Title
Investigation of Gold Nanoprobe Extraction from Various Aqueous Two-Phase System Regions to Improve Lateral-Flow Immunoassay Detection of Protein and Bacterial Targets

Permalink
https://escholarship.org/uc/item/24d794qm

Author
Thach, Alison Vivian

Publication Date
2016

Peer reviewed|Thesis/dissertation
Investigation of Gold Nanoprobe Extraction from Various Aqueous Two-Phase System Regions
to Improve Lateral-Flow Immunoassay Detection of Protein and Bacterial Targets

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Bioengineering

by

Alison Vivian Thach

2016
The objective of this thesis was to investigate aqueous two-phase systems (ATPSs) as a concentration method for improving the lateral-flow immunoassay (LFA) detection of proteins and bacteria at the point-of-care (POC). In the first portion of the thesis, the polyethylene glycol (PEG)-potassium phosphate (salt) ATPS was used to concentrate transferrin (Tf), a model protein for C-reactive protein (CRP), to the interfacial region between the two bulk phases of the ATPS. In the second portion of the thesis, the PEG-salt ATPS was used to concentrate the bacterium *Streptococcus mutans* (*S. mutans*) into the bottom PEG-poor, salt-rich phase of the ATPS. For both studies, the ATPS concentration step was integrated with LFA in order to improve the detection of protein and bacterial targets. Our technologies therefore have the potential to improve POC
solutions that require target concentration, such as detecting CRP and *S. mutans* in the respective oral disease diagnostic tests for periodontal disease and dental caries.

The detection of proteins at the POC allows for the chairside detection of diseases such as periodontal disease, which is associated with the presence of CRP in the body. A detection assay that is rapid, inexpensive, portable, and easy to use is ideal for this application, whereby the LFA is one such assay that fulfills these requirements. However, the sensitivity of LFA is inferior to laboratory-based assays, such as the enzyme-linked immunosorbent assay (ELISA), and needs to be improved. In order to improve the protein sensitivity of LFA, we utilized the PEG-salt ATPS to concentrate our model protein Tf prior to detection. Due to the size of proteins and other small biomolecules, these biomolecules will partition evenly between the two phases of the ATPS. To address this issue, we developed a novel approach that utilized larger colloidal gold nanoparticles decorated with anti-Tf antibodies that partitioned preferentially to the interfacial region between the two phases due to a delicate balance of the components comprising the nanoparticles. These nanoparticles bound to Tf and aided its transport to the interfacial region where Tf was consequently concentrated. Since the interfacial region represents a very small volume region that forms irrespective of the volume ratio, the volume ratio that reached equilibrium in the shortest time was chosen, reducing the sample extraction time to 10 min for phosphate-buffered saline (PBS) and to within 15-25 min for the complex solutions of fetal bovine serum (FBS) and synthetic urine. By concentrating Tf prior to LFA detection, the detection limit of LFA was improved by 100-fold from 1 ng/µL to 0.01 ng/µL in PBS, FBS, and synthetic urine. Thus, the ability to concentrate Tf bound to colloidal gold nanoparticles in a shorter duration of time provides a novel approach for improving LFA detection of small biomolecules.
POC detection of bacteria also allows for the chairside detection of diseases such as dental caries, which is caused by the presence of *S. mutans*. As in protein detection, the rapid time to result, low cost, portability, and ease of use make LFA an appropriate detection assay for the detection of bacteria. However, the bacterial sensitivity of LFA is inferior to laboratory-based methods, such as the use of cell culture with subsequent colony counts, polymerase chain reaction (PCR), and ELISA. Thus, we utilized the PEG-salt ATPS with an extreme volume ratio to concentrate *S. mutans* prior to LFA detection. Unlike proteins, *S. mutans* and other bacterial cells are larger in size and will experience greater steric, excluded-volume interactions with the PEG polymers present at higher concentrations in the PEG-rich, salt-poor phase of the ATPS, causing extreme partitioning to the PEG-poor, salt-rich phase. The *S. mutans* bacteria concentrated in the PEG-poor, salt-rich phase was extracted, mixed with colloidal gold nanoparticles decorated with anti-*S. mutans* antibodies, and applied to an LFA test strip. When *S. mutans* was concentrated prior to LFA detection, the detection limit of LFA was improved by slightly less than 10-fold from approximately $10^7$ colony forming units (CFU)/mL to approximately $10^6$ CFU/mL in PBS. The results of this study confirmed the applicability of using a PEG-salt ATPS to concentrate bacteria into one phase prior to extracting that phase for subsequent addition to an LFA test strip. Therefore, these results form the basis for future studies in which the longer phase separation time associated with extreme volume ratios will be addressed to allow for improved bacterial detection assay sensitivity within a shorter period of time.
The thesis of Alison Vivian Thach is approved.

Benjamin M. Wu

Tzung Hsiai

Daniel T. Kamei, Committee Chair

University of California, Los Angeles

2016
This thesis is dedicated to my parents.
# Table of Contents

Chapter 1: Motivation and Background

1.1 Introduction .................................................................................................................................................. 1
  1.1.1 Periodontal Disease and Protein Detection .......................................................................................... 1
  1.1.2 Dental Caries and Bacterial Detection ............................................................................................... 6
1.2 The Lateral-Flow Immunoassay (LFA) ....................................................................................................... 8
  1.2.1 Competitive Assay .............................................................................................................................. 9
  1.2.2 Sandwich Assay ................................................................................................................................... 10
1.3 Aqueous Two-Phase Systems (ATPSs) ....................................................................................................... 11
1.4 Applications for ATPSs in Diagnostic Detection ....................................................................................... 12

Chapter 2: An Aqueous Two-Phase System for the Concentration and Extraction of Proteins from the Interface to Enhance the Lateral-Flow Immunoassay Detection of a Model Protein

2.1 Introduction .................................................................................................................................................. 15
2.2 Materials and Methods ................................................................................................................................. 19
  2.2.1 Radiolabeling the anti-Tf antibody ...................................................................................................... 19
  2.2.2 Preparing GNPs ....................................................................................................................................... 20
  2.2.3 Partitioning GNPs ................................................................................................................................. 22
  2.2.4 Preparing the LFA Test Strip ............................................................................................................... 23
  2.2.5 Performing LFA with Tf but Without Pre-Concentration ..................................................................... 25
  2.2.6 Combining the ATPS Interface Extraction with LFA for Tf ............................................................... 25
  2.2.7 Quantitative Analysis of LFA Results ................................................................................................. 27
2.3 Results and Discussion ................................................................................................................................. 28
  2.3.1 Engineering of the GNPs for Optimal Interface Partitioning ............................................................. 28
  2.3.2 Identifying the Optimal Volume Ratio ............................................................................................... 30
  2.3.3 Improving LFA Detection by Using Interface Extraction .................................................................. 31
2.4 Conclusions .................................................................................................................................................. 35

Chapter 3: Use of Aqueous Two-Phase Systems to Enhance the Lateral-Flow Immunoassay Detection of *Streptococcus mutans* ............................................................................................................. 37

3.1 Introduction .................................................................................................................................................. 37
3.2 Materials and Methods ................................................................................................................................. 39
  3.2.1 *S. mutans* cells .................................................................................................................................... 39
3.2.2 Preparing Dextran-Coated Gold Nanoprobes (DGNPs) .................................................. 39
3.2.3 Preparing the LFA Test Strip .................................................................................. 40
3.2.4 Performing LFA Alone with S. mutans ................................................................. 42
3.2.5 Combining the ATPS Concentration of S. mutans with LFA ............................... 42
3.3 Results and Discussion ............................................................................................. 43
  3.3.1 Concentration of S. mutans in the ATPS ............................................................... 43
  3.3.2 Improving LFA Detection by Pre-Concentrating S. mutans Using an ATPS ......... 44
3.4 Conclusions .............................................................................................................. 46
Bibliography .................................................................................................................. 48
Acknowledgments

This work would not have been accomplished without the help and support of many people. First and foremost, I would like to thank my advisor and mentor Dr. Daniel T. Kamei. Over the past three years, I have been fortunate to have the opportunity to learn and work under such a passionate person. I have never before been challenged as much as I have under his instruction, but I have also never before felt that I have grown so much as a student and researcher. Both as an undergraduate and graduate, I cherished my time in the Kamei Laboratory as it has been an unbelievable experience. Dan has been an integral part of my time at UCLA and I could never repay him for all of the life lessons I have taken away. He has been a magnificent role model with his defined morals, work ethic, honesty, and attention to detail. Thank you Dan for all that you have taught me, I truly appreciate all that you have ever done for me.

I would also like to thank Dr. Ricky Chiu who was my graduate student mentor. Working with Ricky has been a privilege as I got to experience first-hand what it meant to be an innovative researcher. His mentorship helped provide me with a strong foundation and his kind words were always supportive. Thank you Ricky for teaching me to think on my feet and for sharing your knowledge of the LFA technologies.

Additionally, I would like to thank the other members of the Kamei Laboratory who I have had the chance to work alongside. Brian Lee, my senior mentor, trained me when I first started as an undergraduate researcher. Allison Yip, my name twin, has always been a great mentor to me. Together with Brian and Allison, we worked on drug delivery experiments that led to my first scientific article publication. David Pereira has been a great help as a supportive mentor who has helped me troubleshoot many LFA tests. Working alongside Chloe Wu, we constructed many ATPSs for interface extraction over an extended duration of time that lead to my second scientific
article publication. Coco Pearce has been a great partner with whom I ran countless LFA tests alongside. I would also like to thank the rest of my friends in the Kamei Laboratory who made the office space feel less like a workspace. I will always remember the family environment that we have established with our countless fun, inside jokes, and snack times.

Last but not least, I would like to thank my family and friends for supporting me through my time at UCLA. Thank you Mom and Dad for always being supportive of my career aspirations, letting me figure out my own path in my own time. Much thanks to my sister and brother-in-law who have been tremendous role models with great advice. And to all of my friends, both old and new, that have been a part of this journey, thank you for your encouragement, kind words, and supportive shoulders as you all stood by my side.

