cm$^{-1}$ of the charge-transfer complex at 652 nm. For the acid-stopped reactions, the extinction coefficient of 59,000 M$^{-1}$ cm$^{-1}$ at 450 nm was used for the diimine product. Because these substrates can react without the presence of HRP, we also did control experiments without enzyme to ensure that no appreciable reaction was observed. The standard Michaelis–Menten equation was used to fit the data in order to determine $K_M$ and $V_{\text{max}}$ using Igor CarbonPro nonlinear regression analysis (Equation 4-1). The error bars indicate the standard deviation of three measurements for each substrate concentration.

\[
V_0 = \frac{V_{\text{max}} [S]}{K_M + [S]} \tag{Equation 4-1}
\]

### 4.5.3 Validation of Extinction Coefficients

We verified that the DAP extinction coefficient was valid in the different media by dissolving a known amount of DAP in each medium. For TMB, initial oxidation of the substrate leads to two intermediates, a diamine/diimine charge-transfer complex (blue) and a radical cation (colorless). Addition of acid or further oxidation will ultimately convert the charge-transfer complex and the radical cation to the diimine product (yellow) (Scheme 4-1). The extinction coefficient for the blue charge-transfer complex could not be verified directly because dilution will cause re-equilibration and spectral changes. We verified the extinction coefficient of the yellow diimine by reacting 12.5 $\mu$M TMB in buffer using 4.5 nM HRP and 8.8 mM peroxide and converting to acid by
addition of an equal volume of 2 M H$_2$SO$_4$. The reaction mixture was diluted in 1 M H$_2$SO$_4$ that also contained 30% of the crowder or cosolute. To verify that the extinction coefficient of the charge-transfer complex (blue) was valid in the PEGs, dextran, and glucose, we converted the amount of blue complex and the colorless radical cation to the yellow diimine and measured the rate of formation. Because the ratio of blue to yellow made was approximately 2× for each medium, we used the extinction coefficient of the blue product to monitor the reaction in real time (Figure 4-5). We also examined the absorbance spectra of the charge-transfer complex in each medium to detect any changes in the peak positions, and no change was observed. We did observe some spectral changes in the PEG 400 spectra at high weight percents in the area where the yellow product appears. Small molecular weight PEG solutions have been shown to contain trace impurities such as peroxide, formaldehyde, and organic acids,$^{70}$ which could lead to background oxidation of the charge-transfer complex to the yellow product. We used only the beginning linear portion of the kinetic trace for rate calculation to minimize this effect as much as possible.
Scheme 4-1. Reaction schemes for HRP with the substrates used (A) OPD and (B) TMB.
Figure 4-5. Comparison of the reaction rates for the blue product and the yellow product in each of the 30% crowding agents and cosolutes.

4.5.4 NMR Experiments

NMR experiments were conducted on a Bruker DRX spectrometer operating at 400.01 MHz for $^1$H nuclei. All experiments were performed at 25.00 ± 0.01 °C, and the spectra were processed by the Bruker TopSpin software package. The concentration of OPD in each sample was 2.5 mM, and the TMB was 250 μM in PEG 8k and buffer and 1 mM in dextran 10k in D$_2$O buffer.

$^1$H NMR spectra were recorded with water suppression in the buffer and dextran samples and PEG suppression in the PEG samples using the following acquisition
parameters: 16 scans and 4 dummy scans, 64 K data points (TD), 90° pulse angle, relaxation delay of 2 s, and spectral width (SW) of 10 ppm. A polynomial fourth-order function was applied for baseline correction in order to achieve accurate quantitative measurements upon integration of signals of interest. The spectra were acquired without spinning the NMR tube in order to achieve better water suppression and avoid artifacts, such as spinning side bands of first or higher order. Chemical shifts are reported in ppm from HDO (δ = 4.7). Diffusion coefficients (D) were obtained by fitting the peak area to Equation 4-2 using the TopSpin software.

\[
I(g) = I_0 \exp \left\{ -D \left( \frac{\gamma \delta g}{3} \right)^2 \left( \frac{1}{3} \delta - \frac{1}{2} \tau \right) \right\}
\]

(Equation 4-2)

\(I(g)\) and \(I_0\) are the integrated peak areas, \(g\) is the gradient pulse amplitude, \(\delta\) is the gradient duration, \(\gamma\) is the gyromagnetic ratio of the nucleus, \(\Delta\) is the separation between gradient pulse pairs, and \(\tau\) is the time allowed for gradient recovery before the next pulse.

\(^1\)H diffusion experiments were performed using the ledbpgrp2s pulse sequence with presaturation, which uses bipolar gradients and an eddy current reduction delay. A total of 32 scans of 16 data points were collected using a 90° pulse angle, a relaxation delay of 15 s to ensure full relaxation, and a SW of 10 ppm. The maximum gradient strength produced in the z direction was 5.35 G mm\(^{-1}\). The duration of the magnetic field pulse gradients (\(\delta\)) was optimized for each diffusion time (\(\Delta\)) in order to obtain a 2% residual signal with the maximum gradient strength. The values of \(\delta\) ranged from 800 to 1100 µs, and \(\Delta\) was 200 ms. The pulse gradients were incremented from 2 to 95% of the maximum gradient strength in a linear ramp. The temperature was set and controlled to
298 K with an air flow of 400 L h\(^{-1}\) in order to avoid any temperature fluctuations due to sample heating during the magnetic field pulse gradients.

4.6 References


Chapter 5

When Does Colocalization of Enzymes Lead to Rate Enhancements? Experiments and Modeling of Sequential Enzyme Activity in an Aqueous Biphasic System

This chapter has been submitted for publication to a biophysical chemistry journal.

The author of this dissertation and Bradley Davis contributed equally. The author and Bradley Davis expressed and purified the enzymes, designed the experiments, collected and analyzed the data, and wrote the manuscript. Bradley wrote the initial draft and the author wrote the final draft. Negar Hashemian developed the mathematical model and contributed to the writing of the manuscript. Songon An assisted in expressing and purifying the enzymes.

5.1 Abstract

Subcellular compartmentalization of biomolecules and their reactions is common in biology and provides a general strategy for improving and/or controlling kinetics in metabolic pathways that contain multiple sequential enzymes. Enzymes can be colocalized in multiprotein complexes, on scaffolds, or inside subcellular organelles. “Liquid organelles” formed by intracellular phase coexistence could provide an additional means of sequential enzyme colocalization. Here we use experiment and computation to explore the kinetic consequences of sequential enzyme compartmentalization into model liquid organelles in a crowded polymer solution. Two proteins of the de novo purine biosynthesis pathway, adenylosuccinate lyase (ASL, step 8) and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase (ATIC, steps 9 and 10), were studied in a polyethylene
glycol/dextran aqueous two-phase system. Dextran-rich phase droplets served as model liquid compartments for enzyme colocalization. In this system, which lacks any specific binding interactions between the phase-forming polymers and the enzymes, we did not observe significant rate enhancements from colocalization for the overall reaction under our experimental conditions. The experimental results were used to adapt a mathematical model to quantitatively describe the kinetics. The mathematical model was then used to explore additional, experimentally inaccessible conditions to predict when increased local concentrations of enzymes and substrates can (or cannot) be expected to yield increased rates of product formation. Our findings indicate that colocalization within these simplified model liquid organelles can lead to enhanced metabolic rates under some conditions, but that very strong partitioning into the phase that serves as the compartment is necessary. In vivo, this could be provided by specific binding affinities between components of the liquid compartment and the molecules to be localized within it.

5.2 Introduction

Enzymes of metabolic pathways often exist as multienzyme complexes that are spatially organized within different cellular compartments or organelles.1 Substrate channeling through multienzyme complexes offers many advantages such as the transfer of metabolites from one active site to the next without diffusing throughout the rest of the cell, the sequestration of toxic or labile intermediates, and the reduction of competing reactions from other enzymes.2-4 Sequential enzymes can colocalize by binding to each other or to a scaffold such as another protein, membrane, or cytoskeletal component.5-7
The citric acid cycle is a hallmark example of a scaffold-bound multienzyme complex in which all of the enzymes are bound to the inner mitochondrial membrane. Recent reports of liquid-like organelles in both cytoplasm and nucleoplasm of eukaryotic cells suggest additional means of colocalization. These “liquid organelles,” are thought to be the result of intracellular phase separation, and therefore simple partitioning of enzymes into one of the coexisting phases could offer a means of enzyme colocalization in which enzymes need not be bound to a scaffold. Hence, specific or nonspecific binding interactions with components of the compartment would not be required, but could also be present.

