Improving the phage-displayed peptide probes for pathogen detection

by

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Abstract

In order to minimize the danger to the public from pathogen outbreaks, it is imperative to rapidly identify the infectious agent so appropriate response can be mounted and prevent dissemination of the organism. An ideal detection technology should be fast (minutes rather than hours), accurate, have a long shelf life, inexpensive, and relatively easy to use without extensive training or elaborate equipment. The magnetoelastic (ME) biosensors using phage-displayed oligopeptides as probes have the advantage over other technologies because they are fast, cost effective and require minimal sample manipulations. In the continuing effort to advance the phage-ME biosensors, I made several improvements for this technology with focus on phage displayed molecular probe isolation and attachment to the ME sensor platform. Firstly, I implemented a stringent negative selection procedure that enhanced isolation of molecular probes with higher selectivity while minimizing cross reactivity. Secondly, I improved the classic ELISA procedure against live cells to more accurately assess the interaction between molecular probes and bacterial cells. These results demonstrated that liquid phase ELISA yields results with less background and more clearly distinguishes selective probes from less selective probes. Using these two improved approaches, I isolated highly selective phage-displayed oligopeptide probes for detection of an important foodborne pathogen, *Salmonella enterica* serovar Typhimurium, that recognize the bacterium with 600% higher affinity than other *S. enterica* serovars,

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Escherichia coli or *Shigella* species. In addition, I devised a selection strategy and isolated phage displayed molecular probes that can recognize multiple serovars of pathogenic *S. enterica*. Based on recent *Salmonella* data, it is obvious that many *S. enterica* serovars can cause outbreaks. Thus, these isolated general *Salmonella* recognition probes can serve as the initial detection technology to demonstrate the presence of many of the common pathogenic serovars of *S. enterica* including *S.* Heidelberg, *S.* Newport, *S.* Javiana, S. Dublin, *S.* Braenderup*, and S.* Montevideo. Furthermore, I developed a genetic scheme to improve immobilization of the phage displayed molecular probes on ME biosensor platforms. The strategy is based on tagging 100% of the phage particles with Strep-tag II and allowing them to bind to streptavidin coated ME platform as a monolayer. The strategy can be used to tag any phage probes with any affinity tag to improve binding of the probes to the sensor platform. Finally, I utilized these improvements to construct a *S.* Typhimurium specific ME biosensor. I tagged the highly selective *S.* Typhimurium phage-displayed oligopeptide probe TA1 with Strep-tag II and immobilized it on a 0.028 mm x 0.4 mm x 2 mm magnetoelastic (ME) particle. TA1 biosensor was highly sensitive and I were able to detect between 10^2–10^6 bacterial cells. I expect the sensitivity to increase when the TA1 phage probe is used with a smaller ME particle. The TA1 biosensor was highly selective and only recognized *S.* Typhimurium. When directly compared to the previously used E2 phage biosensor, the TA1 biosensor proved to be more sensitive by 2–4 fold for detection of *S.* Typhimurium between 10^2–10^6 bacterial cells and validated all of the hypotheses and efforts. In summary, the data presented in this dissertation improved the efficacy of a promising ME biosensor technology for accurate and rapid detection of bacterial pathogens.

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Literature Review

1. Foodborne illness in the United States

Foodborne illness remains a serious public health threat even under a 'farm-to-fork' approach in the control of foodborne diseases in the United States (1). Several nationwide foodborne outbreaks caused by bacteria, viruses, parasites or chemical and toxic agents in food products emphasized this fact in recent years (2). For example during 2009-2010, a total of 1,527 foodborne disease outbreaks were reported (3). In 2013, the Foodborne Diseases Active Surveillance Network (FoodNet) identified a total of 19,056 laboratory-confirmed cases of infection, 4,200 hospitalizations, and 80 deaths (4). The population episodes of foodborne illnesses estimates for 2013 are not available yet. However in 2011, the Centers for Disease Control and Prevention reported that there were 9.4 million (5, 6) cases caused by known foodborne pathogens and estimated 48 million illnesses related to foodborne pathogens in the United States each year (7).

In all outbreaks with known etiology, 45% are caused by bacteria (2), such as *Salmonella enterica, Campylobacter* species*, Listeria monocytogenes, Shiga* toxin–producing *Escherichia coli* (STEC) O157 and non-O157, *Shigella* species*,* and *Vibrio* species

(8). *S. enterica* is the leading causative agent of both the number and incidence of laboratory-confirmed foodborne infections. CDC estimated that approximately 1 million infections in the United States per year are caused by various serovars of this bacterium (6). Among these, *S. enterica* continues to be the leading cause of bacterial gastroenteritis (8).

1.1.Salmonella enterica threat to modern foodnet

Salmonella is frequently associated with contaminated poultry and poultry products. The latest *Salmonella* outbreak related to poultry was on May 27th, 2014. A total of 26 states reported having *S. enterica* serovar Infantis and *S. enterica* serovar Newport outbreaks that were linked to live poultry in backyard flocks. In fact, six months previous to that outbreak, the same serotypes of *Salmonella* also caused other multistate outbreaks in 30 states (9). Although the genus *Salmonella* encompasses only two major species, *S. enterica* and *S. bongori*, over 2500 serovars of *S. enterica* subspecies *enterica* have been identified (10). Many of the *S. enterica* serovars are pathogenic and are named after the city or region in which they were isolated. The most famous *S. enterica* is the *S.* Typhi which causes typhoid fever. *S.* Typhi is unique because it only infects humans (11). The foodborne *Salmonella* outbreaks are caused by non-typhoidal *S. enterica*.

Currently *Salmonella* contamination can spread rapidly through the modern food industry system. In 2010, the largest *S.* Enteritidis (*Salmonella enterica* subsp. enterica sv Enteritidis) outbreak ever reported from shell eggs occurred in United States and resulted in recall of over one half billion eggs (12, 13). This event successfully refocused national attention on food safety because almost 2,000 people were infected by *S.* Enteritidis. Another

large recall of a poultry product happened on October 12, 2013, when Costco El Camino retail store located in San Francisco, CA, recalled more than 40,000 pounds of rotisserie chicken products contaminated with *S*. Heidelberg. Only 2 month later on January 10, 2014, Tyson Foods, Inc. recalled approximately 33,840 pounds of mechanically separated chicken products that may have been contaminated with *S*. Heidelberg (14).

In the modern foodnet, various foods, in addition to poultry and other meat products could be contaminated by *S. enterica*. In addition to well known pathogenic serovars of *S. enterica* such as Typhimurium, Enteritidis, Newport, and Heidelberg, other serovars including Braenderup (15), Hadar (16), Montevideo (17), Mbandaka (9), and Lille (18, 19) have been responsible for outbreaks associated with various foods. Outbreaks associated with contamination of peanut butter by *S. enterica* serovars Tennessee, Typhimurium, and Bredeney brought nationwide attention to the dangers of *Salmonella* because of the popularity of peanut butter (20-22). *S. enterica* associated foodborne outbreaks are ongoing and more and more different foods are being contaminated by this pathogen. There were several outbreaks caused by various serovars of *S. enterica* linked to health benefit food alfalfa sprouts, such as *S*. Newport in 1996 and 2010 (23, 24), *S*. Stanley in 1996 (25), *S*. Saintpaul in 2009 (26), *S*. Enteritidis in 2011 (27).

1.2. Salmonella enterica

Salmonellae are facultative anaerobic, Gram-negative, non-spore-forming, rods belonging to the family Enterobacteriaceae (11). Approximately 100 million years ago, the

genus *Salmonella* diverged from a common ancestor with *Escherichia* (28). *Salmonella* was first identified as a causative agent of typhoid fever in France in the early 19th century and it was isolated by Salmon and Smith in 1885 from a swine with hog cholera. Because it was isolated from a swine, the bacterium was named *S. choleraesuis*. Since its discovery, *Salmonella* has had several nomenclature changes. Until recently, *Salmonella* genus was considered to have many different species. Then, in the early 1970s, based on molecular analysis, the *Salmonella* genus was reclassified as having only one species, *S. enterica* (29). However, more recently, the *Salmonella* genus was reclassified into two species (30) with the addition of *S. bongori,* which is predominantly associated with cold-blooded animals (31). Based on the molecular relationships, *S. enterica* is divided into 6 subspecies, *S. enterica* subsp*. enterica, houtenae, arizonae, diarizonae, indica* and *salamae*. All known foodborne *Salmonella* outbreaks are caused by non-typhoidal *S. enterica.*

 S. enterica has over 2,500 identified serovars. Most of the serovars are named based on the disease caused or to location of the outbreak (32). Although *Salmonella* infections are usually mild and only lasts four to seven days for a healty person, having 1,500 outbreaks in 10 years remain a heavy public health burden in the United States (1, 8). The most common agents causing gastroenteritis in humans are *S.* Enteritidis and *S.* Typhimurium (8). *S.* Enteritidis is the leading causative agent of salmonellosis worldwide (33). *S.* Enteritidis is unique because it produces a specialized O-antigen of lipopolysaccharide (LPS) that contributes to long-term survival in eggs (34). *S.* Typhimurium is associated with a wide variety of food commodities (35) and, therefore, a proper identification is critical for efficient epidemiological investigation of *Salmonella* outbreak source tracking (36, 37). *S.*

Typhimurium is the second most common *Salmonella* serotype in the United States and exhibits one of the highest prevalence of antimicrobial resistance (38). *S.* Typhimurium is the best characterized *S. enterica* and, is frequently the model organism in a microbiology laboratory studying *Salmonella* biology.

One of the reasons *S. enterica* is successful as a pathogen is the bacterium's antigenic variability of the somatic (O) and flagella (H) antigens (39). Due to these variations, it is difficult to detect and prevent *S. enterica* from contaminating various foods in our foodnet. From eggs to poultry, from peanut butter to fresh produce, and from hedgehogs to small turtles, pathogenic *S. enterica* is transmitted to humans in many different ways (11, 31, 35, 40). *S. enterica*'s natural habitat is the gastrointestinal tract of animals, as a part of the microflora of the digestive tract (41, 42). Typically, foods become contaminated by exposure to noncomposted animal manure, untreated sewage or irrigation water (43, 44).In addition, foods can become contaminated in food industry during the collection, washing, or packaging steps. Salmonellosis infections could arise from a low dose of 10–1000 colony forming units (CFU) depending on the serovar and the host (1). Thus, due to the prevalence of *Salmonella* in our food systems and relatively low infectious dose, it is not surprising that salmonellosis is an ongoing problem.

Throughout history, humans have attempted to preserve food by removing water content via drying, treating with high concentrations of salt or sugar, and storing them at lower temperatures. These are effective treatments for slowing down microbial metabolism and prolonging the storage life of various foods (45-47). However, *Salmonellae* have adapted various ways to survive and growth of *Salmonella* has been demonstrated to be significantly

robust after treatments such as lowering pH (48), high salt concentration (49), or heat or cold treatments (50, 51). For example, *Salmonella* could form filaments under low water conditions caused by treatment with NaCl, glycerol, or sucrose (46). Filament formation increases *Salmonella* tolerance to low pH (52) and high osmotic stress (53). The filamentation mechanism may might result from inhibition of cell division production, which contained multiple chromosomal copies distributed in an unusually long cell (46). However this adaptive response for bacteria survival is still unclear, but filamentous *Salmonella* could not invade cells due to down regulation of SP-1 gene expression (54). *Salmonella* also readily forms biofilms on food to protect itself from desiccation and other stresses (55).

In order for *S. enterica* to cause disease, it has to overcome multiple barriers present in human digestive tract (56). The first barrier is made up of ~10^{14} residents of the microbiota from more than 400 species that forms a microbial ecosystem in our digestive tract (57) that inhibits space and resources for foreign pathogens (58). The second barrier is the extracellular mucus on the surface of epithelial cells that have antimicrobial function and contains digestive enzymes, bile salts, secretory immunoglobulin A (IgA) and antimicrobial peptides (59). Furthermore, macrophages and other immune cells encounter pathogens that get through the initial barriers.

Non-typhoid *S. enterica* has evolved a wide range of strategies for circumventing the host's defense barriers in the digestive tract (60). *S.* Typhimurium can outcompete other bacteria in the gut by being able to utilize thiosulfate ($S_2O_3^{2-}$) as a terminal electron acceptor and ethanolamine or 1,2-propanediol as carbon sources for anaerobic growth in inflamed human intestine (1, 9, 10). The thiosulfate ($S_2O_3^{2-}$) most likely originates from hydrogen

sulfide (H_2S) secreted by resident flora in an inflamed gut.   In addition, *S*. Typhimurium can oxidize $S_2O_3^{2-}$ to tetrathionate ($S_4O_6^{2-}$) which selectively inhibits *E. coli* of the resident microflora (61). Intracellular lifestyle is another important way for *S*. Typhimurium to successfully evade the host's immune system. Microfold (M) cells on the intestine epithelium is *S*. Typhimurium's preferred target for invasion. M cells pinocytose and transport antigens to lymphoid cells in the underlying Peyer's patches. M cell-mediated uptake allows *Salmonella* to breach the epithelial barrier to invade and reside in intestinal macrophages. Recent microarray studies suggest approximately 900 *S*. Typhimurium genes are differentially regulated between nutrient-rich extracellular environment and the intracellular environment. Some of the most important differentially regulated genes encode for the Type III secretion system (T3SS) (62). This system has evolved from the flagellar export system and is present in multiple Gram-negative pathogens.   In *S*. Typhimurium, two T3SS are encoded on two pathogenicity islands SPI1 and SPI2 (60, 63). SPI1-encoded T3SS is required for bacterial contact with non-phagocytic cells for translocation of bacterial proteins across the plasma membrane to initiate intestinal inflammatory responses. SPI2-encoded T3SS is important for bacterial survival in macrophages and establishment of systemic disease. Infections with non-typhoidal serovars such as *S*. Typhimurium in healthy human adults is usually limited to the intestine due to intestinal lumen polymorphonuclear leukocytes' release of cytotoxic granules and chemokine interleukin (Il)-8 to stimulated innate immune inflammatory responses (60, 64).

2. Detection of food-borne pathogens

2.1. Reducing foodborne illnesses

In order to reduce outbreaks of foodborne pathogens, continued surveillance is required. Up to date reporting of foodborne disease outbreaks provides information that may aid in reducing foodborne illnesses by allowing public health care organizations to mount appropriate responses. Unfortunately, approximately 2 to 4 weeks are currently needed to confirm identity of the causative agent in an outbreak. Such a long delay in identification of the causative agent inhibits a quick response. Thus, rapid detection of foodborne pathogens is essential to initiate a quick response for public safety. To that end, it is imperative to develop accurate, fast, sensitive, cost effective, and user-friendly detection mechanisms. Such a detection mechanism can be used as the first screen to ascertain the presence or absence of particular foodborne pathogens so that appropriate therapeutic and preventive measures can be implemented.

2.2. Importance of effective detection

F. Widal who developed an assay to diagnose typhoid fever developed one of the first clinical tests for Salmonella. The Widal agglutination test was based on the interaction of agglutinin (antibodies) in the serum of an infected patient against the H (flagellar) and O (somatic) antigens of dead *S. typhi* cells. Today, such a simple serological test alone is frequently thought to be inaccurate and several reports have seriously questioned its reliability (65, 66). However, the Widal test was a highly effective and rapid test for its time

because it did not require isolation and purification of the infectious agent.

Presently, the traditional approach of culturing, isolating, and characterizing microbial pathogens is still considered to be the gold standard for identification. The classic culture-based techniques are based on various enrichment media to isolate the purified pathogen. Several selective media based on enzymatic and chromogenic substrates exist today for detection of specific pathogens (67). For example, the Miller-Mallinson agar (MM) allows selective growth and detection of *S. enterica* (68). However, because *S. enterica* contains over 2,500 serotypes, such media are unable to distinguish between the serotypes to identify the specific causative agent for the outbreak. In order to be more serovar specific, serotyping, phage-typing, and antimicrobial resistance typing are performed to identify the specific *S. enterica*. The major drawbacks of these approaches are that antisera could be costly, the assays are time-consuming, and some isolates are non-typeable by these methods. Although phage typing is highly specific, limited number of specific phages and reproducibility problems between laboratories present major drawback to this approach (69), 52, 53, 54]. Antimicrobial resistance typing has been used less frequently during recent years due to the problems associated with spontaneous mutations (70) and horizontal transfer of resistance genes (71).

In order to overcome some of the limitations of the above techniques, Pulsed-Field Gel Electrophoresis (PFGE) was widely adopted in 1990s to identify and track *Salmonella* (72). PFGE is still considered to be one of the most reliable techniques for *Salmonella* subtyping by CDC (73). Unfortunately, because there is very little variation in the genome of various serovars of *S. enterica*, PFGE may not be the best method for differentiating various

S. enterica involved in outbreaks

The most serious drawback to the traditional methods of identifying bacterial pathogens is the need for a large quantity of the organism for differential growth, serotyping, phage typing, antibiotic resistance typing, and PFGE. Thus, although traditional methods are highly accurate, the time required (up to several days) to acquire large quantities of the pathogen from foods or clinical samples make them less than ideal for timely prevention of outbreaks.

2.3. New technology and modern culture-independent molecular approaches

In order to circumvent major problems associated with traditional culture dependent diagnostics, culture-independent diagnostic tests (CIDTs) including immunoassays, nucleic acid assays, and label-free biosensor assays were developed. These modern assays are fast, sensitive and highly accurate. Unfortunately, they are also costly and require extensive training of personnel.

2.3.1. Immunoassays

Immunoassay based techniques for identifying pathogenic agents have been highly useful and include enzyme immunoassay (EIA) and Lateral Flow immunoassay (74). Among EIA, enzyme-linked immunosorbent assay (ELISA) has been a great platform for serological diagnoses with almost 50,000 articles quoting the technique between 2001-2005 (75, 76). Today, ELISA with commercially available lipopolysaccharide (LPS) of *Salmonella* is

frequently used to detect of *S.* Typhimurium and *S.* Enteritidis isolates (77).

2.3.2. Nucleic acid based detection

Since 1990s, many nucleic acid-based detection mechanisms have been developed to identify *Salmonella*. One of the most popular and accurate detection mechanisms is based on Polymerase Chain Reaction (PCR) amplification of specific nucleic acid sequence and determination of the sequence. PCR-based methods are fast, sensitive, and requires very little sample (80). The most commonly targeted sequence is the 16S rRNA gene which has highly conserved as well as variable regions that allow identification of the organism at the species or, in the case of *S. enterica*, serovar level (78). Although the PCR-based methods were effective, they still took days to determine the identity of the organisms. Thus, realtime qPCR-based methods were developed. In this approach, highly specific DNA primers for organisms are used to quantitatively amplify the nucleic acid of interest. For example, the most frequently utilized target genes for the specific detection of *Salmonella* in food sample is *Salmonella* invasion protein gene *invA* (79). In traditional PCR method detection for *Salmonella* sample, the primers designed for *invA* will produced a 284 bp size specific DNA fragment (80). By utilizing several primer sets with each set labeled with different fluorescent dye, this target gene *invA* was wildly utilized in qPCR detection for *Salmonella* (81, 82, 83).

In addition to PCR-based methods, DNA (84) or RNA (85, 86) aptamers have been used as the affinity probes for the detection and identification of specific organisms (87), such as *S.* Typhimurium (88), *S.* Enteritidis (88), *E. coli* (89), *Staphylococcus aureus* (90).

The major disadvantage of the nucleic acid based detection approaches is that these techniques require costly instruments and highly trained personnel. Moreover, these techniques still take hours to perform and thus are not suitable for realtime monitoring of pathogens. Thus, an alternative detection approach is necessary to facilitate accurate and rapid detection of pathogens.

3. Biosensors

Biosensor technology combines the accuracy and sensitivity of other approaches with a great enhancement in the rapidity of detection. A typical biosensor is composed of molecular probes immobilized on a platform with characteristics that can be easily measured based on the probe:analyte interactions (91). As long as the molecular probe is selective, biosensors can be used even in complex matrices (blood, stool, urine or food) with minimum sample pretreatment (92, 93). Based on the method of signal transduction, biosensors can be divided into four basic groups: optical, mass, electrochemical, and thermal sensors (94, 95).

The success of glucose oxidase (GOx) biosensor in 1962 initiated the age of biosensors. The last two decades saw an effort on developing biosensors that are highly selective, precise, reproducible, small, biocompatible, capable of real-time analysis, and manageable under complex physical or chemical environment have been developed (91, 93, 95). More recently, many efforts have been devoted to development of biosensors for the detection of foodborne pathogens. These biosensors include single-stranded DNA aptamer based noncovalent self-assembly of single-walled carbon nanotubes (SWNTs) for *Salmonella* Paratyphi (96), spectroscopy based Fourier transform (FT) Raman spectroscopy for *E. coli*

O157:H7 (97), and optical based two-dimensional angle-resolved light scattering (ALS) for individual rod-shaped bacteria (98).

3.1. The magnetoelastic (ME) platform

The magnetoelastic (ME) particles are a great choice as biosensor platforms because they can be easily manipulated, made at micro- or nano-size, cost-effective, and highly sensitive to mass change for detecting probe:analyte interactions (99). To detect a mass change, a time-varying external magnetic field (driving field) is used to induce vibrational resonance in the particles. The vibrational resonance of the ME particle results in the emission of a magnetic signal that can be remotely detected using a pick-up coil. Recognition and binding of a target agent to a ME particle act as a mass load causing the characteristic resonant frequency to shift to lower frequencies. ME particles can be made very small so that the attachment of a single spore, a bacterium or, in near future, a virus can be detected. MEs can be deployed in large numbers to increase the probability of binding, increase the sensitivity of detection, and reduce detection times. ME particles can be simultaneously moved (guided in a given direction) through a liquid using a DC field, driven into resonance with an AC field and interrogated for binding using a pickup coil. An example of a ME particle biosensor detection system is illustrated in Figure 1.1.