Chapter 2 is a version of Chiu RYT, Thach AV, Wu CM, Wu BM, Kamei DT, An Aqueous Two-Phase System for the Concentration and Extraction of Proteins from the Interface for Detection Using the Lateral-Flow Immunoassay. PLoS ONE, 2015. 10(11): e0142654. doi:10.1371/journal.pone.0142654. Copyright © 2015. Published as an open access article distributed under the terms of the Creative Commons Attribution License. This work was supported by UCLA School of Dentistry funds to D.T. Kamei who was the PI. Experiments were conducted by R.Y.T. Chiu, A.V. Thach, and C.M. Wu while writing of the manuscript was done by R.Y.T. Chiu, A.V. Thach, C.M. Wu, B.M. Wu, and D.T. Kamei.
Curriculum Vitae

EDUCATION

University of California, Los Angeles
Bachelor of Science in Bioengineering, June 2015
GPA: 3.619

James Logan High School
Union City, California
Class of 2011

PUBLICATIONS


PODIUM PRESENTATIONS

Chapter 1: Motivation and Background

1.1 Introduction

Oral disease hinders an individual’s general health and quality of life. The prevalence of such disease establishes conditions in which individuals may encounter mouth and facial pain, oral infection, and tooth loss. These conditions can then limit an individual’s capacity to bite, chew, smile, and speak. Among the most common forms of oral disease reported by the World Health Organization are periodontal disease and dental caries.

1.1.1 Periodontal Disease and Protein Detection

Affecting up to 90% of the population worldwide, periodontal disease occurs in a variety of forms from simple gum inflammation to major soft tissue and supporting bone damage [1]. From 1999 to 2004, periodontal disease was present in 8.52% of American adults between the ages of 20 and 64 [2]. Periodontitis is known as inflammation around the tooth, and persists when gingivitis, or gum inflammation caused by a bacterial biofilm (dental plaque), is not treated. The development and maturity of the biofilm results in the succession of bacteria in which there is a pathogenic shift of bacteria toward increasing numbers of gram-negative anaerobes. As the gums recede from the teeth, bacteria, such as Porphyromonas gingivalis, Prevotella intermedia, and Actinobacillus actinomycetemcomitans, infect the resulting pockets, allowing for infection below the gum line [3]. Consequently, the body’s immune system responds to the growing bacterial biofilm and its generated products, causing injury to the connective tissue holding the teeth in place. Potential bacterial toxins produced include hydrogen sulfide, polyamines, butyrate and propionate fatty acids, endotoxins, and destructive enzymes [4]. The general immune response
involves attacking the bacteria with neutrophils, an abundant white blood cell in mammals, and antibodies. However, a hyper response and hypersensitivity of the immune system will result in tissue destruction. Other inflammatory factors contributing to tissue destruction include the release of cytokines, prostaglandins, and numerous enzymes. In periodontal disease, interleukin 1 (IL-1), tumor necrosis factor alpha (TNFα), and interferon gamma (IFNγ) are cytokines known to control the bone resorption and connective tissue destruction processes while prostaglandin E2 (PGE2) has been investigated for its central role in tissue destruction and for its correlation with disease susceptibility [4,5]. Moreover, prostaglandins and collagenases are attributed to activating osteoclasts and degrading collagen, respectively, resulting in bone and tooth attachment loss [6].

For a less progressed disease, the bacterial biofilm can be removed through a deep-cleaning method called scaling and root planning that involves scraping off any calcified plaque and removing rough spots on the tooth root where bacteria can accumulate. Additionally, antimicrobial and antibiotic medications can be used in conjunction with deep-cleaning to control bacteria growth. When periodontal disease is far more progressed, flap surgery is performed to remove the calcified plaque by lifting back the gums as well as to reduce the depth of the existing periodontal pocket. Bone and tissue grafts can also be performed to promote regeneration of bone or gum tissue that has been lost. If left untreated, periodontal disease may lead to eventual tooth loss.

Treatment to control the infection may be given when the disease has been detected during a dental exam. Aside from visual inspection, conventional detection methods performed by dentists and hygienists include evaluation of gum recession and tooth mobility, observation of bleeding upon probing, and the use of a probe to measure the depths of gum pockets, in which case a healthy pocket depth is defined to be between 1 and 3 millimeters [7]. Dentists and hygienists will also use radiographs to determine if any bone loss has occurred. Additional diagnostic
methods available to aid in the diagnosis of periodontal disease include diagnostic casts, microbial and other biologic assessments, and other relevant medical laboratory tests [8]. Currently available commercial diagnostic tests are shown in Table 1.1. In the US, one available test for identifying the type and concentration of periodontal disease-causing bacteria is the OralDNA Labs’ MyPerioPath salivary diagnostic test [9]. The results of this test allow for the classification of high and low risk cases of periodontal disease as well as for the development of a personalized targeted therapy treatment for patients. Other commercial products available from OralDNA Labs in the US are the MyPerioID IL-1 and MyPerioID IL-6 salivary tests that identify the genetic susceptibility of individuals to periodontal disease. The MyPerioID IL-1 test analyzes the patient’s ability to overexpress the production of inflammatory cytokines IL-1 α and β since there is a positive correlation between individuals with this genetic variation and their susceptibility to disease [9,10]. Similarly, results from the MyPerioID IL-6 test identify individuals with genetic susceptibility by examining production of IL-6. However, the MyPerioPath, MyPerioID IL-1, and MyPerioID IL-6 tests require deoxyribonucleic acid-polymerase chain reaction (DNA-PCR) testing of saliva samples, delaying results for four to five days. An alternative simple screening method available in Japan that provides results within 5 min is the Salivary Occult Blood Test (SOBT) which is based upon detecting hemoglobin in saliva samples by using gold-labeled anti-human hemoglobin monoclonal antibodies [11]. Hemoglobin is a protein found in red blood cells and can be detected when bleeding occurs in poor periodontal health. The SOBT was not found to be sufficiently specific, but it provided a low cost, paper-based method for evaluating an individual’s periodontal status.
Table 1.1. Summary of current diagnostic tests available for detection of periodontal disease.

<table>
<thead>
<tr>
<th>Diagnostic Test</th>
<th>Method of Detection</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>OralDNA Labs MyPerioPath</td>
<td>DNA-PCR testing</td>
<td>4-5 days</td>
</tr>
<tr>
<td>Oral DNA Labs MyPerioID IL-1 and IL-6</td>
<td>DNA-PCR testing</td>
<td>4-5 days</td>
</tr>
<tr>
<td>Salivary Occult Blood Test (SOBT)</td>
<td>Immunochromatography assay utilizing monoclonal antibodies</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Moreover, there has been evidence that periodontal disease produces higher systemic levels of C-reactive protein (CRP), leading our group to envision developing a diagnostic test for the detection of CRP [12–16]. CRP is a pentameric plasma protein synthesized by liver hepatocytes and is present in systemic responses to inflammation, particularly the acute phase response to acute and chronic inflammation [17]. Normally, CRP is present in relatively low serum levels between 1.0 to 3.0 mg/L, but these levels rise rapidly within 72 hours of tissue injury or infection as CRP targets bacteria for destruction via complement-binding and subsequent activation of the complement system [18,19]. Thus, detection of high levels of CRP can provide a potential method to identify the presence of periodontal disease. However, increases in CRP levels have also been found to be caused by such factors as aging, high blood pressure, and smoking. In order to confirm that increased CRP levels are in fact caused by periodontal infection, the periodontal treatment must be effective in lowering the CRP levels detected. As a diagnostic test, the measurement of CRP can be used to screen for periodontal disease as well as monitor the extent and activity of disease and help detect and manage recurrent infection. Through current high-sensitivity laboratory assays, CRP levels in serum have been detected as low as 0.15 mg/L [18]. These high-sensitivity assays utilize labeled monoclonal or polyclonal anti-CRP antibodies in an enzyme-linked immunosorbent assay (ELISA) or an immune-fluorescent assay [18,20].
In the field of diagnostics, it has been challenging to develop a detection assay for proteins that is rapid, portable, and also sensitive [21–23]. Laboratory-based immunoassays, such as the ELISA, display good sensitivity and are the gold standard for detecting protein targets. However, laboratory-based assays are not practical for use in resource-poor settings that lack power, equipment, and trained personnel. On the other hand, the paper-based lateral-flow immunoassay (LFA) is inexpensive, rapid, portable, and easy to use. However, the sensitivity of LFA is lower than that of laboratory-based assays, and LFA therefore cannot be used to detect target proteins that are present at low concentrations [24,25]. Hence, while LFA is very popular and effective in detecting the glycoprotein human chorionic gonadotropin (hCG), a biomarker for pregnancy which exists abundantly in urine from a pregnant woman [26], LFA is not widely used for applications where the target proteins in sample solutions are not as abundant, such as in the detection of infectious and biowarfare agents [23,27,28].

The detection limit of LFA is typically 1-2 orders of magnitude higher than ELISA [22]. While concentrating targets in a sample prior to detection can improve the detection limit, concentrating proteins generally requires laboratory-based equipment, and therefore, typically cannot be combined with point-of-care (POC) assays. Our group, however, has been focusing on concentrating the target analytes into one of the bulk phases of an aqueous two-phase system (ATPS). This concentration procedure can be performed prior to LFA detection so that a concentrated analyte sample may be tested, improving the detection limit of LFA. In addition to concentrating the target into one of the bulk phases, the target can also be concentrated into the interfacial region between the two bulk phases. In Chapter 2, we used transferrin (Tf) as our model protein for CRP to investigate the concentration of proteins into the interfacial region of an ATPS prior to LFA detection.
1.1.2 Dental Caries and Bacterial Detection

Commonly known as tooth decay, dental caries is a prevalent worldwide chronic disease affecting individuals over the course of their lifetime. From 2011 to 2012, dental caries were found in 23% of children 2 to 5 years old, 60% of adolescents 12 to 19 years old, and in 91% of adults 20 to 64 years old in the US [29,30]. It is a slow progressing disease that begins with a microbiological shift in the bacterial biofilm on teeth and is affected by salivary flow and composition, fluoride exposure, dietary sugar consumption, and preventative teeth cleaning measures. The presence of dental caries is marked by the localized destruction of dental hard tissues, affecting the outer covering of the tooth crown (enamel), the outermost layer of the tooth root (cementum), and the tissue beneath the enamel and cementum (dentine) [31]. This localized destruction is recognized as cavitation if the demineralization caused by the acidic byproducts from bacterial fermentation of carbohydrates continues to allow calcium, phosphate, and carbonate to diffuse out of the tooth [32]. Any existing tooth damage can be treated using surgical-restorative procedures to drill out the damaged portion before filling it in with composite or amalgam.