Artificial colocalization of sequential enzymes has been achieved using a variety of scaffolds, such as proteins, nanoparticles, nanostructured DNA, nanostructured RNA, and microfluidic channels. Many of these approaches have resulted in increased reaction rates and product flux and have provided insight into the design of artificial multienzyme complexes for industrial synthesis of valuable molecules, such as biofuels. Colocalization of two enzymes does not always guarantee that a large increase in overall reaction rate will be observed. The interenzyme distance, enzyme active site orientation, and relative activities of the enzymes can affect the catalytic efficiency of an artificial multienzyme complex. There has been considerably less effort towards the study of in vitro liquid compartments for multiple enzyme colocalization. Freely-diffusing enzymes concentrated together within a micron-scale liquid phase compartment are presumably not as fully colocalized as are enzymes bound to a shared scaffold, and they have neither fixed separation nor orientation with
respect to each other. Thus, differences in the kinetic consequences of colocalization can be anticipated between these systems.

An aqueous two-phase system (ATPS) where biomolecules concentrate into one phase by partitioning can serve as an in vitro model system for artificial “liquid organelles.”26-28 One or both phases in which biochemical reactions can occur is macromolecularly crowded, providing excluded volume and chemical interactions, which is reminiscent of the intracellular milieu. Macromolecular crowding can alter enzyme conformation, substrate binding, etc., and has been reported to increase, decrease, or not change enzyme activity, depending on the system.29-33 In addition to crowding, enzymes can be concentrated into one phase of the ATPS, resulting in higher local concentration and concomitant rate increases.34, 35 Few examples of sequential enzyme colocalization in ATPS have appeared. Mann and coworkers observed an 18-fold increase in product yield when the actinorhodin polyketide synthase complex and its substrate were partitioned in poly(diallyldimethylammonium chloride)/ATP coacervates; the coacervate matrix helped stabilize the complex.36 When glucose oxidase and horseradish peroxidase were partitioned in a PEG/citrate ATPS, sequential activity was limited due to accumulation of the enzymes in a different phase from the substrate, such that reaction could only occur at the interface.37 In both sequential reaction examples, only one of the phases was macromolecularly crowded, unlike intracellularly where all liquid phases are expected to contain substantial biopolymer concentrations. Here, we used a PEG/dextran ATPS in which both phases are crowded with macromolecules. When mixed, the result is dextran-rich phase droplets dispersed within a continuous PEG-rich phase. The dextran-rich phase
droplets serve as model liquid compartments that contain a local high concentration of enzyme because of simple partitioning, rather than any specific biorecognition.

We investigate two sequential enzymes from the de novo purine biosynthetic pathway in the PEG/dextran ATPS. The purine pathway in humans consists of six proteins with ten enzymatic steps, with the final step being the synthesis of inosine monophosphate (IMP). The enzymes of this pathway have been shown to reversibly co-localize in vivo,\textsuperscript{38} forming structures termed “purinosomes.” Purinosomes are visualized as cytoplasmic puncta that vary in size and shape (0.2 – 0.9 µm).\textsuperscript{39} Purinosome assembly is thought to be mediated by interactions with microtubules and protein-protein interactions in which several pathway enzymes serve as a scaffold for assembly of the rest.\textsuperscript{40-42} These structures have not been considered to be liquid organelles, and indeed some researchers suggest that they could be aggregated protein/stress bodies.\textsuperscript{43, 44} Purinosomes, nonetheless, were recently shown to share some important features with liquid phase domains in terms of compartment size, absence of a delimiting membrane, and their transient nature.\textsuperscript{39, 42} Additionally, purinosome formation correlates with pathway activity, indicating a kinetic advantage to enzyme colocalization in these bodies.\textsuperscript{4, 40, 45} The local concentration increase of these enzymes due to purinosome formation has not been quantified, but published images indicate that while the signal due to labeled enzymes is strongest in the purinosomes, some enzyme remains free in the cytoplasm.\textsuperscript{38, 42} In addition, unrelated to this pathway, incomplete compartmentalization has been quantified for other proteins, for example proteasome subunit Pre9 which partitions between the cytoplasm and membrane of yeast.\textsuperscript{46} Our model system allows us
to investigate the effect of incomplete compartmentalization on sequential enzyme reactions.

Two proteins from the pathway, adenylosuccinate lyase (ASL, step 8) and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase (ATIC, steps 9 and 10) are investigated here. ASL and ATIC were chosen for this study because, in addition to partitioning in vivo between the purinosomes and their surroundings, both enzymes are relatively stable in buffer solution, and they have been expressed, purified, and characterized previously. They are found to be associated with each other (and the rest of the purinosome), and the AICAR substrate is commercially available. The enzymes partition into the dextran-rich phase of a PEG/dextran ATPS. By decreasing the relative volume of the dextran-rich phase to the PEG-rich phase, the enzymes were increasingly colocalized to that phase. The resulting sequential enzyme activity was similar, regardless of the volume of the dextran-rich phase. Experimental results could be understood in the framework of a mathematical model that also enabled prediction of additional conditions not amenable to experiment.

5.3 Results and Discussion

Scheme 5-1 illustrates the sequential reaction investigated here, and the complex reaction medium, in which both enzymes are concentrated within dextran-rich phase droplets of the PEG/dextran ATPS. ASL is a tetramer with four active sites that cleaves 5-aminoimidazole-4-(N-succinocarboxamide) ribonucleotide (SAICAR) to form 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and fumarate. ATIC is a
bifunctional dimer with single active sites for each enzyme activity. The first step uses 10-formyl tetrahydrofolate (10f-THF) as a cofactor and transfers a formyl group to the AICAR amine, forming 5-formamidoimidazole-4-carboxamide ribonucleotide (FAICAR) and tetrahydrofolate (THF). The second step catalyzes the internal cyclization of FAICAR to make IMP, and releases a water molecule. The AICAR Tfase activity is reversible and the FAICAR to AICAR reaction is 2-3 fold faster, but the IMPCH activity is essentially a forward reaction, which drives the AICAR Tfase reaction toward IMP. The bifunctionality may be advantageous in driving the reaction forward. It is also worth noting that there is a lack of substrate channeling between the two active sites.

Scheme 5-1. (A) The sequential reaction of purine biosynthesis enzymes ASL and ATIC with the substrate, intermediates, cofactors and product shown. (B) An illustration of the partitioning of ASL, ATIC, SAICAR and IMP in the PEG/dextran ATPS from the start of the reaction to near completion.
5.3.1 Compartmentalization in the ATPS

We used a 10% (w/w) PEG 8k, 10% (w/w) dextran 10k ATPS that was prepared in a 33 mM Tris buffer, pH 7.4 and 25 mM potassium chloride. A phase diagram for this system is given in Figure 5-1. This composition resulted in a roughly 3:1 volume ratio of PEG-rich phase: dextran-rich phase (abbreviated PEG:dextran going forward) consisting of an upper PEG-rich phase and a lower dextran-rich phase. The physical properties of the phases differed, with the dextran-rich phase being approximately twice as viscous as the PEG-rich phase due to the higher overall polymer concentration of ~28% (w/w) compared to ~18% (w/w) (Table 5-1). To control the local concentration of enzymes, experiments were performed at specific, non-native PEG:dextran volume ratios, prepared by mixing desired amounts of each phase from a large volume stock ATPS. Most experimental ATPS used in this work had smaller volume dextran-rich phase (i.e., PEG-rich:dex-rich phase volume ratios 9:1, 19:1, 49:1), such that upon mixing, this phase occurred as droplets surrounded by a continuous PEG-rich phase.
Figure 5-1. Phase diagram of PEG 8 kDa and dextran 10 kDa. Samples prepared above the coexistence curve (blue) phase separated while those below (red) were a single phase. The 10% (w/w) PEG 8 kDa and 10% (w/w) dextran 10 kDa composition used here is marked with black diamond.