The schematic of ME sensor detection system.

phage probe

Amplitude

f Mass   f No Mass

Frequency

Magnetic field
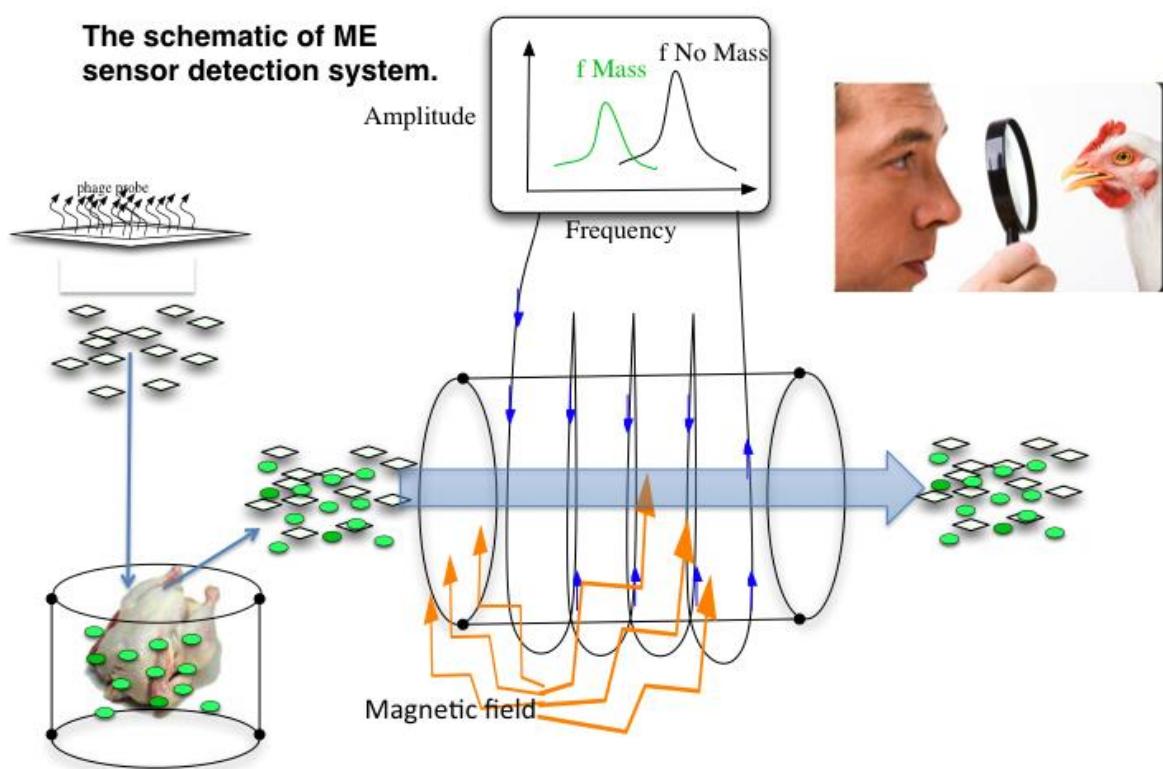
Figure 1.1. The schematic of magnetoelastic sensor detection system.

Illustrated is the proposed detection of contaminated chicken carcass using magnetoelastic biosensor for pathogenic *Salmonellae*.

3.2. Bacteriophage as sensor probes

Typical molecular probes used in biosensors include enzymes, nucleic acids, antibodies, live cells, and phage-displayed oligopeptides (91). The use of biosensors with immobilized antibodies to detect HIV (100) and *Salmonellae* (101, 102) has been reported since 1990s with the relative selectivity and sensitivity that is comparable to ELISA (102, 103). For *S*. Typhimurium, a biosensor using an antibody as the probe was highly specific and gave a linear response from 10^5–10^9 cells/ml microbial suspension with the limitation of detection at 5×10^3 CFU/ml (104). Although highly valuable due to the specificity, the main disadvantages of antibody probes are poor environmental stability (105), and the cost and time associated with production of specific antibodies. In contrast, phages displaying peptides or proteins can be selected, isolated, and rapidly reproduced at low cost to be used as molecular probes. In addition, the filamentous phage-displayed oligopeptide probes are robust because they are resistant to heat (up to 80°C) (106), organic solvents like acetonitrile (107), urea (up to 6 M), acids, alkali and other chemicals (108, 109). Thus, phage probes bound to ME particles are likely to form viable biosensors.

3.3. Phage display Technology

Phage display, developed by George Smith, is a variation of combinatorial chemistry in which random peptides are fused to bacteriophage capsid proteins and expressed as a part of the phage particle (110). The phage display technology offers an ideal approach to develop

specific peptide-based molecular probes that are stable and inexpensive for detection of analytes including live bacterial cells (111-115). Phage-displayed oligopeptides are attractive as probe molecules because they are highly stable and resistant to environmental stressors while maintaining the ability to interact specifically with analytes.

3.3.1. Filamentous bacteriophages

Filamentous bacteriophages are commonly associated with various members of Gram-negative bacteria, including *Escherichia*, *Salmonella*, *Pseudomonas*, *Xanthomonas*, *Vibrio*, *Thermus* and *Neisseria* species (117). They are typically composed of circular, single-stranded DNA (116) surrounded by a cylinder of 5 different coat proteins: pIII, pVI, pVII, pVIII, and pIX (117). Among 60 different filamentous phages that have been characterized to date, two different classes have been identified based on their life cycle (117). First class is designated as episomally replicating phages and these phages do not integrate into the genome of the host. This class includes well-characterized Ff class of phages such as M13 and f1. The second class is designated as chromosomally integrated temperate phages and include the CTXφ of *Vibrio cholerae* (118). The episomal phages are preferred for phage-display technology because their genomes are small and produce high titer of phage lysates (10^{12-13} per ml). The chromosomally integrated phages have large genomes and produce lower titer of phage particles (10^{7-8} per ml) (119).

Ff class of F pilus-specific phage group contains f1, M13 and fd. These phages are very closely related to each other and share almost 99% identity at the genome level according to the International Committee on Taxonomy of Viruses database (ICTVdB, http://ictvdb.bio-mirror.cn/ICTVdBintro.htm). Although M13 is the best characterized and most utilized phage among the Ff class of phages, fd is the phage that had been developed for the phage-display technology (120). However, because M13 and fd are almost identical at the genome level, they will be treated as equals in this discussion. A diagram of M13 phage (M13KE) that is used for construction of a commercial phage-display library by New England Biolabs (Beverley, MA) is shown in Figure 1.2.

All five of fd or M13 phage capsid proteins have been used for phage-display. Peptides displayed on GpIII, GpVI, GpVII, and GpIX are represented at five copies per capsid and are located at polar ends of the phage particle. Among the four proteins, GpIII has been utilized the most and has been used to construct commercially available libraries. Some of the commecially available phage display libraries include 7-mer and 12-mer oligopeptide libraries by New England Biolabs (Beverley, MA), short chain fragement variable (ScFv) library by Creative Biolabs (Shirley, NY), and ScFv and Fab libraries by Phage Display (Cambridge, UK). The New England Biolab's Ph.D.-7 and Ph.D.-12 libraries have been used to isolate highly selective probe for detection of *B. anthracis* spores (121, 122) (Chen et al. unpublished data).

The major capsid protein, GpVIII, has also been used to display peptides (123). The major advantage of displaying peptides on GpVIII is that because there are approximately

2800 copies of the protein used to cover the genomic DNA of the phage, the avidity of the probe is significantly increased due to multivalent binding.

In addition, due to the close proximity of each GpVIII and displayed peptides to each other on the surface of the phage, they form interactions that result in formation of a "landscape" (123). The landscape phages have been highly successful in yielding probes that specifically recognize *Bacillus anthracis* spores (124-129) and *S.* Typhimurium (115, 130-136). The major weakness of the landscape phage-display peptide probes is that because their affinity depends on interaction with neighboring peptides, individual peptides cannot be isolated and used apart from the phage capsid. Another weakness of GpVIII display is that it is limited to peptides of nine amino acids or less (123). In contrast, much larger peptides or proteins can be displayed on GpIII. Thus, GpIII has been the preferred capsid protein for displaying antibodies or ScFv fragments. Another advantage of displaying a peptide or a protein on GpIII is that because it is located only at one end of the phage, the other end with GpVII and GpIX can be used to immobilize the phage on an abiotic surface. As described in Chapter 4, I took advantage of this property of GpIII-displayed peptide probes and developed a novel affinity-immobilization method for attaching our molecular probes to magnetoelastic particle sensors. A major disadvantage of displaying on GpIII is that because GpIII mediates phage attachment to F-pilus of *E. coli*, the recombinant phages are at a disadvantage compared to the wild-type phage for infection and replication. Thus, one must be careful during amplification because it is easy to select for the wildtype phage in lieu of the recombinant phages that display peptides on capsid.
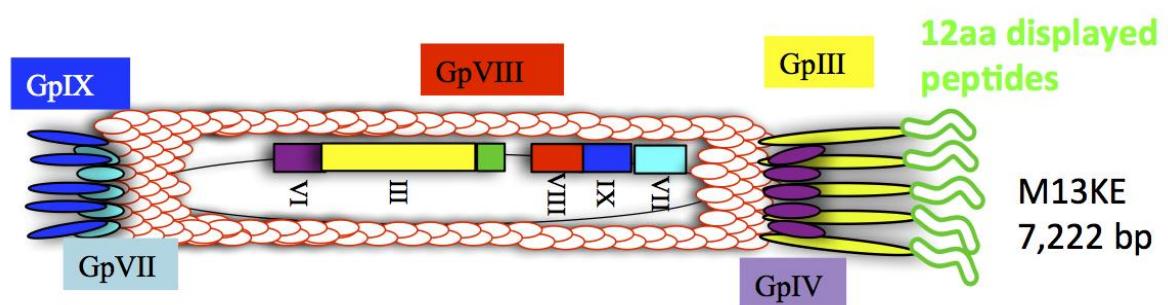
Figure 1.2. New England Biolabs' Ph.D.-12 phage displayed oligopeptide. A library of random oligonucleotides encoding 12 amino acids were cloned in-frame to gpIII of M13KE to generate a 10^9 variation of displayed peptide population (NEB, Beverly, MA)

Phage-displayed peptides on Ff class of filamentous phages have been used successfully to isolate probes against various target analytes including bacterial pathogens such as *B. anthracis* spores (121, 124), *Staphylococcus aureus* (137), and *S.* Typhimurium (113, 115).

3.3.3. Selection of phage-displayed molecular probes

A phage expressing a peptide of interest is identified from the library via biopanning which allows rapid partitioning based on binding affinity to a given target molecule. A standard phage-display library contains 10^9 different combinations of peptides. Thus, it is important to develop a selection scheme in which only the desired peptides that bind to the analyte of interest are isolated. In general, biopanning is performed by incubating a library of phage-displayed peptides on a plate coated with the target, and after several washing steps to eliminate unbound phages, eluting to recover bound phages (138, 139). The eluted phages are then amplified in a bacterial host and the biopanning is repeated for several cycles to enrich for the pool of probes that bind to the target. After several rounds of biopanning, putative probes are characterized by ELISA followed by DNA sequence analysis to identify the peptide sequence. A schematic of a biopanning procedure is illustrated in Figure 1.3.
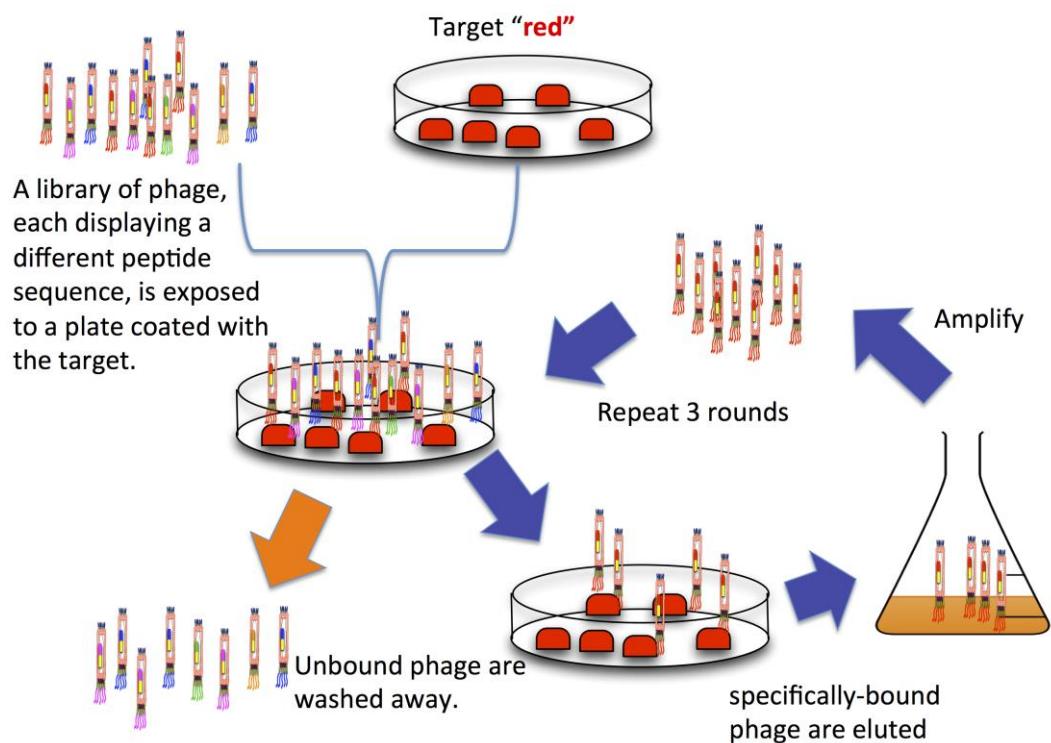
Figure 1.3. The standard biopanning scheme

3.4. Current state of magnetoelastic biosensors

Magnetoelastic biosensors using phage-displayed peptides as molecular probes have produced some very good results for rapid detection of important bacterial pathogens including *B. anthracis* spores (121, 122, 124, 140) and *S.* Typhimurium (115, 133, 136, 141). Recent efforts to improve the detection method has further enhanced the ease of use of magnetoelastic biosensors by allowing remote sensing (141-144). Improvements are also needed on the molecular probe side to increase the selectivity and stability of the sensor. Currently used standard biopanning method frequently yields probes with cross reactivity to closely related targets (Tong and Suh, unpublished data; see also Chapters 2 and 3 of this dissertation) and therefore a new approach is needed to improve the selectivity of phage probes. In addition, the standard biopanning step of immobilizing the target on a plastic surface greatly affects isolation and enrichment of specific peptide probes because of the variations in the analyte binding to plastic. This problem is compounded when live bacterial cells are used as the target because each bacterium binds to abiotic surface with different affinity. Thus, it is better to eliminate this variation by performing biopanning in liquid using freely suspended target cells and the probe. The variations in target attaching to plastic surface also affects the ELISA to determine the relative affinity of the probe:target interactions. Thus, a new approach to minimize this variation is needed. Furthermore, better methods are needed to attach the phage-displayed probes to the magnetoelastic platforms because the current physical adsorption method results in haphazard binding of the phages that may interfere with optimal capture of analytes. A standard phage attachment via physical adsorption method is illustrated in Figure 1.4A.

This dissertation describes efforts to improve the molecular recognition part of the magnetoelastic biosensors including developing a better probe immobilization on the sensor platform as illustrated in Figure 1.4B.
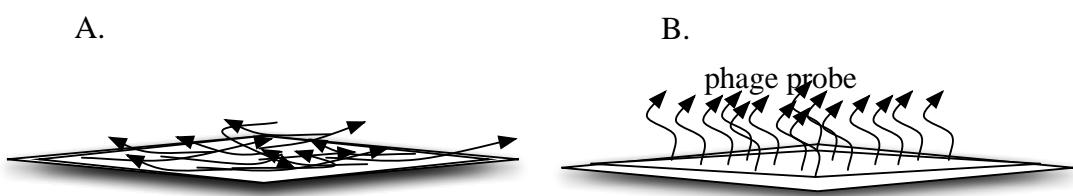
Figure 1.4. Two different modes of phage probe immobilization on magnetoelastic platform. A. Currently used physical adsorption. B. Newly developed affinity immobilization (for details, see Chapter 4).

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Chapter 2

Improved strategies for isolation of highly selective phage displayed oligopeptide probes for detection of *Salmonella enterica* serovar Typhimurium

Abstract

Phage-display technology has been highly useful for studying peptide:macromolecule interactions including protein:protein, protein:nucleic acids, antibody:toxin, and peptide:cell. It has been especially widely used to isolate peptide probes for the detection of various pathogenic organisms. A typical procedure for isolating phage-displayed peptide probes for recognizing of live cells involves biopanning followed by enzyme-linked immunosorbent assay (1) or other quantitative determination for interaction between the probe and the target. Although this approach has yielded several successful probes, the standard procedure suffers when the target cells bind to plates with varying efficiency and results in isolation of probes with low selectivity and affinity. Thus, I developed a probe selection procedure which minimizes variations and results in isolation of highly selective probes against live bacterial cells.Myprocedure utilizes stringent negative selections against closely related target organisms to remove cross-reacting probes and a variation of the pull down experiment for biopanning and ELISA (liquid phase) to bypass

targets' different proclivities for binding to plates. Using the modified approach, highly selective phage-displayed 12-amino acid oligopeptide probes were isolated against *Salmonella enterica* serovar Typhimurium that recognize the bacterium with 600% higher affinity than other *S. enterica* serovars, *Escherichia coli* or *Shigella* species. This procedure can be easily adapted for isolation of highly selective probes against other live cells including pathogenic organisms.

Introduction

As demonstrated by frequent outbreaks of salmonellosis and recalls of *Salmonella* contaminated foods, reducing contamination by this pathogen is important for health of the consumers as well as the food industry in the United States of America. The costs associated with *Salmonella* contamination of foods fall into two broad categories. The first is related to expenses associated with human illnesses caused by the consumption of contaminated food. The total combined costs associated with medical care and loss of productivity in the United States alone has been estimated at approximately $3.3 billion (2). The second category involves loss of revenue for the food industry, including farmers and distributors, as a consequence of *Salmonella* contamination of their products. Serotype Typhimurium were associated with a wide variety of food commodities (3). Accurate identification is critical for efficient epidemiological investigation of Salmonella outbreaks source tracking (4, 5). Thus, it is imperative to develop rapid and accurate detection methods so proactive measures can be taken to reduce consumers' exposure to *S*. Typhimurium.

Pathogen monitoring is one of the most important steps in implementing effective controls against outbreaks. Rapid and reliable identification of pathogens allows for timely application of protective measures such as antibiotic treatment, eradication of infected animals, and recall of contaminated product. Thus, rapid detection of *Salmonella* during each stage of preparation essential to minimize outbreaks of foodborne salmonellosis.

The traditional approach of isolating and identifying pathogens via culturing is still considered to be the gold standard. Unfortunately, this approach requires large quantities of the pathogen, extensive training in microbiological techniques and several days (6). Even the most recently developed *Salmonella* identification method via culturing (Rainbow Agar, Biolog, Hayward, CA) requires at least 24 hours to obtain results. Moreover, the Rainbow Agar cannot differentiate between species or serovars of *Salmonella* and has a limited shelf life of 12 weeks at 4°C. Several molecular approaches are also available to identify *Salmonella* including polymerase chain reaction (7) amplification of *invA* (8), SNP (9), sequence analysis of 16S rDNA (10), and ELISA (11). While these methods are highly sensitive and accurate, they require costly instruments, highly trained staff, and several hours to yield results. Due to these limitations, it is imperative to develop a rapid, cost effective, and easy to use method for detection of *Salmonella*.

An ideal detection technology should be fast (minutes rather than hours), accurate, have a long shelf life, inexpensive, and relatively easy to use without extensive training or elaborate equipment. The magnetoelastic (ME) biosensors using phage-displayed oligopeptides as probes have the advantage over other technologies because they are fast, cost effective and require minimal sample manipulations. For the magnetoelastic biosensors to function properly, it is essential for them to be coupled with molecular probes that are highly specific for the target of interest.

Phage display technology offers an alternative approach to antibodies and has been extensively used for cell surface molecular recognitions (1, 12, 13, 14, 15). Phage display is a variation of combinatorial chemistry in which random peptides are fused to bacteriophage

capsid proteins and expressed as a part of the phage particle (16). Cloning randomized

oligonucleotides in-frame to a phage capsid gene results in generation of a phage library in

which approximately 10^9 phage particles express unique oligopeptides. Phages that express

oligopeptides specific against a target analyte are then selected from the library via several

rounds of biopanning. Selected phages can then be used directly as probes on detection

platforms (1, 13, 17, 18). Alternatively, the peptides identified from the phages can be used

independently of the viral particle as oligopeptide probes (15, 19, 20). Phage-displayed

oligopeptides are attractive and viable alternatives to antibody probes because they are more

resistant to environmental insults including high temperature and long term storage (21). In

addition, they are easier to prepare and are more cost effective than antibodies. Phage

display has been used successfully to isolate probes against various bacterial targets analytes

including bacterial pathogens such as *Bacillus anthracis* spores (17, 22), *Staphylococcus*

aureus (23), and *Salmonella enterica* serovar Typhimurium (1, 14). Although this procedure

has been successful, it was some problems that should to be addressed when live cells are

used as targets for probe selection on solid surface. First, it is important to minimize the

variations caused by the different affinity of bacterial cells to plastic surface. The most

common method to attach bacterial cells to a solid surface involves drying cells on a plastic

surface. A large variation is observed among closely related bacteria for their ability to

attach to the solid surface and this affects enrichment of specific probes. Second, drying the

cells may alter the cell envelope conformation and affect isolation of probes that recognize

native cell envelope. The problems associated with the solid phase biopanning can be

avoided by using the liquid phase biopanning procedure. Third, a similar problem in

variability of cells binding to plastic surface plagues the traditional ELISA for assessing the binding affinity of the probes to the live cell targets due to cells getting washed off. To rectify some of these problems, I developed an improved overall strategy that facilitates isolation of highly selective phage-displayed probes against live cells by implementing stringent negative selections against closely related organisms and performing the target capture as well as ELISA purely in liquid phase (an adaptation of the pull down experiment) to eliminate the variations caused by target binding to solid surface.

Utilizing my improved strategy, I isolated highly selective phage-displayed oligopeptide probes for *S*. Typhimurium that can be immobilized on ME biosensor platform for rapid and accurate detection of the pathogen.

