Title
Ionic Liquid Aqueous Two-Phase Systems for Diagnostic Use in Point-of-Care Settings

Author
Yee, Matthew Foosing

Publication Date
2018

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Ionic Liquid Aqueous Two-Phase Systems for Diagnostic Use in Point-of-Care Settings

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Bioengineering

by

Matthew Foosing Yee

2018

ABSTRACT OF THE THESIS

Ionic Liquid Aqueous Two-Phase Systems

for Diagnostic Use in Point-of-Care Settings

by

Matthew Foosing Yee

Master of Science in Bioengineering

University of California, Los Angeles, 2018

Professor Daniel T. Kamei, Chair

The objectives of this thesis were to investigate ionic liquid aqueous two-phase systems (ATPSs) as a concentration method for improving the lateral-flow immunoassay (LFA) detection of antigens, and demonstrate potential advantages of these ATPSs over conventional polymer-salt and micellar ATPSs used in the past. ATPSs have been widely utilized for liquid-liquid extraction and purification of biomolecules, with some studies also demonstrating their capacity as a biomarker concentration technique for use in diagnostic settings. As the limited polarity range of conventional ATPSs can restrict their use, ionic liquid (IL)-based ATPSs have been recently proposed as a promising alternative to polymer- and micellar-based ATPSs, since ILs are regarded as tunable solvents with excellent solvation capabilities for a variety of natural compounds and proteins. This study demonstrates the first application of IL ATPSs to point-of-care diagnostics. ATPSs consisting of 1-butyl-3-methylimidazolium tetrafluoroborate

([Bmim][BF_4]) and sodium phosphate salt were utilized to quickly concentrate biomarkers prior to detection using the LFA. We found the phase separation speed of the IL ATPS to be very rapid and a significant improvement upon the separation speed of both polymer-salt and micellar ATPSs. This system was successfully applied to both sandwich and competitive LFA formats and enhanced the detection of both *Escherichia coli* bacteria and the transferrin protein up to 8-fold and 20-fold, respectively. This system's compatibility with a broad range of biomolecules, rapid phase separation speed, and tunability suggest wide applicability for a large range of different antigens and biomarkers.

The thesis of Matthew Foosing Yee is approved.

Benjamin M. Wu

Dean Ho

Daniel T. Kamei, Committee Chair

University of California, Los Angeles

2018

Dedicated to my family.

Table of Contents

List of Figures

List of Tables

Acknowledgments

This work could not have been accomplished without the help of many people. First and foremost, I would like to thank Dr. Daniel T. Kamei, my advisor and mentor. It's been a privilege to work under his guidance for the past three years, and he has challenged me to grow and improve in ways I hadn't expected in first joining his lab. Throughout my undergraduate and graduate time in this lab, Dan has inspired me with his philosophy of discipline and integrity that I am sure will continue to guide me after I graduate. Joining this lab is one of the best decisions I've made at UCLA, and I can't thank him enough for all of the lessons I've learned from him and everything he's done for me.

I would also like to thank Sherine Cheung, who has been my graduate mentor during my time as an undergraduate student. She has taught me a lot about research, not just in how to perform it but also about the rigors of research and the mentality needed to succeed. Even as a graduate student, I have continued to look to her for guidance, and she has always been a source of positive support throughout my time in the lab.

I would also like to thank everyone I have worked with over the years in this lab. Grace Emmel and Eric Yang both helped me in the work done this year towards this thesis. Working with Nguyen Le, Eumene Lee and Justin Paek, we performed my first work as a graduate student in developing LFAs for the detection of HPV biomarkers. Sherine Cheung, Elizabeth Gomes and Nguyen Le were great team members who I worked with on developing a food pathogen spot test, which led to my first scientific publication. Sherine Cheung and Nguyen Le also were on my very first project in enhancing the portability of nucleic acid amplification tests, which really set the foundation for me as a researcher. Although it took some time, it was nice to see the manuscript on this work be recently accepted for publication. I'd also like to thank all my

Capstone team members, with whom I got to experience a self-driven project for the first time. Thank you to all my other friends and lab mates in the Kamei Laboratory who really helped make the long days and nights feel more fun and enjoyable. It's a fantastic, supportive community that I will always remember.

Last but not least, I would like to thank my family and all my friends who have helped support me during my time at UCLA. Thank you to my parents and my brother, who have continuously inspired me and supported me to be the best student and person I can be. Finally, thank you to the friends I've made here, my teammates, and everyone I call my family at UCLA, who have made these past five years some of the best of my life.

Chapter 2 is a version of Yee MF, Emmel GN, Yang EJ, Lee E, Paek JH, Wu BM, Kamei DT, *Use of Ionic Liquid Aqueous Two-Phase Systems for the Enhanced Detection of Transferrin and Escherichia Coli*. Submitted to Frontiers in Chemistry, 2018. This work was supported by National Institutes of Health funds to D.T. Kamei who was the PI. Experiments were conducted by M.F. Yee, G.N. Emmel, E.J. Yang, E. Lee, and J.H. Paek, while writing of the manuscript was done by M.F. Yee, G.N. Emmel, E.J. Yang, E. Lee, J.H. Paek, B.M. Wu and D.T. Kamei.

Chapter 1: Motivation and Background

1.1 Introduction

Infectious disease continues to be a leading cause of death worldwide, particularly prevalent in resource-poor settings. These diseases, encompassing a wide range of various pathogens, can greatly reduce quality of life and even lead to death. Furthermore, treatment of these illnesses places a large economic burden on both the individual and country. Contributing to both loss of life and economic strain, infectious diseases comprise a large burden on resource-poor settings globally.

1.1.1 Prevalence of infectious diseases in resource-poor settings

While mortality due to infectious disease has fallen significantly over the past 20 years, infectious disease still constitutes the majority of deaths in low-income countries (1). Furthermore, the bulk of these deaths, and the majority of illnesses, are caused by just a few pathogens; roughly 20 species caused two-thirds of infection-related deaths in 2010 (2). While the number of major infectious diseases is relatively small, these diseases still encompass a large range of different pathogens, and therefore different biomarkers, to detect. Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV), which afflicted approximately 36.7 million people worldwide at the end of 2016 (3). Malaria is caused by the *Plasmodium* parasite, infecting more than 212 million new people in 2015 alone (4). Tuberculosis is a respiratory disease caused by the bacterium *Mycobacterium tuberculosis* and infected over 10 million people in 2016 (5). This variety of disease-inducing pathogens requires either 1) a robust diagnostic system to detect for variations of a single biomarker type

(i.e., proteins, nucleic acids) across all pathogens, or 2) many diagnostic systems to individually target detection of specific pathogens. As the latter is logistically formidable, most current gold-standard diagnostics focus on the first.

Current gold-standard diagnostic tests are primarily laboratory based. Cell culture remains a popular method of detecting for bacterial infections, able to detect at low pathogen concentrations and also give information on bacteria morphology and species. It is commonly used as a standard test for screening of *Chlamydia trachomatis* (6). While highly sensitive, these tests take a long time to yield results, one study indicating a median time of 14 days between testing and the beginning of therapy, the length of which can reduce the number of patients returning for treatment (6). Meanwhile, nucleic acid amplification tests have also been utilized to detect for pathogens, specifically for genetic material belonging to viral or bacterial agents. They are advantageous as they are pathogen-specific down to the nucleotide level, since genetic sequence is unique to each species, and can detect rapidly at low pathogen concentrations. This high specificity has been utilized to screen for tuberculosis in patients as well as differentiate between infection and environmental bacterial contamination of cell culture tests (7). One final commonly used diagnostic test is the enzyme-linked immunosorbent assay (ELISA), which detects for disease-related biomarkers utilizing antibodies specific to the target of interest, followed by signal enhancement. It is a faster technique than cell culture, producing results in hours rather than days, and also more robust, being able to detect for biomarkers corresponding to toxins, parasites and viruses, not being limited to detection of bacteria. ELISA coupled with microscopy has been used to detect for malaria, displaying similar sensitivities and specificities to PCR, while being less expensive and faster than cell culture (8).

While all these tests are highly specific and sensitive, they are often difficult to access or are underutilized in resource-poor settings. This limited access is due to several factors. All require trained laboratory personnel, and often require expensive and bulky equipment, such as thermocyclers and incubators, and even lab space to perform. These resources are often unavailable in the peripheral health centers that serve the majority of the population, due to poor infrastructure and limited assets (9). Resource-poor areas also often lack proper infrastructure to ensure proper quality control of available diagnostics, further limiting the proficiency and accuracy of these tests (10). Even areas that can produce reliable results generally see low patient return for treatment due to the time needed to transport and perform tests at central laboratories, as well as stigma surrounding illnesses like sexually transmitted diseases (11). In response to these concerns, the development of point-of-care diagnostics that can detect for illnesses at high sensitivity and specificity has gained much focus, with the World Health Organization establishing characteristics of an ideal diagnostic test as the ASSURED criteria (**Table 1.1**). One such device that satisfies most of these criteria is the lateral-flow immunoassay.

Table 1.1. World Health Organization's ASSURED criteria for ideal point-of-care diagnostics.

A – Affordable
S – Sensitive
S – Specific
U – User-friendly (easy to perform without training)
R – Rapid and robust (under 30 minutes)
E – Equipment-free
D – Deliverable to end users

1.2 The lateral-flow immunoassay (LFA)

The lateral-flow immunoassay (LFA) is a portable, paper-based diagnostic device that is ideal for point-of-care settings, as it meets many criteria set forth by the World Health Organization's ASSURED criteria. It has been widely utilized as a pregnancy test, for which it has been very successful. The LFA usually consists of a sample pad, a detection zone, and an absorbent pad, often comprised of different paper materials. These materials can be adjusted based on the necessary application. Cellulose or fiberglass materials are often used for the sample pad. This sample pad can be pretreated with blocking agents in order to promote flow through the LFA strip as well as reduce any nonspecific interactions between sample components and the paper material. The sample pad can also be used as a filter in order to remove large contaminants in the sample. Following flow through the sample pad, the next component is the detection zone, which is typically made of nitrocellulose paper, to allow for immobilization of the detection components. This region can also be pretreated with blocking agents for similar reasons to the sample pad. Finally, high-density cellulose paper is generally used as the absorbent pad material at the end of the strip to wick excess solution and act as a sink for fluid flow.

An LFA test consists of applying the sample solution to the sample pad, usually by dipping the LFA strip into the solution. As the device is paper-based, the solution will wick up the strip due to capillary flow. The solution will often contain a colorimetric probe, usually colloidal gold that is conjugated with antibodies specific to the target of interest. Before the strip is added, or during travel up the strip, these probes will interact with the target of interest, and these complexes will in turn interact with the detection zone, visually indicating a positive or negative result. The solution is drawn fully over the detection zone by the absorbent pad, which

collects sample that has passed the detection zone and provides capillary pressure to continue flow up the strip from the sample pad.

There are two formats for the LFA: competitive and sandwich. While both utilize the same core technology, they serve different applications. The sandwich assay is primarily utilized for larger biomarkers, which consist of multiple binding sites for antibodies. Meanwhile, the competitive assay is typically used for small biomarkers like proteins, which may not contain the many antigen binding spots required for the sandwich assay, and therefore would be difficult to capture between two antibodies.

1.2.1 Competitive assay

A competitive format LFA has two lines of protein printed on the detection zone. The bottom line, closest to the sample pad, constitutes the test line, and contains the immobilized biomarker of interest. If the sample contains enough of the target biomarker, the biomarker will saturate all binding spots on the antibodies immobilized to the gold probes. These saturated complexes then will have no free binding spots to interact with the immobilized biomarker on the detection zone, and the probes will bypass the test line without binding to it. If the sample does not contain enough of the target biomarker to saturate the binding spots, the probes will bind to the immobilized biomarker, leading to the formation of a colored line. The second line, called the control line, consists of secondary antibodies that will bind to the primary antibody on the gold probes. Therefore, regardless if there is the target biomarker in the sample solution, the gold probe will be able to bind to the control line, leading to the formation of visual band. This control line validates the test by demonstrating that the fluid was able to flow up the strip. Therefore, the formation of two lines indicates a negative test, and the formation of one line indicates a positive test (**Figure 1.1**).