Table 5-1. Physical Properties of the 10% PEG 8 kDa and 10% dextran 10 kDa ATPS

<table>
<thead>
<tr>
<th>Phase</th>
<th>wt% PEG</th>
<th>wt% dextran</th>
<th>Viscosity (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-rich</td>
<td>13.32 ± 0.04</td>
<td>4.37 ± 0.02</td>
<td>11.4 ± 0.2</td>
</tr>
<tr>
<td>dextran-rich</td>
<td>1.5 ± 0.2</td>
<td>26.8 ± 0.2</td>
<td>24.6 ± 0.2</td>
</tr>
</tbody>
</table>

Partitioning was the mechanism used to achieve high local concentration of enzymes in the dextran-rich phase. Enzymes typically partition to the dextran-rich phase of a PEG/dextran ATPS because of their higher affinity for the more hydrophilic dextran-rich phase. Many factors influence how a protein will partition in a PEG/dextran ATPS such as: protein size, protein shape, surface and overall charge, and weak affinity interactions with the phase forming components. While weak correlations have been found (as molecular weight and net positive charge of the protein increases, partitioning
partitioning of individual enzymes of interest must be experimentally determined. Partitioning is quantified in terms of the partitioning coefficient, $K$, where $K = C_P/C_D$. $C_P$ is the concentration in the PEG-rich phase; $C_D$ is the concentration in the dextran-rich phase. We measured the partitioning of the enzymes and substrates at a 1:1 volume ratio (Table 5-2). The metabolites, SAICAR, AICAR and IMP all partitioned weakly to the dextran-phase. 10$\text{f}^{-}$THF concentration was the same in both phases. The partitioning coefficient of ASL and ATIC was measured individually and in the presence of the other enzyme because any associations between the enzymes could change their partitioning.$^{54, 55}$ In this case, neither enzyme’s partitioning was significantly affected by the presence of the other. ASL partitioned 8.7$\times$ to the dextran-rich phase, while ATIC partitioned much more strongly; it was $\sim$250$\times$ more concentrated in the dextran-rich phase. We also measured the enzyme partitioning after 12 hours to determine any changes from being in the ATPS for an extended time; no significant differences were observed. Figure 5-2 shows the distribution of fluorescently-labeled ASL and ATIC enzymes in a 9:1 volume ratio ATPS; both enzymes accumulate in the dextran-rich droplet phase and the continuous PEG-rich phase appears dark. Some aggregation can be seen as bright spots in the images for both enzymes, particularly for ASL. Aggregation of ASL has been observed previously using static light scattering in a buffer solution.$^{49}$
Table 5-2. Partitioning Coefficients of Enzymes and Substrates

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Partitioning Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASL&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Individual</td>
<td>0.115 ± 0.007</td>
</tr>
<tr>
<td>With ATIC</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>ATIC&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Individual</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td>With ASL</td>
<td>0.0056 ± 0.0005</td>
</tr>
<tr>
<td>SAICAR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.318 ± 0.009</td>
</tr>
<tr>
<td>AICAR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.426 ± 0.009</td>
</tr>
<tr>
<td>10-THF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>IMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43 ± 0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup>ASL and ATIC partitioning was measured at 100 nM.
<sup>b</sup>Small molecule partitioning measured at 100 µM.

Figure 5-2. Confocal fluorescence microscopy of the fluorescently labeled enzymes in the ATPS at a 9:1 volume ratio. Droplets correspond to the dextran-rich phase, surrounded by a continuous PEG-rich phase. (left) Transmitted light (DIC); (center) ASL – Alexa Fluor 647; (right) ATIC – Alexa Flour 488. Images have been contrast-adjusted and false-colored to aid visualization.
5.3.2 Michaelis-Menten Enzyme Kinetics in the Phases

Michaelis-Menten parameters for ASL and AICAR T\textsubscript{f}ase in the PEG-rich and the dextran-rich phases, as well as non-crowded buffer solutions, are reported in Table 5-3. ASL activity showed a 5-fold difference in $K_M$ and a small increase in $k_{\text{cat}}$ in the dextran-rich phase while the $K_M$ and $k_{\text{cat}}$ AICAR T\textsubscript{f}ase are within error. Many factors can influence enzyme activity in macromolecularly crowded solutions, both favorably and unfavorably, such as: changes in active site of the enzyme, changes in substrate chemical activity, or decreased diffusion in the sample.$^{29,32}$ Because there was no difference for $K_M$ of AICAR with respect to ATIC activity and it is similar in size and structure to SA
ICAR, the most likely explanation is differences in ASL structure in the PEG-rich phase, dextran-rich phase and buffer. Further evidence is the orders of magnitude differences in $k_{\text{cat}}$ for ASL in buffers compared to the crowded phases. ASL is sensitive to solution composition, even in two different buffered solutions, which underscores the importance of determining enzyme activity in crowded media. At 100 nM of each enzyme, the activity of ATIC was approximately 7$\times$ the activity of ASL. This was chosen because enhanced catalysis of a coupled enzyme reaction can only be observed when the rate of the second enzyme is greater than the rate of the first.$^{25}$
Table 5-3. Michaelis-Menten Constants of ASL and ATIC within PEG-rich phase and dextran-rich phase

<table>
<thead>
<tr>
<th></th>
<th>$K_M$ (µM)$^a$</th>
<th>$V_{max}$ (µM/min)$^b$</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASL (SAICAR)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 mM Tris buffer, pH 7.4$^{49}$</td>
<td>1.8 ± 0.1</td>
<td>-</td>
<td>90.2 ± 1.9</td>
</tr>
<tr>
<td>50 mM buffer pH 7.5, 150 mM total ionic strength (NaCl)$^{56}$</td>
<td>12.8 ± 2</td>
<td>-</td>
<td>259.8 ± 7.8</td>
</tr>
<tr>
<td>PEG-rich phase</td>
<td>24 ± 8</td>
<td>5.4 ± 0.5</td>
<td>0.90 ± 0.08</td>
</tr>
<tr>
<td>dextran-rich phase</td>
<td>5 ± 1</td>
<td>6.9 ± 0.1</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td><strong>AICAR TFase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(AICAR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66 mM Tris, pH 7.4, 50 mM KCl buffer$^{51}$</td>
<td>10 ± 1</td>
<td>-</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>33 mM Tris, pH 7.4, 25 mM KCl buffer$^{57}$</td>
<td>16.8 ± 1.5</td>
<td>Different units</td>
<td>-</td>
</tr>
<tr>
<td>PEG-rich phase</td>
<td>19 ± 6</td>
<td>39 ± 3</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>dextran-rich phase</td>
<td>16 ± 5</td>
<td>42 ± 3</td>
<td>7.0 ± 0.5</td>
</tr>
</tbody>
</table>

$^a$ $K_M$ measured at 50 nM enzyme

$^b$ $V_{max}$ adjusted to match activity at 100 nM enzyme used in the other assays (See Experimental Section)

5.3.3 Kinetics of the Sequential Reaction

We next measured the sequential reaction kinetics in the individual phases and in the ATPS. We used volume ratios 1:1, 9:1, 19:1 and 49:1 so that as the volume of the dextran-rich phase decreased, the local concentration of enzyme in that phase would increase. This is advantageous because the individual phase composition remained
constant, and it allowed us to change the enzyme stoichiometry in each phase without changing to total number of moles of enzymes in the system. SAICAR, AICAR, and IMP have overlapping UV spectra; high-performance liquid chromatography (HPLC) was used to distinguish them (Figure 5-3). An initial substrate concentration of 100 µM SAICAR was used, as this is an upper estimate of cellular SAICAR concentration (20-100 µM). The final product, IMP, is in approximately the same concentration range. Total enzyme concentrations were 100 nM for ASL and ATIC because these concentrations gave reproducible results and provided sufficient IMP formation over the time course of the reaction. Reaction time points were collected by removing aliquots and adding them to an equal volume of 1 M sodium hydroxide solution to stop the reaction and dilute the ATPS to one phase. Metabolite concentrations were quantified at each time point (data points, Figure 5-4). For AICAR, we did not observe any appreciable concentration in the HPLC chromatograms, but this was expected given the much higher activity of ATIC compared to ASL. We observed a slight increase in product formation at 20 minutes for the ATPS samples compared to the individual phases, with the exception that the 49:1 volume ratio was similar to the dextran-rich phase. The concentration of IMP at 20 minutes in the PEG-rich phase was 78 ± 5 µM, while the dextran-rich phase was 87 ± 2 µM. The ATPS samples were similar: 95 ± 1 µM for 1:1, 99 µM ± 1 µM for 9:1, 98 ± 1 µM for 19:1, and 86 ± 7 µM for 49:1. Overall, there was a modest effect from colocalization in the ATPS compared to the sequential reaction in the individual phases.
Figure 5-3. HPLC- UV chromatogram at 267 nm of the purine substrates and products at 100 µM. The peak at 22 minutes was caused by the salt gradient (See Experimental Section).
**Figure 5-4.** Sequential reaction kinetics in the individual phases and ATPS. The panels are (A) PEG-rich phase, (B) dextran-rich phase and the volume ratios (C) 1:1, (D) 9:1, (E) 19:1, and (F) 49:1. Red open circles are experimentally determined SAICAR concentrations. Blue closed circles are experimentally determined IMP concentrations. The solid lines represent the model predictions of SAICAR (red), AICAR (green), and IMP (blue) and the dashed lines represent the upper and lower limit error of each trace, based on the Michaelis-Menten parameters in Table 5-2. Initial total reaction concentrations were 100 nM ASL, 100 nM ATIC, 100 µM SAICAR and 400 µM 10-THF; these molecules were distributed throughout the biphasic solutions according to their partitioning (Table 5-2).
5.3.4 Mathematical Modeling