Detection of dental caries is performed by clinical and radiographic methods. The clinical method involves utilizing subjective criteria such as color, softness, and ease of plaque removal while the radiographic method is used to visualize lesions by examining the x-ray images for dark spots indicative of demineralization. Clinically, the detection of a lesion is referred to as a cavity and indicates that the tooth’s surface has decayed. Visible tooth decay indicates that the disease is fairly advanced, causing oral pain and possible tooth loss. However, early changes in the bacterial biofilm of the enamel cannot be detected through clinical and radiographic methods. The progression of dental caries may be halted and reversed at any stage, but this treatment is dependent upon the amount of biofilm that is able to be removed, particularly when the dentine or enamel are
destroyed. Additionally, the effect of treatment in terms of stopping, reversing, or maintaining the current disease state is dependent on the balance between demineralization and remineralization [32]. Remineralization of the tooth structure involves the diffusion of calcium and phosphate into the tooth and can be promoted to occur through the use of fluoride treatment [31]. If the dental caries can be detected earlier, there is a higher likelihood of having less demineralization occur and of being able to remove enough biofilm to halt disease progression. Thus, there is a need for diagnostic tests that can detect the presence of cariogenic bacteria, which are the bacteria known to cause tooth decay.

Cariogenic bacteria persist in a complex community as a biofilm adhering to tooth surfaces. In dental caries, acid-tolerant, gram-positive bacteria species present in the biofilm include *Lactobacillus* and *Streptococcus mutans* (*S. mutans*), in which *S. mutans* is the predominate species [33]. In fact, *S. mutans* is the cariogenic bacteria most strongly correlated with the formation of dental caries [34,35]. There is evidence that *S. mutans* can be detected at levels lower than those capable of causing permanent tooth enamel damage, making *S. mutans* an ideal bacterium to detect in order to predict for the formation of future caries [36,37]. However, these low concentrations of *S. mutans* currently requires culturing or the use of laboratory equipment to perform PCR or ELISA [38,39]. These techniques are time consuming and are not viable as rapid, POC diagnostic tests like the LFA. The LFA is an ideal assay for developing a diagnostic test that can be used for chairside detection of *S. mutans*. Currently, there are commercial LFA tests available, such as the GC America SALIVA-CHECK MUTANS, but none are available in the US that are FDA or CLIA approved [40].

As in the case of LFA protein detection, the sensitivity of LFA is inferior to the current gold standard methods, specifically, performing cell culture for bacterial counts, PCR, or ELISA.
In order to improve the sensitivity of LFA, the bacterial cells can be concentrated prior to LFA detection by using an ATPS to concentrate the cells into one of the two bulk phases. For our studies, we investigated the use of an ATPS for concentrating *S. mutans* in order to improve the detection limit and, subsequently, the sensitivity of LFA.

1.2 The Lateral-Flow Immunoassay (LFA)

The lateral-flow immunoassay (LFA) utilizes antibodies specific to target antigens and colorimetric indicators, such as colloidal gold nanoparticles and latex nanoparticles, to provide a visible result. A typical commercial LFA test strip is comprised of a sample pad, conjugate pad, detection zone, and an absorbent pad. Each of these regions is usually comprised of a different type of paper material.

In an LFA test, a sample solution is applied to the sample pad of the test strip. Typically, cellulose or glass fiber materials are used for the sample pad. Pretreatment of the sample pad with blocking agents can be performed to enhance lateral flow as well as to prevent nonspecific binding of target antigens to the paper material. The sample pad as a whole can be used as a filter for the removal of large contaminants present in the sample tested. Downstream from the sample pad is a conjugate pad that contains dried and immobilized colorimetric indicators that have been conjugated with antibodies specific for the target antigen. Following the flow of the sample through the conjugate pad, the next sequential component is the nitrocellulose membrane on which the test and control lines correspond to the detection zone. The nitrocellulose membrane can also be treated with blocking agents for purposes similar to those for the sample pad. Finally, a high-
density cellulose paper is present at the most downstream end of the LFA test strip to wick the excess sample solution and to act as a sink to drive fluid flow across the entire test strip.

There are two assay formats available for the LFA. The competitive assay is an inhibitive format while the sandwich assay is a direct format. In both formats, our group utilizes gold nanoparticles (GNPs), or colloidal gold nanoparticles conjugated with antibodies specific for the target antigen, as our LFA colorimetric indicator. The visual appearance of the positive and negative results for each format is specific for the format being used.

1.2.1 Competitive Assay

The competitive assay format is typically used to detect small molecules or molecules that only have one antigenic binding site that does not allow for the simultaneous binding of two antibodies. This format has the whole target antigen or a portion of the target, such as a nontoxic chain of a toxin molecule, immobilized along the test line of the nitrocellulose membrane. Primary antibodies specific to the target are conjugated to the GNPs, while secondary antibodies specific to the primary antibodies are immobilized along the control line of the nitrocellulose membrane. If a sample contains a high enough concentration of the target, the target-specific antibodies on the GNPs will become saturated, preventing binding of the GNPs to the test line. In this case, a positive result is indicated by the absence of a test line due to the inability of the GNPs to bind to the immobilized targets (Figure 1.1). Alternatively, a negative result is indicated by the presence of a red-colored test line as a consequence of the GNPs binding to the immobilized targets. Whenever the GNPs pass the control line, they will bind to the immobilized secondary antibodies to produce a red-colored control line. The presence of a control line is then used as an indication that a test is valid since the sample solution and GNPs have flown through the strip.
1.2.2 Sandwich Assay

The sandwich assay format is typically used for the detection of targets that have multiple antigenic binding sites, such as hCG, viruses, and bacteria. In this format, the test line is comprised of immobilized primary antibodies specific to the target. These primary antibodies may either be the same or similar to the antibodies conjugated onto the GNPs. The secondary antibodies immobilized on the control line must then be specific to the primary antibodies present on the GNPs. If the target antigen is present in the sample, they will first bind to the antibodies on the GNPs, forming a nanoprobe-antigen complex. As these complexes flow along the LFA test strip, the target will also bind to the primary antibodies present on the test line, producing a red-colored test line. In this case, a positive result is indicated by the visualization of the test line while a negative result is indicated by the absence of the test line (Figure 1.2). As in the competitive assay format, the immobilized secondary antibodies on the control line will bind to the primary
antibodies on the GNPs, producing a visible red-colored control line and validating that the sample solution and GNPs have flown through the test strip.

![Schematic of the sandwich LFA assay format](image)

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

### 1.3 Aqueous Two-Phase Systems (ATPSs)

Aqueous two-phase systems (ATPSs) are biphasic systems that can be used to improve the sensitivity of the LFA for the detection of biomolecules. If biomolecules are present in an ATPS solution, they will distribute, or partition, between the two bulk phases based on their physical and chemical properties, such as size and hydrophobicity. By utilizing the separation behavior of cells, proteins, viruses, and other biomolecules in ATPSs, the biomolecule targets can be concentrated prior to their LFA detection.

The ATPS is adaptable to a POC test since it is portable, easy to use, and does not require laboratory equipment for phase separation to occur. It is a liquid-liquid extraction system, and
therefore, can be easily scaled down to handle small volumes. In comparison to conventional oil-water systems, both phases of an ATPS are predominately composed of water, providing a mild environment for biomolecules. Two of the most common types of ATPSs used are the micelle-based and polymer-salt systems. A commonly used polymer-salt system is the polyethylene glycol (PEG)-potassium phosphate (salt) system. The PEG-salt system undergoes phase separation when a critical salt concentration has been exceeded. Microscopically, the addition of salt ions disrupts the hydrogen bonds between water molecules and PEG polymers [41]. These hydrogen bonds exist in a hydration shell surrounding PEG polymers such that the disruption of bonds allow nearby PEG polymers to interact with each other and experience greater attractive van der Waals interactions. As the interactions between PEG polymers become more favorable and the interactions between PEG and water molecules become less favorable, pockets of PEG-rich and salt-rich domains form in solution. The coalescence of these respective domains will continue to occur over time, causing migration of the collective domains in accordance to their relative densities. Due to their greater density, the salt-rich domains will move downward relative to the PEG-rich domains, producing a top PEG-rich, salt-poor phase and a bottom PEG-poor, salt-rich phase.

1.4 Applications for ATPSs in Diagnostic Detection

Our group has previously concentrated biomolecules by adjusting the operating conditions of the ATPS to establish a volume ratio, defined as the ratio of the volume of the top phase to that of the bottom phase, that was much greater or much less than 1. This reduced the volume of the phase where the target molecules partitioned, effectively concentrating the target molecules in a small volume phase that was then extracted and applied to the subsequent detection assay.
Specifically, micellar and PEG-salt ATPSs were used to concentrate a model virus by 10-fold, and subsequently, improved the detection limit of LFA by 10-fold [42,43]. For protein biomarkers, which are smaller than viruses and thus require the use of different concentration techniques, the protein of interest in the sample was captured using GNPs. The large size of the GNPs was then used to concentrate the model protein by 10-fold, which improved the detection limit of LFA by 10-fold [44,45].

While the combination of ATPS with LFA has been demonstrated, the improvement of LFA depends on the fold-concentration that can be achieved in the ATPS, and this depends entirely on how small of a volume can be achieved for the target-rich phase. A more extreme volume ratio therefore will yield a more concentrated target biomolecule. However, more extreme volume ratios result in longer phase separation times since it takes longer for the microscopic domains that form the smaller phase to find each other, coalesce, and travel to the respective top or bottom phase [46].

In Chapter 2, the concentration of proteins is optimized using a single ATPS step by driving the target biomolecules towards the interface between the two bulk phases. Since the interfacial region represents a very small volume region that can form irrespective of the volume ratio, this novel approach allows for the concentration of the targets without dependence on extreme volume ratios, which have long phase separation times. Instead, the volume ratio that can reach equilibrium in the shortest time was chosen, and this reduced the extraction time to within 10 min in phosphate-buffered saline (PBS), a significant improvement over our previous approach, as well as to within 15-25 min in fetal bovine serum (FBS) and synthetic urine. This approach was also viewed as moving towards the maximum fold-concentration that can be achieved in a single ATPS step since the volume of the interface is much smaller than the two macroscopic bulk phases. Interface
extraction also allows the sample volume to be increased. Increasing the sample volume is favorable as it would increase the total number of target molecules that could promote saturation of the antibodies for a given fixed amount of GNPs. However, in a typical LFA test, increasing sample volume is not favorable as the volume that can flow through the strip is limited by the size of the strip, leading to the majority of the GNPs not reaching the detection zone. With interface extraction, this would not be a problem as the volume to be added to the LFA strip is small as the interfacial region represents a small volume.