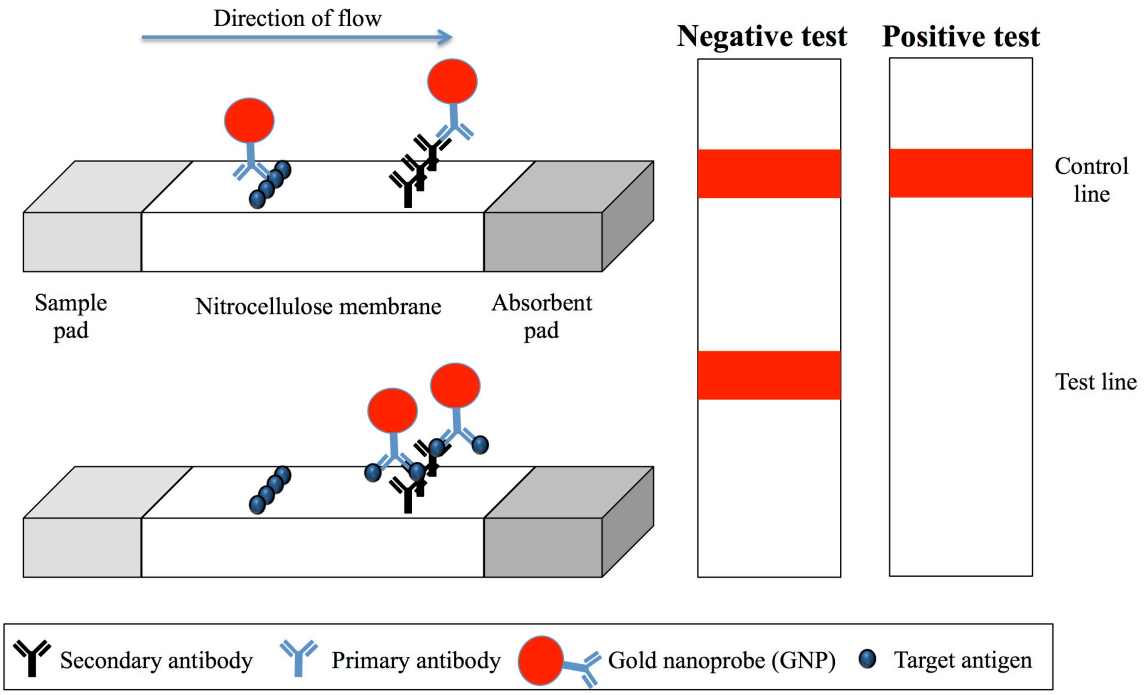

Figure 1.1. Schematic of negative and positive results for the competitive LFA format.

1.2.2 Sandwich assay

The other type of LFA is the sandwich format LFA. In this format, the control line still contains immobilized secondary antibodies that will bind to the primary antibody immobilized on the probe. The formation of a visual band at the control line would therefore still indicate a valid test. However, the test line contains immobilized primary antibodies specific to the target biomarker. If there is biomarker within the sample solution, it will interact with the antibodies on the probes, forming target-antibody-probe complexes. These complexes will pass over the test line, where the immobilized primary antibody will bind to the target, immobilizing the complex. Thus, the formation of a visual line at the test line would indicate the presence of the target biomarker. Conversely, if there is no target biomarker in solution, the non-complexed probe will be unable to interact with the test line, and no line will develop. Considering this mechanism, the presence of two bands would indicate a positive result in this format, while the presence of only

one band would indicate a negative result (**Figure 1.2**). Through the use of both competitive and sandwich format assays, a wide variety of different molecules can be detected using the LFA.

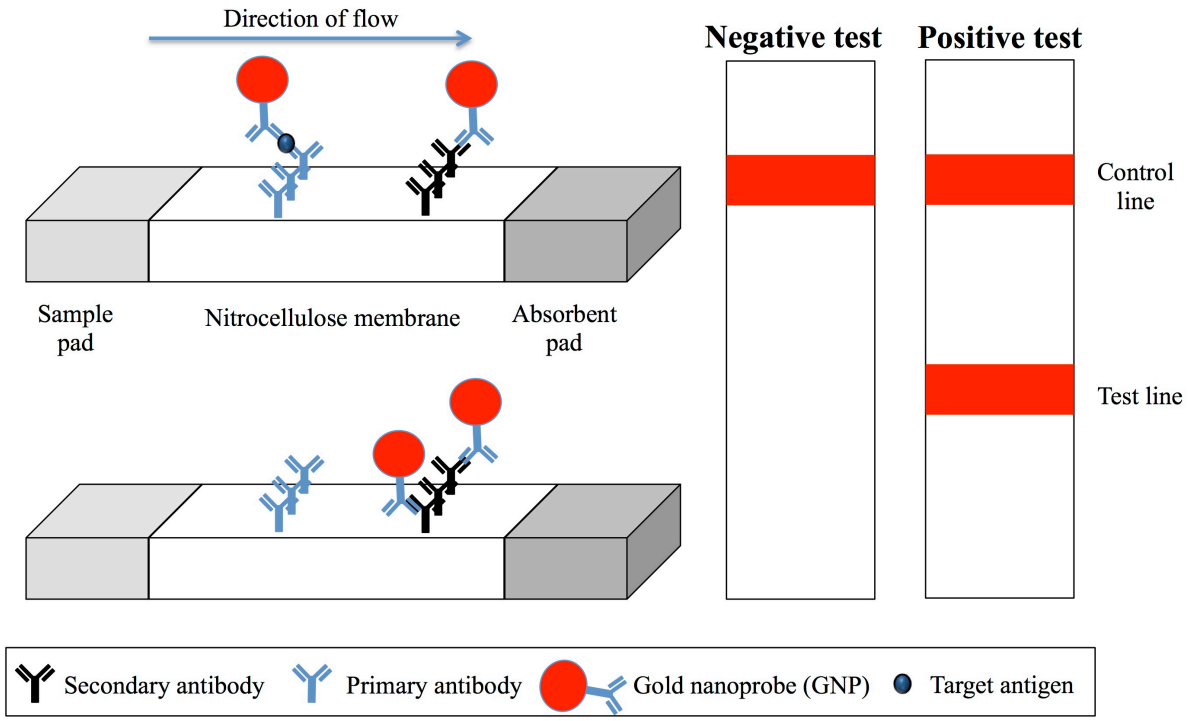

Figure 1.2. Schematic of negative and positive results for the sandwich LFA format.

1.3 Aqueous two-phase systems (ATPSs)

While the LFA is generally ideal for use in point-of-care settings, it does suffer from a lack of sensitivity compared to laboratory-based tests. In order to make the LFA a more viable test for broad use in diagnostics, the sensitivity of the test needs to be enhanced. Generally, there are two methods of doing this. The first is improving the core technology itself, in this case the LFA, which can be technically challenging. The second is to concentrate the biomarker prior to detection, improving the sensitivity without requiring change to the LFA itself.

The aqueous two-phase system (ATPS) is a solution for this second method. ATPSs are liquid-liquid extraction systems that consist of two immiscible phases. They are similar to oil-water systems in this way; however, they are beneficial for use with biological molecules as they provide a mild environment for these molecules, which can be damaged or denatured in oil-based systems. Furthermore, they allow for the extreme partitioning of biomolecules to one of the two phases, due to excluded-volume, hydrophobic, and electrostatic interactions that the biomolecules experience with the components of each phase. For these reasons, ATPSs can be used to enhance the sensitivity of the LFA by concentrating biomarkers in a sample prior to application to the LFA.

The ATPS is also well-suited for use in point-of-care settings as it is lightweight, scalable (so as to minimize sample volume) (12), easy-to-use, and does not require power or equipment to use. Two common types of ATPSs used for diagnostic enhancement are polymer-salt ATPSs and micellar ATPSs. One example of a polymer-salt ATPS is the polyethylene glycol (PEG)-potassium phosphate (salt) system. This PEG-salt ATPS undergoes phase separation at a critical concentration of salt. Initially, PEG molecules introduced to an aqueous solution participate in hydrogen bonding with neighboring water molecules, which form hydration shells around the PEG molecules. These shells prevent PEG molecules from closely approaching each other. The addition of salt to a certain concentration disrupts the directionality of these hydrogen bonds, pulling the water molecules away from the PEG molecules. This disrupts the hydration shell, allowing PEG molecules to approach each other more closely and interact through favorable van der Waals interactions. The combination of the PEG-water interactions becoming less favorable and the PEG-PEG interactions becoming more favorable leads to the formation of microscopic PEG-rich domains and salt-rich domains. Due to the density difference between the domains and

also to reduce the total interfacial energy of the system, the PEG and salt domains will eventually coalesce and macroscopically phase separate into a top PEG-rich phase and a bottom salt-rich phase. Micellar systems separate in a similar manner, responding primarily to a raise in temperature. For example, at a critical temperature, the surfactant Triton X-114 will phase separate into a bottom micelle-rich phase and a top micelle-poor phase.

1.3.1 Applications for ATPSs in diagnostics

Partitioning of target hydrophilic biomolecules in micellar and polymer-salt systems primarily relies on excluded-volume interactions. When large, bulky biomarkers are added to a polymer-salt ATPS, they will experience greater steric interactions with the more abundant polymers in the polymer-rich phase than in the salt-rich phase. Thus, they will partition extremely into the salt-rich bottom phase. Similarly, when large biomolecules are added to micellar systems, they will experience greater steric interactions with the more abundant and larger micelles in the bottom micelle-rich phase than in the top phase and thus partition extremely into the top phase.

Our group has previously demonstrated the use of these systems to concentrate biomarkers prior to detection with the LFA. Specifically, polymer-salt and micellar systems have been used to enhance sensitivity 10-fold in detecting biomarkers related to parasites (13) and viruses (14). In a similar manner, polymer-salt ATPSs were also demonstrated to improve sensitivity and decrease time-to-detection of a paper-based spot immunoassay (15). Furthermore, the degree of concentration of these biomarkers can be finely tuned by controlling the ratio of the volume of the top phase to the volume of the bottom phase, which is known as the volume ratio. More extreme volume ratios would lead to greater concentrations in the target; however, this would occur at the expense of phase separation speed. As the volume ratio of the ATPS becomes

more extreme, it becomes more difficult for microscopic domains of the smaller phase to find each other, and thus time to phase separation is increased. This becomes very apparent in the use of micellar systems, which can take 12 hours to phase separate for 1:9 volume ratio ATPSs, due to the high viscosity of the components and low interfacial tension between the phases (13).

To address this issue, our group has previously demonstrated the use of 3-D paper architecture to enhance phase separation speed. This is based on the phenomenon that the rate of macroscopic phase separation of micellar and polymer-salt ATPSs is increased when applied to porous media (13, 16). This enhanced speed is believed to occur due to a combination of viscosity differences and interactions with the porous media; as the polymer-rich and micellar-rich phases are more viscous than their counterpart phases, they travel through the media more slowly, while the other phase travels more quickly, facilitating phase separation. This 3-D paper architecture was utilized to improve phase separation time of a polymer-salt ATPS from 1 hour to 6 minutes and the separation time of a micellar ATPS from 8 hours in a tube to 3 minutes on paper. While these results indicate drastic improvements in phase separation speed, it is important to note that this technique does require modification to the existing LFA structure. Due to this, more modifications will be necessary to use ATPSs with more extreme volume ratios that would yield the greatest degree of concentration.

Furthermore, in these studies, large biomolecules were specifically utilized so excluded-volume interactions could be used to partition the biomarkers. For the protein biomarkers, which were smaller and did not experience strong steric interactions with the polymers or micelles, probes were utilized to capture the biomarker. The larger size of the probes then allowed for partitioning of the probe-target complex and subsequent concentration of biomarker, leading to improvements in the detection of the protein (17). However, there remain limited options to

concentrate hydrophilic protein biomarkers themselves within conventional micellar and polymer-salt ATPSs. This is due to the limited range in polarity between the two phases, which limits the effect of electrostatics in partitioning behavior. Therefore, despite the effectiveness of the ATPS in enhancing the sensitivity of LFAs, there are still areas to improve, specifically regarding phase separation speed and partitioning behavior. One way to achieve this improvement is through the use of ionic liquids.