Materials and methods

Bacteria, media, and chemicals

S. Typhimurium (ATCC 13311), *S*. Typhimurium (BAA-712), *S*. Typhimurium (BAA-215), *S*. Typhimurium (ATCC 15277=LT2), *S*. Typhimurium (ATCC 51812), *S*. Typhimurium 14a, *S*. Enteritidis (BAA-708), *S*. Newport j1890, *S*. Javiana k2674, *S*. Braenderup k2679*, S*. Heidelberg*, S*. Minnesota, *S*. infantis, *S*. Thompson, and *S*. Panama were grown in nutrient broth (NB) and confirmed for identity, propagated, and maintained by MacConkey Agar (ACUmedia, Lansing, MI). *E. coli* ER2738, *E. coli* O157:H7 (ATCC43888), *E. coli* O157:H7 (ATCC43894), *E. coli* O157:H7 (ATCC43895), *E. coli* O157:H7 (BAA-460), *Shigella sonnei* (ATCC 9290), *S. sonnei* (ATCC 29031), *S. sonnei*

(ATCC 29030), and *S. sonnei* (ATCC 29930) were grown in Lysogeny broth (24, 25) at 37°C with aeration at 220 rpm unless otherwise indicated. When necessary, media were solidified MacConkey Agar (ACUmedia, Lansing, MI). Antibiotics and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Sigma-Aldrich and 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was purchased from Gold Biotechnology (St. Louis, MO). Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). The following chemical concentrations were used in this study (per milliliter): tetracycline(26), 20 μg; X-gal, 50 μg/ml; and IPTG, 40 μg/ml.

All bacteria were grown in an appropriate growth medium at 37°C for overnight in a New Brunswick (Enfield, CT) shaking water bath incubator. 10 ml of cell culture were collected by centrifugation at 5000 rpm for 10 min, washed twice with 10 ml of sterile phosphate-buffered saline (PBS, pH 7.0), and adjusted to OD_{600} nm of 1.0 with PBS.

Phage library and pre-clearing of the library to eliminate plastic and BSA binding phages

M13 pIII phage displayed peptide library (Ph.D.[TM]-12) and M13 vector phage were purchased from New England Biolabs (Beverly, Mass., USA). Phages were propagated in *E. coli* ER2738 and titer was determined according to the manufacturer's recommendations (New England Biolabs, Beverly, Mass.).

Prior to biopanning, the phage library was pre-cleared of nonspecific population that binds to plastic and Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO). Briefly, 80 μl of Ph.D.™ Phage Display Library (1 x 10^{13} pfu/ml) from New England Biolab (Ipswich, MA) was diluted in 1 ml PBST (phosphate buffered saline supplemented with 0.1% Tween

20) and added to a 35 mm sterile petri plate and shaken for 30 min at room temperature at 20 rpm/min to induce binding of the phage probes to the plastic. Unbound phages were removed into fresh petri plate to further eliminate plastic binding phages. This procedure was repeated for a total of three times. In order to remove those phages that bind to BSA, 35 mm petri plates were first blocked with 1 ml of 5 mg/ml BSA solution overnight at 4°C. Then the phage lysate that had been pre-cleared of plastic binding virions were added to the BSA coated petri plate and incubated for 30 minutes at 4°C with gentle shaking at 20 rpm/min to promote phage binding to the BSA coated plate. The unbound phages were transferred to a fresh BSA coated plate to further remove BSA binding virions for a total of three times and finally collected.

Depletion of phages binding to Escherichia coli *and* Shigella spp.

The pre-cleared phage library was further depleted of oligopeptides that bind to closely related bacteria *Escherichia coli* and *Shigella* species before being biopanned for *S.* Typhimurium binding probes. Briefly, *E. coli* O157:H7 (4 strains), *S. sonnei* (4 strains)*,* and *S. flexneri* (3 strains) were grown overnight in LB, washed twice with PBS, and diluted to OD_{600} of 0.5 in PBS. For each bacterium, equal volumes of different strains were combined to generate a mixture of that organism used for the subsequent experiments. In a 2 ml microcentrifuge tube, 500 μl of *E. coli* was centrifuged, resuspended in 1 ml of the pre-cleared phage library, and incubated for 30 minutes at room temperature with gentle shaking at 50 rpm/min on a rotator. Following incubation, cells and phages bound to cells were collected by centrifugation at 5000 rpm for 5 minutes. Unbound phages were

transferred to a new microcentrifuge tube containing 500 µl of *S. sonnei* suspension and the procedure was repeated. Finally, phages were incubated with *S. flexneri* cells and unbound phages were collected by a final centrifugation at 8000 rpm for 10 minutes. This pre-cleared phage pool constituted the phage library for biopanning.

Biopanning of phage-displayed peptides for S. *Typhimurium specific probes*

Solid-phase biopanning was performed as described previously (1). Briefly, six strains of *S.* Typhimurium were grown overnight in LB, 37°C shaking at 220 rpm, 1 ml of each culture was mixed, and 1 ml of the mixed culture was added to a 35 mm diameter petri plate and allowed to dry in a fume hood overnight. The following morning, 2 ml of 5 mg/ml of BSA was added to the plate and incubated at room temperature for 2 hours with gentle agitation (20 rpm/min) to block non-cell bound sites on the plate. Following blocking with BSA, the plate was washed twice with 1 ml of PBS to remove unbound BSA and cells. Then 1 ml of the pre-cleared phage library (1×10^{11} pfu/ml) was added to the cell coated plate and allowed to bind to *S.* Typhimurium cells at room temperature for 40 min. Unbound phages were removed by washing the plate ten times with 1 ml of PBS containing 0.02% Tween 20. Following the final wash, phages that bound to *S.* Typhimurium cells were eluted with 0.8 ml of 0.2 M Glycine elution buffer (pH 2.2) for 10 min at room temperature and neutralized with 150 µl of 1M Tris-HCl (pH 9.0).

For liquid-phase biopanning, in lieu of 35 mm petri plates, 2 ml microcentrifuge tubes that had been coated with 2 ml of 5 mg/ml BSA overnight at 4°C were used as the vessel to capture *S.* Typhimurium specific phage probes. Briefly, six strains of *S.*

Typhimurium were grown overnight in LB at 37°C with 220 rpm of shaking, equal volume of each culture was mixed, and 1 ml of the mixed culture was added to a 2 ml microcentrifuge tube that had been coated with BSA and gently centrifuged at 5000 rpm for 3 min to harvest cells. The pelleted cells were resuspended in 1 ml of pre-cleared phage library (1 x 10^{11} pfu/ml) and incubated at room temperature for 40 min with gentle rotation to promote cell:phage binding. Cell:phage complex was collected by centrifugation at 5000 rpm for 3 min, unbound phages removed, and the complex was resuspended in 1 ml 1X PBS containing 0.02% Tween 20 to wash. The wash step was repeated for a total of five times. Phages that remained bound to *S.* Typhimurium cells were eluted with 0.8 ml of 0.2 M Glycine elution buffer (pH 2.2) by vigorously tapping on the tube for 10 min at room temperature followed by centrifugation at 7500 rpm for 3 min to remove cells, and neutralized with 150 µl of 1 M Tris-HCl (pH 9.0).

The eluted phages were titered and amplified in *E. coli* ER2738 as per NEB's instructions.

ELISA

The solid-phase ELISA was performed as described previously (1). Briefly, the wells of a 96-well EIA/RIA plate (Corning 3591, Corning, NY) were coated with 100 µl of a mixture of six strains of *S.* Typhimurium cells that had been grown in LB overnight at 37°C with 220 rpm/min shaking. All assays were performed in triplicate. The plates were incubated in the fume hood overnight to dry the cells in each microtiter well. The following morning, 100 µl of blocking buffer (5 mg/ml BSA) was added to each well and incubated

for 1 hour at room temperature and washed three times with PBST to remove unbound BSA. 100 µl of isolated phage pool (5×10^{10} pfu/ml) was then added to each well, incubated for 1 hour at room temperature to promote binding, and washed six times to remove unbound phage with 100 µl of PBST. Bound phages were probed first with 1:3000 diluted anti-Fd phage IgG rabbit antibody (Sigma-Aldrich) followed by 1:5000 diluted anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich). Both reactions were performed at room temperature for 1 hour with gentle shaking. The reaction was developed with 100 µl of 2 mg/ml *p*-NPP (*p*-nitrophenylphosphate) and the kinetic signals were measured on BioTek Synergy HT microtiter plate reader (BioTek, Winooski, VT, USA) for a reaction time of 60 min at 405 nm. The alkaline phosphatase activity was measured as the linear slope of the reaction.

The liquid-phase ELISA is based on the liquid-phase biopanning scheme and the steps of liquid-phase biopanning were followed until the phage elution step. Following the final wash, in lieu of eluting the phage, the cell:phage pellet was resuspended in 250 µl of 1:3000 diluted anti-Fd phage IgG rabbit antibody and incubated at room temperature for 30 min with gentle shaking on a rotator. Cell:phage complex was pelleted by centrifugation at 6000 rpm for 3 min and washed twice with 1 ml of PBST. The complex was then resuspended in 250 µl of 1:5000 diluted anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase and incubated for 30 min at room temperature. The cell:phage:antibody:antibody complex was harvested by centrifugation at 6000 rpm for 3 min, washed twice with 1 ml of PBST, and resuspended in 180 µl of fresh 0.1 M glycine buffer supplemented with 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ (pH 10.2). 50 µl of the

resuspended complex was transferred to the wells of a EIA/RIA plate, reaction developed with 50 μl of 4 mg/ml *p*-NPP (*p*-nitrophenylphosphate), and the kinetic signals were measured on BioTek Synergy HT microtiter plate reader (BioTek, Winooski, VT, USA) for a reaction time of 60 min at 405 nm. The alkaline phosphatase activity was calculated as the linear slope of the reaction. All assays were performed in triplicate.

Polymerase chain reaction (PCR) and DNA sequencing

A region of the pIII gene carrying the cloned oligonucleotides was sequenced to determine the identity of the displayed peptides from the isolated phage probes. Briefly, phage DNA for sequencing was acquired via polymerase chain reaction from either the lysogen carrying the phage in replicative form or from the purified phage genomic DNA. The phage DNA was purified from individual phage clones as described previously (17). The PCR was performed with the following primers: -115 pIII (5'- CCA CAG ACA GCC CTC ATA GTTA -3') and +384bp for M13pIII (5'- TTC TTT TGC CTC TTT CGT TTTA -3') with Phusion® DNA Polymerase (NEB. Beverly, Mass., USA) on an Eppendorf MasterCycler gradient (Hauppauge, NY). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced from the 3'-end of the pIII gene with the primer (5'- CCA CAG ACA GCC CTC ATA GTTA -3') at the Auburn University DNA Sequencing Facility. DNA sequences were analyzed with Geneious (www.geneious.com) and Lasergene software suite (DNA Star, Madison, WI).

Strains	Genotype and relevant characteristics	Source
ER2738	F′ $proA^+B^+$ $lacI^q$ Δ(lacZ)M15 zzf::Tn10 (Tet^R)/fhuA2 glnV thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 (rk$^-$ mk$^-$ McrBC$^-$)	New England Biolabs
SS2205	*Escherichia coli* O157:H7 (ATCC 43888)	ATCC
SS2206	*Escherichia coli* O157:H7 (ATCC 43894)	ATCC
SS2207	*Escherichia coli* O157:H7 (ATCC 43895)	ATCC
SS2208	*Escherichia coli* O157:H7 (BAA-460)	ATCC
SS2210	*Salmonella enterica* subsp. *enterica* sv Typhimurium (BAA-215)	ATCC
SS2211	*Salmonella enterica* subsp. *enterica* sv Typhimurium (BAA-712)	ATCC
SS2212	*Salmonella enterica* subsp. *enterica* sv Typhimurium (ATCC 15277=LT2)	J. Roth via J. Escalante-Semerena
SS2213	*Salmonella enterica* subsp. *enterica* sv Typhimurium (ATCC 51812)	ATCC
SS2235	*Salmonella enterica* subsp. *enterica* sv Typhimurium 14a (Environmental Isolate)	CDC (D. Schaffner)
SS2275	*Salmonella enterica* subsp. *enterica* sv Typhimurium (ATCC 13311)	ATCC
SS2183	*Salmonella enterica* subsp. *enterica* sv Heidelberg	ATCC (J. Barbaree)
SS2190	*Salmonella enterica* subsp. *enterica* sv Infantis	ATCC (J. Barbaree)
SS2191	*Salmonella enterica* subsp. *enterica* sv Minnesota	ATCC (J. Barbaree)
SS2193	*Salmonella enterica* subsp. enterica sv Thompson	ATCC (J. Barbaree)
SS2236	*Salmonella enterica* subsp. *enterica* sv Newport J1890	CDC (D. Schaffner)
SS2237	*Salmonella enterica* subsp. *enterica* sv Javiana K2674	CDC (D. Schaffner)
SS2238	*Salmonella enterica* subsp. *enterica* sv Braenderup K2679	CDC (D. Schaffner)
SS2218	*Shigella sonnei* (ATCC 9290)	ATCC
SS2219	*Shigella sonnei* (ATCC 29031)	ATCC
SS2220	*Shigella sonnei* (ATCC 29030)	ATCC
SS2221	*Shigella sonnei* (ATCC 29930)	ATCC
SS2215	*Shigella flexneri* (ATCC 12023)	ATCC
SS2216	*Shigella flexneri* (ATCC 25929)	ATCC
SS2217	*Shigella flexneri* (ATCC 49070)	ATCC

Table 2.1. Bacterial Strains.

SS strain designations are for the Suh laboratory use only. ATCC: American Type Culture Collection; CDC: Centers for Disease Control and Prevention.

Results

<u>*Comparison between solid-phase and liquid-phase biopanning against live cell*</u>

A standard protocol for biopanning includes both solid-phase (surface panning) and liquid-phase (solution-phase) biopanning procedures. However, the most commonly used approach is the solid-phase biopanning. The liquid-phase is reserved for cases in which the ligand is affected during surface coating (27). The two procedures have not yet been directly compared to determine which approach yields better probes. During these attempts to deplete the phage library of non-selective probes by biopanning against organisms that are closely related to *S.* Typhimurium, significant differences were observed between bacteria for binding to the plastic surface. Differences in affinity to plastic affected these results because it changed the number of targets available to capture phage probes. Thus, I performed both solid- and liquid-phase biopannings were performed and then compared them to determine the effectiveness of each approach when using live bacterial cells as targets.

Both solid- and liquid-phase biopannings were initiated with the same batch of the phage display library that had been pre-cleared against plastic, BSA, *E. coli*, *S. sonnei*, and *S. flexneri*. I mixed six different strains of *S.* Typhimurium (Table 2.1) were mixed and used as the target bacterium to minimize isolation of probes that are strain specific. Following each round of biopanning, the isolated phage pools were amplified. As demonstrated in Table 2.2, liquid-phase biopanning consistently resulted in more phages binding to *S.* Typhimurim in

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rounds 2 through 4 than solid-phase biopanning. Solid-phase biopanning did allow recovery of what appeared to be an equal number of *S.* Typhimurium specific phages in round 4 of the liquid-phase. Unfortunately, analysis of the pool, following amplification of the isolated phages demonstrated that over 60% of the phages in the amplified pool were Lac$^-$ as indicated by white colonies on plates containing the chromogenic substrate X-gal. This suggested that solid-phase biopanning was not specific after round 4 and resulted isolation and amplification of the vector phage M13KE (data not shown). In contrast, liquid biopanning did not result in further amplification of the vector phage or *S.* Typhimurium specific phages following round 4.

Phage pools recovered from each round of biopanning were assayed for specificity to *S.* Typhimurium via ELISA (Figure 2.1). As shown in Figure 2.1, liquid-phase biopanning yielded a great numbers of specific phage probes that were enriched through successive rounds of biopanning.

	Pre-clearing		Selection					
Target	Plastic/BSA	*E. coli*, *S. sonnei*, *S. flexneri*	*S.* Typhimurium[*]					
			Rounds of Biopanning (Solid-phase)					
			1	2	3	4	5[#]	6[2]
Input Phage	8.0×10^{11}	6.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	N.D.	N.D.
Output Phage	6.0×10^{11}	3.5×10^{11}	7.6×10^{5}	7.0×10^{5}	9.0×10^{5}	2.0×10^{8}	N.D.	N.D.
			Rounds of Biopanning (Liquid-phase)					
Input Phage	8.0×10^{11}	6.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}
Output Phage	6.0×10^{11}	3.5×10^{11}	8.6×10^{5}	7.9×10^{6}	1.0×10^{8}	2.4×10^{8}	2.0×10^{8}	2.1×10^{11}

Table 2.2.   Comparison of solid-phase versus liquid-phase biopanning for isolation of S. Typhimurium specific probes.
[*]Six strains of *S.* Typhimurium were mixed in equal volume and used as the target organism.
[#]For solid-phase biopanning, > 60% of the lysogens were Lac⁻ after being amplified following round 4 to indicating that a majority of the phages after round 4 were vectors.

55
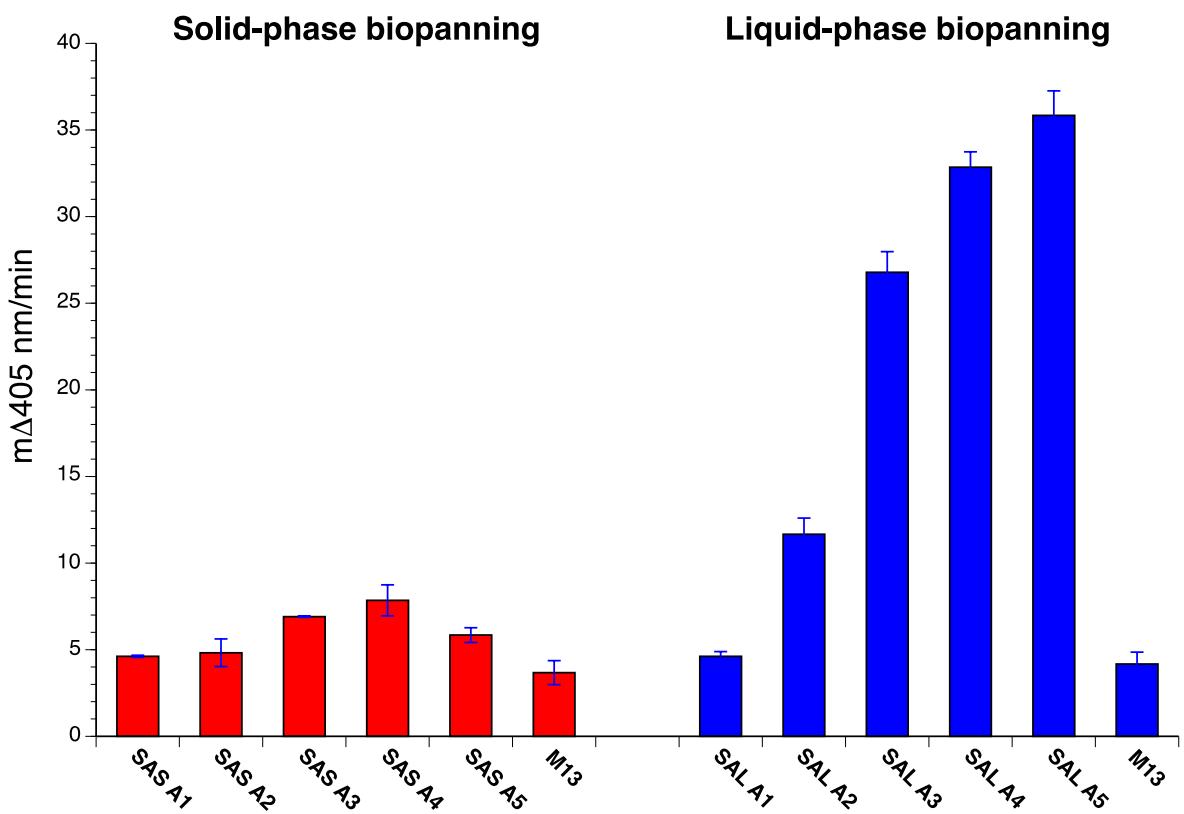
Figure 2.1. ELISA results of solid-phase biopanning versus liquid-phase biopanning. For ELISA, amplified pool of phages following each round of biopanning was used as the target substrate. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as $m\Delta OD_{405}$ which is ΔOD_{405} x 1000. SAS: *Salmonella* phage surface-phase biopanned; SAL: *Salmonella* phage liquid-phase biopanned. Shown is a representative of at least two independent experiments.

Initial characterization of S. *Typhimurium* phage probes

From the phage pool 5 (SAL A5 in Figure 2.1.), I randomly selected 24 individual clones and tested their affinity to *S.* Typhimurium, *S.* Enteritidis, *E. coli* O157:H7, and *S. sonnei* by ELISA. Five representative clones are illustrated in Figure 2.2. The other 19 clones demonstrated a similar selectivity (data not shown). Although the initial isolation of the phage probes via liquid-phase biopanning appeared to be promising as shown in Table 2.2 and in Figure 2.1, none of the 24 individual clones demonstrated a high-level of selectivity for *S.* Typhimurium without also having high cross-reactivity to *S.* Enteritidis. In addition, although the phage library against *E. coli* O157:H7 and *Shigella* species was pre-cleared, all of these 24 clones still appeared to have high affinity to both closely related bacteria.
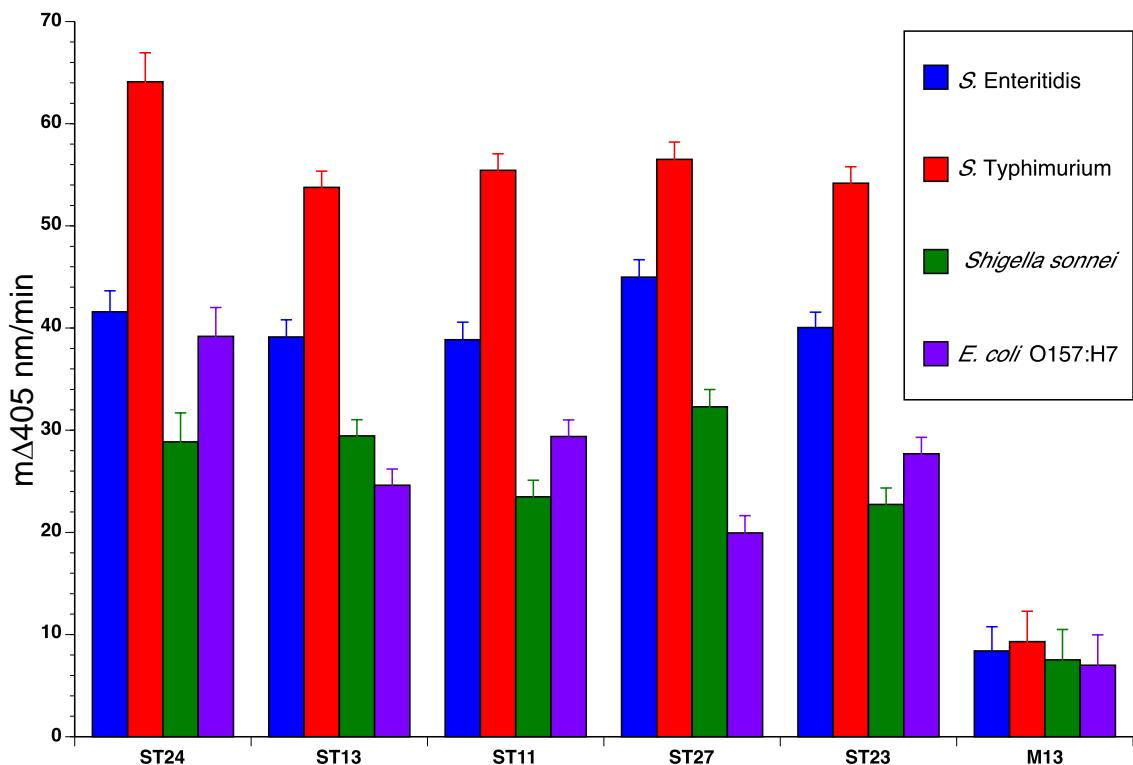
Figure 2.2. Selectivity of *S*. Typhimurium probes. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as $m\Delta OD_{405}$ which is ΔOD_{405} x 1000. Data are representative of at least two independent experiments.