In Chapter 3, the application of using the ATPS for diagnostic detection is extended to bacterial targets. Unlike proteins, bacterial cells are large by nature and will experience greater steric, excluded-volume interactions with the PEG polymers present at higher concentrations in the PEG-rich, salt-poor phase of the ATPS, causing extreme partitioning to the PEG-poor, salt-rich phase. GNPs are therefore not needed for the capture and concentration of the bacterial targets. Instead, the targets present in a sample may be directly concentrated by using an extreme volume ratio. Although this has been previously achieved for viral targets, we extended this approach to bacteria. In Chapter 3, we discuss our investigation of the applicability of using an ATPS to concentrate bacteria prior to the extraction of the phase containing the bacteria for subsequent addition to an LFA test strip. The results of this study form a basis for future studies in which the longer phase separation time associated with extreme volume ratios will be addressed by using a 3-D paper architecture to allow for simultaneous phase separation and target concentration as the ATPS flows through the paper membranes [47,48]. Effectively, the extraction step from the ATPS can then be bypassed by utilizing a 3-D paper design which can be integrated with a downstream LFA detection test. Use of this integrated system will then allow for the detection of low concentrations of target bacteria, improving assay sensitivity.
Chapter 2: An Aqueous Two-Phase System for the Concentration and Extraction of Proteins from the Interface to Enhance the Lateral-Flow Immunoassay Detection of a Model Protein

2.1 Introduction

Periodontal disease is a prevalent gum disease worldwide. It persists in a variety of forms from simple gum inflammation to major soft tissue and supporting bone damage. The underlying cause of these disease states is not treating the inflammation caused by the bacterial biofilm. Consequently, the development and maturity of the biofilm results in a pathogenic shift of bacteria toward the gram-negative anaerobes, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*, that will infect the pockets created by gum recession [3]. In response, the body’s immune system will cause injury to the connective tissue holding the teeth in place as the body attacks the bacteria. Individuals that are more susceptible to periodontal disease are those with a genetic susceptibility as well as those who smoke and have diabetes [7].

Current methods to detect for periodontal disease include visual inspection, evaluation of gum recession and tooth mobility, observation of bleeding upon probing, the use of a probe to measure the depths of gum pockets, and the use of radiographs to determine if any bone loss has occurred. Additional available diagnostic methods include diagnostic casts, microbial and other biologic assessments, and other relevant medical laboratory tests. Amongst the diagnostic tests currently available are the OralDNA Labs’ MyPerioPath, MyPerioID IL-1, and MyPerioID IL-6 tests as well as the Salivary Occult Blood Test (SOBT) [9–11]. However, the OralDNA Labs’ tests required a four- to five-day delay for results to be obtained, and the SOBT was not found to be sufficiently specific.
There has been evidence indicating that periodontal disease produces higher systemic levels of C-reactive protein (CRP), making CRP a potential target for periodontal disease diagnostic tests [12–16]. CRP is a pentameric plasma protein synthesized by liver hepatocytes and is present in systemic responses to inflammation. Normal serum levels of CRP are between 1.0 to 3.0 mg/L, but these levels will rise rapidly within 72 hours of tissue injury or infection as CRP targets bacteria for destruction via complement-binding and subsequent activation of the complement system [18]. Thus, detection of high levels of CRP can help identify patients with periodontal disease. However, since increased levels of CRP can also be associated with aging, high blood pressure, and smoking, confirmation that periodontal disease is responsible for the high levels of CRP must be obtained by effective reduction in CRP levels following periodontal treatment. As a diagnostic test, the measurement of CRP can be used to screen for periodontal disease as well as monitor the extent and activity of disease, while also helping to detect and manage recurrent infection. Currently, high-sensitivity laboratory assays allow for the detection of CRP at levels as low as 0.15 mg/mL [18]. These high-sensitivity assays utilize labeled monoclonal or polyclonal anti-CRP antibodies in an enzyme-linked immunosorbent assay (ELISA) or an immune-fluorescent assay [18,20].

In the field of diagnostics, it has been challenging to develop a detection assay for proteins, such as CRP, that is rapid, portable, and also sensitive [21–23]. Laboratory-based immunoassays, such as the ELISA, display good sensitivity and are the gold standard for detecting protein targets. However, laboratory-based assays are not practical for use in resource-poor settings that lack power, equipment, and trained personnel. On the other hand, the paper-based lateral-flow immunoassay (LFA) is inexpensive, rapid, portable, and easy to use. However, the sensitivity of
LFA is lower than that of laboratory-based assays, and therefore, LFA cannot be used to detect target proteins that are present at low concentrations [24,25].

In order to improve the sensitivity of LFA, the proteins can be concentrated prior to application to the test strip. We optimized the concentration of biomolecules using a single aqueous two-phase system (ATPS) step by driving the target biomolecules towards the interfacial region between the two bulk phases. This novel approach allows for concentration of the proteins without dependence on extreme volume ratios since the interfacial region represents a very small volume region that forms irrespective of the volume ratio. There is then no need to wait for the long phase separation times associated with extreme volume ratios. Instead, the volume ratio that reached equilibrium in the shortest time was chosen, reducing the extraction time to within 10 min in phosphate-buffered saline (PBS). We also view this approach as moving towards the maximum fold-concentration that can be achieved in a single ATPS step since the volume of the interface is much smaller than the two macroscopic bulk phases. Furthermore, interface extraction allows the sample volume to be increased. By increasing the sample volume, the total number of target molecules would be increased and this could promote saturation of the antibodies for a given fixed amount of gold nanoprobes (GNPs). However, the volume that can flow through a typical LFA test strip is limited by the size of the strip. Interface extraction would not have this problem since the interfacial region only represents a small volume that can applied to the test strip. Thus, interface extraction allows for the sample volume to be increased without increasing phase separation time in order to detect low concentrations of target proteins, improving the sensitivity of an assay. Figure 2.1 pictorially compares interface extraction with extraction of one of the two bulk phases.
Figure 2.1: Summary of the technical innovation of engineering particles capable of partitioning to the interface of an ATPS to concentrate a target and the improvements in PBS relative to our previous proof-of-concept studies.

<table>
<thead>
<tr>
<th>Extraction type</th>
<th>Bulk phase extraction</th>
<th>Interface extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schematic</td>
<td>[Schematic image]</td>
<td>[Schematic image]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bulk phase extraction</th>
<th>Interface extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase separation time</td>
<td>&gt; 6 h</td>
<td>10 min</td>
</tr>
<tr>
<td>Extraction time</td>
<td>&gt; 30 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Volume ratio</td>
<td>1:9 or 9:1</td>
<td>1:1</td>
</tr>
<tr>
<td>Concentration factor</td>
<td>~10</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Temperature</td>
<td>25-37 °C</td>
<td>25-37 °C</td>
</tr>
</tbody>
</table>

PEG-rich phase | Salt-rich phase | Gold Nanoprobe | Target analyte

The technological innovation described in this chapter is the development of nanoprobes that can localize at the interface and also serve as the colorimetric indicator for LFA. We investigated the volume ratio for the polyethylene glycol (PEG)-potassium phosphate (salt) ATPS that phase separated the fastest and also allowed for the greatest recovery of GNPs. Subsequently, using transferrin (Tf) as our model protein for CRP, we demonstrated that our novel method of combining LFA with the ATPS interface extraction step is an effective yet rapid approach by
improving the detection limit of LFA for Tf by 100-fold. We then extended our studies to more closely approach real-world applications, and reoptimized the system for fetal bovine serum (FBS) [49,50] and synthetic urine [51,52], in smaller volumes, which are preferable for blood sampling. Our data shows that, even in the more complex systems which required a few procedural modifications such as increasing the incubation time allotted for phase separation to occur, ATPS interface extraction can be performed within 15–25 min to concentrate the target 100-fold. This led to a 100-fold improvement in the detection limit of LFA for Tf, which allowed us to detect concentrations as low as 0.01 ng/μL, closing the gap in sensitivity between laboratory-based and paper-based immunoassays. An improved LFA with increased sensitivity would improve point-of-care solutions that require concentration of the target ligand, such as improving the detection of CRP for diagnostic tests in order to identify patients with periodontal disease. Overall, the ATPS interface extraction protocol is a general pre-concentration technique applicable to LFA and other detection methods when the concentration of targets is low.

2.2 Materials and Methods

2.2.1 Radiolabeling the anti-Tf antibody

All reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. Iodine-125 (\(^{125}\)I) was used to radiolabel the tyrosine residues of goat anti-human Tf polyclonal antibody (Catalog # A80-128A, Bethyl Laboratories, Montgomery, TX). Briefly, Na\(^{125}\)I (MP Biomedicals, Irvine, CA) was activated by IODO-BEADS (Pierce Biotechnology, Rockford, IL). Subsequently, the activated \(^{125}\)I was reacted with goat anti-Tf antibodies for 15 min. The radiolabeled proteins were purified, and free \(^{125}\)I was removed using a Sephadex G10 size-
exclusion column. The phosphotungstic acid assay was used to quantify the radioactivity and concentration of the radiolabeled proteins.

2.2.2 Preparing GNPs

The naked gold nanoparticles were prepared using a protocol described by Frens [53,54], resulting in a clear, cherry-colored solution with particle sizes approximately 20 nm in diameter, measured using transmission electron microscopy (TEM). Specifically, 27 mg of sodium citrate was added to 50 mL of filtered ultrapure water and 500 μL of 1% gold (III) chloride that was maintained at 100°C while stirring at 400 revolutions per minute for 2 min. The absorption (A) wavelength of the maximum Plasmon peak of the gold particles was found using a UV-Vis spectrophotometer. The diameter of the particles was found using dynamic light scattering and was compared to a molar decadic extinction coefficient (ε) chart provided by BBInternational Life Sciences to determine the corresponding ε value. Some of the ε values from this chart are shown in Table 2.1. For a path length (l) of 1 cm, we were able to calculate the molar concentration (C) of the gold particles by rearranging Beer’s law (A = εlC). TEM was then used to image the naked gold nanoparticles (Figure 2.2). 2.5 μL of the sample was placed on an EMS carbon film 200 mesh grid (Electron Microscopy Sciences, Hatfield, Pennsylvania) and filter paper was used to wick away any excess. The grid was left to air dry at ambient temperature prior to being imaged using a FEI TF20 transmission electron microscope (FEI Company, Hillsboro, Oregon) at 200 kV. The average diameter of the gold nanoparticles was found to be 20.0 ± 3.0 nm from analysis of the TEM image in Figure 2.2 using ImageJ.
Table 2.1: Colloidal gold nanoparticle extinction coefficients.