1.4 Ionic liquids (ILs)

Ionic liquids (ILs) are salts that are molten at low temperatures due to their low charge density, and thus are solvents consisting entirely of ions. They were first discovered in 1914 (18) and have been investigated as alternatives to traditional organic solvents, which are generally toxic, flammable, and volatile. While initial ionic liquids were primarily investigated for applications in extractions and separations, second-generation room temperature ILs were soon developed, these air- and water-stable ILs offering applications beyond just extraction processes (19). Other uses for them include roles as catalysts, reagents, and even electrolytes for use in batteries, displaying a wide range of applications due to their unique nature (20). Furthermore, they are relatively inexpensive to produce and easy to modify, the R group of the cation being readily variable, and the identity of the anion easily adjusted (21). This potential for modularity presents these liquids as designer solvents, whose properties can be fine tuned to solvate a variety of different compounds and molecules, including organic, inorganic, and organometallic compounds. ILs have also been reported to display miscibility with both water and organic solvents, including benzene and toluene (22).

Room temperature hydrophilic ILs are particularly promising for protein extraction, as they can be used at low temperatures and are easily recycled. Many of these ILs display thermosensitive behavior and separate themselves from co-solvents and other materials at higher temperatures, allowing for reuse of the IL solvent (23) A popular example is the methylimidazolium-based ILs. These are prepared through the alkylation of methlimidazole with an akyl halide, the alkyl halide constituting the variable R group of the IL cation (24). Alkylation is followed by halogen exchange if the anion species is a halogen, or with addition of salt if the anion species is not a halogen. The identity of the halogen or salt thus dictates the anion species. As seen from this process, IL preparation can be varied readily, with both the cation and anion exhibiting modular behavior. Additionally, while imidazolium-based ILs are the most characterized IL for protein partitioning, other ILs have been investigated for use in protein isolation, including cholinium-based ILs, ammonium-based ILs, phosphonium-based ILs, and Good's Buffer ILs. These ILs demonstrate varying characteristics for use in different settings, such as different protein interactions that promote improved partitioning, buffer capabilities that prevent the need for additional buffers, and different phase separation behavior, i.e., phase separating with the use of different salts, polymers, or carbohydrates.

There are six principle techniques reported in the literature for IL-assisted protein separation (25). The first involves the use of hydrophobic ILs in liquid-liquid extraction, which is more widely used for non-biological compounds. There have been several reported uses of protein extraction from aqueous solutions to an IL phase due to enhanced solubility of specific proteins in the IL (26, 27). However, compared to hydrophilic ILs, hydrophobic ILs are less biodegradable, more expensive, and more toxic, which limits the number of hydrophobic ILs that can be used. Additionally, the partitioning of these proteins is extremely dependent on favorable

hydrophobic interactions with hydrophobic IL, limiting the number of proteins that can be extracted. The second technique, three-phase partitioning, focuses on the concentration of proteins to an interface to achieve greater fold improvements in protein concentration. However, the number of IL-based systems that can promote the formation of this third phase is limited, and there has been little industrial interest in this (28-30). A third technique consists of microemulsions using ILs, which have been demonstrated to achieve extraction of proteins similar to liquid-liquid extraction with hydrophobic ILs, but with improved selectivity (31, 32). In a fourth technique, ILs have also been proposed to be used for protein extraction from solid phases, such as algae (33) and yeast cells (34); however, few studies have been performed fully exploring this concept. In a different way, ILs were also used to modify adsorbent materials to achieve more efficient solid-phase extraction. In this fifth technique, IL moieties were immobilized to different polymers (35, 36), the new chemical characteristics of which allowed for greater adsorption of specific proteins to the polymers. This phenomenon was highly dependent on pH and ionic strength of the solution, indicating the role of electrostatics in separation regarding ILs. The last and most robust technique reported was the use of IL-based ATPSs.

1.4.1 Application of ILs to ATPS

The first reported use of an IL in an ATPS was by Rogers and coworkers in 2003 (37). By mixing imidazolium-based ILs with kosmotropic salts, two aqueous phases were reported to form. Being primarily aqueous-based, these IL ATPSs have been recognized as efficient protein extraction alternatives compared to other IL-based separation processes. In addition to traditional ATPS advantages such as mild operating environment and biocompatibility, which is shared with polymer-salt and micellar systems, these IL ATPS also offer two distinct advantages in fast

macroscopic phase separation equilibrium time as well as increased partitioning contributions from electrostatic interactions (38).

As opposed to the phase separation mechanism of polymer-salt and micellar systems, in which the combination of reduced hydrogen bonding with water and increased van der Waals interactions between polymers or between micelles promotes the formation of different domains, phase separation of IL systems is primarily due to a salting-out effect of the salt component on ILs (37). Compared to conventional salts, ILs exhibit a lower charge density, due to their large size versus charge, and thus do not interact as strongly with water molecules compared to salt molecules. When these higher charge density salts are added to a solution of IL and water, the salts are preferentially solvated and hydrated by the water molecules, which reduces the solubility of IL in the water (39). This excludes the IL to an IL-rich phase, which exists adjacent to the salt-rich phase. Domains will coalesce for similar reasons to conventional ATPSs to form two macroscopic bulk phases.

While the most common IL ATPSs are composed of IL and salt, several alternatives have been utilized, with their own advantages and disadvantages. IL and carbohydrate systems have been investigated, with the carbohydrate used as the salting-out agent instead of salt (40). While the use of carbohydrates limits the amount of ionic exchange between phases, which increases the capacity to reuse ILs, there are significantly less carbohydrates available to induce phase separation, as they are a weaker salting-out agent (41). Systems composed of IL and polymer have also been considered for the partitioning of enzymatic proteins, with the polymer serving as the primary phase-forming component and the IL acting as a promoter for separation (42). These systems allow for more precise control over the phases' polarities compared to polymer-salt

systems, yet are still less tunable than IL-salt systems. However, these systems allow for lower IL concentrations than IL-salt ATPSs, which improves their biocompatibility.

The degree of IL salting-out, which is controlled by the specific species of salts and ILs used, can dictate the polarity of each phase. Thus, through tunable hydrophobic/electrostatic interactions with the protein surface chemistry, proteins that normally wouldn't be concentrated through excluded-volume interactions can be partitioned extremely via these IL ATPSs (43). Unlike separation in polymer-salt and micellar systems, in which steric interactions dominate partitioning, there are multiple factors that dictate partitioning in IL ATPSs.

One factor is protein salting-out. Specifically when referring to IL-salt ATPSs, kosmotropic salts in the ATPS will interact with water molecules more than ILs will, due to the disparity in charge density. These kosmotropic salts will then structure water molecules around them, forcing them into 'cage-like' configurations. As the salt-rich phase consists of a high concentration of these salts, the water molecules present in this phase will be less able to interact with proteins introduced into the system. Therefore, there will be less solvent in the salt-rich phase, promoting protein partitioning to the IL-rich phase, in which these proteins will be more soluble (44). Another factor that drives protein partitioning is hydrophobic interactions between the protein of interest and the cation species of the IL. For instance, it was found that imidazolium-based ILs are very useful for partitioning of bovine serum albumin, lysozyme, and hemoglobin, as the aromatic π system of these ILs favorably interacts with the hydrophobic residues on these proteins, promoting partitioning to the IL-rich phase (45). Additionally, these proteins can be more precisely concentrated by adjusting the system pH to closely match the pI of the protein. It was demonstrated that the closer the pH of the system is to the pI of the protein, the more significant the hydrophobic interactions and thus the easier to partition these proteins

(45). Finally, electrostatic interactions are also thought to play a large role in partitioning. One example is through the use of Ammoeng ILs, which are acyclic ammonium-based ILs. As opposed to imidazolium-based ILs, in which hydrophobic interactions with proteins are described to have a much larger factor in partitioning, protein partitioning in Ammoeng-salt ATPSs is primarily controlled by electrostatic interactions between negatively charged protein residues and the cation of the IL (46). From these studies, the primary factors that govern partitioning in IL ATPSs are hydrophobic interactions between proteins and ILs, electrostatic interactions between proteins and ILs, and the salting-out effect. The prevalence of each interaction depends on the protein of interest and the specific ATPS components used.

Through these factors, ATPSs have been demonstrated in several studies to achieve high levels of protein extraction, while also retaining protein function regarding the purification of enzymes and other functional proteins. Most work in demonstrating the isolation of enzymes are focused on lipase enzymes, as lipases are highly relevant for use in industry, having roles in petrochemical, pharmaceutical, food, paper, and waste management industries. Lipase from *Thermomyces lanuginosus* was demonstrated to be extracted from aqueous solutions, while maintaining its native structure and function, using an appropriate imidazolium-based IL (47). While these authors initially performed extraction of purified enzyme, extraction from complex media such as fermentation broth was also demonstrated to be successful using IL ATPSs (48). It was found that optimized IL ATPSs were more efficient than conventional polymer ATPSs for extraction, yielding higher purification factors and enzyme recoveries. While lipases are the most well-studied enzyme, isolation of different enzymes, including papain, horseradish peroxidase, wheat esterase, and superoxide dismutase have also been demonstrated (25), suggesting these IL-based systems can be widely applicable for the partitioning of proteins. Furthermore, as protein

structure can be maintained in these systems, IL ATPSs have great potential to be used effectively in immunoassays.

With ILs generally considered as alternatives to organic solvents for use in industrial separations, there have been few studies investigating their use in enhancing diagnostics. Most applications for ILs in diagnostics involve the use of electrochemical sensors that respond to a change in chemical environment or other bioelectronics that utilize ILs primarily as a material to perform detection on (49). Despite the low number of studies, it has been reported that antibodies can retain their function within IL solutions. This was demonstrated through the performance of a fluorescence quenching immunoassay between IgG antibodies and a fluorescein-like protein (50). Binding between this antibody and protein pair was retained for high IL concentration in phosphate buffer. Furthermore, immobilized antibodies were shown to also retain affinity for the target when exposed to pure IL as a solvent, with minimal loss in activity.

In Chapter 2, application of the IL ATPS for the enhancement of the LFA was demonstrated. Despite the high concentration of IL in the ATPS, gold nanoprobes and dextran-coated gold nanoprobes were both found to be stable in the system and demonstrate suitable binding capabilities for use in LFA. The IL ATPS was found to phase separate very quickly; complete phase separation was achieved in less than 1 minute for the 1:1 volume ratio ATPS and in less than 5 minutes for the 1:9 volume ratio ATPS. This exhibits similar phase separation times to phase separation on paper using polymer-salt and micellar systems, without necessitating the use of 3-D paper architecture. Furthermore, the IL ATPS was compatible with both competitive and sandwich format assays, demonstrating improvements in sensitivity over LFA tests alone. The IL ATPS also produced greater enhancements in competitive assay detection limits than seen in polymer-salt ATPSs, due to a screening effect that was beneficial

for competitive assay tests. This study is the first use of IL ATPS for enhancing diagnostics and can be used as a basis for future studies investigating the use of IL ATPS for precise protein partitioning in point-of-care diagnostics.

Chapter 2: Use of Ionic Liquid Aqueous Two-Phase Systems for the Enhanced Paper-Based Detection of Transferrin and *Escherichia Coli*

2.1 Introduction

While global health has improved over the last few decades, health pandemics in resource-poor settings remain a large problem (51-53). These health issues include chronic health conditions, such as diabetes (54) and infectious diseases, such as tuberculosis (5). In countries like the U.S, many of these illnesses are readily treatable, especially when diagnosed early; however, in resource-poor settings, patients lack easy access to standard laboratory-based tests such as the enzyme-linked immunosorbent assay (ELISA), nucleic acid amplification tests, and serology tests (55). With issues such as poor infrastructure and limited funding already leading to underutilization of central laboratories in these resource-poor settings (55) (56), there is a growing interest in developing point-of-care techniques to diagnose a variety of diseases. Devices, such as miniaturized bioelectronics and microfluidic tests like the lateral-flow immunoassay (LFA), have received much attention over recent years due to their ease-of-use, portability, and limited need for power (57). However, in comparison to the gold standard laboratory-based tests, these devices are still restricted by their limited sensitivity, indicating an increasing need for enhanced detection capabilities at the point-of-care.

One technique for enhancing point-of-care detection is the aqueous two-phase system (ATPS), a liquid-liquid extraction system that has previously been demonstrated to concentrate biological markers (58). ATPSs consist of two immiscible phases, similar to oil-water systems; however, both phases of an ATPS are aqueous-based. Molecules introduced into an ATPS can

experience extreme partitioning between the two phases based on the excluded-volume, hydrophobic, and electrostatic interactions they experience with the components of each of the two phases. Furthermore, ATPSs are much more biocompatible than conventional oil-water systems, and have been widely utilized for the purification of proteins and nucleic acids (58). While the ATPS has been traditionally used in large-scale, industrial bioseparations, it also lends itself well for applications in point-of-care settings, as it is easy-to-use, can be rapid, and is scalable (to minimize sample volume) (12). In addition, ATPSs do not require laboratory equipment and are low in cost compared to more conventional laboratory tests such as the ELISA and nucleic acid amplification.