Enhancing selectivity by implementing more stringent negative selections

In order to improve the chance of isolating phage probes with higher selectivity, I modified the negative selection steps were modified associated. The strategy and the data are summarized in Table 2.3. The strategy relied on repeating the negative selection against three other closely related bacteria, *E. coli*, *S. sonnei*, and *S. flexneri* to further deplete the nonspecific phage probes, as well as implementing negative selections against several other serovars of *S. enterica*. The eluted phage pools were tested for selectivity and cross-reactivity to *S.* Typhimurium, *E. coli* O157:H7, and *S. sonnei* via ELISA. As demonstrated in Figure 2.3, the modified approach resulted in successive amplification of the phage population specific for *S.* Typhimurium. The phage probes that bind *E. coli* and *S. sonnei* stayed relatively same throughout successive biopanning rounds suggesting that the modified approach may have been more successful than the previous attempt. After four rounds of biopanning, the affinity of phage pools for *S.* Typhimurium was 6-fold higher than the M13KE control (Figure 2.3).

	Pre-clearing		Selection							
			Biopanning	Depletion[#]	Biopanning	Depletion[#]	Biopanning	Depletion[#]	Biopanning	Biopanning
Target	Plastic/ BSA	*E.coli, S. sonnei, S. flexneri*	*S.* Typhimurium*	*E.coli, S. sonnei, S. flexneri*	*S.* Typhimurium*	*S.* Newport, *S.* Javiana, *S.* Enteritidis*, *E. coli* O157:H7, *S. sonnei*	*S.* Typhimurium*	*S.* Thompson, *S.* Javiana, *S.* Enteritidis*, *S.* flexneri	*S.* Typhimurium*	*S.* Typhimurium*
			Rounds of Biopanning							
			1		**2**		**3**		**4**	**5**
Input phage	8×10^{11}	6×10^{11}	1×10^{11}	6×10^{11}	1×10^{11}	1×10^{11}	1×10^{10}	1×10^{11}	2×10^{10}	7.5×10^{7}
Output phage	6×10^{11}	3.5×10^{11}	8.6×10^{5}	4×10^{11}	7.9×10^{6}	2×10^{10}	5×10^{6}	4×10^{10}	1.25×10^{8}	1.6×10^{7}

Table 2.3. Enhanced biopanning with extensive negative selection steps.
I performed the first round of liquid-phase biopanning using the phage pool that had been pre-cleared against the mixture of six strains of *S.* Typhimurium. The eluted phages from round 1 were amplified and biopanned against the mixture of *E. coli*, *S. sonnei*, and *S. flexneri* again to further deplete the phages that bind these bacteria. The phages that did not bind to *E. coli* and *Shigella* spp. were recovered and used in the second round of biopanning against *S.* Typhimurium. The eluted phages from round 2 were amplified and biopanned against the mixture of *S.* Newport, *S.* Javiana, *S.* Enteritidis, *E. coli* O157:H7, and *S. sonnei* to further deplete those phages that bind to closely related organisms including other serovars of *S. enterica*. Unbound phages were then used in the third round of biopanning against *S.* Typhimurium. The eluted phages from round 3 were then biopanned against the mixture of *S.* Thompson, *S.* Javiana, *S.* Enteritidis, and *S. flexneri*. The phages that did not bind to these bacteria were then used to biopan against *S.* Typhimurim in the fourth round. Finally, the eluted phages from round 4 were used directly without amplification in another round of biopanning against *S.* Typhimurium to enrich for specific phage probes against that bacterium. *Six strains of *S.* Typhimurium, four strains of *E. coli* O157:H7, four strains of *S. sonnei,* and three strains of *S. flexneri* were used for each bacterium. #Depletion: biopanning against other bacteria to remove cross-reacting probes.
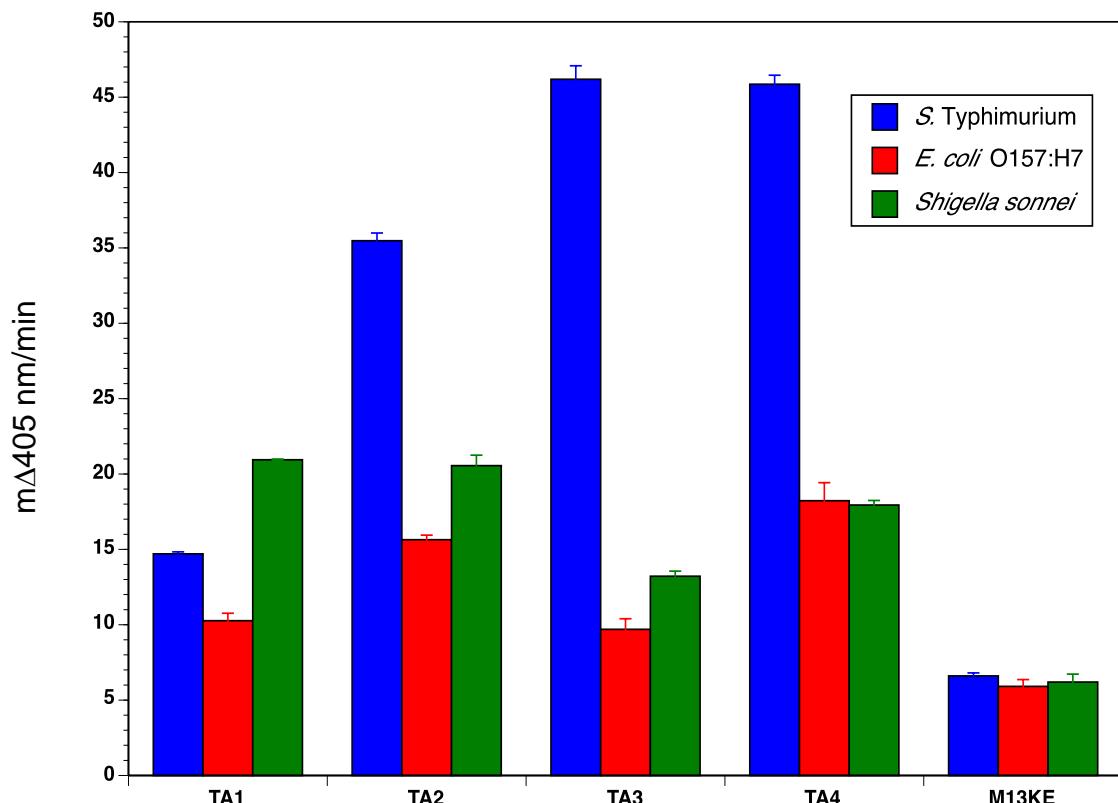
Figure 2.3. Phage pool selectivity following more stringent negative selections. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as mΔOD_{405} which is ΔOD_{405} x 1000. Shown is a representative of at least two independent experiments.

From the last phage pool (round 5), 26 individual phage probes were randomly selected, PCR amplified the segment of the phage DNA encoding the displayed oligopeptide, and determined the sequence. As shown in Table 2.3, I successfully enriched for several dominant phage clones such as TA1 (VHNTMWWGRQQP), which represented 38% of the 26 clones. The second most dominant clone was TA2 with 15%, followed by TA3 with 12% of frequency. Others were represented only once out of 26 to yield a total of nine classes from 26 clones.

In order to determine each phage clones' selectivity to *S.* Typhimurium, I performed phage capture ELISA to characterize nine phage clones. As shown in Figure 2.4, all nine phage clones bound *S.* Typhimurium at least 350% higher than did the M13KE control vector phage. The dominant phage clone TA1 bound *S.* Typhimurium almost 7-fold better than did the M13KE phage. Among the nine isolated phage clones, TA1 had the best selectivity with 220% and 300% better binding to *S.* Typhimurium than to *E. coli* and *S. sonnei*, respectively, followed by TA5. Unfortunately, all nine clones, including TA1, also showed good affinity to *E. coli* O157:H7 and *S. sonnei* suggesting that more stringent negative selections may not haven been sufficient on their own for isolation of highly selective clones.

Clone	Frequency	Amino acid sequence											
TA1	10/26	Y	H	N	T	M	W	W	G	R	Q	Q	P
TA2	4/26	N	S	F	W	A	N	W	E	H	L	W	S
TA3	3/26	H	G	A	K	W	H	N	Q	Y	N	P	K
TA4	1/26	V	D	N	N	M	W	K	E	P	Q	Q	P
TA5	1/26	L	V	T	P	K	E	T	N	S	I	A	Y
TA6	1/26	V	P	L	T	S	P	I	V	R	S	T	E
TA7	1/26	T	S	T	R	T	A	Q	M	D	F	R	V
TA8	1/26	K	C	C	Y	S	L	N	A	Q	G	H	M
TA9	1/26	H	S	I	R	S	D	G	W	V	L	P	L

Table 2.4. Amino acid sequence of the 26 phage-displayed peptides.
26 phage clones randomly selected from last round biopanning phage pool.
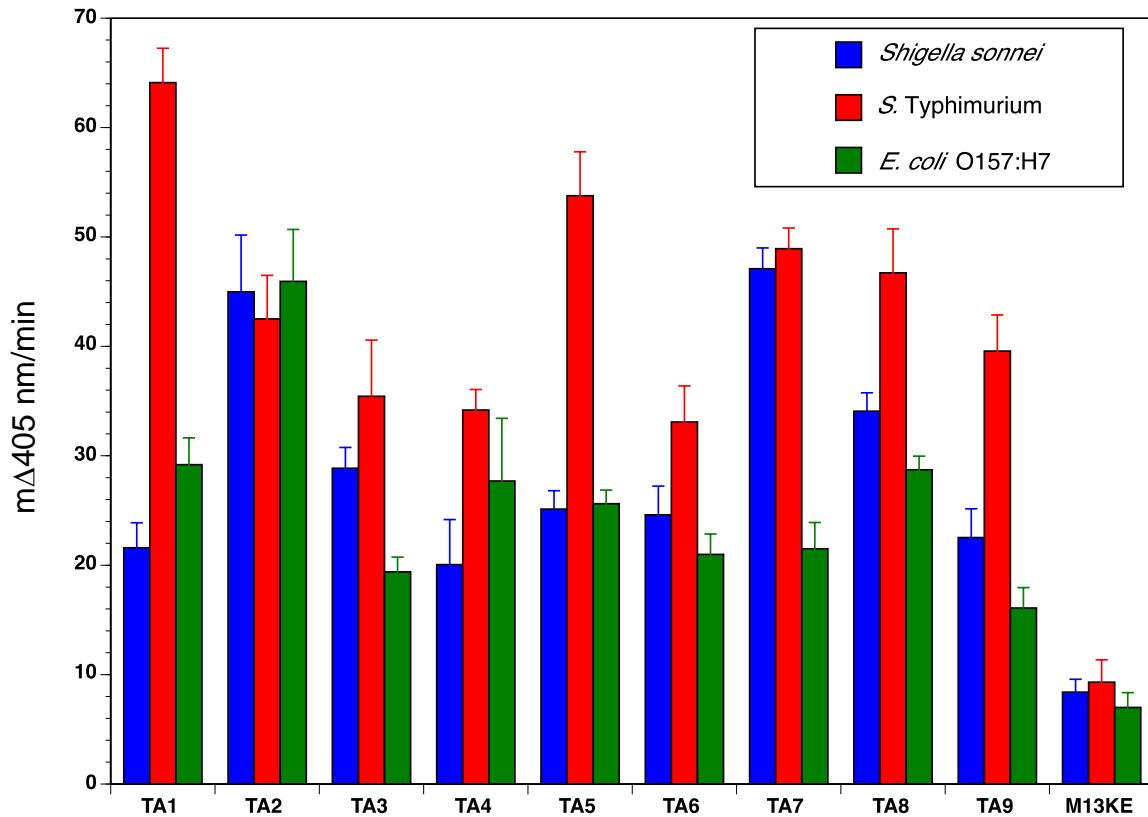
Figure 2.4. Relative selectivity of nine *S.* Typhimurium phage clones. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as $m\Delta OD_{405}$ which is ΔOD_{405} x 1000. Shown is a representative of at least two independent experiments.

Development of the liquid-phase phage capture ELISA system

In order to eliminate potential biases associated with cell or phage binding to plastic during ELISA that could affect the ability to accurately determine the affinity of these phage clones, I developed a liquid-phase ELISA system. This assay is based on the liquid-phase biopanning and the same binding steps are taken to select phages that bind to the target cells. However, in lieu of phage elution following five washing steps with PBS containing 0.02% Tween 20, the cell:phage complex is reacted with the primary antibody, washed, and reacted with the secondary antibody. The cell:phage:antibody:antibody complex is harvested by centrifugation, washed, and transferred to a EIA/RIA plate. The reaction is then developed as for the solid-phase ELISA.

Figure 2.5 shows the differences in performance of the TA2 phage probe between the liquid phase ELISA and the solid phase ELISA. These data clearly demonstrate the effectiveness of the liquid-phase ELISA system in minimizing the background caused by phage binding to plastic. In solid-phase ELISA, TA2 probe only showed a maximum of 150% difference between its affinity for *S.* Typhimurium and *E. coli* O157:H7. However, in liquid-phase ELISA, I observed a difference of up to 670% in its affinity for *S.* Typhimurium and *E. coli*. With bacterial cells at OD_{600} of 2.0 and phage concentrations of 5 x 10^{10}, 1 x 10^{11}, and 2 x 10^{11} pfu/ml, TA2 bound *S.* Typhimurium better than *E. coli* O157:H7 by 580%, 560%, and 670%, respectively. In contrast, under the same phage and bacterial cell concentrations, the solid-phase ELISA resulted in differences of 170%, 160%, and 130%, respectively, in TA2 binding to *S.* Typhimurium and *E. coli*. In addition, a linear relationship was not observed between the ELISA signal and increasing phage and bacterial cell concentrations in solid-phase ELISA. This suggested that some of the ELISA signals in solid-phase ELISA were due to non-specific

A. Liquid-phase phage capture ELISA for phage probe TA2
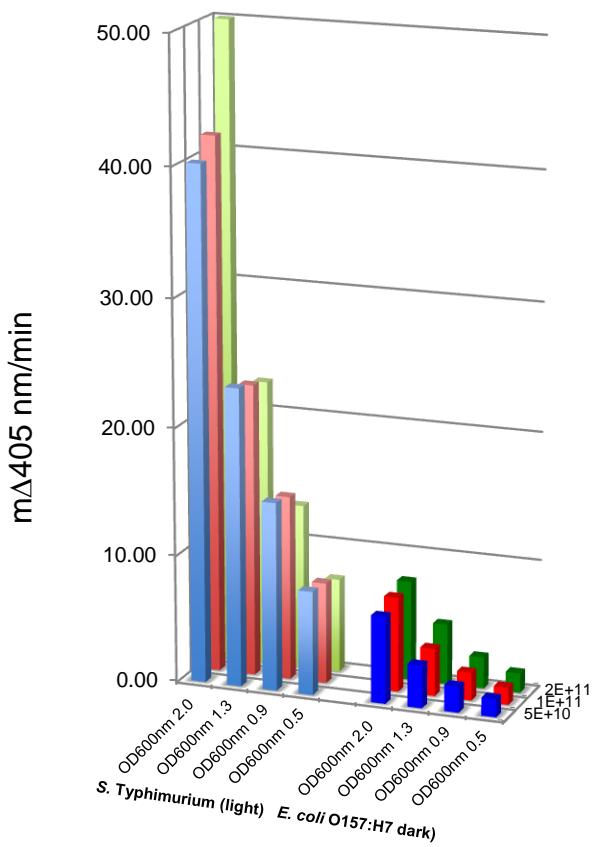
B. Solid-phase phage capture ELISA for phage probe TA2
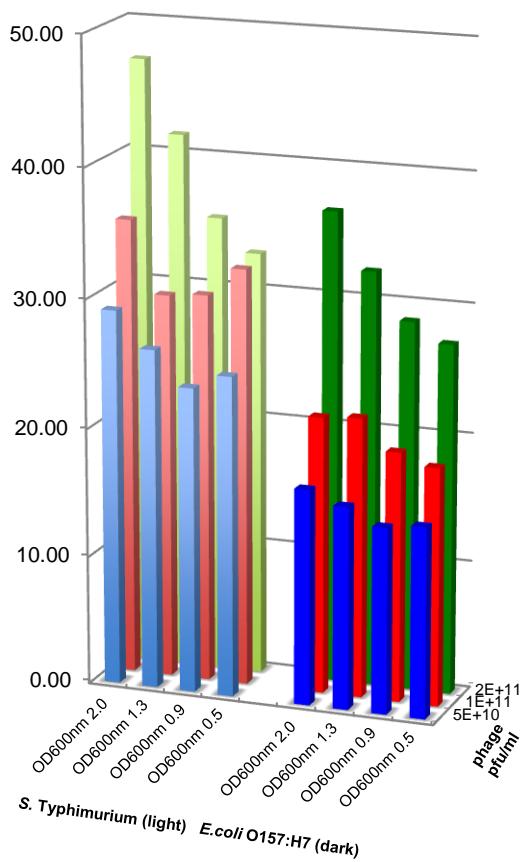
Figure 2.5. The TA2 phage different performance between the liquid phase ELISA and optimized solid phase ELISA. In both assays, three different phage concentrations (5 x 10^{10}, 1 x 10^{11}, and 2 x 10^{11}pfu/ml) and serial dilutions of bacterial culture were used. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as mΔOD$_{405}$ which is ΔOD$_{405}$ x 1000. Shown is a representative of at least two independent experiments.

binding of phages to plastic. Analysis of the displayed oligopeptide sequence of TA2 probe suggested a possible reason for relatively high plastic binding of the phage because it contained the WxxWxxxW motif. This motif has been implicated in strengthening binding of peptides to plastic (28, 29). However, as demonstrated by the results, the liquid-phase ELISA system was able to overcome this potential pitfall and clearly demonstrate the selective affinity of TA2 phage probe for *S.* Typhimurium. Thus, the results in Figure 2.5 clearly indicate that although TA2 binds to *S.* Typhimurium with much higher affinity than to *E. coli*, the data in Figure 2.4 are skewed due to the phage binding to plastic. Based on these results, it is clear that the liquid-phase ELISA minimizes background and is more accurate for determination of probe:target interaction than the solid-phase ELISA.

Analysis of S. *Typhimurium Probes*

Two *S.* Typhimurium phage clones that demonstrated the most selectivity via solid-phase ELISA, TA1 and TA5, were further analyzed via liquid-phase ELISA. In this assay, both TA1 and TA5 were tested for their selectivity for *S.* Typhimurium over nine other *S. enterica* serovars as well as *E. coli* O157:H7. As demonstrated in Figure 2.6, both TA1 and TA5 had high selectivity for *S.* Typhimurium over other *S. enterica* serovars, *S. sonnei*, and *E. coli* O157:H7 when assayed by liquid-phase ELISA. When the same two phages were tested by solid-phase ELISA (Figure 2.4), relative affinity for *S.* Typhimurium over *S. sonnei* was 290% and 230%, respectively. However, by liquid-phase ELISA, relative affinity *S.* Typhimurium over *S. sonnei* was 660% and 640%, respectively. Based on theses data, the liquid-phase ELISA is believed to be more accurate for analysis of the phage probes for their affinity and selectivity because the assay minimizes the background associated with target cells and phage probes binding nonspecifically to plastic surfaces.
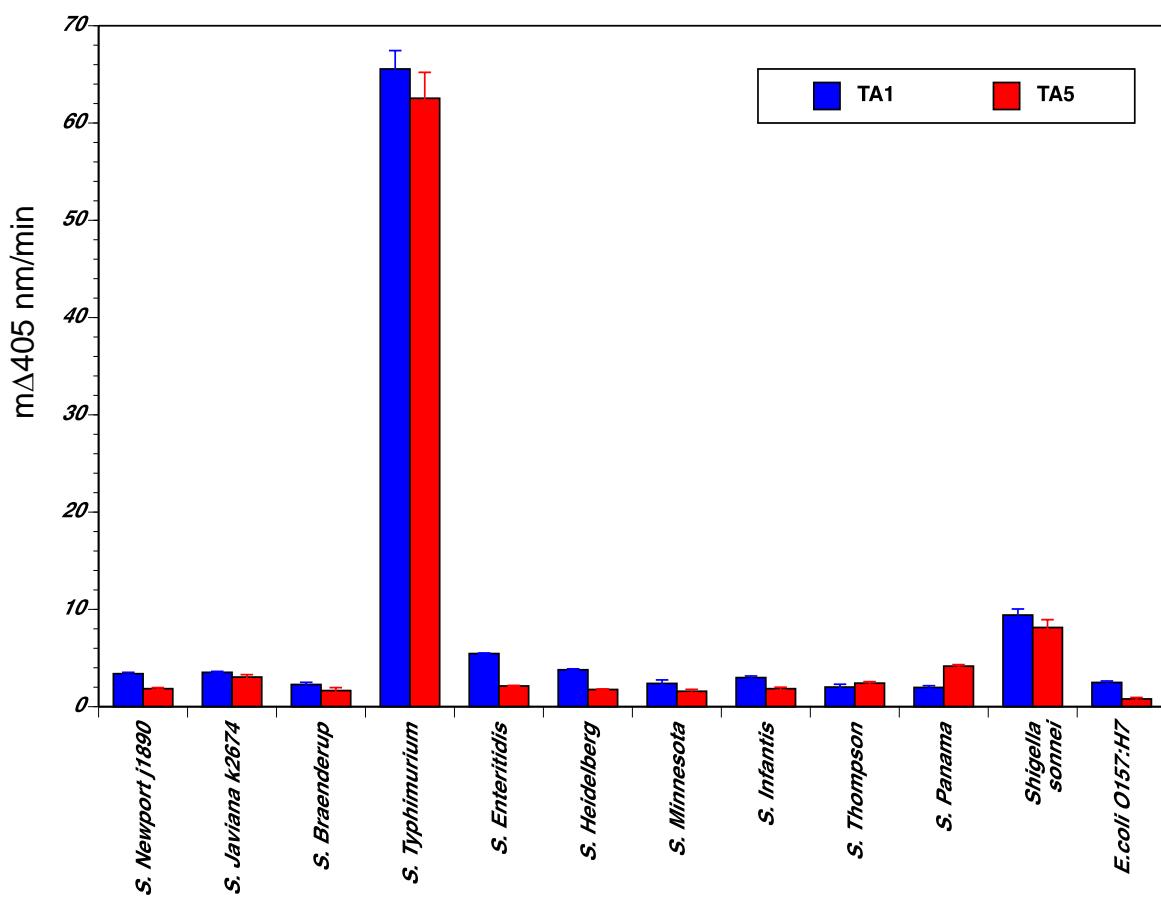
Figure 2.6. Relative selectivity of the TA1 phage and TA5 phage to 10 *Salmonella enterica serovars* **as assayed by liquid-phase ELISA.**
Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as $m\Delta OD_{405}$ which is ΔOD_{405} x 1000. Shown is a representative of at least two independent experiments.