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>$5.502 \times 10^8$</td>
</tr>
<tr>
<td>18</td>
<td>$6.644 \times 10^8$</td>
</tr>
<tr>
<td>19</td>
<td>$7.941 \times 10^8$</td>
</tr>
<tr>
<td>20</td>
<td>$9.406 \times 10^8$</td>
</tr>
<tr>
<td>21</td>
<td>$1.105 \times 10^9$</td>
</tr>
<tr>
<td>22</td>
<td>$1.288 \times 10^9$</td>
</tr>
<tr>
<td>23</td>
<td>$1.492 \times 10^9$</td>
</tr>
<tr>
<td>24</td>
<td>$1.717 \times 10^9$</td>
</tr>
<tr>
<td>25</td>
<td>$1.964 \times 10^9$</td>
</tr>
</tbody>
</table>

Figure 2.2: Transition electron microscopy (TEM) image of naked gold nanoparticles. Nanoparticles were suspended in filtered ultrapure water. Length of the scale bar corresponds to 40 nm. ImageJ analysis indicated the particle diameter to be $20.0 \pm 3.0$ nm ($n = 275$).
To prepare the GNPs, 320 mg of goat anti-Tf antibody was incubated with $3.60 \times 10^{18}$ colloidal gold particles, prepared in an NaOH buffer adjusted to pH 9, for 30 min, followed by the addition of 0.1 mg/mL thiolated-PEG5000, using a molar ratio of 3500:1 for PEG:GNP and an additional incubation of 30 min. To prevent nonspecific binding of other proteins to the surfaces of the colloidal gold, 2 mL of a 10% bovine serum albumin (BSA, Sigma Aldrich catalog #B4287, lyophilized crystal form dissolved in filtered ultrapure water) solution was added to the mixture and mixed for an additional 10 min. The resulting solution was gently mixed on a shaker during the incubation period. To remove free (unbound) antibodies, PEG, and BSA, the mixture was subsequently centrifuged for 30 min at 4°C and 9,000 g. The pellet of GNPs was washed twice with a 1% BSA solution. Finally, the recovered GNPs were resuspended in 2 mL of a 0.1 M sodium borate buffer at pH 9.0.

2.2.3 Partitioning GNPs

The GNPs decorated with radiolabeled anti-Tf antibodies were partitioned in the ATPS at the different conditions shown in Table 2.2 to determine the volume ratio that could yield the fastest and highest GNP recovery. For each partitioning experiment, 3 identical PEG-salt solutions in Dulbecco’s phosphate-buffered saline (PBS; Invitrogen, pH 7.4, ionic strength 154 mM) were prepared to a total volume of 1500 μL. PEG-salt ATPS solutions with three different volume ratios (1:1, 6:1 and 1:6) were prepared using specific concentrations of PEG and potassium phosphate. Subsequently, 10 μL of GNP decorated with radiolabeled anti-Tf antibodies were added to each ATPS solution. The suspensions were equilibrated at 0°C to ensure that the suspensions were homogeneous. Once equilibrium at 0°C was attained, the suspensions were incubated in a water bath at 37°C to induce phase separation, and the GNPs were found to partition between the two coexisting phases. The GNPs at the interface were withdrawn carefully using a pipette, and 30 μL
of the interface solution were withdrawn to ensure most, if not all, of the GNPs at the interface were collected. The two coexisting phases were also completely withdrawn separately using pipettes. The amounts of GNPs at the interface and in the two coexisting phases were quantified by measuring the amount of radioactivity in each region using the Cobra Series Auto-Gamma Counter since the GNPs were bound to radiolabeled anti-Tf antibodies. The quantified amount of GNPs in each of the three regions was used to calculate the recovery percentage of the GNPs at the interface using a mass balance equation.

Table 2.2: Recovery of the GNPs as a function of different phase volume ratios.$^a$

<table>
<thead>
<tr>
<th>Volume ratio (top phase:bottom phase)</th>
<th>1:1</th>
<th>6:1</th>
<th>1:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase separation time (min)</td>
<td>10</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Radioactivity of GNP at interface (cpm ± SD)</td>
<td>4130 ± 290</td>
<td>3240 ± 200</td>
<td>2390 ± 520</td>
</tr>
<tr>
<td>GNP recovery at interface (%)</td>
<td>84.1 ± 1.8</td>
<td>64.8 ± 1.8</td>
<td>70.9 ± 6.6</td>
</tr>
<tr>
<td>Radioactivity of GNP in top phase (cpm ± SD)</td>
<td>371 ± 80</td>
<td>940 ± 71</td>
<td>547 ± 140</td>
</tr>
<tr>
<td>GNP recovery in top phase (%)</td>
<td>7.5 ± 1.3</td>
<td>18.8 ± 1.5</td>
<td>16.1 ± 0.9</td>
</tr>
<tr>
<td>Radioactivity of GNP in bottom phase (cpm ± SD)</td>
<td>408 ± 61</td>
<td>818 ± 110</td>
<td>450 ± 180</td>
</tr>
<tr>
<td>GNP recovery in bottom phase (%)</td>
<td>8.3 ± 1.1</td>
<td>16.3 ± 1.8</td>
<td>13.0 ± 3.2</td>
</tr>
</tbody>
</table>

$^a$Studies were performed using 1500 μL of ATPS. Data are reported as mean ± standard deviation (SD), where n = 3.

2.2.4 Preparing the LFA Test Strip

A competitive mechanism was implemented for the LFA (Figure 2.3). In the competitive assay [45], the target of interest is immobilized on a nitrocellulose membrane to form the test line. Immobilized secondary antibodies against the primary antibodies on the GNPs make up the control line. The antibodies on the GNPs will always bind to the immobilized secondary antibodies, creating a visible control line which indicates a valid test. The GNPs form a visual band at the test line if their conjugated antibodies are not saturated with the target. As shown in Figure 2.3, a
positive result will be indicated by the presence of one visual band while a negative result will be indicated by the presence of two visual bands.

Figure 2.3: Schematic representation of the integration of ATPS interface extraction with competitive-based LFA and the interpretations of the positive and negative results. An ATPS solution was constructed and allowed to phase separate for 10 min in PBS and 25 min in FBS and synthetic urine in a glass tube prior to the extraction of 30 μL of the interface containing GNPs. The extracted sample was then applied to an LFA test strip and results were read after 10 min for the PBS system and after 25 min for the FBS and synthetic urine systems. The appearance of only the control line indicated a positive result while the appearance of both the control and test lines indicated a negative result.

The lateral-flow strip consists of a nitrocellulose membrane, as well as cellulose paper for the sample pad and absorbent pad. 50 μL sucrose solutions were prepared to be printed across the nitrocellulose membrane using a Becton Dickinson plasti-pak syringe and a Harvard Apparatus PHD 2000 microfluidic syringe pump set at an infuse rate of 250 μL/min. The control line was printed using a 0.5 mg/mL solution of anti-goat IgG (Bethyl Laboratories). The test line was printed using a 2.5 mg/mL solution of Tf. The three paper components of the lateral-flow strip were connected through an adhesive backing.
2.2.5 Performing LFA with Tf but Without Pre-Concentration

Tf stock solutions containing varying concentrations of Tf were prepared in PBS. Subsequently, 20 μL of each Tf stock solution were added to 10 μL of the GNP suspension and 20 μL of test buffer (0.2% BSA, 0.3% Tween20, 0.2% sodium azide, 0.1% PEG, 0.1 M Trizma buffer, pH 8), which were used to aid the flow of the samples through the test strips. A total of 5 sample suspensions (50 μL each) with various concentrations of Tf were prepared (0 (negative control), 0.001, 0.01, 0.1, and 1 ng/μL). A test strip was dipped vertically into each sample suspension, where the sample pad would come in contact with the suspension. After 10 min, the test strips were taken out, and an image of each strip was immediately taken by a Canon EOS 1000D camera (Canon U.S.A., Inc., Lake Success, NY).

In the experiments performed in FBS (HyClone, characterized, pH 7.4), a 270.6 ng/mL GNP suspension was used so that the volume of GNP could be scaled down appropriately for the lower-volume experiments. The concentrations of Tf in the FBS stock suspensions were adjusted to achieve the same final Tf concentrations used in the PBS experiments by adding 5 μL of a Tf stock solution to 5 μL of GNP suspension, followed by 40 μL of test buffer. Similarly, experiments were conducted using synthetic urine prepared with a method described by Martinez [55].

2.2.6 Combining the ATPS Interface Extraction with LFA for Tf

A volume ratio of 1:1 was used for the study conducted in PBS based on the findings from the Partitioning GNPs experiment. By utilizing anti-Tf antibodies, the GNPs first captured Tf in the sample, followed by the entire Tf-GNP complex being concentrated at the interface. A similar protocol to that described in the Partitioning GNPs section was used except that various concentrations of Tf were also spiked into the ATPS solutions. Briefly, 10 μL of the 69.7 ng/mL GNP suspension were added to 4990 μL of the Tf-spiked ATPS solution that yielded a 1:1 volume
ratio and that contained various Tf concentrations (0 (negative control), 0.001, 0.01, and 0.1 ng/μL). The solutions were equilibrated at 0°C to ensure that the solutions were homogeneous. Once equilibrium was attained, the solutions were placed in a water bath at 37°C to trigger phase separation. After 10 min, 30 μL of the interface suspension, which contained the Tf and the 19 ng/mL concentrated GNPs that were concentrated approximately 42-fold, were withdrawn. This interface suspension was mixed with 20 μL of test buffer to form the 50 μL sample suspensions. The lateral-flow strip was inserted vertically into a tube containing the suspension, and the tube container held the strip. After 10 min, the test strips were taken out, and an image of each strip was immediately taken by a Canon EOS 1000D camera.