For these reasons, our research group has recently demonstrated the use of the ATPS as a pre-concentration tool to improve the sensitivity of portable paper-based diagnostic tools such as the LFA. Through the use of conventional polymer-salt and micellar systems, ATPSs combined with existing detection technologies have been shown to enhance sensitivity in detecting various biomarkers, including ten-fold improvements in LFA detection of parasitic biomarkers (13) and viruses (14). In a similar manner, the ATPS was also shown to improve sensitivity and decrease time-to-detection of a paper-based spot immunoassay (15). However, despite the efficacy of these systems, the limited polarity range of these ATPSs can restrict their use (38), particularly regarding the partitioning of small hydrophilic proteins. As micellar and polymer ATPSs predominantly rely on excluded-volume interactions to partition hydrophilic biomolecules to a particular phase, smaller hydrophilic biomarkers such as proteins can be difficult to partition extremely. One approach to improve upon this issue is to fine-tune the polarity of the ATPS components and introduce electrostatic effects as a more significant factor in partitioning.

One potential solution is through the use of ionic liquids (ILs), which are salts that are molten at low temperatures. ILs have been investigated as alternatives to traditional, volatile organic solvents as they exhibit non-flammability and negligible volatility (38) due to their ionic nature. In addition, they are particularly promising for use in an ATPS as they are highly tunable and have excellent solvation capabilities (23) for a variety of natural compounds and proteins. This has led to their use in various extraction and separation processes (23, 59, 60) including ATPSs. These systems were found to phase separate with the mixture of kosmotropic salts and imidazolium-based ILs (37). Since then, different classes of ILs have been discovered, developed, and utilized in the formation of ATPSs (39, 61); this variety in ILs, combined with an even greater variety in salts, could potentially allow researchers to precisely concentrate smaller biomolecules that would otherwise be difficult to partition extremely into one phase through excluded-volume interactions alone.

In this study, IL ATPSs consisting of 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) and sodium phosphate salt were utilized to demonstrate the compatibility of the IL ATPS with LFA and the ability of this technique to improve the sensitivity of LFA tests. This enhancement was applied to the model protein transferrin, using the competitive LFA format, and the model pathogen *Escherichia coli* O157:H7, using the sandwich LFA format. To our knowledge, this is the first application of an IL ATPS for the enhancement of point-of-care diagnostics. The IL ATPS demonstrated very fast phase separation and was found to be directly compatible with LFA, requiring no additional modification to existing LFA structure; by utilizing these benefits and also a significant enhancement effect, our system addresses limitations faced by existing paper-based portable diagnostics regarding the concentration of small biomarkers.

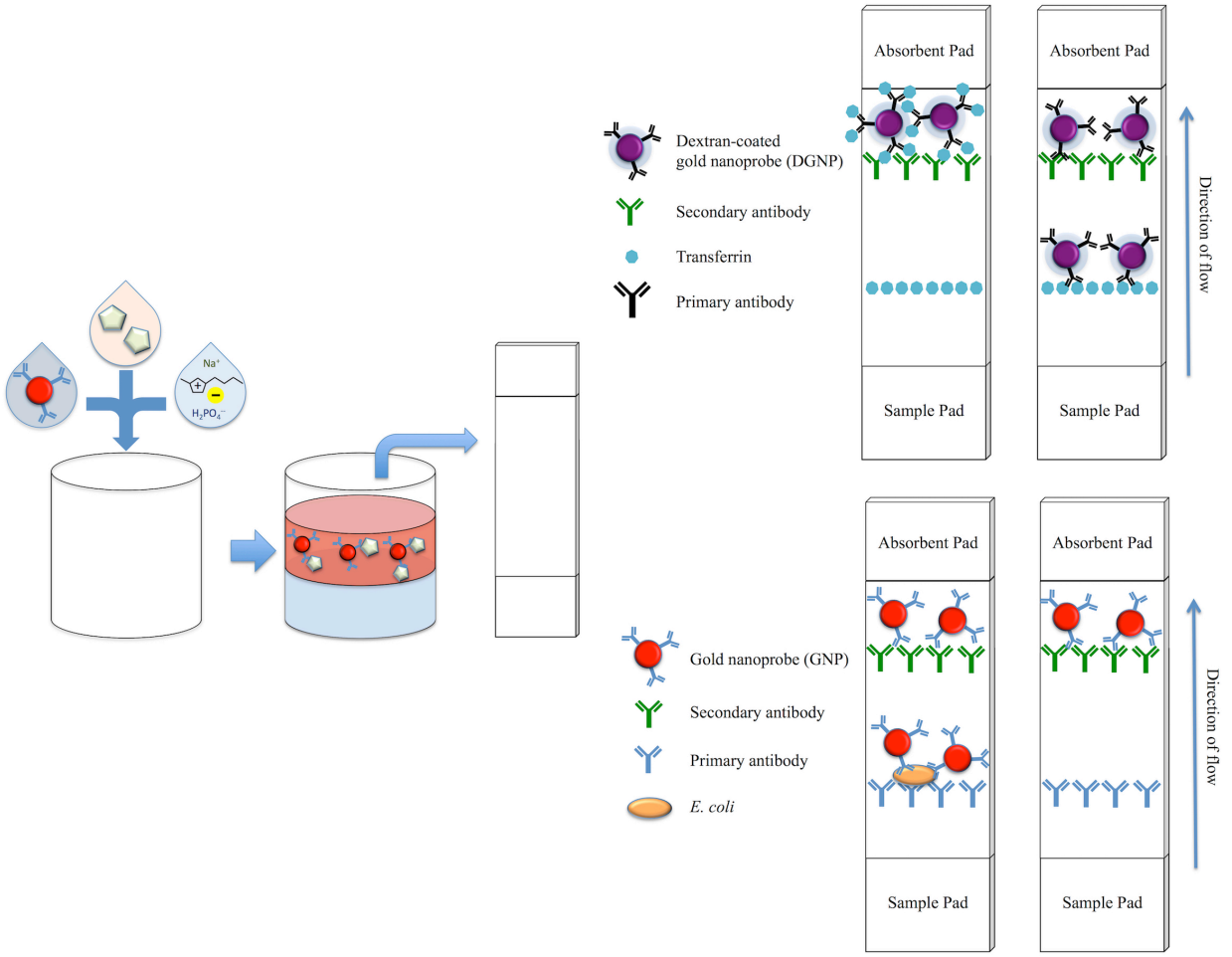

Figure 2.1. Schematic of an IL ATPS when used for diagnostics. Following separation, the top phase is applied to both competitive format LFAs for detection of transferrin and sandwich format LFAs for the detection of *E. coli*.

2.2 Materials and Methods

2.2.1 Preparation of bacterial cell cultures

Escherichia coli O157:H7 bacteria (*E. coli*) (ATCC® 700728™) were grown and cultured according to manufacturer protocol (ATCC, Manassas, VA) and plated onto Difco Nutrient Agar (Becton, Dickinson and Company, Sparks, MD) plates. Plated cells were subsequently incubated at 37°C aerobically overnight. The incubated plates were then sealed

with Parafilm and stored at 4°C until use. To prepare bacterial suspensions for use in ATPS and LFA tests, single colonies were picked from the agar plate and cultured in 5 mL of Difco Nutrient Broth (Becton, Dickinson and Company, Sparks, MD). The cell suspension was then incubated in a shaker-incubator at 37°C and 200 rpm for 16 hours. After use in LFA tests, the concentrations of bacteria in the suspensions were determined through plating of bacteria following serial dilutions. These bacteria were then incubated at 37°C aerobically overnight, after which the colonies were counted in order to quantify the bacterial concentrations used in the tests.

2.2.2 Preparation and visualization of IL ATPSs

Compositions of IL and salt necessary to achieve the desired equilibrium volume ratios, i.e., the volume of the top phase divided by the volume of the bottom phase, were determined by varying the initial concentrations of both [Bmim][BF_4] (Sigma-Aldritch, St. Louis, MO) and sodium phosphate (2:1 dibasic:monobasic) in solutions of Dulbecco's phosphate-buffered saline (PBS; Invitrogen, Grand Island, NY, pH 7.4). Conditions for 1:1 and 1:9 volume ratio ATPSs were found to be 35% [Bmim][BF_4] and 3% salt, and 65% [Bmim][BF_4] and 0.5% salt, respectively. Additionally, 0.01% Triton X-100 surfactant (Sigma-Aldritch, St. Louis, MO) was added to the 1:9 IL ATPS to facilitate phase formation. These conditions were used for all of the following experiments.

For visualization of phase formation in the IL ATPSs, 44 µL or 8.8 µL of bovine serum albumin-coated dextran-coated gold nanoparticles were added to 1.5 g 1:1 volume ratio or 1:9 volume ratio ATPSs, respectively. These ATPSs were well-mixed to ensure a homogenous mixture. The ATPSs were then incubated at room temperature. Time-to-equilibrium was

established when the visible domains arrived at their respective macroscopic phases, and the location of the interface remained stable.

2.2.3 Detection of transferrin (Tf)

2.2.3.1 Preparation of dextran-coated gold nanoprobes (DGNPs)

Purple colored dextran-coated gold nanoparticles (DGNs) were synthesized according to Min and coworkers with slight modifications (62, 63). Briefly, 0.75 g of dextran (M_w 15000-25000) were dissolved in 9.9 mL of filtered UltraPure sterile water (Rockland Immunochemicals Inc., Gilbertsville PA). The solution was stirred and heated to a boil, after which 135 μL of 1% w/v gold (III) chloride hydrate were added. The color of the reaction mixture became dark purple, and the solution was stirred and boiled for 20 more minutes. The particles were stored at 4°C until use.

The dextran-coated gold nanoprobes (DGNPs) were prepared as follows. A 1 mL aliquot of dextran-coated gold nanoparticles was adjusted to pH 9.0 using 0.5 M NaOH. Subsequently, 4 μg of anti-transferrin (anti-Tf) antibodies were added to the solution. The mixture was placed on a shaker for 30 min to facilitate the formation of dative bonds between the antibodies and the dextran-coated gold nanoparticles. Free antibodies were removed by centrifugation. The pellet was resuspended in 100 μL of 0.1 M of sodium borate buffer at pH 9.0.

2.2.3.2 Preparation of competitive LFA strips

LFA test strips utilizing the competitive assay format were assembled in a similar manner to our previous studies (17). In this format, immobilized Tf constitutes the test line and immobilized secondary antibodies specific to the primary anti-Tf antibody constitute the control line. If enough Tf is present to saturate the antibodies immobilized to the DGNPs in a sample,

the Tf-DGNP complexes flowing through the LFA strip will not bind to the immobilized Tf on the test line. This results in the absence of a visible purple band at the test line region. If Tf is not present, unbound antibodies on the DGNPs will bind to the immobilized Tf, and a visual band will form at the test line. In either case, the antibodies on the DGNPs will bind the secondary antibodies immobilized at the control line and form a visible line, indicating successful sample flow through the strip. Therefore, a positive result is indicated by only one purple band at the control line, while a negative result is indicated by two purple bands at both the test line and control line (**Figure 2.1**).

2.2.3.3 Detection of Tf with LFA only

To verify the detection limit of Tf with LFA only tests, anti-Tf DGNPs were added to a sample solution in a test tube and allowed to bind to Tf present in the sample to form Tf-DGNP complexes. Tests consisted of 50 μL sample solution, which was composed of 3 μL of anti-transferrin DGNPs and 47 μL of a known amount of Tf dissolved in PBS, or only PBS for the negative control. The solution was incubated for 10 min at room temperature to allow the DGNPs to capture the Tf in solution. The LFA test strip was then inserted vertically into the sample solution, which wicked through the strip via capillary action towards the absorbent pad. Images of the test strips were taken immediately after 20 min with a Nikon D3400 camera in a controlled lighting environment. Triplicates of each test were obtained and analyzed with a custom MATLAB program.