Discussion

Phage-display has been very useful for studying various molecular interactions including peptide:protein, peptide:nucleic acid, peptide:carbohydrate, and peptide:cell. A typical approach is to isolate the desired phage probes displaying peptides of interest from a phage-display library and characterize the affinity/selectivity of the probes via ELISA. During the course of isolating phage-displayed oligopeptide probes for detection of *S.* Typhimurium, it became clear that there were several potential problems with the traditional approach. Thus, I optimized the phage-displayed probe isolation and characterization was optimized in this study.

One of the most obvious potential problems is isolating strain specific probes rather than species specific probes. In order to minimize this problem, a mixture of multiple isolates and strains as the target was used when possible. Another potential problem was isolating probes with high level of cross-reactivity against other closely related bacteria. I resolved this issue by implementing stringent negative selection steps against other closely related organisms such as various serovars of *S. enterica*. However, during the negative selection steps, another problem of live cell biopanning was realized when working with live cells. In working with multiple serovars of *S. enterica*, it was observed that not all serovars bound to the plastic with equal affinity. In fact, some serovars such as *S.* Newport and *S.* Infantis frequently washed off during the washing steps and left very few cells behind. This affected phage binding to the cells and highlighted a potential problem associated with the solid-phase biopanning. Thus, the two different biopanning approaches were compared: solid-phase versus liquid-phase. Although the traditional biopanning protocols recommend both

approaches, no one had done a direct comparison of the two methods for isolation probes against live bacterial cells has not been reported. A major advantage of the liquid-phase biopanning is that it bypasses the target cell binding to the plastic surface because phage library is incubated with a freely suspended cell culture in a microcentrifuge tube. As shown in Figure 2.1, after four rounds of liquid-phase biopanning, an enriched phage probe pool was isolated that bound to the mixture of 4 isolates of *S*. Typhimurium 600% better than did the M13 vector phage. In contrast, the isolated phage pool from solid-phase biopanning only bound *S*. Typhimurium mixture 150% better than did the M13 vector phage. A major disadvantage of the liquid-phase biopanning is that it is much more labor intensive.

An unforeseen problem was encountered when tested for the selectivity of the isolated *S*. Typhimurium specific phage probes. Despite the stringent negative selections and liquid-phase biopanning that enriched for *S*. Typhimurium specific probes (Figure 2.3), when individual phage clones were tested for selectivity, they demonstrated a high level of cross-reactivity (Figure 2.4). This unexpected result suggested that the stringent negative selections were not enough to eliminate or minimize isolation of probes with high level of cross reactivity. Another possibility was that despite the stringent blocking of the EIA/RIA plates with BSA, the phages were still binding to the plastic to give false positive ELISA results. A likely reason was suggested from the oligopeptide sequences of the phage probes. TA2 contained a motif WxxWxxxW which had been implicated in increasing binding to plastic surface (28, 29). If the phage probes were binding to the plastic, then that would explain the relatively high cross reactivity of the probes against various bacteria. Thus, in order to test this hypothesis, a liquid-phase ELISA system was developed based on the liquid-phase biopanning. In this ELISA, the target binding to the plastic surface of EIA/RIA by promoting cell:phage was eliminated as well as primary and secondary antibody captures in free suspension. The cell:phage:antibody:antibody complex was collected via centrifugation and developed the reaction in EIA/RIA plate wells. Assessing the probe:target

interaction via liquid-phase ELISA proved to be much more sensitive than the traditional solid-phase ELISA. A major disadvantage of the liquid-phase ELISA is that it is very labor intensive because each reaction has to be performed in a microcentrifuge tube and treated individually rather than in a 96-well plate. Thus, the number of samples that can be tested at a given time is limited. However, given the significant difference in the results as demonstrated by Figures 2.4 and 2.6, I believe the liquid-phase ELISA is the better approach to assess the affinity and selectivity of the phage probes against live bacterial cells.

In summary, I recognized and resolved several problems associated with the traditional biopanning approach for isolation and characterization of phage-displayed probes against bacterial cells in this study. The data suggest that to isolate highly selective phage-displayed peptide probes against a live bacterial cell, it is best to use liquid-phase biopanning and liquid-phase ELISA to prevent and minimize the bias caused by the bacterial attachment to plastic surface.

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Chapter 3

Isolation of phage-displayed oligopeptide probes for detection of multiple serovars of

pathogenic *Salmonella enterica*

Abstract

Recent outbreaks of salmonellosis by multiple serovars of *S. enterica,* including Enteritidis, Typhimurium, Newport, Braenderup, and Heidelberg, indicate the necessity to develop a detection system that is not limited to recognizing only one or two common serovars. Magnetoelastic biosensors equipped with phage-displayed oligopeptide molecular recognition probes have been demonstrated to be viable candidates for accurate, sensitive, cost-effective, user-friendly and rapid detection of pathogens. In order to develop a magnetoelastic biosensor that can detect multiple serovars of pathogenic *S. enterica,* but not closely related bacteria such as *Escherichia coli* O157:H7 and *Shigella* species. The four different biopanning were devised approaches to isolate phage-displayed oligopeptide probes. The biopannings were performed using 14 different serovars of *S. enterica* divided into five groups based on the relative frequency of outbreaks as targets. Group A was composed of six strains of *S.* Typhimurium; Group B was composed of four strains of *S.* Enteritidis; Group C was composed of *S.* Newport, *S.* Javiana, and *S.* Heidelberg; Group D was composed of *S.* Montevideo, *S.* Braenderup, *S.*

Thompson, and *S.* Minnesota; and Group E was composed of *S.* Infantis, *S.* Dublin, *S.* Mission, *S.* Derby, and *S.* Panama. In the first approach, successive serial biopannings were performed using each group of *S. enterica* as targets. In this approach, the phage-displayed probes that bound to the first group were to be collected and used to biopan against the next group until all five groups were biopanned and probes with the potential to recognize all 14 serovars isolated. Unfortunately, this approach resulted in the preferential enrichment of the wildtype phage after four rounds. In the second approach, the biopan was against a mixture of five groups mixed in equal quantity. For each round, bound phages were collected, amplified, and biopanned against the mixture for a total of five rounds of biopanning to isolate and enrich for those probes with the potential to bind to all 14 serovars. This approach resulted in preferential isolation of phage probes that were bound to *S.* Enteritidis and *S.* Typhimurium because of their high representation in the bacterial mixture. In the third and fourth approaches, I developed two different parallel biopanning schemes. In the third approach, the phage library was biopanned individually against each of the five groups of *S. enterica*. The bound phages from each biopanning were collected, amplified and combined into one pool. These phages were then biopanned against each individual pools of *S. enterica* for the second time and bound phages with the potential to recognize all 14 serovars were collected. The third approach yielded 61 potential phage probes with 24 genotypes. However, probes were not found that recognized all fourteen serovars equally. Thus, the approach was modified and further divided the five groups of *S. enterica* into a total of 12 subgroups and used them as targets for biopanning. Each of the

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twelve subgroups was biopanned with the phage library, bound phages were collected, amplified, and mixed into one phage pool. This combined phage pool was used to biopan each of the twelve subgroups of bacteria to isolate probes with the potential to bind to all 14 serovars of *S. enterica*. The fourth approach was successful and yielded two excellent phage-displayed oligopeptide probes that recognize all 14 serovars used in this study at least 200% higher than the two closely related bacteria *E. coli* O157:H7 and *S. sonnei*.

Introduction

Salmonella enterica is a pathogenic bacterium that poses a serious public health concern as foodborne and zoonotic pathogens(1-6). This fact was emphasized by several recent outbreaks of *S. enterica* in the United States(7-20). From eggs to poultry, peanut butter to fresh produce such as tomatoes and cucumbers, and hedgehogs to small turtles, pathogenic *S. enterica* is transmitted to humans in various ways to cause disease, predominantly gastroenteritis. One of the difficulties of combating salmonellosis is that although the causative agent, *S. enterica*, is only one of two species of the genus *Salmonella*, there are multiple subspecies and serovars of *S. enterica* that are pathogenic to humans (21, 22). Thus, it is imperative to develop rapid, accurate, and cost effective monitoring methods that can detect various pathogenic *S. enterica* serovars. This detection mechanism could be used as an accurate initial screen to ascertain the presence or absence of pathogenic *Salmonella* so that appropriate therapeutic and preventive measures can be implemented to minimize the spread of pathogens.

One of the most important first steps in implementing effective control measures against outbreaks is pathogen monitoring. Although there are multiple systems to accurately detect the presence of *Salmonella* from contaminated foods and environments, most of the rapid technologies are geared towards identifying a specific serovar of *S. enterica* (as described in Chapter 2). Several technologies that have the capability to detect various serovars of *S. enterica* include qPCR using specific oligonucleotide primers for each serovar

(23-30), sequence analysis of 16S rDNA (31, 32), and ELISAs (33-35). Unfortunately, a qPCR instrument is still quite expensive and all of the technologies listed above require extensive training of personnel. Another technology that can detect multiple serovars of *S. enterica* is the Rainbow Agar (Biolog, Hayward, CA). However, this traditional culturing requires at least 24 hours of growth and a relatively large quantity of the pathogen to obtain results. Thus, it is standard to spend the initial 24-48 hours to enrich the pathogen before plating them on the selective medium for additional 24 hours. Furthermore, Rainbow has a limited shelf life of only 12 weeks at 4°C. Based on these limitations, it is imperative to develop a rapid, cost effective, and easy to use method to detect *Salmonella*.

An ideal detection technology should be fast (minutes rather than hours), accurate, inexpensive, have a long shelf life, be easy to use without extensive training of personnel, and do not require elaborate equipment. Magnetoelastic (ME) biosensors using phage-displayed oligopeptides as probes fill this role because they are fast, cost effective, user-friendly and require minimal sample manipulation (36-51). For magnetoelastic biosensors to function properly, it is essential for them to be coupled with molecular probes that are highly specific for the target of interest. To this end, I sought to develop a phage-displayed probe based magnetoelastic biosensor that could detect multiple *S. enterica* serovars.

Phage display technology offers an alternative approach to antibodies and has been used extensively for cell surface molecular recognitions (52-56). Unlike the antibody probes, phage-displayed peptide probes are highly resistant to environmental stressors including high temperature and long-term storage (57). In addition, they are easy to prepare in large quantities and are cost-effective because the only costs associated with preparing phages are growth

medium, incubator, and a centrifuge. Several phage-displayed peptide probes have been isolated for detecting pathogens including *Bacillus anthracis* spores (58, 59), *Staphylococcus aureus* (60), and *Salmonella enterica* serovar Typhimurium (54, 56) (also see Chapter 2). In order to isolate phage-displayed oligopeptide probes for detection of pathogenic non-typhoid serovars of *S. enterica*, I focused on 14 serovars that have recently caused outbreaks: *S.* Enteritidis, *S.* Typhimurium, *S.* Newport, *S.* Javiana, *S.* Heidelburg, *S.* Montevideo, *S.* Minnesota. *S.* Branderup, *S.* Thompson, *S.* Infantis, *S.* Dublin, *S,* Mission, *S.* Derby, and *S.* Panama (21).

Materials and Methods

Bacteria, media, and chemicals

All bacteria used in this study are listed in Table 3.1.   *S.* Typhimurium (ATCC 13311), *S.* Typhimurium (BAA-712), *S.* Typhimurium (BAA-215), *S.* Typhimurium (ATCC 15277=LT2), *S.* Typhimurium (ATCC 51812), *S.* Typhimurium 14a, *S.* Enteritidis (BAA-708), *S.* Enteritidis (ATCC 4931), *S.* Enteritidis (ATCC49214), *S.* Enteritidis (BAA-1045), *S.* Newport j1890, *S.* Javiana k2674, *S.* Braenderup k2679*, S.* Heidelberg*, S.* Minnesota, *S.* infantis, *S.* Thompson, and *S.* Panama were grown in nutrient broth (NB), confirmed for identity, propagated, and maintained on MacConkey Agar (ACUmedia, Lansing, MI). *E. coli* ER2738, *E. coli* O157:H7 (ATCC43888), *E. coli* O157:H7 (ATCC43894), *E. coli* O157:H7 (ATCC43895), *E. coli* O157:H7 (BAA-460), *Shigella sonnei* (ATCC 9290), *S. sonnei* (ATCC 29031), *S. sonnei* (ATCC 29030), and *S. sonnei* (ATCC 29930) were grown in Lysogeny broth (61) at 37°C with aeration at 220 rpm unless otherwise indicated. When necessary, the medium

was solidified with 1.5% Difco Bacto Agar (BD, Franklin Lakes, NJ).. Antibiotics and

isopropyl-β-D-thiogalactoside (IPTG) were purchased from Sigma-Aldrich and

5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was purchased from Gold

Biotechnology (St. Louis, MO). Oligonucleotide primers were purchased from Integrated

DNA Technologies (Coralville, IA). The following chemical concentrations were used in this

study (per milliliter): tetracycline, 20 µg; X-gal, 50 µg/ml; and IPTG, 40 µg/ml.

All bacteria were grown in an appropriate growth medium at 37°C overnight in a

New Brunswick (Enfield, CT) shaking water bath incubator. Ten ml of bacterial cells were

collected by centrifugation at 5000 rpm for 10 min, washed twice with 10 ml of sterile

phosphate-buffered saline (PBS, pH 7.0), and adjusted to OD_{600} nm of 1.0 with PBS.

Phage-display library and pre-clearing of the library to eliminate plastic and BSA binding

phages

M13 GpIII phage displayed peptide library (Ph.D.[TM]-12) and M13 vector phage were

purchased from New England Biolabs (Beverly, MA, USA). Phages were propagated in *E.

coli* ER2738 and the titer was determined according to the manufacturer's recommendations

(62).

Prior to biopanning, the phage library was pre-cleared of its nonspecific population that

bound to plastic and the blocking agent, Bovine Serum Albumin (Sigma-Aldrich, St. Louis,

MO). Briefly, 80 µl of the Ph.D.™ Phage Display Library (1 x 10^{13} pfu/ml) from New England

Biolabs was diluted in 1 ml PBST (PBS supplemented with 0.1% Tween 20), added to a 35 mm

sterile petri plate and shaken for 30 min at room temperature at 20 rpm/min to induce the

binding of the phage probes to the plastic. Unbound phages were then transported into a fresh petri plate to further eliminate plastic binding phages. This procedure was repeated for a total of three times. In order to remove the phages that bound to BSA, 35 mm petri plates were first coated with 1 ml of 5 mg/ml BSA solution overnight at 4°C. The phage lysate that had been pre-cleared of plastic binding virions were then added to the BSA coated petri plate and incubated for 30 minutes at 4°C with gentle shaking at 20 rpm/min to promote phage binding to the BSA coated plate. The unbound phages were transferred to a fresh BSA coated plate to further remove BSA binding virions for a total of three times. The collected unbound phage pool represents the pre-cleared library.

Depletion of phages binding to Escherichia coli *O157:H7 and Shigella spp.*

The pre-cleared phage library was further depleted of oligopeptides that bind to the closely related bacteria *E. coli* and *Shigella* species before being biopanned for *S.* Typhimurium binding probes. Briefly, *E. coli* O157:H7 (4 strains), *S. sonnei* (4 strains)*,* and *S. flexneri* (3 strains) were grown overnight in LB, washed twice with PBS, and diluted to OD_{600} of 0.5 in PBS. For each bacterium, equal volumes of different strains were combined to generate a mixture of that organism used for the subsequent experiments. In a 2 ml microcentrifuge tube, 500 μl of *E. coli* was centrifuged, resuspended in 1 ml of the pre-cleared phage library, and then incubated for 30 minutes at room temperature with gentle shaking at 50 rpm/min on a rotator. Following incubation, cells and phages bound to cells were collected by centrifugation at 5000 rpm for 5 minutes. Unbound phages were transferred to a new

microcentrifuge tube containing 500 μl of *S. sonnei* suspension and the procedure was repeated. Finally, phages were incubated with *S. flexneri* cells and unbound phages were collected by a final centrifugation at 8000 rpm for 10 minutes. This phage pool constituted thephage library for biopanning against *Salmonellae*.

Biopanning

All biopanning steps were performed via liquid-phase biopanning described in Chapter 2. Briefly, 2 ml microcentrifuge tubes that had been coated with 2 ml of 5 mg/ml BSA overnight at 4°C were used as the vessel to capture *Salmonella* specific phage probes. All serovars of *Salmonella* were grown overnight in NB at 37°C with 220 rpm of shaking, equal volume of each strain/serovar of a group was mixed together to generate the five groups (Table 3.2), and 1 ml of the mixed culture (10^9 CFU) representing a group was added to a 2 ml microcentrifuge tube that had been coated with BSA and gently centrifuged at 5000 rpm for 3 min to harvest cells. The pelleted cells were resuspended in 1 ml of pre-cleared phage library (1 x 10^{11} pfu/ml) and incubated at room temperature for 40 min with gentle rotation to promote cell:phage binding. The cell:phage complex was collected by centrifugation at 5000 rpm for 3 min, unbound phages were then removed, and the complex was resuspended in 1 ml PBS containing 0.02% Tween 20 to wash. The washing step was repeated for a total of five times. Phages that remained bound to *Salmonella* cells were eluted with 0.8 ml of 0.2 M Glycine elution buffer (pH 2.2) and vigorous tapping on the tube for 10 min at room temperature followed by centrifugation at 7500 rpm for 3 min to remove cells. The phage solution was

neutralized with 150 µl of 1 M Tris-HCl (pH 9.0) and the eluted phages were titered and amplified in *E. coli* ER2738 according to the NEB's instructions.

I took four different biopanning approaches to isolate phage-displayed oligopeptide probes that could recognize multiple serovars of *Salmonella*. As the first step, I separated 14 non-typhoid *S. enterica* serovars into five groups (Table 3.2) based on the frequency of outbreaks caused by each organism between the years 2009 to 2012 (2) (21).

In the first biopanning approach, each group of *Salmonellae* was biopanned in successive order to isolate only those phage probes that bound to all five groups. Briefly, the pre-cleared phage library was incubated with Group A and bound phages were eluted and amplified. The amplified phages were then incubated with Group B and the elution and amplification were repeated. This biopanning and amplification steps were repeated until Group D cells. Biopanning against Group E was not performed for reasons described in the Results. In the second approach, all 5 groups of *Salmonellae* were combined and used as the target in six successive rounds of biopanning. Following each round of biopanning, the eluted phages were amplified in *E. coli* ER2738. In the third approach, each group of *Salmonellae* was biopanned independently with the phage library, the eluted phages were amplified and combined into one pool, and biopanning was repeated against each individual *Salmonella* groups. The fourth biopanning approach was focused on optimizing the third approach. The major improvement was subdividing the 14 *S. enterica* serovars into 12 subgroups which were then individually biopanned with the pre-cleared phage library for a total of three rounds. The 12 subgroups in each round of biopanning were selected from the initial five groups as follows: 2 strains from Group A, 2 strains from Group B, 3 serovars from Group C, 3 serovars from

group D, and 2 serovars from Group E. The exact composition of each subgroup per round of biopanning is shown in Figure 3.7. For each round, eluted phages were amplified individually then mixed with the other eluted phages in equal concentration before proceeding to do the next round biopanning.

ELISA

The liquid-phase ELISA is based on the liquid-phase biopanning scheme and the steps of liquid-phase biopanning were followed until the phage elution step. Each ELISA was performed with 10^{11} PFU of phage and 10^8 of bacterial cells. Following the final wash, in lieu of eluting the phage, the cell:phage pellet was resuspended in 250 μl of 1:3000 diluted anti-Fd phage IgG rabbit antibody and incubated at room temperature for 30 min with gentle shaking on a rotator. The cell:phage complex was pelleted by centrifugation at 6000 rpm for 3 min and washed twice with 1 ml of PBST. The complex was then resuspended in 250 μl of 1:5000 diluted anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase and incubated for 30 min at room temperature. The cell:phage:antibody:antibody complex was harvested by centrifugation at 6000 rpm for 3 min, washed twice with 1 ml of PBST, and resuspended in 180 μl of fresh 0.1 M glycine buffer supplemented with 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ (pH 10.2). 50 μl of the resuspended complex was transferred to the wells of a EIA/RIA plate and the reaction was developed with 50 μl of 4mg/ml *p*-NPP (*p*-nitrophenylphosphate) and the kinetic signals were measured on BioTek Synergy HT microtiter plate reader (BioTek, Winooski, VT, USA) with a reaction time of 60 min at 405 nm. The alkaline phosphatase activity was calculated as the linear slope of the reaction. All assays

were performed in triplicate unless otherwise indicated.