For the studies conducted in FBS and synthetic urine, the PEG and potassium phosphate concentrations needed to first be adjusted to achieve the 1:1 volume ratio. The ATPS in FBS also phase separated more slowly, and instead of the 10 min incubation used in the PBS system, the solutions were kept in a 37°C water bath for 25 min. In addition, as mentioned earlier, the volumes were reduced to more closely resemble a practical application. Therefore, rather than show a detection limit increase using 5000 μL (100 times more volume than the 50 μL Tf stock solution used in the LFA only experiments), the studies performed in FBS and synthetic urine showed an equivalent improvement using 1000 μL (100 times more volume than the 10 μL Tf stock solution used in the LFA only experiments). The protocol previously described for PBS was modified for the lower volumes, so that 5 μL of the more concentrated gold suspension were added to 995 μL of the Tf-spiked ATPS solution in FBS or synthetic urine. 20 μL of the interfacial region were extracted, followed by the addition of 30 μL test buffer. Each LFA strip was dipped in the suspension for 15 min before being taken out and imaged.
2.2.7 Quantitative Analysis of LFA Results

The images taken of the LFA test strips were analyzed using a custom MATLAB script. To quantify the line intensities of our results, the images were cropped and converted to 8-bit grayscale matrices. These matrices were split in half in order to produce one matrix containing the control line and the other containing the test line. Each matrix was then analyzed separately to determine the location of the control or test line by identifying the darkest spot with minimum intensity using vectors perpendicular to the line of interest. The average location of the minima found was centered on a 15 pixel-high rectangular region that spanned the length of the control and test lines, where the average grayscale intensities were denoted as $I_{\text{control}}$ and $I_{\text{test}}$, respectively. In order to normalize the intensities of the control and test lines, the average grayscale intensity of a reference region, denoted as $I_{\text{reference}}$, was used to remove the effects of any background color present. The reference region was defined to be 15 pixels wide and 50 pixels upstream from the test line. Signal intensities of the control and test lines were then found using equations 2.1 and 2.2:

$$\text{Signal}_{\text{control}} = I_{\text{reference}} - I_{\text{control}} \quad (2.1)$$

$$\text{Signal}_{\text{test}} = I_{\text{reference}} - I_{\text{test}} \quad (2.2)$$

Signal intensity of each test line was then converted to relative test signal intensity through division by the maximum test signal intensity in the corresponding set of images. Plots of relative test signal intensity versus transferrin concentration were then made.
2.3 Results and Discussion

2.3.1 Engineering of the GNPs for Optimal Interface Partitioning

In order to combine the ATPS interface extraction with the paper-based LFA detection assay, the GNPs developed in this study possessed three functions. First, the decorated specific antibodies on the surfaces of the GNPs captured the target proteins present in the sample. Second, the optimized formulation of PEG and proteins on the surfaces of the GNPs caused the GNPs to partition to the interface and not the bulk phases. Lastly, the GNPs acted directly as the colorimetric indicator for LFA, and hence allowed the subsequent detection assay to be performed immediately without extra washing or other preparation steps. A schematic of the GNP is shown in Figure 2.4.

The GNP has 3 main components: the PEG polymers, the gold nanoparticle, and the anti-Tf antibodies. Each component by itself would drive the nanoparticle into one of the two bulk phases. First, decorated PEG drives the nanoparticle into the top PEG-rich phase due to the favorable PEG-PEG interactions between the polymer on the particle surface and the abundant polymers in the top phase (Figure 2.5 A). Specifically, increasing the molar ratio of PEG:GNP changes the conformation of the bound PEG to more closely resemble a “brush” conformation, expanding the amount of surface area exposed to increase PEG-PEG interactions [56]. On the other hand, the large size of the gold nanoparticle causes the nanoparticle to partition into the bottom PEG-poor phase where it experiences fewer repulsive, excluded-volume interactions with the PEG polymers. The hydrophilic proteins (anti-Tf Ab and BSA) on the GNP increase the hydrophilicity of the GNP, and also cause it to partition into the bottom PEG-poor phase, which is more hydrophilic than the top PEG-rich phase (Figure 2.5 B). In combination, the 3 components of the GNP can be varied and delicately balanced to ultimately drive the GNP to the interface in our ATPS (Figure 2.5 C).
Figure 2.4: Surface modification of GNP to influence partitioning behavior in ATPS. Schematic of GNP and the functionality of each component.

<table>
<thead>
<tr>
<th>PEG</th>
<th>Gold Nanoparticle</th>
<th>Anti-Tf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevents aggregation of GNPs in high salt environment of PEG-poor phase</td>
<td>Greater excluded-volume interactions with PEG in the PEG-rich phase drive the nanoparticles into the PEG-rich phase</td>
<td>Increases the hydrophilicity of the nanoparticles to drive the nanoparticles into the PEG-poor phase</td>
</tr>
</tbody>
</table>

Figure 2.5: Demonstration of the partitioning behavior of GNPs in our PEG-salt ATPS. Various amounts of PEG were conjugated to the GNPs to manipulate their partitioning behavior: (A) Using a molar ratio of 5800:1 PEG:GNP during conjugation, the resulting GNPs partitioned preferentially into the PEG-rich top phase. (B) Using a molar ratio of 1200:1 PEG:GNP during conjugation, the GNPs partitioned into the PEG-poor bottom phase. (C) Using a molar ratio of 3500:1 PEG:GNP during conjugation, the resulting GNPs partitioned exclusively to the interface. For (A), (B), and (C), the red observed at the very top of the liquid-air interface was due to a reflection and not due to the presence of nanoprobe. Studies were performed in glass tubes 12 x 75 mm in size.
2.3.2 Identifying the Optimal Volume Ratio

Three volume ratios were tested to determine the optimal volume ratio that could recover the most GNPs within the shortest period of incubation. The results are shown in Table 2.2. It is not surprising to observe that the 1:1 volume ratio phase separated the fastest and allowed for the greatest recovery of the GNPs. When phase separation is triggered by increasing the temperature, microscopic PEG-rich and PEG-poor domains are formed, and similar domains will find each other and coalesce. As the domains coalesce, they travel and eventually form the macroscopic PEG-rich, salt-poor phase on top and the macroscopic PEG-poor, salt-rich phase on the bottom due to the interfacial tension and the density difference between the two phases. A 1:1 volume ratio phase separates faster than the 6:1 or 1:6 volume ratios since the domains have an easier time finding each other and coalescing when there is a significant amount of each phase. For more uneven volume ratios, domains of the smaller volume phase can be entrained in the larger continuous phase due to the domains experiencing difficulty coalescing. Moreover, the 6:1 volume ratio is expected to phase separate more slowly than the 1:6 volume ratio since the PEG-rich phase is the continuous phase for the 6:1 volume ratio, and the PEG-poor domains experience more difficulty finding each other and moving to their respective macroscopic phase in the more viscous PEG-rich continuous phase.

Since the GNPs do not partition into either domain, they remain between the domains as the domains coalesce. Eventually, the GNPs appear as a thin red film at the interface when phase separation is completed. The recovery of GNPs is more efficient when using the 1:1 volume ratio as entrainment is minimized at this volume ratio and less of the GNPs would therefore be lost to the interfaces that are present between the entrained domains and the continuous phase. Since the
1:1 volume ratio phase separated the fastest while yielding the highest GNP recovery, it was used in the subsequent experiments.

**2.3.3 Improving LFA Detection by Using Interface Extraction**

To demonstrate the enhancement of LFA by incorporating the ATPS interface extraction step, we utilized the model protein transferrin (Tf). Tf is a serum protein for iron transport, and in addition to both Tf and its antibody being commercially available and inexpensive, we have experience radiolabeling the Tf antibody, which was important in determining GNP recovery. To establish the detection limit of Tf in LFA, we performed a series of LFA tests with various Tf concentrations without any prior concentration step. If a sample contained enough Tf molecules to saturate the anti-Tf antibodies decorated on GNP, then these anti-Tf antibodies did not bind to the immobilized Tf on the nitrocellulose membrane at the test line and therefore did not form a visual band at the test line. This indicated a positive result, which was observed when testing the sample with a Tf concentration of 1 ng/μL (Figure 2.6 A, top panel). On the other hand, if insufficient or no Tf was present in the sample to saturate the anti-Tf antibodies, then these anti-Tf antibodies did successfully bind to the immobilized Tf on the nitrocellulose membrane and therefore formed a visual band at the test line. This indicated a negative result, which was observed when testing samples with Tf concentrations less than 1 ng/μL. Since 1 ng/μL is the lowest Tf concentration that showed a true positive result, this indicated a detection limit of approximately 1 ng/μL for Tf when performing LFA without the prior concentration step.

To determine if the ATPS interface extraction step could improve the detection limit of Tf by 100-fold using LFA, we applied the same amount of the GNPs to the ATPS solutions with Tf concentrations that were 100 times lower than the detection limit of LFA (0.01 ng/μL). Since we had an idea of the number of Tf molecules required to saturate the antibodies, we increased the
sample volume 100-fold from 50 μL to 5000 μL to keep the total number of Tf molecules the same. Since only a limited amount of sample (50 μL) could be applied to an LFA test strip, the diluted GNPs in this larger sample solution needed to be concentrated and applied to LFA to obtain a valid result. To recover these GNPs that were saturated with the target proteins, we placed the solution in a water bath at 37°C to collect the GNPs at the interface within 10 min. The GNPs were then extracted and applied directly to the LFA test strip. The results of this study are shown in the bottom panel of Figure 2.6 A. We were able to obtain a true positive result at 0.01 ng/μL, which showed a 100-fold improvement in the detection limit. The test line intensities of the false negative result at 0.001 ng/μL using this approach were lighter than those without the prior concentration step when comparing samples with the same Tf concentration, indicating that more Tf was captured to make it difficult for the GNPs to bind to the test lines. The test line intensities also increased as the Tf concentration decreased, which was expected as the amount of Tf available to saturate the antibodies decreased. If GNPs were lost to either of the two domains prior to interface extraction, the line intensities of the subsequent LFA test would be expected to be diminished, improving the limit of detection of the assay. However, the loss of too many GNPs would produce a control line that is too faint in intensity and the LFA test result would be invalidated.
Figure 2.6: Results of LFA for detecting Tf in PBS. (A) Images of test strips without (top panel) and with (bottom panel) the prior concentration step using the ATPS interface extraction step. 50 µL sample solutions were applied to each LFA test strip. (B) MATLAB quantification of test signal intensity where a value above a threshold of 0.25 corresponded to a negative test.