2.2.3.4 Detection of Tf with the IL ATPS/LFA setup

For detection of Tf with the 1:1 volume ratio ATPS, 120 mg of a well-mixed 1:1 ATPS containing 3.6 μL of anti-Tf DGNPs and a known concentration of Tf were added into a test

tube. The solution was incubated for 10 min at room temperature to allow the DGNPs to capture the Tf in solution and to allow the ATPS to phase separate. The top phase was extracted and placed in a new test tube, and the LFA test strip was inserted vertically into the sample solution as explained previously (**Figure 2.1**). For detection of Tf with the 1:9 ATPS, 600 mg of a well-mixed 1:9 volume ratio ATPS containing 4.8 µL of anti-Tf DGNPs and a known concentration of Tf were added into a test tube. These overall ATPS volumes were chosen to maintain the sample volume applied to the LFA at 50 µL. The remainder of the procedure follows the methods outlined for detection with the 1:1 ATPS. Images of the test strips were taken immediately after 20 min with a Nikon D3400 camera in a controlled lighting environment. Triplicates of each test were obtained and also analyzed with a custom MATLAB program.

2.2.4 Detection of *E. coli*

2.2.4.1 Preparation of gold nanoprobes (GNPs)

Cherry-colored gold nanoparticles of diameter 40 nm (Nanocomposix, San Diego, CA) were obtained and stored at 4°C until use. To prepare functional probes for use in the LFA tests, the pH of the gold nanoparticle solution was adjusted to pH 9.0 using 0.5 M NaOH. For every 1 mL of gold nanoparticle solution, 8 µg of anti-*E. coli* antibodies were added. The reaction mixture was placed on a shaker for 30 min to facilitate formation of dative bonds between the antibodies and gold nanoparticles. Free antibodies were removed through centrifugation. The pellet was resuspended in 100 µL of 0.1 M sodium borate buffer at pH 9.0 and subsequently stored at 4°C until use.

2.2.4.2 Preparation of sandwich LFA strips

LFA test strips utilizing the sandwich style assay were prepared in a similar manner to our previous studies (14). For the sandwich style format, anti-*E. coli* antibodies specific for the target *E. coli* are immobilized at the test line, while secondary antibodies against the primary anti-*E. coli* antibody are immobilized at the control line. If enough *E. coli* is present in the sample, *E. coli* will bind to the antibodies on the GNPs, producing *E. coli*-GNP complexes. These will bind to primary antibodies on the test line, trapping the particles and forming a visual red band. Alternatively, if the target biomarker is not present, the colloidal gold will bypass the test line without binding. Regardless, antibodies immobilized on the GNPs will bind the secondary antibodies on the control line, forming a visual band and therefore indicating a valid test. Thus, the presence of one line at the control line indicates a negative test, while the presence of two lines at both the control line and test line indicates a positive test (**Figure 2.1**).

2.2.4.3 Detection of *E. coli* with LFA only

Tests with LFA only were performed as now described. Solutions containing *E. coli* suspensions in Nutrient Broth were first prepared, with *E. coli* concentrations serially diluted from an initially prepared culture in Nutrient Broth to achieve a range of concentrations for detection. Five microliters of diluted *E. coli* suspension, or 5 μL of pure Nutrient Broth for the negative control, were added to 40 μL of PBS and 5 μL of anti-*E.coli* GNPs for a constant sample volume of 50 μL. The resulting solutions were mixed and incubated for 10 min to allow for binding between *E. coli* and the GNPs. A test strip was dipped vertically into each solution, and the sample was allowed to wick up the LFA. After 20 min, the LFA strips were taken out of the solution, and an image of each strip was immediately taken with a Nikon D3400 camera in a

controlled lighting environment. Triplicates of each test were obtained; images were analyzed visually and quantified using a custom MATLAB program.

2.2.4.4 Detection of *E. coli* with the IL ATPS/LFA setup

For tests combining the ATPS with LFA, 120 mg of a 1:1 ATPS containing 5 μL GNPs and 12 mg of an *E. coli* suspension were added to a test tube. The suspension was incubated for 10 min, after which the top phase was extracted and tested as described previously for the LFA only tests. For the 1:9 ATPS, 600 mg of an ATPS containing 5 μL GNPs and 60 mg of an *E. coli* suspension were added to a tube and tested in a similar manner to the 1:1 ATPS runs. These overall volumes were chosen to maintain the sample volume applied to the LFA at 50 μL. The tests were also run for 20 min and immediately imaged with a Nikon D3400 camera in a controlled lighting environment. Triplicates of each test were obtained, and the images were analyzed via a custom MATLAB program.

2.2.5 LFA Quantification

A custom MATLAB script was written with an approach similar to Yager and coworkers (64) to quantitatively analyze the LFA tests. Images of the test strips were taken with a Nikon D3400 camera under controlled lighting, with each strip oriented the same way. The images were cropped and converted to an 8-bit grayscale matrix. The intensity was averaged along the axis perpendicular to the flow, and therefore parallel to both the control and test lines, generating a one-dimensional intensity map. The two maxima were identified as the control and test lines, with the distance between the two lines calibrated by using a reference LFA image with strong test and control lines. In the case of the transferrin competitive assay, this corresponded to the

negative control, and in the case of the *E. coli* sandwich assay, this corresponded to the positive control.

To obtain test line intensity from our sample data, the location of the control line was determined from the reference LFA image, and its distance from the test line was calibrated as described above. The test line region was set as a 15 pixel-wide region centered at this location. The baseline for the measurement was determined by averaging the signal from two 25 pixel wide boxes beginning 25 pixels before and 25 pixels after the center of our determined test line region. The test line intensity was then calculated as the area under the curve for this test line region.

2.3 Results

2.3.1 Visualization of IL ATPS phase separation

Several criteria were used to determine a suitable ATPS for use in this study. Our anticipated design involved taking advantage of rapid phase separation speeds of IL-based systems to avoid issues faced using polymer or micellar systems. To achieve ease-of-use and minimize extra user handling steps, we sought an ATPS where our probes would partition to the top phase. Additionally, maintaining low ionic content and physiological pH were considered, to preserve antibody function for use in the LFAs. With these considerations, [Bmim][BF_4] and sodium phosphate salt (2:1 dibasic:monobasic) were chosen as the components of the ATPS. These components successfully phase separated, and allowed for relatively low salt concentrations as well as a pH of 7.0 in the top phase, which was optimal for our applications. A schematic of the IL ATPS, along with competitive and sandwich LFA formats, can be found in **Figure 2.1**.

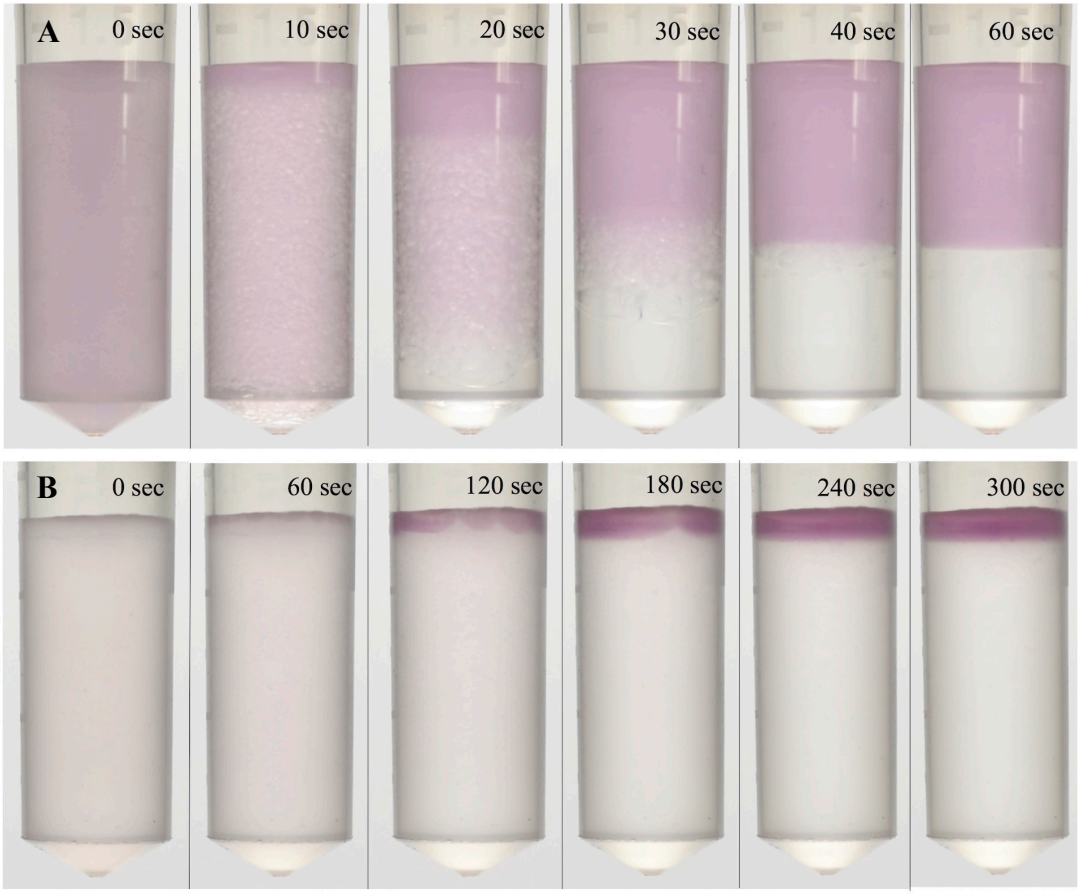

Figure 2.2. Visualization of phase separation and speed of an IL ATPS. Purple-colored dextran-coated gold nanoparticles partitioned extremely to the top phase and were used to visualize separation. ATPSs separated within **(A)** 1 minute for the 1:1 ATPS, and **(B)** 5 minutes for the 1:9 ATPS.

1:1 and 1:9 volume ratio ATPSs were achieved, and phase separation was visualized through the addition of bovine serum albumin-coated DGNs. The DGNs partitioned preferentially into the top phase, indicated by the purple-colored top phase, while the bottom phase remained clear due to the absence of DGNs. The particles were found to be stable in the ATPS, exhibiting no signs of aggregation over several days. These visualization experiments were also performed utilizing bovine serum albumin-coated gold nanoparticles (GNs), which exhibited similar partitioning and stability behavior. In all cases, phase separation was found to be quite rapid, occurring in 1 min for the 1:1 ATPS (**Figure 2.2A**) and in 5 min for the 1:9 ATPS

(**Figure 2.2B**). As 9:1 polymer-salt ATPSs can take an hour or so to phase separate, and 1:9 micellar systems even longer, this marked a great improvement in phase separation time compared to conventional ATPSs.

2.3.2 Detection of Tf

Upon identifying compositions for 1:1 and 1:9 IL ATPSs, we investigated the degree of improvement in utilizing these ATPSs for the detection of the model biomarker Tf. To do this, we sought to determine the limit of detection of Tf utilizing an LFA-only set-up, and then compare it directly with the limit of detection obtained utilizing the IL ATPS/LFA setup. As previously mentioned, these experiments were performed as competitive assays; two bands would indicate a negative test, as the antibodies on the DGNPs would be able to bind the immobilized Tf at the test line, and a single band at the control line would indicate a positive test, as DGNP antibody binding sites would be saturated and therefore unable to bind the Tf on the test line. With these mechanisms in mind, the limit of detection was defined as the lowest concentration of Tf at which the test line was not visible.