Next, we quantitatively described the enzyme kinetics in the individual phases and the ATPS to make predictions about the enzyme activity under other conditions. A mathematical model that we had previously developed for the enzyme kinetics of glucose oxidase and horseradish peroxidase in a PEG/citrate ATPS (Chapter 3)\textsuperscript{37} was adapted to fit this system.

5.3.4.1 Computational Domain

The ATPS reaction medium was modeled as spheres (droplets) of the dextran-rich phase uniformly distributed within a continuous PEG-rich phase. Dextran-rich phase droplet radius for the PEG:dextran volume ratios 1:1, 9:1, 19:1, and 49:1 were measured by microscopy after mixing and determined to be $90 \pm 40 \, \mu m$, $40 \pm 20 \, \mu m$, $18 \pm 5 \, \mu m$, and $7 \pm 2 \, \mu m$, respectively. Also, since the domain space was symmetric, we used a subsection of the domain to simplify the calculations.

5.3.4.2 Mass Conservation

We next described the mass conservation of the system that involved two phenomena: the enzyme reactions and the diffusion of the species. The assumption was that the diffusion coefficient of the components was constant within a phase, and since the average velocity of species was close to zero, the convective forces could be neglected in the simulation volume. The mass conservation of specie $i$ was described by the partial differential equation:
\[
\frac{\partial c_{i,j}}{\partial t} - D_{i,j} \nabla^2 c_{i,j} = r_{i,j}
\]  
(Equation 5-1)

where, \( i \) denoted the substrate species, i.e. \( i = \{ s,a,f \} \) which represented SAICAR, AICAR, and IMP (i.e. \( f = \text{IMP} \)). The phase \( j = \{ P,D \} \) represented the PEG-rich or dextran-rich phase. The concentration and diffusion coefficient of species \( i \) in phase \( j \) were denoted by \( c_{i,j} \), the concentration in the phase, and \( D_{i,j} \), the diffusion coefficient in the phase. These values were calculated using the Stokes-Einstein equation using the viscosities of the phases listed in Table 5-1. The net rates of the reactions that involve species \( i \) in phase \( j \) were represented by \( r_{i,j} \). The Laplace operator was denoted by \( \nabla^2 \) and described the gradient divergence of the function throughout space. The concentration of each species \( i \) in phase \( j \) was dependent on the partitioning coefficient, \( K_i \), and provided the following boundary condition:

\[
c_{i,P}(r,t) \bigg|_{r=R} = K_i c_{i,D}(r,t) \bigg|_{r=R}
\]  
(Equation 5-2)

This boundary conditions ensured that the partitioning was maintained if a species was consumed or produced. Because the model was symmetrical, periodic boundary conditions could be applied to opposite faces of the cube:

\[
c_{i} \bigg|_{a} = c_{i} \bigg|_{b}, \ F_{i,j} \bigg|_{a} = -F_{i,j} \bigg|_{b}
\]  
(Equation 5-3)

where \( a \) and \( b \) were the two opposite faces of the cube. The inward flux, \( F \), to phase \( j \) of the \( i \) component at face \( l \) was represented by:

\[
F_{i,j} \bigg|_{l} = -D_{i,j} \nabla c_{i,j} \bigg|_{l}
\]  
(Equation 5-4)
5.4.3.3 Reaction Rate expressions

We modeled ASL and AICAR Tfase using the Michaelis-Menten equation. The IMPCH activity of ATIC was assumed to be instantaneous and equal to the rate of the AICAR Tfase activity. FAICAR could not be detected in our system by HPLC, so we assumed its concentration was low (< 3 µM). Additionally, the $K_M$ of FAICAR has been reported to be below 1 µM, and it is beyond the sensitivity of most assays. The AICAR Tfase activity required both AICAR and the cofactor 10-fTHF, but we could model with respect to varying AICAR concentration only because 10f-THF was in 4× excess. We anticipated that the concentration of the intermediate, AICAR, would remain relatively low, since it will be used by AICAR Tfase faster than it is produced by ASL; hence in practice 10f-THF will be at even greater excess.

For ASL, the rate of SAICAR consumption was equal to the rate of AICAR production. This rate was solely dependent on ASL and SAICAR concentrations. The rate of IMP production was equal to the rate of AICAR consumption. AICAR, the intermediate of the sequential assay, was first produced by ASL and consumed by ATIC. The Michaelis-Menten reaction rate expressions below described these conditions:

$$r_{s,j} = -\frac{k_{cat,\text{ASL},j}c_{\text{ASL},j}c_{s,j}}{K_{M,s,j} + c_{s,j}}$$  \hspace{1cm} (Equation 5-5)

$$r_{a,j} = \frac{k_{cat,\text{ASL},j}c_{\text{ASL},j}c_{s,j}}{K_{M,s,j} + c_{s,j}} - \frac{k_{cat,\text{ATIC},j}c_{\text{ATIC},j}c_{a,j}}{K_{M,a,j} + c_{a,j}}$$  \hspace{1cm} (Equation 5-6)

$$r_{f,j} = \frac{k_{cat,\text{ATIC},j}c_{\text{ATIC},j}c_{a,j}}{K_{M,a,j} + c_{a,j}}$$  \hspace{1cm} (Equation 5-7)
To aid in the simplicity of the simulation, we non-dimensionalized the mass conservation equation and the reaction rate expressions. These equations and parameter definitions are given as Eqs. 5-8–5-11 and Table 5-4. The simulation output was the concentration profiles of the reactants and products in time.

**Table 5-4. Definitions of Dimensionless Parameters**

<table>
<thead>
<tr>
<th>Dimensionless Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{i,j}$</td>
<td>$c_{i,j}$</td>
</tr>
<tr>
<td>$T$</td>
<td>$t$</td>
</tr>
<tr>
<td>$\alpha_{i,j}$</td>
<td>$D_{i,j} \tau$</td>
</tr>
<tr>
<td>$\beta_{ASL,j}$</td>
<td>$k_{cat, ASL,j}c_{ASL,j} \tau$</td>
</tr>
<tr>
<td>$\gamma_{s,j}$</td>
<td>$K_{M,s}$</td>
</tr>
<tr>
<td>$\beta_{ATIC,j}$</td>
<td>$k_{cat, ATIC,j}c_{ATIC,j} \tau$</td>
</tr>
<tr>
<td>$\gamma_{a,j}$</td>
<td>$K_{M,a}$</td>
</tr>
</tbody>
</table>

$c_{s,0}$ is the initial SAICAR concentration (100 µM)

$\tau$ is the time length of the experiments (20 mins)

\[
\frac{\partial C_{i,j}}{\partial T} - \alpha_{i,j} \cdot \nabla^2 C_{i,j} = R_{i,j} \quad \text{(Equation 5-8)}
\]

\[
R_{s,j} = -\frac{\beta_{ASL,j}C_{s,j}}{\gamma_{ASL,j} + C_{s,j}} \quad \text{(Equation 5-9)}
\]

\[
R_{a,j} = \frac{\beta_{ASL,j}C_{s,j}}{\gamma_{ASL,j} + C_{s,j}} - \frac{\beta_{ATIC,j}C_{a,j}}{\gamma_{ATIC,j} + C_{a,j}} \quad \text{(Equation 5-10)}
\]
\[ R_{f,j} = \frac{\beta_{ATIC,j} C_{a,j}}{\gamma_{ATIC,j} + C_{a,j}} \]  
(Equation 5-11)

5.3.5 Comparison of Simulation and Experimental Results

The simulation concentration profiles are graphed with the experimental data presented earlier in Figure 5-4 for comparison. The solid lines are the average Michaelis-Menten parameters, and the dashed lines represent the upper and lower extremes of the standard deviations of the \( K_M \) and \( V_{max} \) parameters. The simulations show that AICAR reaches a steady state concentration between 0-3 \( \mu \)M, depending on the case, which is consistent with the lack of detection of AICAR in the sequential assays. Generally, we saw good agreement of the modeling results with most of the experimental results. The four curves with deviations were for the SAICAR curve in the PEG-rich phase, the IMP curve of the dextran-rich phase and the 19:1 curves, but the simulation still reasonably predicted them. This was significant because the model accurately described the enzyme kinetics in the individual phases and volume ratios.