To study the effectiveness of ATPS interface concentration in a system more likely to be applied to a future device, we tested lower volume ATPS solutions made with FBS to mimic a small sample blood draw from a patient. Due to the more complicated composition of FBS, the procedure used with the ATPS in PBS was reoptimized for the FBS system. The higher protein content of FBS altered the volume ratio of the ATPS, requiring different concentrations of PEG and salt to form a 1:1 volume ratio. Since the experiments performed in FBS also utilized smaller sample volumes, the volume of GNP had to be scaled down, and a more concentrated GNP stock was made. In addition, the incubation time for the ATPS was extended from 10 to 25 min as the FBS slowed down the phase separation process. Additionally, due to the complex mixture comprising FBS, the time for the LFA test was extended from 10 to 15 min. Despite serum representing a more complex matrix, Figure 2.7 A shows that LFA combined with ATPS interface extraction still yielded a 100-fold improvement in the detection limit compared to LFA without prior concentration. As previously mentioned, we used lower volumes to more closely resemble a
practical application, and used 10 μL of the sample for LFA only and 1000 μL of the sample for LFA combined with ATPS interface extraction. A similar optimization process was performed for the synthetic urine system, ultimately demonstrating an analogous 100-fold improvement in detection limit, as displayed in Figure 2.8 A.

![LFA Results for FBS](image1)

**Figure 2.7: Results of LFA for detecting Tf in FBS.** (A) Images of test strips without (top panel) and with (bottom panel) the prior concentration step using the ATPS interface extraction step. 50 μL sample solutions were applied to each LFA test strip. (B) MATLAB quantification of test signal intensity where a value above a threshold of 0.25 corresponded to a negative test.

![LFA Results for Synthetic Urine](image2)

**Figure 2.8: Results of LFA for detecting Tf in synthetic urine.** (A) Images of test strips without (top panel) and with (bottom panel) the prior concentration step using the ATPS interface extraction step. 50 μL sample solutions were applied to each LFA test strip. (B) MATLAB quantification of test signal intensity where a value above a threshold of 0.25 corresponded to a negative test.
In order to quantitatively analyze our LFA results from Figures 2.6 A, 2.7 A, and 2.8 A, the signal intensities of the control and test lines were assessed using a custom MATLAB script. The relative $\text{Signal}_{\text{test}}$ intensities obtained for each LFA test strip were compared to a threshold value of 0.25 in Figures 2.6 B, 2.7 B, and 2.8 B, where a relative $\text{Signal}_{\text{test}}$ value less than 0.25 indicated positive detection of Tf and a relative $\text{Signal}_{\text{test}}$ value greater than 0.25 indicated negative detection of Tf. When quantitatively analyzing the LFA panels for PBS, FBS, and synthetic urine, the LFA only results indicated a detection of Tf at 1 ng/μL while the results of performing ATPS interface extraction along with LFA indicated a detection of Tf at 0.01 ng/μL. Therefore, the results of the quantitative analysis of the LFA test strips in Figures 2.6 A, 2.7 A, and 2.8 A have confirmed our visually determined 100-fold improvement in the detection limit of LFA for Tf.

2.4 Conclusions

In this study, a novel approach to improve the performance of the LFA paper-based immunoassay was investigated. Specifically, a multi-functional nanoprobe, or the GNP, was developed and utilized to first capture target protein molecules in a sample, then concentrate preferentially to the interface of the ATPS, and finally serve as the colorimetric indicator for LFA. The GNPs were not seen to aggregate when decorated with molar ratios of 5800:1, 3500:1, and 1200:1 PEG:GNP. Different volume ratios of the PEG-salt ATPS were investigated to achieve the fastest and greatest recovery of the GNPs at the interface. A 1:1 volume ratio was found to be optimal since over 80% of the GNPs could be recovered at the interface within only 10 min in an ATPS comprised of PEG, potassium phosphate, and PBS. Using this volume ratio, we subsequently demonstrated the improved performance of detecting a model protein with LFA by
combining LFA with the ATPS interface extraction step. This effectively decreased the detection limit of LFA by 100-fold in PBS, FBS, and synthetic urine. Furthermore, the 100-fold improvement in detection limit demonstrated in complex fluids indicates that this new technology is robust and may eventually be implemented successfully with patient samples. We believe that this innovation will have great impact on the emerging field of paper-based assays since we provide a rapid, inexpensive, and highly effective solution for concentrating proteins with minimal power and no need for laboratory equipment.
Chapter 3: Use of Aqueous Two-Phase Systems to Enhance the Lateral-Flow Immunoassay Detection of *Streptococcus mutans*

3.1 Introduction

Dental caries is a chronic oral disease affecting individuals worldwide. This disease is more commonly referred to as tooth decay or cavities. Individuals that are more susceptible to dental caries include those in poverty as well as those with poor oral hygiene, insufficient fluoride exposure, inadequate salivary flow, and those with high numbers of cariogenic bacteria [31]. Current diagnostic methods used to detect dental caries include the use of subjective clinical criteria to examine the color, softness, and resistance to removal of bacterial plaques as well as the use of radiography to identify dark lesions indicative of tooth demineralization. These methods will only provide a positive result when the disease is fairly advanced in its progression. However, this progression can be stopped and reversed by removing the cariogenic bacterial biofilm.

*Streptococcus mutans* (*S. mutans*) is a cariogenic bacteria known to cause dental caries. In fact, it is the predominate species amongst the acid-tolerant, gram-positive bacteria present in the bacterial biofilms associated with dental caries [33]. *S. mutans* metabolizes sucrose into fructose and glucose. The fructose is fermented as an energy source to support bacterial growth. The glucose is polymerized into an extracellular dextran-based polysaccharide that forms the biofilm matrix and allows for the adherence of *S. mutans* onto the surfaces of teeth. Additionally, the dextran-based polysaccharide can be depolymerized when needed so that glucose can be used as an energy source, resulting in lactic acid production in the bacterial biofilm [57]. Overall, sucrose metabolism by *S. mutans* creates an acidic environment that causes demineralization and makes teeth susceptible to tooth decay. Thus, there is a need for a diagnostic test that allows for the
detection of *S. mutans*, particularly at low concentrations when tooth damage is not yet irreversible.

The detection of *S. mutans* has been successfully detected through early diagnostic tests. Moreover, concentrations of *S. mutans* lower than those capable of causing permanent tooth damage have been successfully detected, indicating that the detection of *S. mutans* could be used as a preventative method for predicting the formation of dental caries [36,37]. However, detection of low concentrations of *S. mutans* has required the use of cell culture with subsequent colony counts or the use of laboratory equipment to perform the polymerase chain reaction (PCR) or the enzyme-linked immunosorbent assay (ELISA) [38,39]. These techniques are time consuming and cannot be used to provide rapid results for a point-of-care (POC) diagnostic test. On the other hand, the lateral-flow immunoassay (LFA) is an ideal assay for developing a rapid diagnostic test since it is simple to use and can be used for chairside detection of *S. mutans*. There are commercial LFA tests currently available, such as the GC America SALIVA-CHECK MUTANS, that allow for detection of *S. mutans*, but the LFA overall is known to have an inferior sensitivity in comparison with the more time-consuming laboratory-based methods [40].

To improve the sensitivity of LFA for detecting *S. mutans*, the bacteria can be concentrated prior to application to the test strip. We wanted to utilize aqueous two-phase systems (ATPSs) to concentrate the bacteria into one of the two bulk phases. After concentrating the bacteria in the ATPS, a sample from the bulk phase containing the *S. mutans* could be extracted and applied to the LFA test strip. ATPSs are ideal for this application since they do not require laboratory equipment and offer a mild environment for the bacteria. Thus, we investigated the use of a polyethylene glycol (PEG)-potassium phosphate (salt) ATPS for its application in concentrating *S. mutans* and potential in improving the LFA detection limit and sensitivity. Our results demonstrated that the integration of ATPS extraction and LFA is an effective method for
improving the LFA detection limit of *S. mutans* by slightly less than 10-fold. Since the sensitivity of the LFA was also improved, this method would allow for more accurate detection of *S. mutans* at low concentrations, providing a potential solution for making a POC diagnostic test that can be used to identify individuals that are at risk of having dental caries.

### 3.2 Materials and Methods

#### 3.2.1 *S. mutans* cells

*S. mutans* bacteria suspended in brain-heart infusion broth were obtained from the Shi group at the UCLA School of Dentistry. The concentration of each bacteria solution was predetermined by traditional colony counting methods. Therefore, the concentration of *S. mutans* was reported in colony forming units (CFU) per mL. For our studies, the *S. mutans* cells were centrifuged at 3,000 g for 5 min and resuspended in Dulbecco’s phosphate-buffered saline (PBS; Invitrogen, pH7.4, ionic strength 154 nM) to the appropriate concentrations. All reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

#### 3.2.2 Preparing Dextran-Coated Gold Nanoprobes (DGNPs)

Dextran-coated gold nanoparticles were prepared according to the protocol used by Min and coworkers with slight modifications [58]. Briefly, 750 mg of dextran (MW 15,000 – 25,000) were dissolved in 10 mL of filtered UltraPure sterile water (Rockland Immunochemicals Inc., Gilbertsville, PA) at 100°C on a heating plate while stirring. Once the dextran was completely dissolved, 135 µL of 1% w/v gold (III) chloride solution was added. The heating plate was then turned off, and the solution was stirred for 20 min during which the color of the solution turned violet. The solution was then cooled to room temperature.
To prepare the dextran-coated gold nanoprobe (DGNPs), 8 mL of the dextran-coated gold nanoparticle solution were first adjusted to pH 7.0 using a 0.1 M sodium borate buffer (pH 9.0) before 64 µg of rabbit anti-*S. mutans* immunoglobulin G (IgG) antibodies (ab31181; Abcam, Cambridge, MA) were added. The mixture was then allowed to react for 30 min at room temperature on a shaker to allow for the formation of dative bonds between the antibodies and the particles. To prevent nonspecific binding of other proteins to the surfaces of the DGNPs, 800 µL of a 10% w/v bovine serum albumin (BSA) in an aqueous solution were added to the mixture. Unbound antibodies and BSA were then removed by centrifuging 3 times for 30 min at 4°C and 12,000 g with 1% w/v BSA in aqueous solution washes in between centrifugation spins. After the final spin, the recovered DGNPs were resuspended in 800 µL of 0.1 M sodium borate buffer.