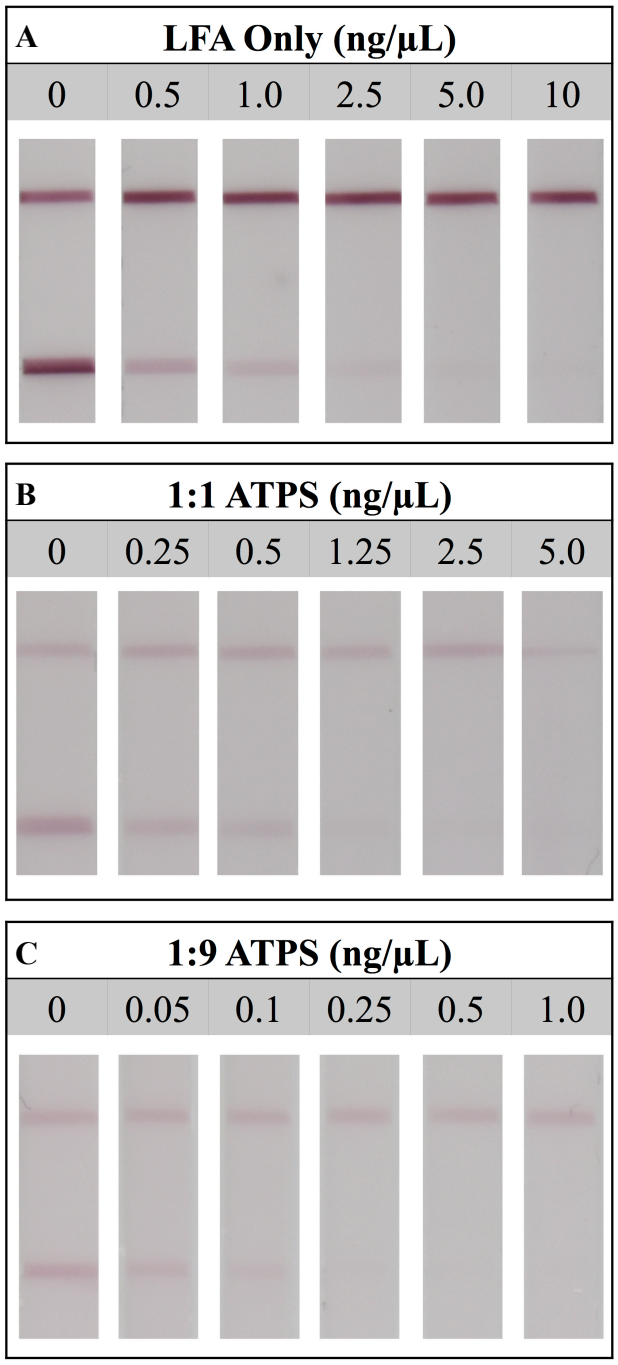

Figure 2.3. Limit of detection for LFA tests detecting for protein transferrin. **(A)** Detection limit for the LFA-only test was found to be 5 ng/µL. **(B)** Detection limit for the LFA when combined with a 1:1 ATPS was 1.25 ng/µL and **(C)** detection limit for the LFA when combined with a 1:9 ATPS was 0.25 ng/µL, indicating 4-fold and 20-fold improvements, respectively.

For the LFA-only tests, when a high concentration of Tf was used (10 ng/μL), test lines did not develop, indicating a true positive result. However, at a lower concentration (2.5 ng/μL), a test line was present, indicating a false negative result. This suggested that the limit of detection for LFA without ATPS enhancement was 5 ng/μL (**Figure 2.3A**). A similar analysis was performed for the 1:1 and 1:9 ATPS setups and the limits of detection were found to be 1.25 ng/μL (**Figure 2.3B**) and 0.25 ng/μL (**Figure 2.3C**), respectively, indicating 4-fold and 20-fold improvements in detection over LFA-only. The improvement was significant, but the test lines were fainter compared to the LFA only tests. The control line intensities were also fainter. These findings were confirmed via our MATLAB analysis; test lines were less developed across all concentrations, including our negative control at 0 ng/μL (**Figure 2.4**). We also observed a large standard deviation in the test line intensity of LFA only for 0.5 ng/μL, which is likely due to variability in the background signal. However, this variability did not have a significant effect on our conclusions from the MATLAB analysis, as the error bars for different tests did not overlap, and the entire range of intensities for this concentration corresponds to very visible lines. Since a less developed test line corresponds to an improvement in the limit of detection for the competitive assay, it was unclear if the improvement we observed was primarily a result of this diminished line intensity or of the ATPS concentration. To determine this, we also investigated the use of the IL ATPS with a sandwich format assay.

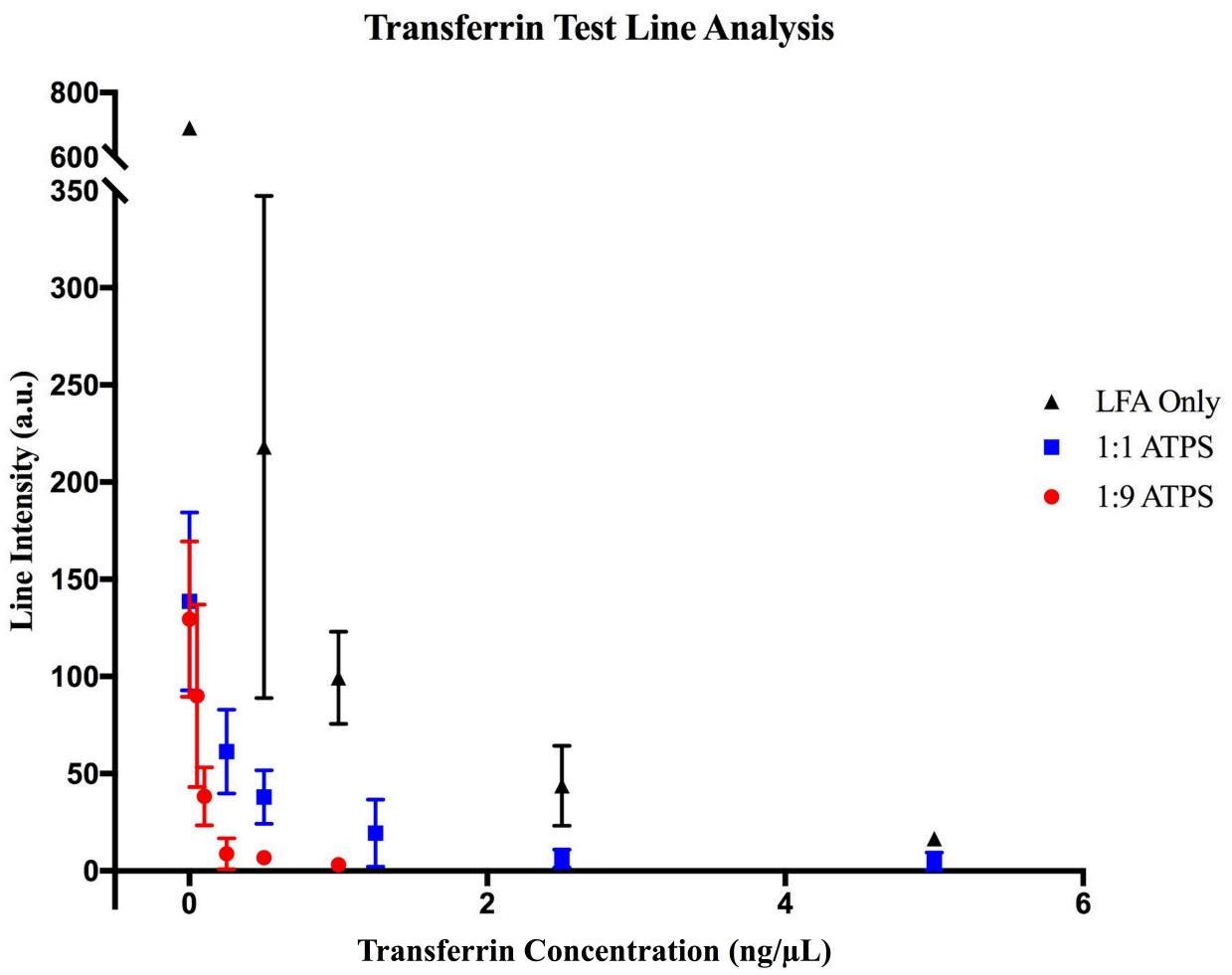

Figure 2.4. MATLAB analysis of transferrin LFA tests. Data for (▲) LFA-only tests without ATPS enhancement, (■) enhancement with a 1:1 ATPS, and (●) enhancement with a 1:9 ATPS. At each concentration, test line intensities in arbitrary units (a.u.) were lower for the 1:1 ATPS/LFA test than the LFA-only test and lower for the 1:9 ATPS/LFA test than the 1:1 ATPS/LFA test, indicating improved detection with more extreme volume ratios.

2.3.3 Detection of *E. coli*

Following the detection of Tf, we studied the detection of *E. coli* as a large biomarker to demonstrate improvement using an IL ATPS in a sandwich-format LFA, where diminishing of line intensities will have a negative impact on the detection limit. We performed experiments in a similar manner to the Tf tests. For this format, the top line still constituted the control line,

indicating a valid test. However, the test line was comprised of primary antibodies specific to the target, rather than the target biomolecule itself. If the sample solution contains the target antigen, the antigen will bind antibodies on the GNPs, forming an antigen-antibody complex. This complex will then bind the immobilized antibodies on the test line, producing a visual red band. Conversely, if there is no target antigen, the antibody-antigen complex will not form and no test line will develop. Thus, for sandwich assays, two lines would indicate a positive test, while only one line would indicate a negative test. In this case, the limit of detection was defined as the lowest concentration of *E. coli* at which the test line was visible.

When testing with LFA only, at a high concentration of *E. coli* (1.8×10^6 cfu/mL), two strong lines developed, indicating a true positive result. At a lower concentration (9×10^4 cfu/mL), only one line formed, exhibiting a false negative result. These results suggest the limit of detection of *E. coli* using LFA only tests is 3.6×10^5 cfu/mL (**Figure 2.5A**). Utilizing this analysis, the limits of detection for the 1:1 ATPS and 1:9 ATPS were determined to be 1.8×10^5 cfu/mL (**Figure 2.5B**) and 4.5×10^4 cfu/mL (**Figure 2.5C**), respectively, indicating 2-fold and 8-fold improvements in the limit of detection. While lighter line intensities than expected were still observed in these tests, the fact that improvement was still achieved in a sandwich LFA indicated that the concentration effect due to the IL ATPS was the dominant contributor to the improvement in detection limit. This analysis was confirmed via MATLAB analysis. As seen in **Figure 2.6**, test line intensity was improved with the application of more extreme volume ratio ATPSs, which corresponds with improvements in the detection limit of these tests.

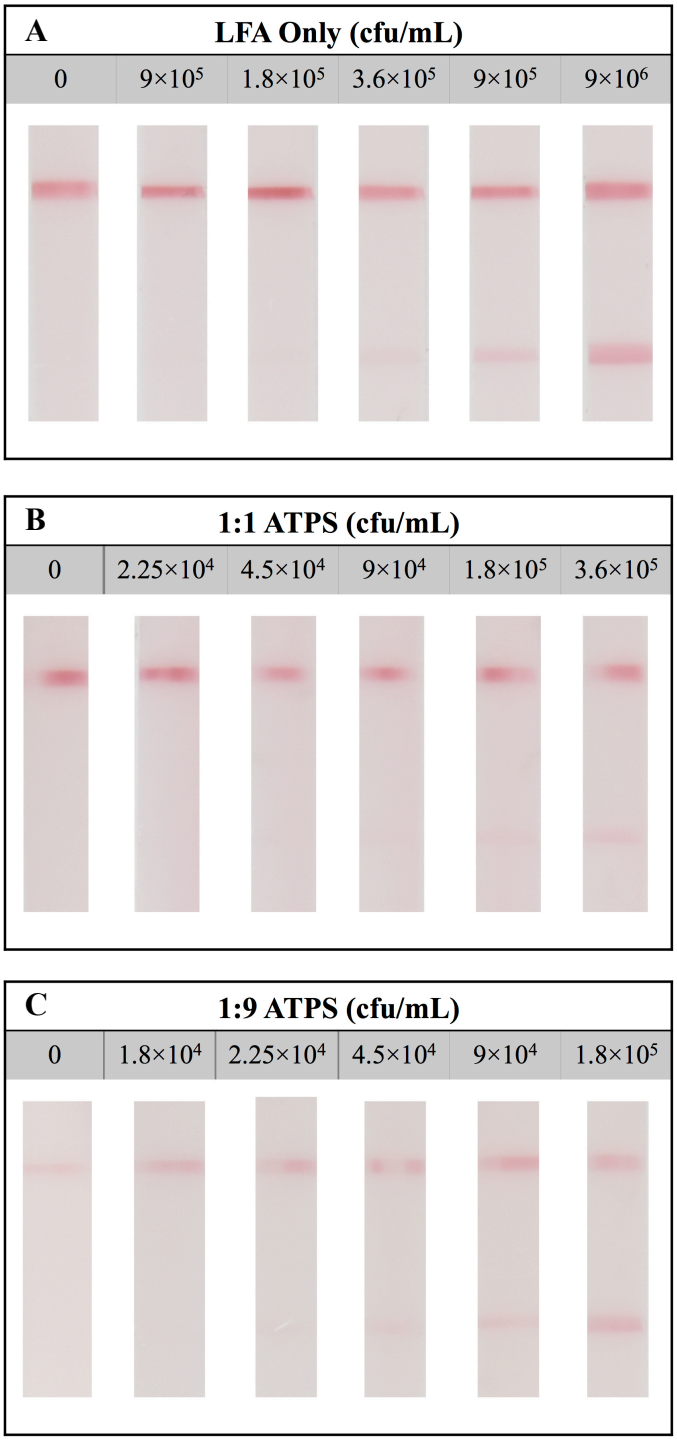

Figure 2.5. Limit of detection for LFA tests detecting for *Escherichia coli*. **(A)** Detection limit for the LFA-only test was found to be 3.6×10^5 cfu/mL. **(B)** Detection limit for the LFA when combined with a 1:1 ATPS was 1.8×10^5 cfu/mL and **(C)** detection limit for the LFA when combined with a 1:9 ATPS was 4.5×10^4 cfu/mL, indicating 2-fold and 8-fold improvements, respectively.