5.3.6 Effect of Compartmentalization on the Sequential Rate

As the volume ratio increased, the local concentration of enzyme (and degree of compartmentalization) in the dextran-rich phase increased. However, this did not significantly improve the rate of final product formation. The results here are in contrast to the enhanced rates of reaction observed for a single RNA ribozyme\(^ {34} \) and the enzyme urease\(^ {60} \) that were each partitioned to the dextran-rich phase in a PEG/dextran ATPS.
The calculated simulation rates for production of IMP for the first 10 minutes (linear portion of each curve) of each phase and volume ratio are given in Table 5-5. The model predicted that among the volume ratios, the 1:1 case gave the fastest rate of IMP production. The rate of IMP production decreased as the volume of dextran-rich phase decreased. Analysis of the enzyme distribution in the ATPS as a function of volume ratio, however, explained the result. Table 5-5 gives the predicted concentrations and number of moles of the enzymes in each phase of the volume ratios. Note that, for constant $K$, although enzyme concentration increased in the dextran-rich phase as the phase volume decreased, the fraction of total enzyme in the dextran-rich phase decreased. At equal volumes of both phases (1:1), the concentration of ASL in the dextran-rich phase was 180 nM, which increased to 782 nM when the relative volume of the dextran-rich phase was reduced to one part in 50 (49:1). At the same time, the total amount of ASL in dextran-rich phase dropped from 90 to 16 pmoles out of the total 100 pmoles of ASL present in the reaction. Since ASL is the slower enzyme (has a smaller $V_{max}$), its concentration has greater impact on the overall rate of the sequential reaction. This contrasts with prior work in which ribozyme cleavage was enhanced 66-fold by compartmentalization in a 100:1 volume ratio PEG/dextran ATPS, compared to dextran-rich phase alone. In the ribozyme experiments, extremely strong partitioning (nearly 3000-fold greater concentration in the dextran-rich phase) meant that nearly all of the total ribozyme remained in the dextran-rich phase even as the volume of this phase became very small.
Table 5-5. Predicted Rate of IMP Formation, Concentrations and Number of Moles of Enzymes in the Individual Phases and Volume Ratios.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate of IMP formation (µM/min)\textsuperscript{b}</th>
<th>C\textsubscript{P}\textsuperscript{c} (nM)</th>
<th>C\textsubscript{D}\textsuperscript{d} (nM)</th>
<th>n\textsubscript{P}\textsuperscript{e} (pmol)</th>
<th>n\textsubscript{D}\textsuperscript{f} (pmol)</th>
<th>C\textsubscript{P}\textsuperscript{c} (nM)</th>
<th>C\textsubscript{D}\textsuperscript{d} (nM)</th>
<th>n\textsubscript{P}\textsuperscript{e} (pmol)</th>
<th>n\textsubscript{D}\textsuperscript{f} (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-rich phase alone</td>
<td>4.0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>dextran-rich phase alone</td>
<td>6.3</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1:1</td>
<td>6.1</td>
<td>20</td>
<td>180</td>
<td>10</td>
<td>90</td>
<td>1</td>
<td>199</td>
<td>&lt;1</td>
<td>100</td>
</tr>
<tr>
<td>9:1</td>
<td>5.2</td>
<td>55</td>
<td>503</td>
<td>50</td>
<td>50</td>
<td>4</td>
<td>965</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>19:1</td>
<td>4.8</td>
<td>71</td>
<td>647</td>
<td>68</td>
<td>32</td>
<td>7</td>
<td>1859</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td>49:1</td>
<td>4.4</td>
<td>86</td>
<td>782</td>
<td>84</td>
<td>16</td>
<td>17</td>
<td>4181</td>
<td>16</td>
<td>84</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Calculated based on constant $K$ values for the individual enzymes listed in Table 5-2, assuming 100 total picomoles of each enzyme and 1 mL total volume
\textsuperscript{b} average rate from 0-10 minutes, as predicted by the model
\textsuperscript{c} $C\textsubscript{P} =$ concentration in the PEG-rich phase
\textsuperscript{d} $C\textsubscript{D} =$ concentration in the dextran-rich phase
\textsuperscript{e} $n\textsubscript{P} =$ number of moles in the PEG-rich phase
\textsuperscript{f} $n\textsubscript{D} =$ number of moles in the dextran-rich phase

We further explored the sequential activity in each of the phases of the ATPS with the mathematical model in which we decreased the activity in the PEG-rich phase by a factor of 100, essentially turning off the reaction in the PEG-rich phase (Figure 5-5). Decreasing the volume of the dextran-rich phase resulted in a decrease in IMP production, which demonstrated that the enzyme in the PEG-rich phase contributed significantly to the amount of IMP produced.
Simulated IMP production in the ATPS in which the enzyme activity in the PEG-rich phases is decreased 100×. The traces are 1:1 (black), 9:1 (red), 19:1 (blue), 49:1 (green) and PEG-rich phase (orange).

5.3.7 Probing Changes in Enzyme and Substrate Partitioning with the Mathematical Model

Next, we used the mathematical model to explore how changes in enzyme partitioning would affect the reaction rate. In this experimental system, the enzymes partition and have no specific interactions with the phase components. In the cell, however, enzyme localization is controlled through a number of different means, such as weak and strong binding interactions, phosphorylation states of enzymes, and conformational changes. We investigated IMP formation by simulating changes in the partitioning of both ASL and ATIC using the mathematical model. Figure 5-6 shows the effect on IMP formation from varying the enzymes from equal concentration in both phases (K=1) to 1,000× more concentrated in the dextran-rich phase (K=0.001). At each volume ratio, as the partitioning of the enzymes increased, the rate of IMP formation
increased. No further enhancement was observed by simulating the partitioning beyond K=0.001. While the enhancements here were rather small, increased local concentrations under these conditions led to increased product formation. Recently, it was reported that purinosome containing cells had increased purine biosynthesis compared to cells without purinosomes; a nearly 3-fold increase in IMP cellular concentration was observed.\textsuperscript{4, 40, 45} These results and our modeling results are consistent with the hypothesis that increased local concentration of the sequential enzymes leads to an increase in final product formation.

![Figure 5-6](image_url)

**Figure 5-6.** Effect of simulated changes in enzyme partitioning at each of the volume ratios. (A) 1:1, (B) 9:1, (C) 19:1, (D) 49:1. From bottom to top - black traces: K=1, red traces: K=0.1, blue traces: K = 0.01, green traces: K= 0.001. As the partitioning is increased to the dextran-rich phase, the IMP production is increased for each volume ratio.

Next, we explored the effect of substrate partitioning. We increased the partitioning of the small molecules (SAICAR, AICAR, 10f-THF, and IMP) to the
dextran-rich phase, using K=0.1 and K=0.01 (Figure 5-7A, B). We observed a trend as we have with the previous conditions in Figure 5-4, in that the rate of IMP decreased as the volume ratio increased. The K=0.01 condition was decreased in IMP formation compared to K=0.1 because there was less substrate available to the active enzyme in the PEG-rich phase. These results indicate that since functional enzymes are present in both phases, there is not a large advantage to substrate localization in this PEG/dextran model system with ASL and ATIC. We simulated changes in which both the enzymes and substrates partitioned strongly to the dextran-rich phase. We held each enzyme partitioning at K= 0.001 and used partitioning of the substrates K=0.01. When enzyme concentration within dextran-rich phase was high, increasing the substrate partitioning did not increase the overall rate to an appreciable extent in the volume ratios (Figure 5-7C, Figure 5-8). Here, substrate location has an impact on the sequential rate. Small molecule localization is important in vivo in the form of substrate channeling. Substrate channeling has been observed under other experimental model conditions, but we do not believe that is happening to an appreciable extent here because the enzymes are free to diffuse independently from each other in the compartment. Cluster-mediated channeling has also been proposed. Metabolites are not likely to be found distributed evenly throughout an entire cell because of specific interactions with other biomolecules, and they are involved with metabolic processes that only occur in specific areas.
**Figure 5-7.** The effect of changes in substrate partitioning. Panel (A) is $K=0.1$ for all small molecules (SAICAR, AICAR, 10-fTHF, IMP) and panel (B) is $K=0.01$. The activity decreased as the volume ratio is increased (from top to bottom): 1:1 (black), 9:1 (red), 19:1 (blue), 49:1 (green). Panel (C) is $K=0.001$ for both enzymes with experimental $K$ (black) and $K=0.01$ (red) for the small molecules.