Polymerase chain reaction and DNA sequencing

A region of the pIII gene carrying the cloned oligonucleotides was sequenced to determine the identity of the displayed peptides from the isolated phage probes. Briefly, phage DNA for sequencing was acquired via polymerase chain reaction from either the lysogen carrying the phage in replicative form or from the purified phage genomic DNA. The phage DNA was purified from individual phage clones as previously described (58). The PCR was performed with the following primers, -115 M13 pIII (5'- CCA CAG ACA GCC CTC ATA GTTA -3') and +384bp for M13 pIII (5'- TTC TTT TGC CTC TTT CGT TTTA -3'), with the Phusion® DNA Polymerase (NEB, Beverly, MA, USA) on an Eppendorf MasterCycler gradient (Enfield, CT). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced from the 3'-end of the pIII gene with the primer (5'- CCA CAG ACA GCC CTC ATA GTTA -3') at the Auburn University DNA Sequencing Facility. DNA sequences were analyzed with Geneious (www.geneious.com) and Lasergene software suite (DNA Star, Madison, WI).

Strains	Genotype and relevant characteristics	Source
ER2738	F′ *proA*$^+$*B*$^+$ *lacI*^{q} □(*lacZ*)M15 *zzf*::Tn*10* (Tet^R)/*fhuA2 glnV*	New England Biolabs

	thi Δ(*lac-proAB*) *Δ*(*hsdMS-mcrB*)5 (rk⁻ mk⁻ McrBC⁻)	
SS2205	*Escherichia coli* O157:H7 (ATCC 43888)	ATCC
SS2206	*Escherichia coli* O157:H7 (ATCC 43894)	ATCC
SS2207	*Escherichia coli* O157:H7 (ATCC 43895)	ATCC
SS2208	*Escherichia coli* O157:H7 (BAA-460)	ATCC
SS2210	*Salmonella enterica* subsp. *enterica* sv Typhimurium (BAA-215)	ATCC
SS2211	*Salmonella enterica* subsp. *enterica* sv Typhimurium (BAA-712)	ATCC
SS2212	*Salmonella enterica* subsp. *enterica* sv Typhimurium (ATCC 15277=LT2)	J. Roth via J. Escalante-Semerena
SS2213	*Salmonella enterica* subsp. *enterica* sv Typhimurium (ATCC 51812)	ATCC
SS2235	*Salmonella enterica* subsp. *enterica* sv Typhimurium 14a (Environmental Isolate)	CDC (D. Schaffner)
SS2275	*Salmonella enterica* subsp. *enterica* sv Typhimurium (ATCC 13311)	ATCC
SS2209	*Salmonella enterica subsp. enterica sv* Enteritidis (ATCC 4931)	ATCC
SS2276	*Salmonella enterica subsp. enterica sv* Enteritidis (ATCC 49214)	ATCC
SS2277	*Salmonella enterica subsp. enterica sv* Enteritidis (BAA-708)	ATCC
SS2278	*Salmonella enterica subsp. enterica sv* Enteritidis (BAA-1045)	ATCC
SS2181	*Salmonella enterica* subsp. *enterica* sv Montevideo	ATCC
SS2183	*Salmonella enterica* subsp. *enterica* sv Heidelberg	ATCC (J. Barbaree)
SS2190	*Salmonella enterica* subsp. *enterica* sv Infantis	ATCC (J. Barbaree)
SS2191	*Salmonella enterica* subsp. *enterica* sv Minnesota	ATCC (J. Barbaree)
SS2193	*Salmonella enterica* subsp. enterica sv Thompson	ATCC (J. Barbaree)
SS2194	*Salmonella enterica* subsp. enterica sv Dublin	ATCC (J. Barbaree)
SS2195	*Salmonella enterica* subsp. enterica sv Mission	ATCC (J. Barbaree)
SS2196	*Salmonella enterica* subsp. enterica sv Derby	ATCC (J. Barbaree)

SS2197	*Salmonella enterica* subsp. enterica sv Panama	ATCC (J. Barbaree)
SS2236	*Salmonella enterica* subsp. *enterica* sv Newport J1890	CDC (D. Schaffner)
SS2237	*Salmonella enterica* subsp. *enterica* sv Javiana K2674	CDC (D. Schaffner)
SS2238	*Salmonella enterica* subsp. *enterica* sv Braenderup K2679	CDC (D. Schaffner)
SS2218	*Shigella sonnei* (ATCC 9290)	ATCC
SS2219	*Shigella sonnei* (ATCC 29031)	ATCC
SS2220	*Shigella sonnei* (ATCC 29030)	ATCC
SS2221	*Shigella sonnei* (ATCC 29930)	ATCC
SS2215	*Shigella flexneri* (ATCC 12023)	ATCC
SS2216	*Shigella flexneri* (ATCC 25929)	ATCC
SS2217	*Shigella flexneri* (ATCC 49070)	ATCC

Table 3.1. Bacterial Strains. SS strain designations are for the Suh laboratory use only. ATCC: American Type Culture Collection; CDC: Centers for Disease Control and Prevention.

Group A	Group B	Group C	Group D	Group E
6 strains of *S.* Typhimurium	4 strains of *S.* Enteritidis	*S.* Newport *S.* Javiana *S.* Heidelberg	*S.* Montevideo *S.* Braenderup *S.* Thompson *S.* Minnesota	*S.* Infantis *S.* Dublin *S.* Mission *S.* Derby *S.* Panama

Table 3.2. Division of 14 non-typhoid *Salmonella enterica* into five biopanning groups. *S. enterica* serovars were divided into five separate groups based on the frequency of outbreaks caused by each organism between the years 2009 to 2012 (2, 21).

A.   B.

| Ph.D.-12 Phage Display Peptide library | | Ph.D.-12 Phage Display Peptide library |

| Group A | 1st round | | 5 Groups | 1st round |
Group B	2nd round		5 Groups	2nd round
Group C	3rd round		5 Groups	3rd round
Group D	4th round		5 Groups	4th round
Group E	5th round		5 Groups	5th round
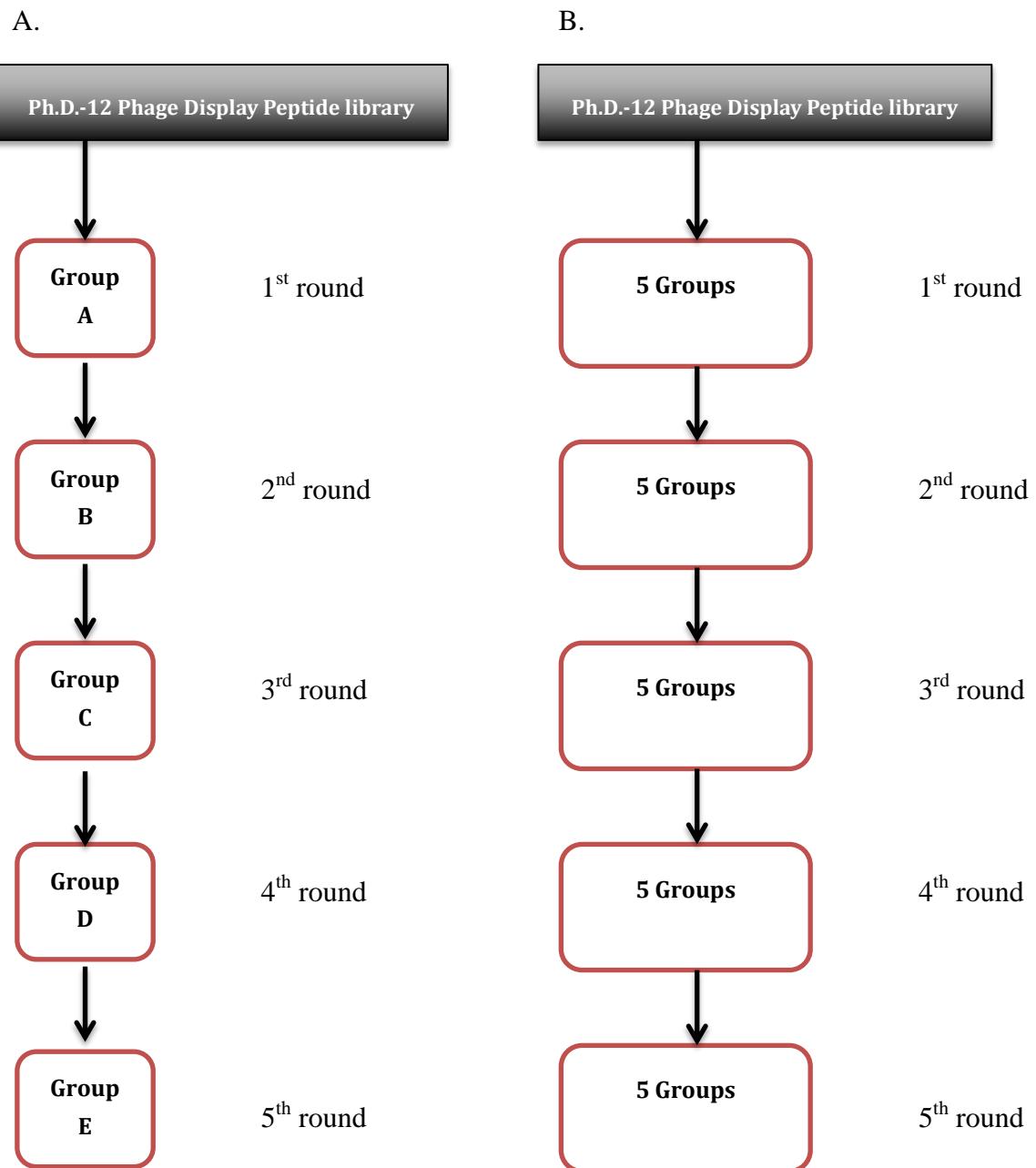
Figure 3.1. Two different serial biopanning approaches. Eluted phages were amplified between each round to increase the concentration of phages used for the subsequent rounds of biopanning.

Results

Serial biopanning approaches

In order to isolate phage-diplayed oligopeptide probes that can recognize multiple serovars of pathogenic *S. enterica*, fourteen serovars were used as targets in biopanning. These fourteen serovars of *S. enterica* were divided into five groups, as shown in Table 3.2, based on the relative frequency of outbreaks. Four different biopanning approaches were devised to isolate phage-displayed probes. The first two approaches were based on serial biopanning and the last two approaches were based on parallel biopanning. The serial biopanning approaches are illustrated in Figure 3.1. In the first approach (Figure 3.1(A)), the phage library was first biopanned against Group A (*S.* Typhimurium). The phages that bound to the six strains of *S.* Typhimurium were collected and were used to biopan against the four strains of *S.* Enteritidis of Group B. The phages that bound to *S.* Enteritidis were used to biopan against Group C. The results from these successive serial biopannings are shown in Table 3.3. Between each round of biopanning, the eluted phages were amplified in *E. coli* ER2738. Although the data on Table 3.3 suggest enrichment of *S. enterica* binding phages in successive biopannings after the second round, there was higher enrichment of the wildtype phage (M13KE) than the recombinant phage probes. After the third round, more than 10% of the amplified phages were wildtype and this population increased to 67% after the fourth round of biopanning with Group D and amplification. Based on these results, it was clear that this first approach was unsuccessful for isolating phage probes against multiple serovars of *S.*

enterica and therefore the procedure was abandoned.

In the second serial biopanning approach, all five groups of *S. enterica* were combined into one group and used as the target, as demonstrated in Figure 3.1B. The results of the biopanning are shown in Table 3.4. Although, enrichment of *S. enterica* binding phages was observed after the second round, this phage population did not enrich further in rounds three through six. However, after six rounds of biopanning, affinity of the phage pool for combined *S. enterica* serovars was 7-fold higher than the M13 control (Figure 3.2). Following the last round of biopanning, 144 individual clones were randomly selected from the eluted phage population and independently propagated. From the ELISA analysis of 144 individual phage clones, 49 phage probes were found to bind to *S. enterica* at least 3-fold better than the M13 phage (Figure 3.3). Five phage probes with the highest affinity (50, 131, 111, 123, 124) were selected for further analysis via ELISA to test the relative selectivity of phage probes for different serovars of *S. enterica* in comparison with binding to *S. sonnei*, *S. flexneri*, and *E. coli* O157:H7. Unfortunately, as demonstrated in Figure 3.4, all five of the phages demonstrated a significant bias in affinity toward *S.* Typhimurium and *S.* Enteritidis. I believe this may have been due to the fact that these two serovars were represented multiple times in biopanning. The relatively low cross-reactivity to *E. coli* O157:H7 and *S. sonnei* demonstrated that the negative-selection procedure was highly successful. However, given the data shown in Figure 3.4, there was a lack of confidence that probes were isolated with high affinity to all 14 serovars of *S. enterica* used in the study.

Target	Pre-clearing		Selection				
	Plastic/BSA	E. coli, S. sonnei, S. flexneri	Group A	Group B	Group C	Group D	Group E
			Rounds of Biopanning (The successive biopanning approach)				
			1	2	3	4	5[#]
Input Phage	8.0×10^{11}	6.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	N.D.
Output Phage	6.0×10^{11}	3.5×10^{11}	7.0×10^{5}	7.0×10^{5}	2.0×10^{6}	1.8×10^{8}	N.D.

Table 3.3. Serial biopanning approach #1. Phages were titered and amplified following each round of biopanning. [#]Group E was not biopanned because of the high percentage of wildtype phage in the recovered phage pool after the Group D biopanning and amplification.

Target	Pre-clearing		Selection						
	Plastic/BSA	*E. coli,* *S. sonnei,* *S. flexneri*	Combined pool of five groups of *S. enterica* representing 14 serovars						
			Biopanning						
			1	2	3	4	5	6	
Input Phage	8.0×10^{11}	6.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}	1.0×10^{11}
Output Phage	6.0×10^{11}	3.5×10^{11}	8.6×10^{5}	7.9×10^{6}	1.0×10^{8}	2.4×10^{8}	2.0×10^{8}	2.1×10^{8}

Table 3.4. Serial biopanning approach #2. Phages were titered and amplified following each round of biopanning.
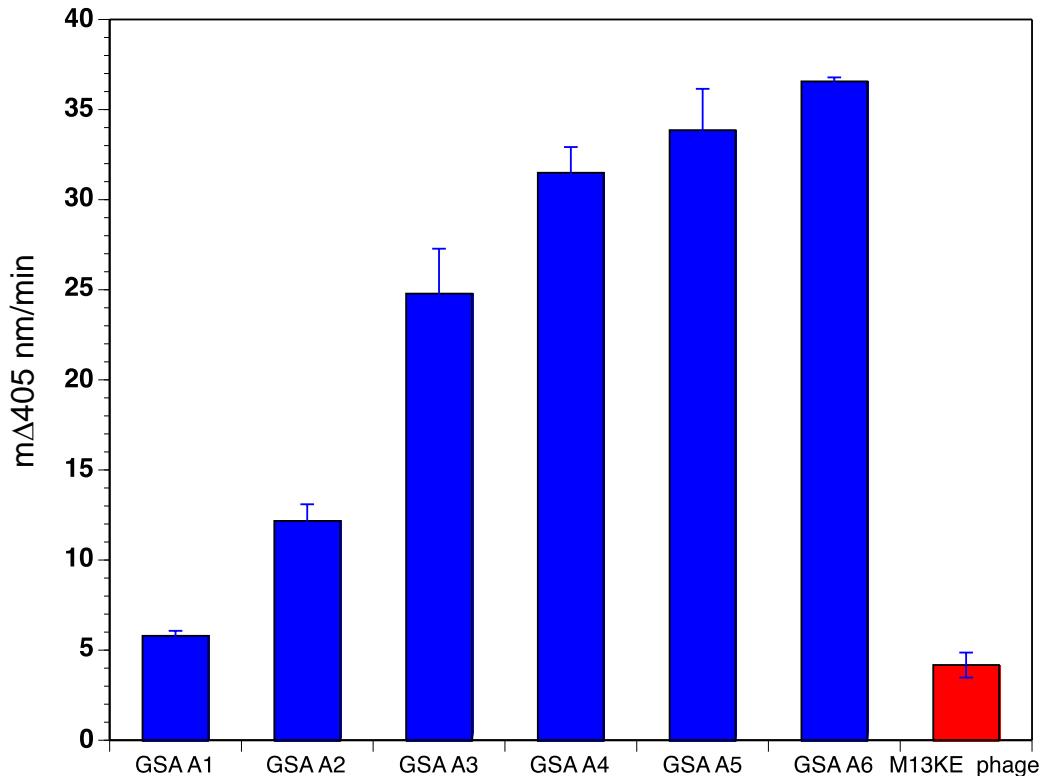
Figure 3.2. Enrichment of phage probes for _S. enterica_. For ELISA, amplified pool of phages following each round of biopanning was used to bind the bacterial pool containing all 14 serovars of _S. enterica_. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as $m\Delta OD_{405}$ which is ΔOD_{405} x 1000. GSA A1: first round; GSA A2: second round; GSA A3: third round; GSA A4: fourth round; GSA A5: fifth round; and GSA A6: sixth round of biopanning as shown in Table 3.4. The data shown are representative of at least two independent experiments with triplicate samples.
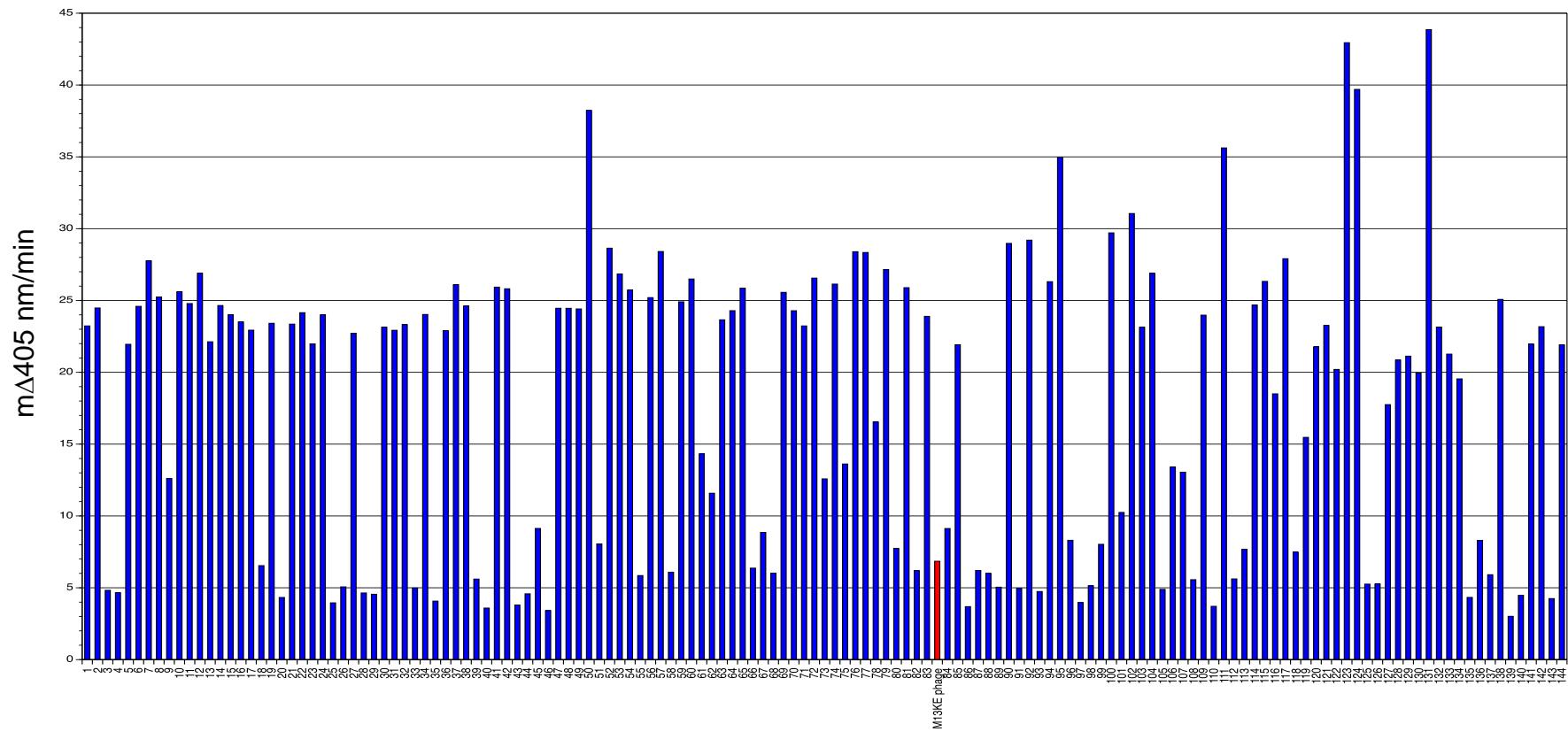
Figure 3.3. Relative affinity of 144 potential phage-displayed oligopeptide probes to the mixture of 14 serovars of *S. enterica*. For ELISA, each phage probe was grown independently and used to bind the bacterial pool containing all 14 serovars of *S. enterica*. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as mΔOD₄₀₅ which is ΔOD₄₀₅ x 1000.   The data shown are representative of at least two independent experiments with duplicate
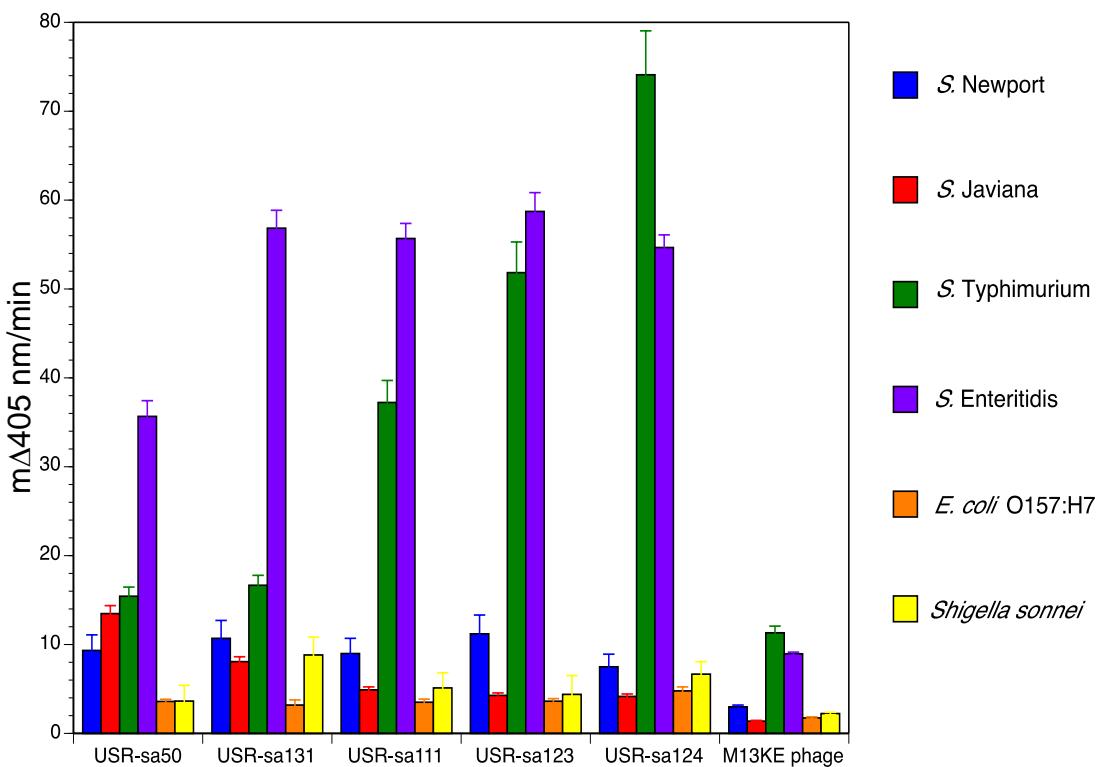
Figure 3.4. Relative selectivity of individual phage probes. Five putative Universal *Salmonellae* Recognition (USR) phage probes with the highest affinity to the mixture of 14 *S. enterica* mix were tested for their affinity to 4 individual *S. enterica* serovars, *E. coli* O157:H7, and *S. sonnei* via ELISA. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as $m\Delta OD_{405}$ which is ΔOD_{405} x 1000. The data shown are representative of at least two independent experiments with triplicate samples.