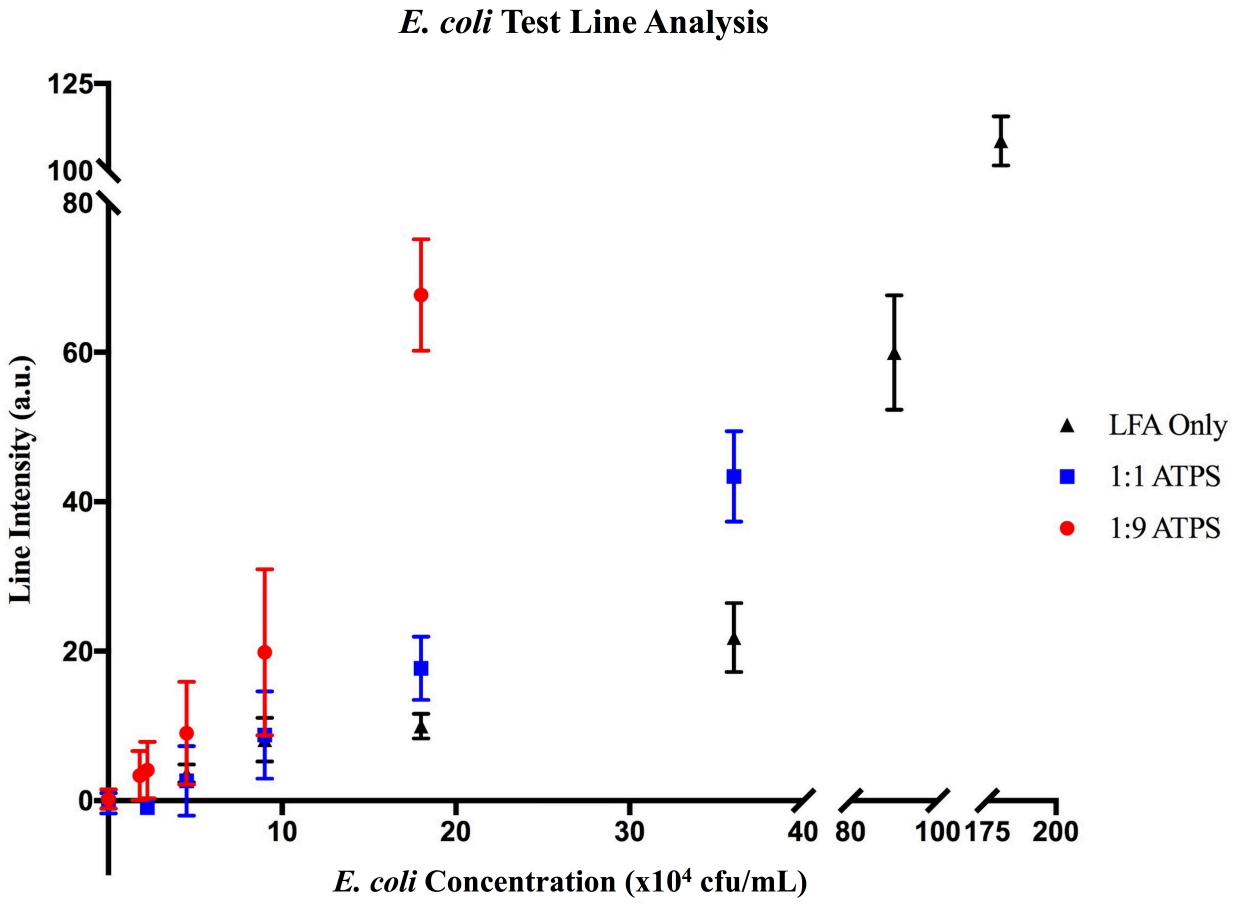

Figure 2.6. MATLAB analysis of *E. coli* LFA tests. Data for (▲) LFA-only tests without ATPS enhancement, (■) enhancement with a 1:1 ATPS, and (●) enhancement with a 1:9 ATPS. At each concentration, test line intensities in arbitrary units (a.u.) were greater for the 1:1 ATPS/LFA test than the LFA-only test and greater for the 1:9 ATPS/LFA test than the 1:1 ATPS/LFA test, indicating improved detection with more extreme volume ratios.

2.4 Discussion

Our group had previously demonstrated enhanced detection through the combination of LFA with ATPSs, specifically using polymer-salt and micellar systems; however, a primary handicap in the direct application of these ATPSs is their long phase separation time. As described previously, these systems can take hours to phase separate, which can limit their

viability at the point-of-care. Our laboratory also demonstrated that the application of an ATPS to 3-D paper architecture drastically improves phase separation time (13). However, this method requires modification to existing LFA devices to accommodate the enhancement technique. As the phase separation time of the IL ATPS for both 1:1 and 1:9 volume ratios were within 1 min and 5 min, this IL ATPS can be directly applied to existing LFA technologies, without necessitating modifications to the LFA. We hypothesize that this rapid phase separation speed can be due to several factors. First, relative to micellar and polymer systems, IL-based systems exhibit low viscosity (65), allowing domains to move more quickly in solution and find similar domains faster, promoting more rapid macroscopic phase separation. Furthermore, preliminary wetting experiments indicate greater hydrophilic/hydrophobic differences between phases than exhibited in polymer or micellar systems, suggesting a greater interfacial tension between the two phases that can facilitate faster phase separation. This is further supported by the work of Gutowski et al. in 2001; kosmotropic salts would increase the difference in dielectric constants between IL and water (37), promoting coalescing of similar domains in response to a high interfacial tension.

In addition to more rapid phase separation, the IL ATPS also displayed a greater degree of enhancement, specifically regarding the competitive LFA tests for Tf. While the degree of concentration for the 1:9 ATPS should be close to 10-fold, the improvement in the limit of detection was found to be 20-fold. We hypothesize this unexpected improvement is most likely due to the high ionic content of the system, which produces a screening effect that influences antibody-antigen binding. However, as use of the IL ATPS for the detection of *E. coli* still improved detection 8-fold, we determined that most of the enhancement seen for Tf is still a result of the ATPS concentration effect. The precise level of test line diminishment, and

therefore the deviance from expected improvement, likely depends on the exact antibodies used in a particular assay. We do note that, in this study, both monoclonal and polyclonal antibodies for different antigens in different assay formats were successful, suggesting this system should still be widely applicable. Additionally, while the system does exhibit diminishing of line intensities, it should be noted that a primary motivation for use of this system would be to apply it to the partitioning of small biomarkers. These smaller biomarkers would generally require detection with the competitive LFA, as they typically do not contain many antigen binding sites required for use with the sandwich assay. Therefore, this screening effect only helps our system.

We envision the IL ATPS to be used for the detection of a wide range of proteins, with the ability to tailor the exact IL and salt system to accommodate the target of choice. To achieve this, we anticipate that a greater understanding of the phase separation mechanism of these IL-based ATPSs will be required. We also investigated the IL ATPS comprised of [Bmim][Cl] and potassium phosphate salt. Surprisingly, despite having similar components to our [Bmim][BF_4] ATPS, this system displayed different phase separation behavior, consisting of an IL-rich top phase and a salt-rich bottom phase. While the exact mechanism of IL ATPS phase separation is not precisely understood, it is commonly believed that separation occurs due to the salting out effect of kosmotropic salts on the IL component. The degree of this salting out is likely a large factor in determining partitioning behavior and relative hydrophobicity/hydrophilicity of each phase. We found that this [Bmim][Cl] system yielded different gold nanoprobe partitioning behavior from the [Bmim][BF_4] counterpart, with GNs partitioning extremely to the top IL-rich phase and DGNs partitioning extremely to the bottom salt-rich phase. While the ability to partition similar particles to different phases is promising, it is clear that a greater understanding

of phase separation and partitioning behavior is needed to take full advantage of these capabilities.

2.5 Conclusions

In summary, we successfully demonstrated the first use of an IL ATPS for the enhanced detection of biomarkers with the LFA. Specifically, a 20-fold improvement in the detection limit for transferrin was achieved utilizing a 1:9 volume ratio ATPS. This improvement can be seen as a combination of biomarker concentration, induced by the ATPS, as well as diminished test line intensity, likely due to screening effects from the ionic content of the ATPS. Despite the effects of the ATPS ionic content, an 8-fold improvement could still be achieved in the detection limit for *E. coli* using the sandwich-format LFA, where diminished test line intensities have a negative impact on the detection limit. Accordingly, most of the enhancement in the detection limit can be attributed to the preconcentration capability of the ATPS. Furthermore, this IL ATPS was found to phase separate very quickly, allowing for direct application to LFA without requiring modifications to existing LFA structure. While we demonstrated functionality using two biomarkers, we anticipate that further investigation into specific IL-salt pairings can enhance improvement for small biomarkers that would be difficult to concentrate otherwise. This combination of tunability and speed presents this system as a flexible, powerful enhancement tool for use with a wide variety of biomarkers and pathogens.

Bibliography

1. Dye C. After 2015: infectious diseases in a new era of health and development. *Philos Trans R Soc Lond B Biol Sci* (2014). doi: 10.1098/rstb.2013.0426.

2. Lozano R. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the global burden of disease study 2010. *Lancet* (2013) 380:2095-128.

3. United Nations AIDS Fact Sheet - World AIDS Day 2017. United Nations, (2017).

4. World Malaria Report 2016. World Health Organization, (2016).

5. Global tuberculosis report 2017. Geneva: World Health Organization, (2017).

6. Hook EW, Spitters C, Reichart CA. Use of Cell Culture and a Rapid Diagnostic Assay for Chlamydia trachomatis Screening. *JAMA* (1994) 272(11):867-70. doi: 10.1001/jama.1994.03520110047027.

7. Greco S, Girardi E, Navarra A, Saltini C. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax* (2006) 61(9):783-90.

8. Noedl H, Yingyuen K, Laoboonchai A, Fukuda M, Sirichaisinthop J, Miller RS. Sensitivity and specificity of an antigen detection ELISA for malaria diagnosis. *Am J Trop Med Hyg* (2006) 75(6):1205-8.

9. Drain PK, Hyle EP, Noubary F, Freedberg KA. Evaluating Diagnostic Point-of-Care Tests in Resource-Limited Settings. *Lancet Infect Dis* (2014) 14(13):239-49. doi: 10.1016/S1473-3099(13)70250-0.

10. Noordhoek GT, van Embden JD, Kolk AM. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. *J Clin Microbiol* (1996) 34:2522-5.

11. Mabey D, Peeling RW, Ustianowski A, Perkins MD. Diagnostics for the developing world. *Nature Reviews* (2004) 2:231-40.

12. Iqbal M, Tao Y, Xie S, Zhu Y, Chen D, Wang X, et al. Aqueous two-phase system (ATPS): an overview and advances in its applications. *Biological Procedures Online* (2016) 18(1):1-18. doi: 10.1186/s12575-016-0048-8.

13. Pereira DY, Chiu RYT, Zhang SCL, Wu BM, Kamei DT. Single-step, paper-based concentration and detection of a malaria biomarker. *Analytica Chimica Acta* (2015) 882:83-9. doi: 10.1016/j.aca.2015.04.040.

14. Mashayekhi F, Chiu RYT, Le AM, Chao FC, Wu BM, Kamei DT. Enhancing the lateral-flow immunoassay for viral detection using an aqueous two-phase micellar system. *Analytical and Bioanalytical Chemistry* (2010) 398(7-8):2955-61. doi: 10.1007/s00216-010-4213-7.