### 3.2.3 Preparing the LFA Test Strip

A sandwich assay mechanism was implemented for the LFA (Figure 3.1). In the sandwich assay, rabbit anti-*S. mutans* IgG antibodies and protein A, an IgG antibody binding protein, were immobilized on the nitrocellulose membrane to form the test and control lines, respectively. The antibodies on the DGNPs will always bind to the immobilized protein A, indicating that a test is valid with a visible band at the control line. When there is enough *S. mutans* present to adequately bind to the conjugated antibodies on the DGNPs, the *S. mutans*-bound DGNP complexes can bind to the immobilized antibodies on the nitrocellulose membrane, producing a visible band at the test line. As shown in Figure 3.1, a negative test result will be indicated by the presence of one visual band while a positive test result will be indicated by the presence of two visual bands.
Figure 3.1: Schematic representation of the integration of ATPS extraction with LFA in the sandwich assay format and the interpretations of the negative and positive results. An ATPS solution was constructed and allowed to phase separate for 90 min before 40 µL of the bottom phase containing concentrated *S. mutans* was extracted and mixed with DGNPs. The mixture was applied to an LFA test strip and results were read after 30 min. The appearance of only the control line indicated a negative test result while the appearance of both the control and test lines indicated a positive test result.

The LFA test strip consists of a glass fiber sample pad, nitrocellulose membrane, and cellulose paper absorbent pad connected through an adhesive backing. Using a Becton Dickinson plasti-pak syringe and a Harvard Apparatus PHD 2000 microfluidic syringe pump set at an infuse rate of 250 µL/min, 50 µL sucrose solutions were printed across the nitrocellulose membrane. The test line was printed using a 2.0 mg/mL solution of rabbit anti-*S. mutans* IgG antibodies, and the control line was printed using a 0.2 mg/mL solution of protein A. To prevent nonspecific binding of *S. mutans* to the membrane, the entire nitrocellulose membrane was treated with a 1% w/v BSA in 0.1 M Tris buffer solution (pH 8).
3.2.4 Performing LFA Alone with *S. mutans*

Various concentrations of *S. mutans* stock suspensions were prepared in PBS. Subsequently, 40 µL of each *S. mutans* stock were added to 10 µL of the DGNP suspension. A total of 5 sample suspensions (50 µL each) were prepared at 0 (negative control), $10^5$, $10^6$, $10^7$, and $10^8$ CFU/mL concentrations. A test strip was dipped vertically into each sample suspension, allowing the sample pad to come into contact with the suspension. After 30 min, images of each test strip were immediately taken using a Canon EOS 1000D camera (Canon U.S.A., Inc., Lake Success, NY).

3.2.5 Combining the ATPS Concentration of *S. mutans* with LFA

PEG-salt ATPS solutions were prepared in PBS to a total volume of 600 µL using PEG and potassium phosphate stock solutions. The PEG stock was prepared in PBS using PEG with a molecular weight of 8,000 g/mol, and the potassium phosphate salt stock was prepared in PBS using dibasic potassium phosphate salt. The prepared ATPSs had a top PEG-rich, salt-poor phase and a bottom PEG-poor, salt-rich phase with a corresponding volume ratio of 10.6:1.

The PEG-salt ATPSs were used to concentrate the *S. mutans* prior to application to an LFA test strip. They were made with 0 (negative control), $10^5$, $10^6$, $10^7$, and $10^8$ CFU/mL concentrations of *S. mutans*. The solutions were allowed to incubate for 90 min to allow for the formation of the top PEG-rich, salt-poor and bottom PEG-poor, salt-rich phases. After 90 min, 40 µL of the bottom phase, which contained the concentrated bacteria, were extracted using a pipette. This extracted solution was then mixed with 10 µL of the DGNP suspension for a resulting 50 µL sample solution. The LFA test strip was dipped vertically into each 50 µL sample and was allowed to develop for 30 min. An image of each strip was then taken after 30 min using a Canon EOS 1000D camera. Additionally, an extracted sample from the bottom phase was measured for its optical density value.
at 600 nm (OD\textsubscript{600}) using a UV-Vis spectrophotometer. The OD\textsubscript{600} value was then compared to the OD\textsubscript{600} value of the control \textit{S. mutans} stock suspension to determine approximately how much the \textit{S. mutans} was concentrated into the bottom phase of the ATPS.

### 3.3 Results and Discussion

#### 3.3.1 Concentration of \textit{S. mutans} in the ATPS

In order to integrate ATPS concentration of \textit{S. mutans} with the paper-based LFA detection assay, we first needed to determine which phase the \textit{S. mutans} partitioned into. Theoretically, \textit{S. mutans} should partition into the bottom PEG-poor, salt-rich phase of the ATPS due to experiencing greater repulsive, steric, excluded-volume interactions with the more abundant PEG polymers present in the top PEG-rich, salt-poor phase. To investigate the partitioning behavior, we constructed an ATPS with a 10\textsuperscript{8} CFU/mL concentration of \textit{S. mutans}. We used a 10\textsuperscript{9} CFU/mL stock suspension, which had a greater opacity than the 10\textsuperscript{8} CFU/mL stock suspension, to construct the ATPS at the desired concentration (Figures 3.2 A and B). After phase separation occurred, the bottom phase had a greater opacity than the top phase, indicating that the \textit{S. mutans} had been concentrated into the PEG-poor, salt-rich phase (Figure 3.2 C). When comparing the opacity of the bottom phase of the 10\textsuperscript{8} CFU/mL ATPS to that of the 10\textsuperscript{8} CFU/mL stock suspension, the greater opacity of the bottom phase suggested that the bacteria now had a concentration greater than 10\textsuperscript{8} CFU/mL. Thus, we determined that the bottom phase of the ATPS should be extracted when integrating the ATPS as a prior concentration step to LFA detection of \textit{S. mutans}.
Figure 3.2: Visualization of *S. mutans* concentration in an ATPS. The opacity of the *S. mutans* stock suspensions in PBS decreased as the concentration decreased from (A) $10^9$ to (B) $10^8$ CFU/mL. (C) When constructing an ATPS with a $10^8$ CFU/mL overall concentration, the bottom phase had a greater opacity than the $10^8$ CFU/mL stock suspension, indicating an increase in concentration.

### 3.3.2 Improving LFA Detection by Pre-Concentrating *S. mutans* Using an ATPS

In order to demonstrate the improvement in LFA detection of *S. mutans* by concentrating the bacteria prior to detection, we ran a series of LFA tests at different concentrations with and without using the ATPS as a concentration step. To establish the LFA detection limit, or lowest concentration at which a true positive result can be obtained, we examined the test results obtained for when only LFA was used (Figure 3.3, top panel). If a sample contained enough *S. mutans* to bind to the anti-*S. mutans* antibodies conjugated to the DGNPs, then the *S. mutans*-bound DGNP complexes could bind to the immobilized antibodies on the nitrocellulose membrane at the test
line, forming a visible band at the test line. This was seen for the tests performed using samples with concentrations of at least 10^7 CFU/mL, indicating that these tests produced positive results. Conversely, if there was an insufficient amount of *S. mutans* present to adequately bind to the anti-*S. mutans* antibodies on the DGNPs, then there would be no binding to the immobilized antibodies on the test line and no visual band would appear. For the LFA tests performed using samples with a concentration less than 10^7 CFU/mL, these tests produced negative results. Since 10^7 CFU/mL was the lowest concentration of *S. mutans* that produced a true positive result, this indicated that the detection limit was approximately 10^7 CFU/mL when performing LFA without using the prior concentration step.

To demonstrate that the use of the ATPS to concentrate the *S. mutans* can improve the LFA detection limit of *S. mutans*, we constructed ATPSs with concentrations of *S. mutans* that were lower than the detection limit of LFA alone (10^7 CFU/mL). The bottom PEG-poor, salt-rich phases of these ATPSs were then extracted so that a concentrated sample of *S. mutans* could be mixed with DGNPs and applied to the LFA test strips. The results of this study are shown on the bottom panel of Figure 3.3. Visual bands at the test lines were obtained for tests performed with concentrations of *S. mutans* of at least 10^6 CFU/mL. The absence of the visual band at the test line for the 10^5 CFU/mL concentration indicated that the detection limit of the integrated ATPS extraction and LFA system was approximately 10^6 CFU/mL.

When comparing the test line intensities of the 10^7 CFU/mL test for the integrated system and the 10^8 CFU/mL test for LFA only, the intensity of the 10^7 CFU/mL integrated system test had a slightly weaker intensity than the 10^8 CFU/mL LFA only test. Likewise, the test line intensity of the detection limit for the integrated system (10^6 CFU/mL) had a slightly weaker intensity than that of the detection limit for LFA only (10^7 CFU/mL). Thus, the use of the ATPS as a prior
concentration step for LFA detection of *S. mutans* produced an improvement in the detection limit that was slightly less than 10-fold due to the differences in test line intensities between the integrated system and LFA only. Moreover, the comparison of the OD$_{600}$ values of the bottom phase and control stock solution indicated that the *S. mutans* was concentrated by slightly less than 10-fold, mirroring the improvement factor in LFA detection with the use of the ATPS concentration step.

<table>
<thead>
<tr>
<th></th>
<th>Negative (0 CFU/mL)</th>
<th>$10^8$ CFU/mL</th>
<th>$10^7$ CFU/mL</th>
<th>$10^6$ CFU/mL</th>
<th>$10^5$ CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LFA only</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>ATPS extraction + LFA</strong></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 3.3: LFA test strip images without (top panel) and with (bottom panel) the prior concentration of *S. mutans* using an ATPS.

### 3.4 Conclusions

In this study, we have demonstrated that the PEG-salt ATPS can be used for the application of concentrating *S. mutans* for improving LFA performance. Specifically, we showed that the bacteria partitioned favorably into the bottom PEG-poor, salt-rich phase of the ATPS constructed
using a 10.6:1 volume ratio. Using this volume ratio, we subsequently demonstrated the improved performance of detecting *S. mutans* with LFA by combining LFA with the prior ATPS concentration step. The detection limit of LFA was effectively improved by a factor slightly less than 10-fold, which was consistent with the approximate *S. mutans* concentration factor obtained from examining OD<sub>600</sub> values. Our results indicated that *S. mutans* are compatible for concentration using the PEG-salt ATPS, allowing for the improved LFA detection of the bacteria and the subsequent potential for improved detection of dental caries.

2. Periodontal Disease in Adults (Age 20 to 64). In: National Institute of Dental and Craniofacial Research [Internet]. 2014. Available: http://www.nidcr.nih.gov/DataStatistics/FindDataByTopic/GumDisease/PeriodontaldisdiseaseAdults20to64.htm


15. Loos BG, Craandijk J, Hoek FJ, Wertheim-van Dillen PME, van der Velden U. Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of


32. Featherstone JDB. The Continuum of Dental Caries-Evidence for A Dynamic Disease


41. Walter H, Brooks D, Fisher D. Partitioning In Aqueous Two-Phase System: Theory, Methods, Uses, And Applications To Biotechnology. Orlando, Florida: Academic Press,


54