In order to enhance isolation of phage probes that can recognize multiple serovars of *S. enterica*, parallel biopanning approaches were developed. In the first parallel biopanning approach, I independently biopanned each of the five groups of *S. enterica* serovars listed in Table 2, amplified each of the eluted phage pools and combined them to a single phage library, and repeated biopanning against each *S. enterica* group. A total of three rounds of parallel biopannings were performed. A schematic of this approach is illustrated in Figure 3.5. In order to ensure each strain/serovar was represented equally in each group, all *Salmonellae* were grown to equal concentration (based on OD_{600} of the culture) in NB and mixed in equal volume for biopanning as described in Materials and Methods. After three rounds of parallel biopanning, the phage pool affinity to twelve serovars of *S. enterica* increased 3-5 fold compared to *E. coli* O157:H7 as shown in Figure 3.6.

I isolated 61 individual phages from the pool and determined sequences of the displayed oligopeptides. Using the neighbor-joining approach, I divided the predicted amino acid sequences into 3 clusters as shown in Figure 3.7. Cluster 1 had 17 probes with the sequence NSFWANWEHLWS, 14 probes with the sequence VHNTMWWGRQQP, and three sequences presented only once. Cluster 2 had a total of 14 sequences of with one sequence that appeared five times (HSIRHDFYYVDS), two sequences appeared three times (HSIRLDVITMRE and HGAKWHNQYNPK), and 11 sequences that appeared only once. Cluster 3 had five probes that appeared only once. The 24 groups of the predicted amino acid sequences of the displayed oligopeptides are shown in Table 3.5. From this analysis, I isolated several phage probes that appeared promising for detection of multiple serovars of pathogenic *S. enterica*. Of the potential phage probes, two GSP (Group *Salmonella* Parallel) phages, GSP-SA36 and GSP-A8, that did not belong to a high frequency peptide group demonstrated the best activities against the *S. enterica* groups (data not shown). Relative selectivity of

GSP-SA36 and GSP-A8 phage probes against 10 *S. enterica* serovars, *E. coli* O157:H7, and *S. sonnei* are shown in Figure 3.8. Although both GSP-SA8 and GSP-SA36 bound to many serovars of *S. enterica*, neither phage bound to *S.* Thompson or *S.* Panama very well.
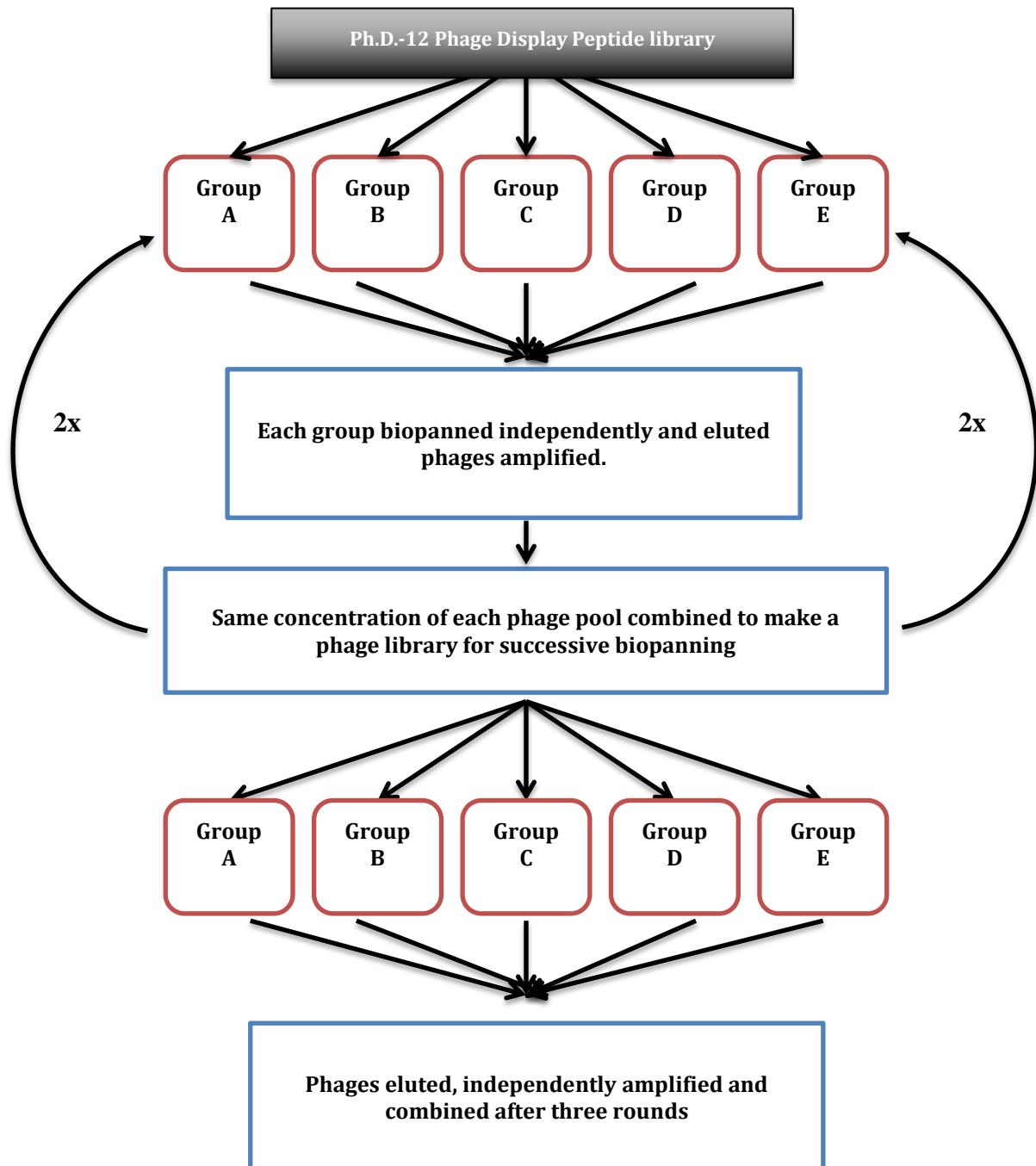
Figure 3.5. Original parallel biopanning approach. Each group of *Salmonellae* in Table 3.2 was biopanned independently with the phage library and the eluted phages from each biopanning were combined to constitute a phage library used in the subsequent biopanning step. The selection steps were repeated for a total of three rounds.
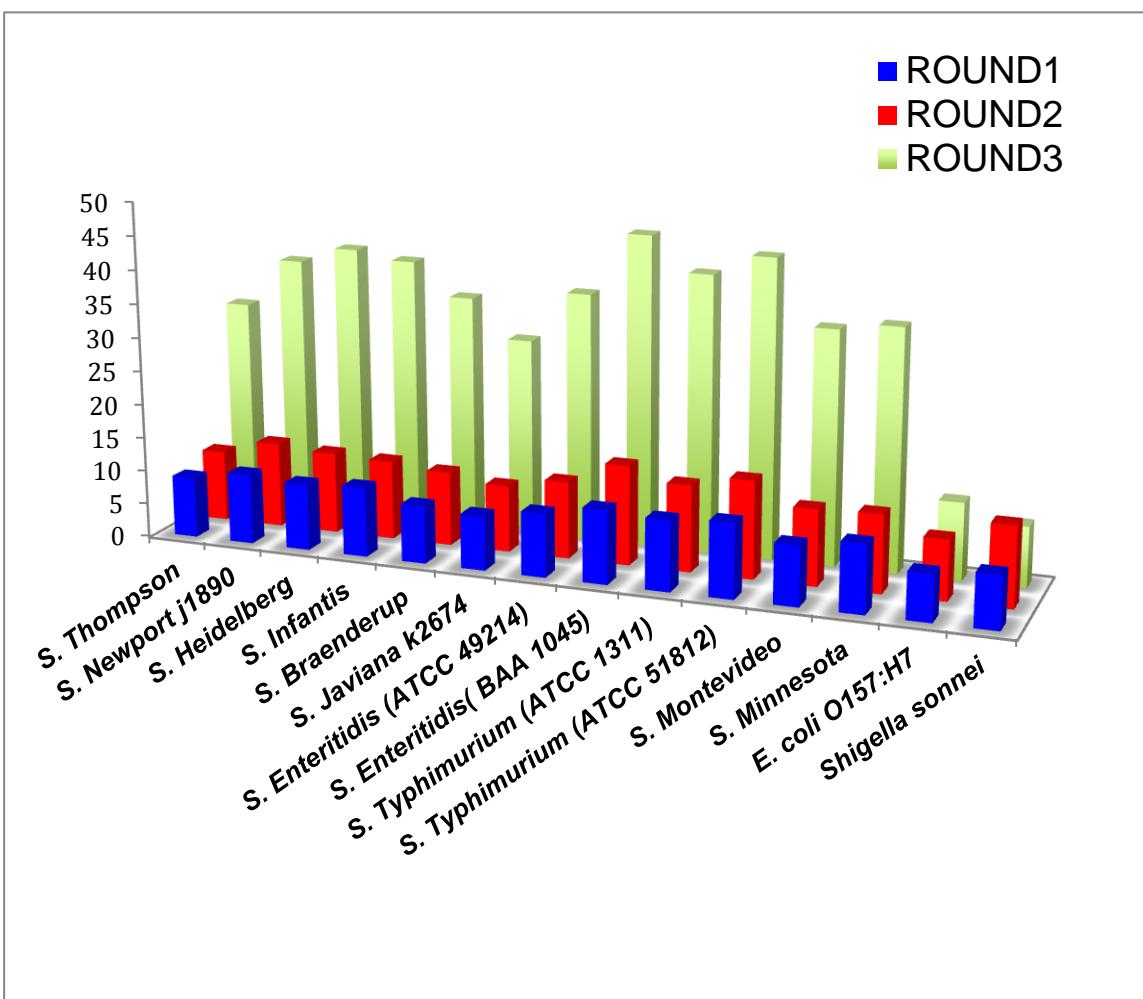
Figure 3.6. Relative selectivity of phage pool from parallel biopanning approach #1. ELISA was performed with the combined phage pool from three rounds of parallel biopanning with twelve serovars of *S. enterica* and *E. coli* O157:H7. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as mΔOD$_{405}$ which is ΔOD$_{405}$ x 1000. The data shown are representative of at least two independent experiments with triplicate samples.
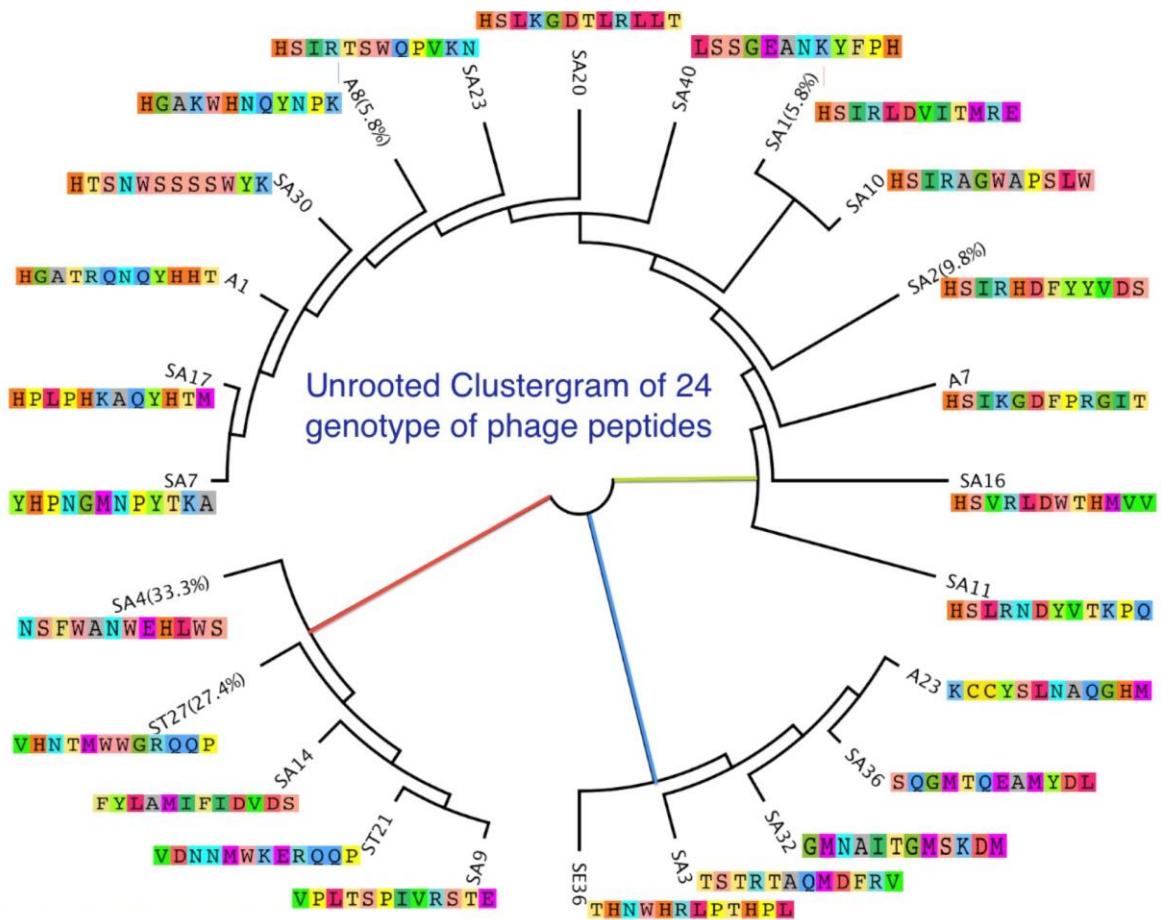
Figure 3.7. Clustering of displayed oligopeptides via neighbor-joining method. The 24 genotypes of the displayed oligopeptides were organized into 3 clusters by the neighbor-joining method (1). The tree alignment cost matrix is Blosum62. (%) is the percentage of how many times a particular sequence was represented out of **6**1sequences. ST: the same sequence was also found in the *S.* Typhimurium phage probe (Chapter 2); SE: the same sequence was found in the *S.* Enteritidis phage probe (data not shown); A: the same sequence was found in the serial biopanning approach; SA: only found in this five group parallel biopanning approach.

Clone#	Frequency	Cluster	Amino acid sequence											
GSP-SA4	17/61	1	N	S	F	W	A	N	W	E	H	L	W	S
GSP-ST27	14/61	1	V	H	N	T	M	W	W	G	R	Q	Q	P
GSP-SA14	1/61	1	F	Y	L	A	M	I	F	I	D	V	D	S
GSP-ST21	1/61	1	V	D	N	N	M	W	K	E	R	Q	Q	P
GSP-SA9	1/61	1	V	P	L	T	S	P	I	V	R	S	T	E
GSP-SA7	1/61	2	Y	H	P	N	G	M	N	P	Y	T	K	A
GSP-SA17	1/61	2	H	P	L	P	H	K	A	Q	Y	H	T	M
GSP-A1	1/61	2	H	G	A	T	R	Q	N	Q	Y	H	H	T
GSP-SA30	1/61	2	H	T	S	N	W	S	S	S	S	W	Y	K
GSP-A8	3/61	2	H	G	A	K	W	H	N	Q	Y	N	P	K
GSP-SA23	1/61	2	H	S	I	R	T	S	W	Q	P	V	K	N
GSP-SA20	1/61	2	H	S	L	K	G	D	T	L	R	L	L	T
GSP-SA40	1/61	2	L	S	S	G	E	A	N	K	Y	F	P	H
GSP-SA1	3/61	2	H	S	I	R	L	D	V	I	T	M	R	E
GSP-SA10	1/61	2	H	S	I	R	A	G	W	A	P	S	L	W
GSP-SA2	5/61	2	H	S	I	R	H	D	F	Y	Y	V	D	S
GSP-A7	1/61	2	H	S	I	K	G	D	F	P	R	G	I	T
GSP-SA16	1/61	2	H	S	L	R	L	D	W	T	H	M	V	V
GSP-SA11	1/61	2	H	S	L	R	N	D	Y	V	T	K	P	Q
GSP-A23	1/61	3	K	C	C	Y	S	L	N	A	Q	G	H	M
GSP-SA36	1/61	3	S	Q	G	M	T	Q	E	A	M	Y	D	L
GSP-SA32	1/61	3	G	M	N	A	I	T	G	M	S	K	D	M
GSP-SA3	1/61	3	T	S	T	R	T	A	Q	M	D	F	R	V
GSP-SE36	1/61	3	T	H	N	W	H	R	L	P	T	H	P	L

Table 3.5. Amino acid sequence of the 61 phage-displayed peptides. 61 phage clones were randomly selected from combined phage pool following the third round of biopanning. The 24 genotypes of the displayed oligopeptides were organized into 3 clusters by the neighbor-joining method (1). The tree alignment cost matrix is Blosum62. Frequency is the number of times a particular sequence was represented out of 61sequences. ST: the same sequence was also found in our *S.* Typhimurium phage probe (Chapter 2); SE: the same sequence was found in our *S.* Enteritidis phage probe (data not shown); A: the same sequence was found in our serial biopanning approach; SA: only found in this five group parallel biopanning approach.
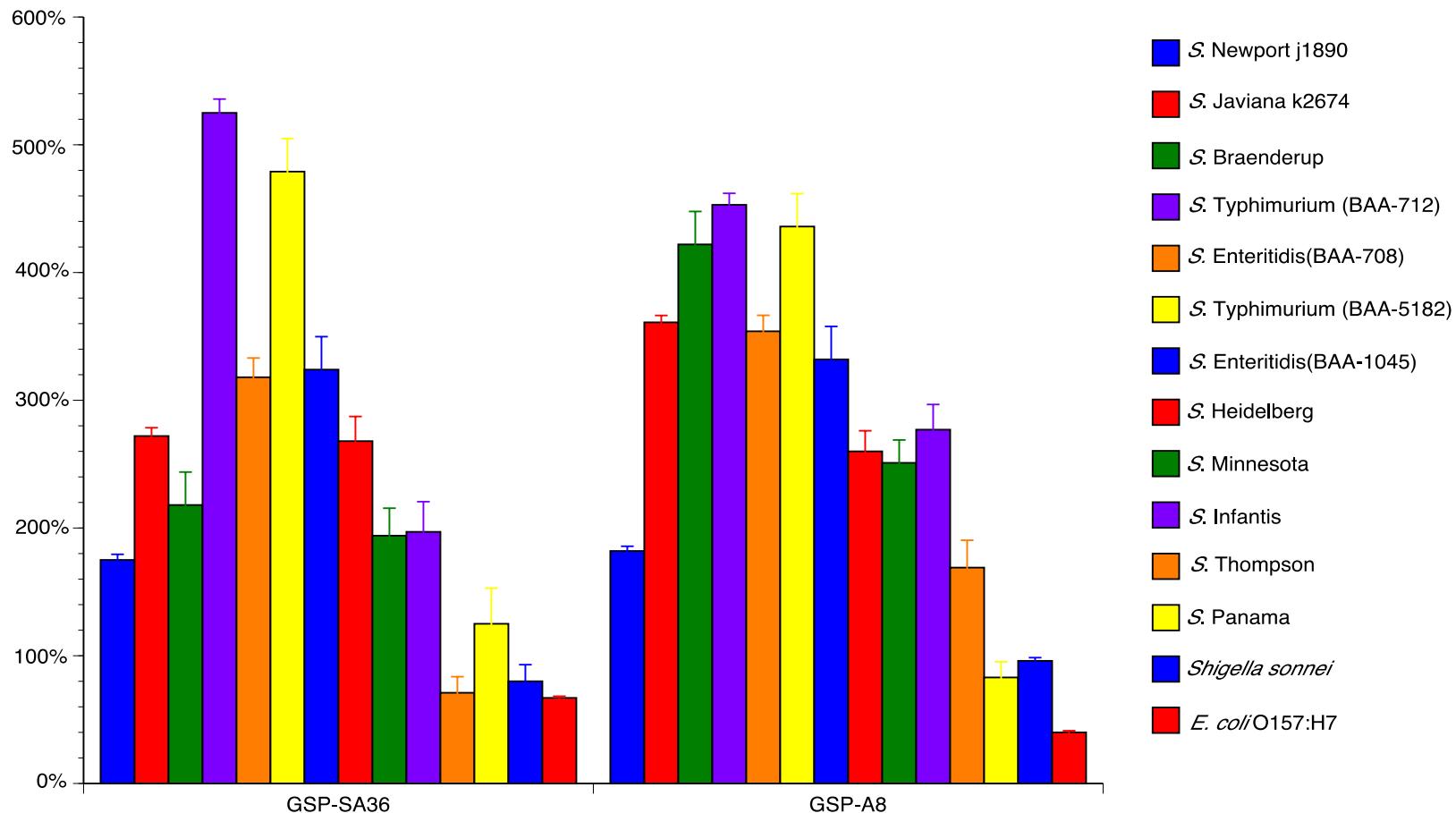
Figure 3.8. Relative selectivity of two probes from parallel biopanning. ELISA was performed with independently isolated and amplified GSP-SA36 and GSP-A8 phages at 10^{11} pfu and indicated bacteria at 10^8 cfu of bacterial cells. Absorbance was measured at 405 nm and converted to relative percentage in which 100% is the rate of ΔOD_{405} in reactions of M13KE with the same bacteria. The data shown are representative of at least two independent experiments with triplicate samples.