15. Cheung SF, Yee MF, Le NK, Gomes EA, Afrasiabi Z, Kamei DT. A Combined Aqueous Two-Phase System and Spot-Test Platform for the Rapid Detection of Escherichia coli O157:H7 in Milk. *SLAS Technology* (2017) 23(1):57-63. doi: 10.1177/2472630317731892.

16. Chiu RYT, Jue E, Yip AT, Berg AR, Wang SJ, Kivnick AR, et al. Simultaneous concentration and detection of biomarkers on paper. *Lab on a Chip* (2014) (16):3021-8.

17. Mashayekhi F, Le AM, Nafisi PM, Wu BM, Kamei DT. Enhancing the lateral-flow immunoassay for detection of proteins using an aqueous two-phase micellar system.

Analytical and Bioanalytical Chemistry (2012) 404(6-7):2057-66. doi: 10.1007/s00216-012-6278-y.

18. Lei Z, Chen B, Koo Y-M, MacFarlane DR. Introduction: Ionic Liquids. *Chemical Reviews* (2017) 117:6633-5.

19. Vekariya RL. A review of ionic liquids: Applications towards catalytic organic transformations. *Journal of Molecular Liquids* (2017) 227:44-60.

20. Parvulescu VI, Hardacre C. Catalysis in Ionic Liquids. *Chem Rev* (2007) 107(6):2615-65. doi: 10.1021/cr050948h.

21. Huddelston JG, Willauer HD, Swatloski RP, Visser AE, Rogers RD. Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction. *Chem Commun* (1998):1765-66.

22. Wilkes JS, Zaworotko MJ. *J Chem Soc, Chem Commun* (1992) 965.

23. Berthod A, Ruiz-Ángel MJ, Carda-Broch S. Ionic liquids in separation techniques. *Journal of Chromatography A* (2008) 1184:6-18. doi: 10.1016/j.chroma.2007.11.109.

24. Branco LCR, João N.; Moura Ramos, Joaquim J.; Afonso, Carlos A. M. Preparation and Characterization of New Room Temperature Ionic Liquids. *Chem Eur J* (2002) 8(16):3671-77. doi: 10.1002/1521-3765(20020816)8:16<3671::AID-CHEM3671>3.0.CO;2-9.

25. Ventura SPM, e Silva FA, Quental MV, Mondal D, Freire MG, Coutinho JAP. Ionic-Liquid-Mediated Extraction and Separation Processes for Bioactive Compounds: past, Present, and Future Trends. *Chemical Reviews* (2017) (117):6984-7052.

26. Cheng DH, Chen XW, Shu Y, Wang JH. Selective Extraction/isolation of Hemoglobin with Ionic Liquid 1-butyl-3-trimethylsilyimidazolium Hexafluorophosphate (BtmsimPF6). *Talanta* (2008) 75:1270-8.

27. Shimojo K, Kamiya N, Tani F, Naganawa H, Naruta Y, Goto M. Extractive Solubilization, Structural Change and Functional Conversion of Cytchrome C in Ionic Liquids via Crowth Ether Complexation. *Anal Chem* (2006) 78:7735-42.

28. Alvarez-Guerra E, Irabien A. Ionic Liquid-Based Three Phase Partitioning (ILTPP) for Lactoferrin Recovery. *Sep Sci Technol* (2014) 49:957-65.

29. Alvarez-Guerra E, Irabien A. Ionic Liquid-Based Three Phase Partitioning (ILTPP) Systems for Whey Protein Recovery: Ionic Liquid Selection. *J Chem Technol Biotechnol* (2015) 90:939-46.

30. Alvarez-Guerra E, Irabien A, Ventura SPM, Coutinho JAP. Ionic Liquid Recovery Alternatives in Ionic LIquid-based Three-phase Partitioning (ILTPP). *AIChE J* (2014) 60:3577-86.

31. Mao QX, Wang H, Shu Y, Chen XW, Wang JH. A Dual Ionic Liquid Microemulsion System for the Selective Isolation of Hemoglobin. *RSC Adv* (2014) 2014(4).

32. Shu Y, Cheng D, Chen X, Wang J. A Reverse Microemulsion of Water/AOT/1-butyl-3-methylimidazolium Hexafluorophosphate for Selective Extraction of Hemoglobin. *Sep Purif Technol* (2008) 64:154-9.

33. Martins M, Vieira FA, Correia L, Ferreira RAS, Abreu H, Coutinho JAP, et al. Recovery of Phycobiliproteins from the Red Macroalga Gracilaria sp. using Ionic Liquid Aqueous Solutions. *Green Chemistry* (2016) 18:4287-96.

34. Ge L, Wang XT, Tan SN, Sai HH, Yong JW, Hua L. A novel method of protein extraction from yeast using Ionic Liquid Solution. *Talanta* (2010) 81:1861-4.

35. Liu Y, Ma R, Deng Q, Zhang L, Liu C, Wang S. Preparation of Ionic Liquid polymer Materials and their Recognition Properties for Proteins. *RSC Adv* (2014) 4:52147-54.

36. Shu Y, Chen XW, Wang JH. Ionic Liquid-polyvinyl Chloride Ionomer for Highly Selective isolation of Basic Proteins. *Talanta* (2013) 81:637-42.

37. Gutowski KE, Broker GA, Willauer HD, Huddleston JG, Swatloski RP, Holbrey JD, et al. Controlling the Aqueous Miscibility of Ionic Liquids : Aqueous Biphasic Systems of Water-Miscible Ionic Liquids and Water-Structuring Salts for Recycle , Metathesis , and Separations. *Journal of American Chemical Society* (2003) 125:6632-3. doi: 10.1021/ja0351802.

38. Freire MG, Cla FM, Coutinho AP, Marrucho IM. Aqueous biphasic systems : a boost brought about by using ionic liquids. *Chemistry Society Review* (2012) 41:4966-95. doi: 10.1039/c2cs35151j.

39. Ventura SPM, Sousa SG, Serafim LS, Lima ÁS, Freire MG, Coutinho JAP. Ionic Liquid Based Aqueous Biphasic Systems with Controlled pH: The Ionic Liquid Cation Effect. *Journal of Chemical & Engineering Data* (2011) 56(11):4253-60. doi: 10.1021/je200714h.

40. Wu B, Zhang YM, Wang HP. Aqueous Biphasic Systems of Hydrophilic Ionic Liquids + Sucrose for Separation. *J Chem Eng Data* (2008) 53(4):983-5.

41. Chen Y, Wang Y, Cheng Q, Liu X, Zhang S. Carbohydrates-tailored phase tunable systems composed of ionic liquids and water. *J Chem Thermodyn* (2009) 41(9):1056-9. doi: 10.1016/j.jct.2009.04.008.

42. Souza RL, Ventura SPM, Soares CMF, Coutinho JAP, Lima A. Lipase Purification using Ionic Liquids as Adjuvants in Aqueous Two-phase Systems. *Green Chemistry* (2015) 17:3026-34.

43. Pei Y, Wang J, Wu K, Xuan X, Lu X. Ionic liquid-based aqueous two-phase extaction of selected proteins. *Sep Purif Technol* (2009) 64(3):288-95.

44. Schröder C. Proteins in Ionic Liquids: Current Status of Experiments and Simulations. *Topics in Current Chemistry* (2017) 375(25). doi: 10.1007/s41061-017-0110-2.

45. Pei Y, Li L, Li Z, Wu C, Wang J. Partitioning Behavior of Wastewater Proteins in Some Ionic Liquids-Based Aqueous Two-Phase Systems. *Separation Science and Technology* (2012) 47(2):277-83.

46. Dreyer S, Salim P, Kragl U. Driving Forces of Protein Partitioning in an Ionic Liquid-based Aqueous Two-phase System. *Biochem Eng J* (2009) 46:176-85.

47. Deive F, Rodriguez A, Pereiro A, Araujo J, Longo M, Coelho M, et al. Ionic Liquid-Based Aqueous Biphasic System for Lipase Extraction. *Green Chemistry* (2011) 13:390-6.

48. Ventura SPM, de Barros RLF, de Pinho Barbosa JM, Soares CMF, Lima AS, Coutinho JAP. Production and Purification of an Extracellular Lipolytic Enzyme using Ionic Liquid-based Aqueous Two-phase Systems. *Green Chemistry* (112) 14:734-40.

49. Wei D, Ivaska A. Applications of ionic liquids in electrochemical sensors. *Analytica Chimica Acta* (2008) 607:126-35.

50. Baker SN, Brauns EB, McCleskey TM, Burrell AK, Baker GA. Fluorescence quenching immunoassay performed in an ionic liquid. *Chemistry Communications* (2006):2851-3. doi: 10.1039/b606473f.

51. Gunasekera DPS, Pathiraja RP. Congenital Zika Infection : A Challenge for Resource Poor Settings. *Clinical Pediatrics* (2016) 1(4):1-2.

52. Tang J, Nour NM. HIV and Pregnancy in Resource-Poor Settings. *Reviews in obstetrics and gynecology* (2010) 3(2):66-71. doi: 10.3909/riog0118.

53. Scarborough M, Thwaites GE. The diagnosis and management of acute bacterial meningitis in resource-poor settings. *The Lancet Neurology* (2008) 7(7):637-48. doi: 10.1016/S1474-4422(08)70139-X.

54. Nugent R. Chronic diseases in developing countries: Health and economic burdens. *Annals of the New York Academy of Sciences* (2008) 1136:70-9. doi: 10.1196/annals.1425.027.

55. Elbireer AM, Opio AA, Brough RL, Jackson JB, Manabe YC. Strengthening Public Laboratory Service in Sub-Saharan Africa: Uganda Case Study. *Laboratory Medicine* (2011) 42(12):719-25. doi: 10.1309/LM2OBNYY9D0UXZJO.

56. Nkengasong JN, Mesele T, Orloff S, Kebede Y, Fonjungo PN, Timperi R, et al. Critical Role of Developing National Strategic Plans as a Guide to Strengthen Laboratory Health Systems in Resource-Poor Settings. *American Journal of Clinical Pathology* (2018) (April):852-7. doi: 10.1309/AJCPC51BLOBBPAKC.

57. Sharma S, Zapatero-Rodríguez J, Estrela P, O'Kennedy R. Point-of-Care diagnostics in low resource settings: Present status and future role of microfluidics. *Biosensors* (2015) 5(3):577-601. doi: 10.3390/bios5030577.

58. Hatti-Kaul R. Aqueous Two-Phase Systems: A General Overview. *Molecular Biotechnology* (2001) 19.

59. Tang B, Bi W, Tian M, Row KH. Application of ionic liquid for extraction and separation of bioactive compounds from plants. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* (2012) 904:1-21. doi: 10.1016/j.jchromb.2012.07.020.

60. Liu Q, Yu J, Li W, Hu X, Xia H. Partitioning Behavior of Penicillin G in Aqueous Two Phase System Formed by Ionic Liquids and Phosphate Partitioning Behavior of Penicillin G in. *Separation Science and Technology* (2007) 41. doi: 10.1080/01496390600786135.

61. Wilkes JS, Levisky JA, Wilson RA, Hussey CL. Dialkylimidazolium Chloroaluminate Melts: A New Class of Room-Temperature Ionic Liquids for Electrochemistry, Spectroscopy, and Synthesis. *Inorganic Chemistry* (1982) 21(3):1263-4. doi: 10.1021/ic00133a078.

62. Jang H, Ryoo SR, Kostarelos K, Han SW, Min DH. The effective nuclear delivery of doxorubicin from dextran-coated gold nanoparticles larger than nuclear pores. *Biomaterials* (2013) 34(13):3503-10. doi: 10.1016/j.biomaterials.2013.01.076.

63. Chiu RYT, Nguyen PT, Wang J, Jue E, Wu BM, Kamei DT. Dextran-Coated Gold Nanoprobes for the Concentration and Detection of Protein Biomarkers. *Annals of Biomedical Engineering* (2014) 42(11):2322-32. doi: 10.1007/s10439-014-1043-3.

64. Fu E, Liang T, Houghtaling J, Ramachandran S, Ramsey SA, Lutz B, et al. Enhanced sensitivity of lateral flow tests using a two-dimensional paper network format. *Anal Chem* (2011) 83(20):7941-46.

65. Huddleston JG, Visser AE, Reichert WM, Willauer HD, Broker GA, Rogers RD. Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazolium cation. *Green Chemistry* (2001) 3(4):156-64. doi: 10.1039/b103275p.