**Figure 5-8.** Probing changes in substrate partitioning. The enzyme partitioning of ASL and ATIC was held at $K=0.001$. Black traces are with the experimental small molecule partitioning, while red traces are $K=0.01$ for all the small molecules.

### 5.4 Conclusion

The presence of liquid-like compartments in cytoplasm and nucleoplasm of biological cells suggests new ways to compartmentalize and potentially control metabolic pathways. These liquid organelles are an attractive possibility for transient multienzyme assembly because they allow for reversible localization of the enzymes based on
formation/dissolution of the liquid compartment. Our experimental and computational model for compartmentalization of the purine enzymes to a liquid compartment did not result in a large increased flux of the sequential reaction. Instead, an increase in the number of total moles of ASL in the PEG-rich phase compared to the dextran-rich phase caused a decrease in rate of the final product, IMP, as the volume ratio was increased. Our computational model allowed us to consider systems beyond those readily accessible to experiment, including conditions more closely relevant to the purine de novo pathway and other multienzyme pathways in vivo. Significantly increased reaction rates could only be realized if essentially all active copies of the enzyme were localized exclusively to compartments. For the PEG/dextran ATPS used here, which intentionally lacked any affinity interactions with the enzymes, although ASL partitioning led to 8.7-fold higher local concentration, this compartmentalization was not strong enough to result in overall rate increases. Recent work by Levy and coworkers has shown that enzymes partition between the cytoplasm and the membrane of yeast cells. They showed that the protein there was an ~9.5 fold difference in concentration of proteasome subunit Pre9 between the cytoplasm and the membrane. 566 other proteins had at least a 4-fold difference in concentration between the two locations. In principle, selective biorecognition partners present within an intracellular liquid organelle could provide the necessary local concentration to increase flux for the sequential reaction in vivo. For the purinosomes, interactions with other purine biosynthetic enzymes and unidentified factors are thought to be important in localization. More generally, intracellular liquid phases are rich in both nucleic acids and proteins that could provide ample opportunity for strong affinity partitioning. Additionally, the cell could modulate an enzyme’s activity depending on its
cellular location. Future work in this area could explore using phase forming components (such as proteins and nucleic acids) that have stronger interactions with the enzymes, which would have the potential to change enzyme activity and local enzyme concentration. In the specific case of the de novo purine pathway, we note that only the last two proteins were included in this study. Greater kinetic advantages may be expected for the full pathway, particularly if it assembles onto a scaffold such as the cytoskeleton, as has been proposed.4,40

5.5 Experimental Section

5.5.1 Materials

Human adenylosuccinate lyase (ASL) plasmid DNA and human AICAR transformylase/IMP cyclohydrolase (ATIC) plasmid DNA were provided by the Stephen J. Benkovic group at Penn State University. Poly(ethylene glycol) 8,000 Da, dextran from L. mesenteroides 9,000-11,000 Da, 5-aminoimazole-4-carboxamide ribonucleotide (AICAR), potassium chloride, glycine, lysozyme, sodium dodecyl sulfate, Coomassie Plus Reagent, DL-dithiothreitol, bromophenol blue, Amicon Ultrace filters (MWCO 10 kDa), Trizma base, and Trizma hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Rosetta 2(DE3)pLysS competent E. coli cells and BL21 (DE3) competent E. coli cells were from Novagen. Yeast extract, tryptone, agar, phenylmethylsulfonyl fluoride (PMSF), acrylamide:bis-acrylamide 19:1, TLC PEI cellulose F plates, ammonium acetate, ammonium hydroxide, methanol, potassium phosphate monobasic,
and potassium phosphate dibasic were acquired from EMD Chemicals (Darmstadt, Germany). Kanamycin sulfate was obtained from Calbiochem (Darmstadt, Germany). Complete EDTA-free protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Ni-NTA His-Bind resin was obtained from Qiagen (Hilden, Germany). Tetramethylethylenediamine was purchased from IBI Scientific (Peosta, IA). Glacial acetic acid and fumaric acid were acquired from Mallinckrodt Baker (Phillipsburg, NJ). Glycerol and sodium chloride were obtained from BDH Chemicals (West Chester, PA). Slide-A-Lyzer dialysis cassettes (MWCO 10 kDa) and albumin standards were acquired from Thermo (Waltham, MA). Alexa Fluor 488 C₅ maleimide, Alexa Fluor 647 NHS ester labeling kit, and 13 mm Secure-Seal Spacers were purchased from Life Technologies (Carlsbad, CA). mPEG-NH₂ MW 5000 was obtained from Shearwater Polymers (Huntsville, AL). Deionized water with a resistivity of 18.2 MΩ·cm from a Barnstead NANOpure Diamond water purification system (Van Nuys, CA) was used for all experiments. Buffers were filtered using a 0.45 µm pore size Nalgene filter units. All reagents were used as received without further purification.

5.5.2 Expression and Purification of Enzymes

N-terminal hexahistidine-tagged ASL plasmid DNA in a pET-28 vector was transformed into Rosetta 2(DE3)pLysS competent E. coli cells according to the Novagen protocol and was expressed and purified as reported by Lee and Colman⁴⁷ with several adaptations. The cell pellet (~1 g wet cell pellet per 500 mL cell culture) was dissolved in 50 mL of 50 mM potassium phosphate, pH 8.0, 300 mM potassium chloride, 10%
glycerol lysis buffer. A protease inhibitor cocktail tablet 10 µg/mL phenylmethylsulfonyl fluoride, and 1 mg/mL lysozyme were added and the mixture was stirred for 30 minutes. The mixture was sonicated on ice using a Misonix S-4000 sonicator. Pulse durations of 15 seconds at 50% amplitude were followed by a 45 seconds delay between pulses where the solution was placed in an ice bath, for a total pulse time of 5 minutes. Cell debris was removed by centrifugation (15 × g for 30 minutes at 4°C). The cell lysate was incubated with Ni(II)-NTA resin and 1% Triton X-100 to inhibit non-specific protein binding for at least 1 hour on an orbital shaker. The cell lysate was transferred to a column and the Ni(II)-NTA resin was washed with one column volume of lysis buffer and subsequently washed with one column volume of 20 mM imidazole to remove non-specific protein binding. A 10 step gradient of lysis buffer to 500 mM imidazole with lysis buffer was used to elute ASL in 10 mL increments and protein purity was determined with sodium dodecyl sulfate polyacrylamide gel electrophoresis. Pure fractions of ASL were concentrated to approximately 20% the original volume with centrifugal filter units (MWCO 10 kDa) and dialyzed against a 50 mM potassium phosphate, pH 7.0, 150 mM potassium chloride, 1 mM dithiothreitol, 10% glycerol storage buffer to be stored in small enzyme aliquots at -80°C. Enzyme concentration was determined by the standard Bradford assay utilizing 1.5 mL Comassie Plus Reagent and 50 µL of sample.

The pET-28 N-terminal hexahistidine-tagged ATIC vector was transformed into BL21 (DE3) competent E. coli cells as instructed by Novagen protocol. ATIC was subsequently expressed and purified as indicated by Wolan et al.48 Cell lysis and enzyme purification was done as described for ASL with lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 20 mM imidazole). After the pure fractions were
concentrated, ATIC was dialyzed against a 20 mM Tris (pH 7.5), 150 mM sodium chloride, 50 mM potassium chloride buffer. ATIC was quantified by the Bradford assay and was stored in aliquots at 4º C.

### 5.5.3 SAICAR Synthesis

SAICAR was prepared and purified as described by Zikánová with several adaptations. Fumaric acid stock solutions were prepared in the reaction buffer and adjusted to pH 7.0 with sodium hydroxide. The 1 mL reaction volume contained 2.6 mM AICAR and 20 mM fumaric acid in a 10 mM Tris, pH 7.5, 10 mM potassium chloride buffer. The final concentration of ASL was 0.4 mg/mL and the reactions were allowed to proceed for 5 hours at room temperature. Enzyme was removed by centrifugal filter units (MWCO 10 kDa) and the reaction mixture was concentrated to 100 µL via a Thermo Savant DNA 120 SpeedVac at ambient temperature. Higher temperature settings yielded the pink compound described by Van den Bergh. Aliquots of 20 µL were spotted on PEI-cellulose TLC plates to provide adequate separation of the concentrated product with 1 M ammonium acetate. Product was eluted with 2 mL of 1 M ammonium hydroxide overnight on an orbital shaker and the supernatant was subsequently evaporated to dryness with a Thermo Savant DNA 120 SpeedVac. SAICAR was resuspended in 33 mM Tris buffer, pH 7.4, 25 mM potassium chloride and the concentration was determined spectrophotometrically at 269 nm using the molar extinction coefficient $13.1 \times 10^3 \text{M}^{-1}\cdot\text{cm}^{-1}$. Aliquots were stored at -80 ºC.
5.5.4 10-THF Synthesis

The cofactor 10-formyltetrahydrofolic acid was prepared as described by Rabinowitz\textsuperscript{64} and Rowe.\textsuperscript{65} After purification, aliquots were stored at -80 °C. Concentrations were determined prior to use by spectrophotometry using the extinction coefficient of 9540 M\textsuperscript{-1}\cdot cm\textsuperscript{-1}.\textsuperscript{66}

5.5.5 ATPS Preparation

For binodal determination, separate 25 % (w/w) PEG 8 kDa and 25 % (w/w) dextran 10 kDa stock solutions were prepared with 33 mM Tris, pH 7.4, 25 mM potassium chloride buffer\textsuperscript{67} and samples with varying percentages of polymers were prepared near the expected binodal to determine at which concentrations phase separation occurred. For assays and partitioning experiments, a 10 % (w/w) PEG 8k and 10 % (w/w) dextran 10k in the same buffer was prepared and physically separated. The phases were then recombined at the desired PEG:dextran volume ratios. Samples were subsequently concentrated by a SpeedVac so that upon addition of enzymes and/or substrates, the ATPS would be diluted to its original concentration. This ensures the reconstituted volume ratios remain on the same tie line and was done for all described experiments. The viscosity of the phases was measured with an Ostwald viscometer.
5.5.6 Phase Composition Determination

The polymer composition of each phase was determined using a combination of refractometry and polarimetry.\(^68\) Refractive index measurements were done using a Leica Abbe Auto Refractometer. Polarimetry measurements were done using a PerkinElmer Model 343 Polarimeter. The concentration of dextran 10 kDa was determined by polarimetry, using a standard curve of known concentrations of dextran. The concentration of PEG was determined using refractometry. Calibration curves of known weight percents of PEG 8 kDa and dextran 10 kDa were prepared, and the refractive index of each of the standards and the PEG-rich and dextran-rich phases was measured. The contribution of the refractive index from dextran was subtracted from the total refractive index and the remaining refractive index was attributed to PEG 8 kDa.

5.5.7 Partitioning Coefficients

To determine enzyme partitioning, ASL was labeled at amines using succinimidyl ester functionalized Alexa Fluor 647 (degree of labeling, DoL): 4.8 dyes/tetramer, and ATIC was labeled at thiols using C5-maleimide Alexa Fluor 488 (DoL: 1.1 dyes/dimer). Both enzymes were labeled according to Invitrogen protocol, with the exception that enzyme concentration was 5 mg/mL instead of the suggested 2 mg/mL to prevent over-labeling. Upon addition of enzyme to the previously concentrated ATPS samples at a final concentration of 100 nM, samples were allowed to mix on a VWR Tube Rotator Unit for one hour or overnight and were phase separated by centrifugation. The partitioning coefficient of each of the enzymes was determined within the ATPS at a 1:1
PEG:dextran volume ratio (bulk fluorescence measurements) and a 9:1 PEG:dextran volume ratio (confocal microscopy measurements), using standard curves of each enzyme (ASL and ATIC) in each phase (PEG-rich phase and dextran-rich phase). Bulk fluorescence was measured using a Horiba Jobin Yvon Fluorolog 3-21 fluorimeter with FluorEssence software. Confocal microscopy images were acquired using a Leica TCS SP5 laser scanning confocal inverted microscope with a 63× oil objective. Samples were vortexed prior to imaging. Enzyme partitioning was measured individually and with both enzymes together. The predicted concentrations of ASL and ATIC in each phase at each volume ratio in Table 5-5 were calculated using the measured partitioning coefficients, as described by Strulson et al. 34

The partitioning of SAICAR, AICAR, 10-THF, and IMP were measured individually in a 1:1 PEG:dextran volume ratio at a final concentration of 100 µM. Samples were mixed and phase separated by centrifugation and the concentration in each phase was determined by HPLC. Before analysis, aliquots from each phase were diluted 2× with 1 M sodium hydroxide.

5.5.8 HPLC analysis of ATPS

The HPLC system consisted of an Agilent 1260 Infinity quaternary pump and 1260 Infinity Autosampler coupled to 1260 Infinity thermostatted column compartment using a Partisil 10-SAX anion-exchange column (0.42 x 22 cm) and an anion guard cartridge purchased from Mac-Mod Analytical (Chadds Ford, PA). Absorbance of SAICAR and AICAR were monitored at 267 nm and IMP was monitored at 250 nm with
a 1260 Infinity diode array detector. The metabolites were eluted using a flow rate of 2.0 mL/min with a 25 minute concave gradient \((t_G)\) where \(n = 7\) was used, going from 7.0 mM potassium phosphate, pH 3.0 to 250 mM potassium phosphate, 500 mM KCl, pH 3.8 (Eq. 5-12)\(^{69}\) Once the gradient reached 100%, the method was completed with a 5 minute flush of the more concentrated eluent before a 5 minute ramp to the initial conditions. Concentrations were calculated by measuring the peak area of the samples and standards of known concentration.

\[
\% \text{ Strong Eluent} = \left( \frac{t}{t_G} \right)^n \times 100
\]

(Equation 5-12)

5.5.9 Enzyme Assays: Michaelis-Menten Kinetics in the Individual Phases

ASL assays were conducted with adaptations from a previously described method\(^{70}\) using the difference extinction coefficient of 700 M\(^{-1}\)cm\(^{-1}\) at 267 nm\(^{71}\) with an Agilent 8453 diode-array UV-visible spectrometer with Agilent ChemStation Software. SAICAR was varied from 0 µM to 100 µM and ASL concentration was 50 nM. Product formation was measured for 5 minutes and the slope of the linear portion of the curve was used to measure activity. The standard Michaelis-Menten equation (Eq. 5-13) was used to fit the data in order to determine \(K_M\) and \(V_{\text{max}}\) using Igor Pro nonlinear regression analysis. The Michaelis-Menten parameters of the AICAR transformylase activity of ATIC were determined by adaptations from a previous method\(^{66}\) using a difference extinction coefficient of 19,700 M\(^{-1}\)cm\(^{-1}\) at 298 nm. The final concentration of ATIC was 50 nM. The \(K_M\) and \(V_{\text{max}}\) of AICAR was determined by varying the AICAR concentration.
from 0 µM to 100 µM in the presence of 200 µM 10-ψTHF. Absorbance measurements were collected for 5 minutes and analyzed as described above. The detection of the inosine monophosphate cyclohydrolase activity of ATIC was beyond the sensitivity of a spectrophotometric assay in the presence of AICAR TFase activity. All assays were conducted in triplicate. The $V_{\text{max}}$ of the enzymes needed to be adjusted for the mathematical model because we observed inconsistent activity rates between the activity of the enzyme in these assays and in the ATPS assays described above, most likely due to variabilities in enzyme batch purity and/or activity loss over time. We accounted for this by adjusting the enzyme activity to match the ATPS data described below and measuring the rate of ASL and ATIC at 100 µM substrate by HPLC, and using that rate for the $V_{\text{max}}$ of the enzymes for the mathematical model, as 100 µM substrate is well above the $K_{M}$ of the enzymes.

$$V_0 = \frac{V_{\text{max}}[S]}{K_M + [S]} \quad \text{(Equation 5-13)}$$

### 5.5.10 Enzyme Assays: Sequential Reaction in Individual Phases and Volume Ratios

Sequential assays were conducted in the PEG:dextran volume ratios in addition to the individual dextran-rich and PEG-rich phases. Concentrations were 100 nM ASL and ATIC, 100 µM SAICAR, and 400 µM 10-ψTHF. Samples were prepared without the initial substrate SAICAR and homogeneously mixed. Upon addition of SAICAR to initiate the reaction, the reaction was mixed on a VWR tube rotator and aliquots were taken at time points and diluted 2× with 1 M sodium hydroxide to both quench the
reaction and dilute the biphasic system to one phase for HPLC analysis. SAICAR and IMP concentrations were determined by standard curves prepared for each metabolite.

5.5.11 Simulation Method

As discussed in the Results and Discussion section, a set of partial differential equations (PDEs) described the system employing the mass balance equations. In order to simulate the underlying ATPS, we used software package COMSOL 4.3b, which solved the coupled PDEs numerically, using the finite element method. The minimum and maximum element size of the created mesh and the dimensionless time element in the simulation were 0.07, 0.5, and $10^{-2}$ respectively. To satisfy the partitioning expressions in Eq. 5-2 at the interface, we exploited a type of change of variables to define new continuous variables at the interface. By solving the new variables, the local concentration profiles during time were created, as discussed in our previous work (Chapter 3).^{37}

Contrary to the ATPS simulation, in PEG-rich or dextran-rich single-phase simulation, due to symmetry, the mathematical model could be simplified. In these cases, at every point, the net rates of diffusion were zero and the concentration of all species was uniform throughout the volume. As a result, the sequential reactions took place uniformly in the domain space, and Eq. 5-1 became a set of ordinary differential equations (ODEs). Also, the boundary conditions expressed in Eq. 5-2 and 5-3 were not considered in these two cases. To solve the ODE system for PEG-rich or dextran-rich
phase, we used software package MATLAB2014a and specifically the Runge-Kutta integration method.

5.6 References


Chapter 6
Conclusions and Future Directions

6.1 Conclusions

The work described here provides valuable insight as to the possible consequences of aqueous phase separation and compartmentalization in biological cells. I demonstrated that nonionic polymer-polymer and polymer-salt phase separation, as well as complex coacervation, are all capable of providing heterogeneous environments to study enzyme activity and compartmentalization. As described in Chapter 1, the liquid nature of some intracellular organelles is an exciting discovery because the same physicochemical concepts that apply to relatively simple phase separated systems can be used to understand cellular liquid compartment phase behavior. For example, the novel RNA/peptide complex coacervation work described in Chapter 2 shows that a long polyanion and short cationic peptide are capable of creating liquid phase droplets reminiscent of liquid-like compartments in cells. Could a simple mechanism based just on electrostatic attraction be enough to create phase droplets in vivo? It is possible, but specific biorecognition interactions and macromolecule structural changes are almost certainly factors that affect compartment formation and molecular contents in modern cells. However, one can imagine early earth scenarios where simple electrostatic attractions would have increased local concentration and facilitated biochemical
reactions, either of the phase forming components themselves or other reactive molecules partitioned to those compartments.

It is clear that many factors can affect enzyme activity in the cell—both favorably and unfavorably. There are advantages to colocalization of enzyme activities to a compartment as described in this dissertation and by others\textsuperscript{1-3} and as demonstrated with the purine biosynthesis enzymes in Chapter 5. But the cell also requires methods that minimize enzyme activity to prevent accumulation of unwanted or unnecessary products.\textsuperscript{4} Chapter 4 shows that this can be achieved by attractive interactions of substrates with other macromolecules in the vicinity of the enzyme, while Chapter 3 demonstrates the substrate can be localized to a separate coexisting environment away from the active enzyme. These mechanisms are in addition to others at the cell’s disposal, such as changing activity through positive or negative feedback,\textsuperscript{5} modifying enzyme structure,\textsuperscript{6} or through post-translational modifications\textsuperscript{7} such as phosphorylation\textsuperscript{8} discussed in Chapter 2. There are instances in which cells need to restrict enzyme activity to a single, specific area, so a scaffold bound enzyme would prove valuable. However, enzymes act on multiple substrates (e.g. HRP from Chapter 4), and may be needed in different locations at different times. So a liquid compartment capable of forming and dissolving as well as the ability to rapidly exchange contents would be advantageous for such enzymes. A liquid environment could allow the enzymes to adopt a specific structure. Why particular enzymes have evolved to be scaffold bound, freely diffusing, or both still remains as an open question.

Compartmentalization is crucial for biological cells. While the focus of this dissertation has been models for non-membrane bound compartments, it should be noted
that there has been extensive work on enzyme compartmentalization using techniques such as fusion enzymes\textsuperscript{9-11} and nanoparticle scaffolds.\textsuperscript{11-14} Until now, there was considerably less effort toward studying enzyme activity in crowded, multiphase systems. This dissertation provides an excellent framework for understanding enzyme activity in crowded, phase-separated environments as well as the possible mechanisms for creating liquid phase separation in cells.

\textbf{6.2 Future Directions}

Going forward, the overall goal should be to create more elaborate model systems to further our understanding of cells. This could be achieved through a number of different means. All of the phase systems described here were two phases, however, cells contain multiple coexisting environments. Multiphase systems could provide many compartments. The poly U/RRASLRRASL coacervates in Chapter 2 could be investigated within a macromolecularly crowded environment, perhaps a PEG/dextran ATPS. This could allow for three coexisting microenvironments, each with a distinct chemical composition. Added solutes would partition to different phase, and the kinase and phosphatase activity are likely to change based on being primarily in a crowded phase rather than the supernatant phase. It may also be possible to create different complex coacervates within a polymer-polymer system using nanostructures (like the silica beads) that have been functionalized with different surface chemistries as nucleation sites.
The creation of more elaborate multienzyme complexes/cascades is another direction that could be taken. One obvious avenue to pursue would be to incorporate even more sequential enzymes into aqueous phase model systems. I expect that there would be more kinetic advantages as the number of sequential enzymes increases. One potential challenge is that the enzyme could partition weakly, and prevent a large kinetic advantage like we observed with the purine enzymes in Chapter 5. Recent experimental work has demonstrated that there are kinetic advantages to purine biosynthesis in vivo, so the pathway may be capable of displaying kinetic advantages in vitro. Another approach would be to combine multienzyme complexes on scaffolds with other freely diffusing enzymes in the same solution to mimic both a scaffold-bound enzyme complex and complex within a separate liquid organelle. Another route to explore is dynamically changing enzyme or substrate location as a result of a reaction. An enzyme could be acting on a substrate in one phase and the product could then change partitioning to another phase and stimulate a reaction in that phase. Phosphorylation is a mechanism that could be used to achieve this goal, either by changing enzyme localization or enzyme structure in the phase systems.

An additional level of complexity could be applied to the phase systems by encapsulation of the phase forming components within a lipid membrane in an attempt to mimic the behavior of membrane bound compartments/organelles within an entire cell. This would allow for more selective transport of components into and out of the compartments. A small molecule such as cyclic-AMP could be transported across a membrane encapsulating a RNA-peptide coacervate system and activate regulatory subunit bound- protein kinase A in the coacervate phase. Phosphatase activity could be
regulated by addition or removal of the metal cofactors or other small molecule inhibitors.

There are also a few specific questions that remain in regard to the work in Chapter 2. We did not specifically look at the enzyme activity within the coacervate phase (because of the small volume and difficulty of extracting the phase), but it would be worth pursuing. A more detailed analysis of the kinetics of the kinase and phosphatase reaction with RRASLRRASL could be explored. Multisite substrates are common in vivo, but experimental kinetic data of multisite substrates in vitro is lacking.16,17

In conclusion, this dissertation has made significant strides in the understanding of biological cell complexity with use of aqueous phase model systems. My hope is that these experiments at the interface of physical chemistry, analytical chemistry, and biochemistry will prove relevant and beneficial not only to other chemists studying model cells, but also to biologists and biochemists studying enzymatic reactions and phase separation in vivo.

6.3 References


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