In order to isolate probes with better ability to recognize all 14 serovar of *S. enterica* used in this study, I further improved the parallel biopanning approach. Because the amino acid sequences of the two best probes, GSP-SA36 and GSP-SA8, were represented only once and at 5.8%, respectively, out of 61 probes analyzed, it appeared that the biopanning scheme still contained some bias. In order to minimize potential bias caused by over representation of *S.* Typhimurium and *S.* Enteritidis in the target mixtures, I further enhanced the parallel biopanning approach and subdivided the five *S. enterica* groups into 12 subgroups. The schematic of the enhanced parallel biopanning is shown in Figure 3.9. After three rounds of individual parallel biopanning against each of the 12 subgroups of *S. enterica*, I isolated 122 potential phage probes and randomly selected 22 for further analysis. The predicted amino acid sequences of the 22 selected phage-displayed oligopeptides are shown in Table 3.6. I found 21 sequences among 22 phage probes sequenced. I performed ELISA with all 21 phage probes individually against 13 serovars of *S. enterica* (data not shown). Results of two best probes, GS04 and GS103, are shown in Figures 3.10 and 3.11. The data demonstrate that both GS04 and GS103 bind to all 13 serovars of *S. enterica* at least 200% better than to *E. coli* O157:H7 and *S. sonnei*. Thus, the enhanced parallel biopanning approach yielded two phage-displayed oligopeptide probes that could potentially be used for rapid detection of pathogenic *S. enterica*.

Clone	Frequency	Amino acid sequence											
GS02	1/22	D	Q	P	Y	N	N	S	Q	L	L	I	H
GS04	**1/22**	**Y**	**Y**	**P**	**L**	**Y**	**H**	**S**	**T**	**N**	**T**	**L**	**T**
GS27	1/22	T	L	R	T	T	A	W	S	W	S	P	T
GS34	1/22	Y	K	A	P	P	Y	P	P	S	H	Y	N
GS35	1/22	F	D	P	S	S	Y	R	M	I	H	Q	P
GS57	1/22	N	P	G	S	V	A	R	P	L	H	N	P
GS58	1/22	T	H	N	G	N	T	I	A	P	P	G	R
GS71	1/22	V	G	N	Y	S	S	P	F	L	W	E	N
GS81	1/22	T	D	M	T	M	Y	W	M	H	T	P	L
GS88	1/22	F	T	A	P	G	S	D	R	L	V	T	L
GS96	1/22	A	L	D	F	T	A	H	R	I	Q	T	L
GS102	1/22	V	H	N	N	S	W	W	G	L	T	Y	G
GS103	**2/22**	**H**	**T**	**L**	**R**	**V**	**D**	**N**	**L**	**H**	**R**	**P**	**L**
GS104	1/22	N	Q	T	D	H	L	F	S	T	F	I	S
GS105	1/22	H	S	L	R	T	D	R	I	L	P	H	M
GS 107	1/22	E	A	K	C	C	S	F	D	W	L	K	P
GS108	1/22	F	P	M	P	G	S	S	F	Q	T	G	S
GS109	1/22	S	G	L	K	V	W	S	I	P	I	S	K
GS111	1/22	S	L	K	C	C	Y	P	M	T	P	A	D
GS114	1/22	H	S	I	R	H	D	F	Y	Y	V	D	S
GS115	1/22	N	H	F	P	P	L	P	A	R	A	T	F

Table 3.6. Amino acid sequence comparison of 22 phage probes isolated from the optimized parallel biopanning approach. 22 phage clones were randomly selected from the combined phage pool following the third round of biopanning and characterized. Two probes, GS04 and GS103, that demonstrated the best binding to 13 *S. enterica* serovars are shown in bold.
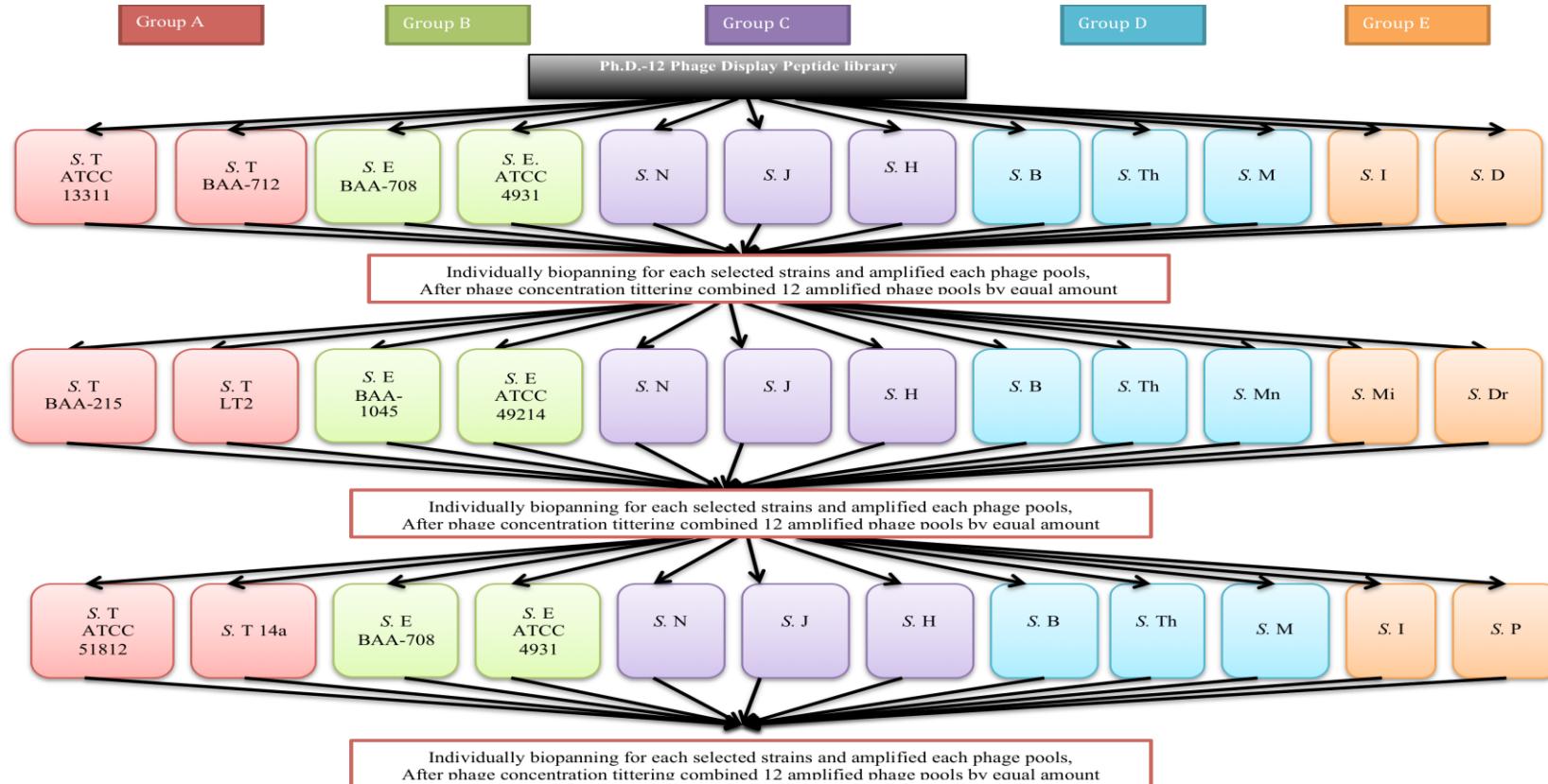
Figure 3.9. Schematic of optimized parallel biopanning. The initial five groups of *S. enterica* serovars were subdivided into twelve subgroups. Each subgroup was composed of two or three strains or serovars from the parental group. The two or three strains/serovars in each of the twelve subgroups per biopanning round were chosen based on their relative importance and frequency of recent outbreaks. *S.* T: *S.* Typhimurium; *S.* E: *S.* Enteritidis, *S.* N: *S.* Newport; *S.* J: *S.* Javiana; *S.* H: *S.* Heidelberg; *S.* B: *S.* Braenderup; *S.* Th: *S.* Thompson; *S.* M: *S.* Montevideo; *S.* Mn: *S.* Minnesota; *S.* I: *S.* Infantis; *S.* Mi: *S.* Mission; *S.* D: *S.* Dublin; *S.* Dr: *S.* Derby; *S.* P: *S.* Panama.

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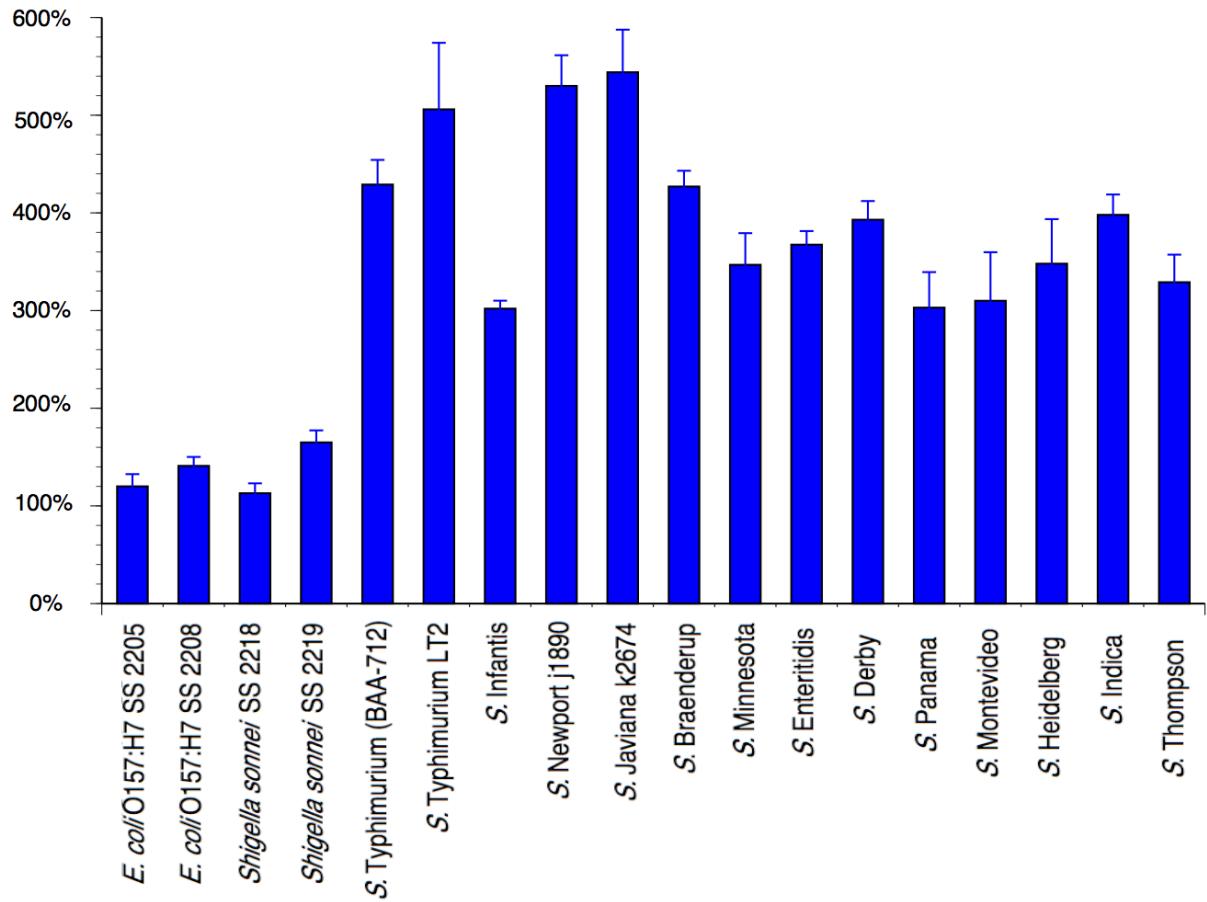
Figure 3.10. Relative selectivity of GS04. ELISA was performed with GS04 phage at 10^{11} pfu and indicated bacteria at 10^8 cfu of bacterial cells. Absorbance was measured at 405 nm and converted to relative percentage in which 100% is the rate of ΔOD_{405} in reactions of M13KE with the same bacteria.
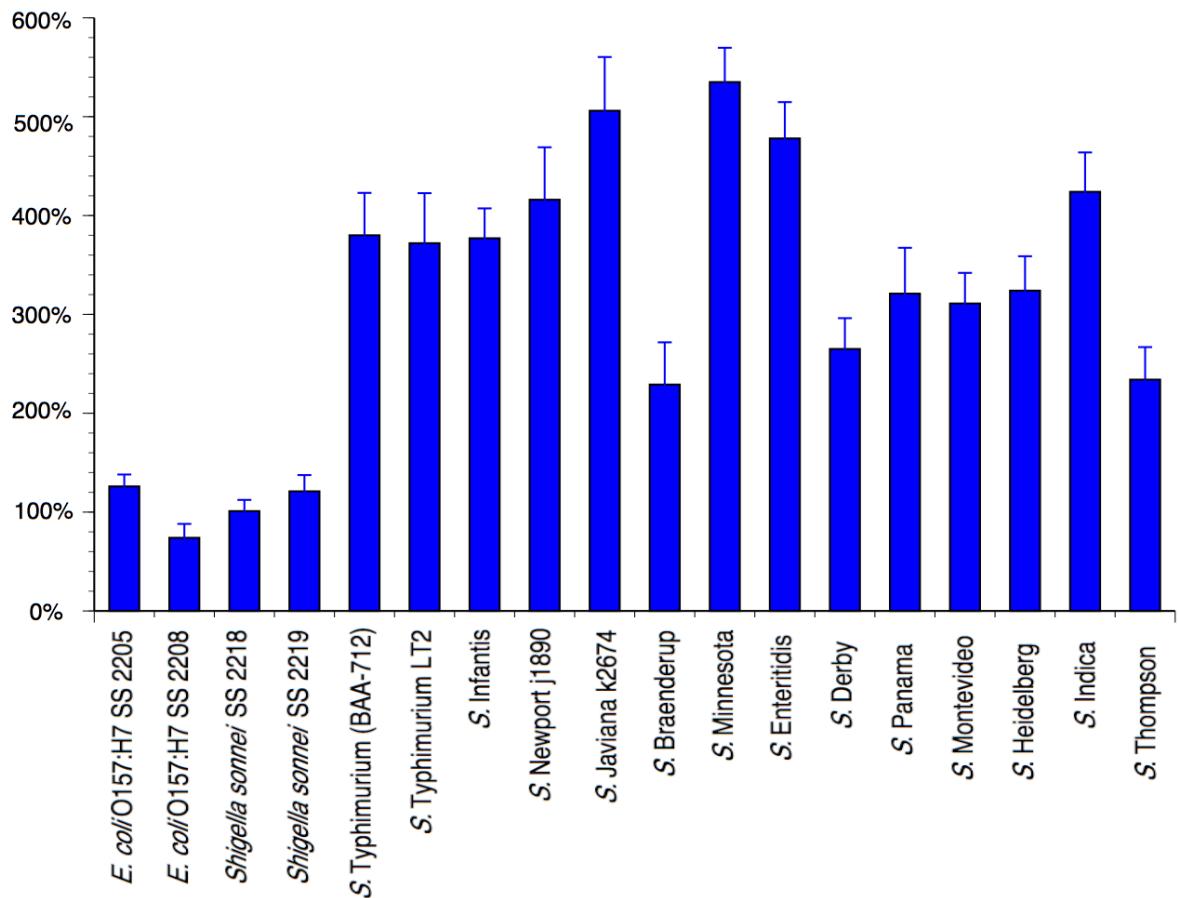
Figure 3.11. Relative selectivity of GS103. ELISA was performed with GS103 phage at 10^{11} pfu and indicated bacteria at 10^8 cfu of bacterial cells. Absorbance was measured at 405 nm and converted to relative percentage in which 100% is the rate of ΔOD_{405} in reactions of M13KE with the same bacteria.

Discussion

Based on recent outbreaks of salmonellosis by various non-typhoid serovars of *S. enterica*, it is obvious that a diagnostic system that can rapidly identify the presence of any number of pathogenic *S. enterica* is needed to implement effective controls. In lieu of costly and time consuming molecular and/or traditional culturing methods, the magnetoelastic biosensors using phage-displayed oligopeptides have demonstrated to be the ideal platform for cost effective and rapid diagnostic tool for pathogen detection (36-51). To achieve this goal, I developed four different approaches to isolate phage-displayed oligopeptide probes for recognition of multiple serovars of pathogenic *S. enterica* to be used with the magnetoelastic biosensor platform. In lieu of using only one or two common serovars such as *S.* Typhimurium and *S.* Enteritidis, I used a total of fourteen serovars that caused recent outbreaks of salmonellosis as the target for these probes.

The first two approaches were based on the traditional serial biopanning and I developed parallel biopanning methods for the second two approaches. Prior to the probe selections, I divided fourteen serovars of *S. enterica* into five groups. The first two groups were composed of multiple strains of *S.* Typhimurium and *S.* Enteritidis, respectively, because these two serovars are the most prevalent cause of foodborne salmonellosis. As demonstrated in the Results, the two serial biopanning approaches failed to yield potential phage probes that can be used for recognition of multiple serovars of pathogenic *S. enterica*. The serial biopanning is the classical strategy that relies on enriching for better binders while eliminating weak binders. Because the number of phages isolated with each round of biopanning is relatively low, phages need to be amplified prior to the next round of selection.

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Unfortunately, amplification may also reduce the binding phages because typically the wildtype phages in the pool have the advantage in infecting the bacterial host. Therefore, presence of any wildtype phage during biopanning may result in the loss of any desired binding phages. In fact, this is exactly what I observed in the first serial biopanning approach. As demonstrated in Figure 3.2, the first approach resulted in over abundance of wildtype phage in the pool following each amplification (Figure 3.2). The second serial biopanning approach resulted in isolation of phages that preferentially bound *S.* Typhimurium and *S.* Enteritidis. This was due to the fact that the mixture of fourteen serovar contained six strains of *S.* Typhimurium and four strains of *S.* Enteritidis. Thus, these two serovars were over-represented in the experiment and resulted in enrichment of phages that preferentially recognized the over-abundant target.

In order to minimize isolating probes with strong bias and to isolate probes that can recognize general epitopes present in all serovars, I developed a parallel biopanning approach. Figure 3.12 illustrates the concept of the parallel biopanning approach. As demonstrated in the figure, the key to the parallel biopanning is simply increasing the ratio of phages that bind to common epitope in each serovar before the next round of selection. Unlike in the serial approach, in parallel biopanning, phages that bound individual group of bacteria are mixed together in equal ratio which increases the total number of phages that bound to the universal epitope. In Figure 3.12, this is represented by the red epitope. Thus, after one round of biopanning, instead of proceeding to the second round with 1 copy of the universal epitope recognizing phage, one now proceeds with three. Several rounds of biopanning in this manner result in enrichment of those phages that recognize the universal epitope as illustrated in the figure. The first parallel approach, in which each of the five groups of *Salmonellae* were used as targets, yielded several probes with the ability to recognize multiple serovars of *S. enterica* except for *S.* Thompson and *S.* Panama (Figure 3.8). Although these two probes

(GSP-SA36 and GSP-A8) could satisfy the general need for a biosensor for recognition of multiple serovars of *S. enterica*, it is possible to improve the parallel approach to isolate probes with even broader activity. The major improvement I made to the parallel biopanning approach was to devise different mix of *S. enterica* for each round of parallel biopanning. In this approach, because each of the subgroup pools were composed of only three organisms, each of the organism is more available to bind to phage. The hypothesis was validated by isolation of two phage probes, GS04 and GS103, that recognized thirteen *S. enterica* serovars. The fourteenth serovar, *S.* Mission, was not tested in this assay.

At present time, identity of the "universal" epitope recognized by GS04 and GS103 is unknown. However, because both phage probes recognize *S. enterica* better than either *E. coli* O157:H7 or *S. sonnei,* the epitope must be specific to *Salmonella*. One possibility is the core-oligosaccharide portion of lipopolysaccharide (LPS) of *S. enterica*. The O-antigen is unlikely to be the universal epitope because there is much variability among serovars. However, the core-oligosaccharide of *S. enterica* is highly conserved and, to date, only one core-oligosaccharide type has been identified in this species (63, 64). Another possibility is a conserved outer membrane protein (OMP), fimbriae, or a flagellin. Of these three possibilities, flagellin is the least likely because, at least for *S.* Typhimurium, there are two different flagellins (H1 and H2) that are expressed by the bacterium as a part of its phase variation (65).

In an attempt to identify a potential universal epitope, I took an in silico approach with Geneious (http://www.geneious.com) to analyze the two oligopeptides displayed by GS04 and GS103. With GS04, I did not find any motif that may suggest a potential binding partner. However, with GS103, I believe there is a precedence that the oligopeptide may be involved in OMP binding. GS103 oligopeptide contains a motif RxxxxxR between residues 4 through 10. This motif is similar to the motif found by Meyer et al. (66) for OmpD binding

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sequences of KLDLYGKVH and DRTNNQVKA. Both of these motifs have arginine in one or two places of lysine found in GS103. However, since both arginine and lysine are positively charge hydrophilic amino acids, they might serve similar functions in these short oligopeptides. Based on this analysis, I can speculate that the motif K/RxxxxxR/K maybe involved in binding to OmpD. However, this is just a hypothesis and needs experimental validation. Additionally, more detailed in silico analysis that considers the positions of the displayed peptides in relation to the fused GpIII is necessary to increase confidence.
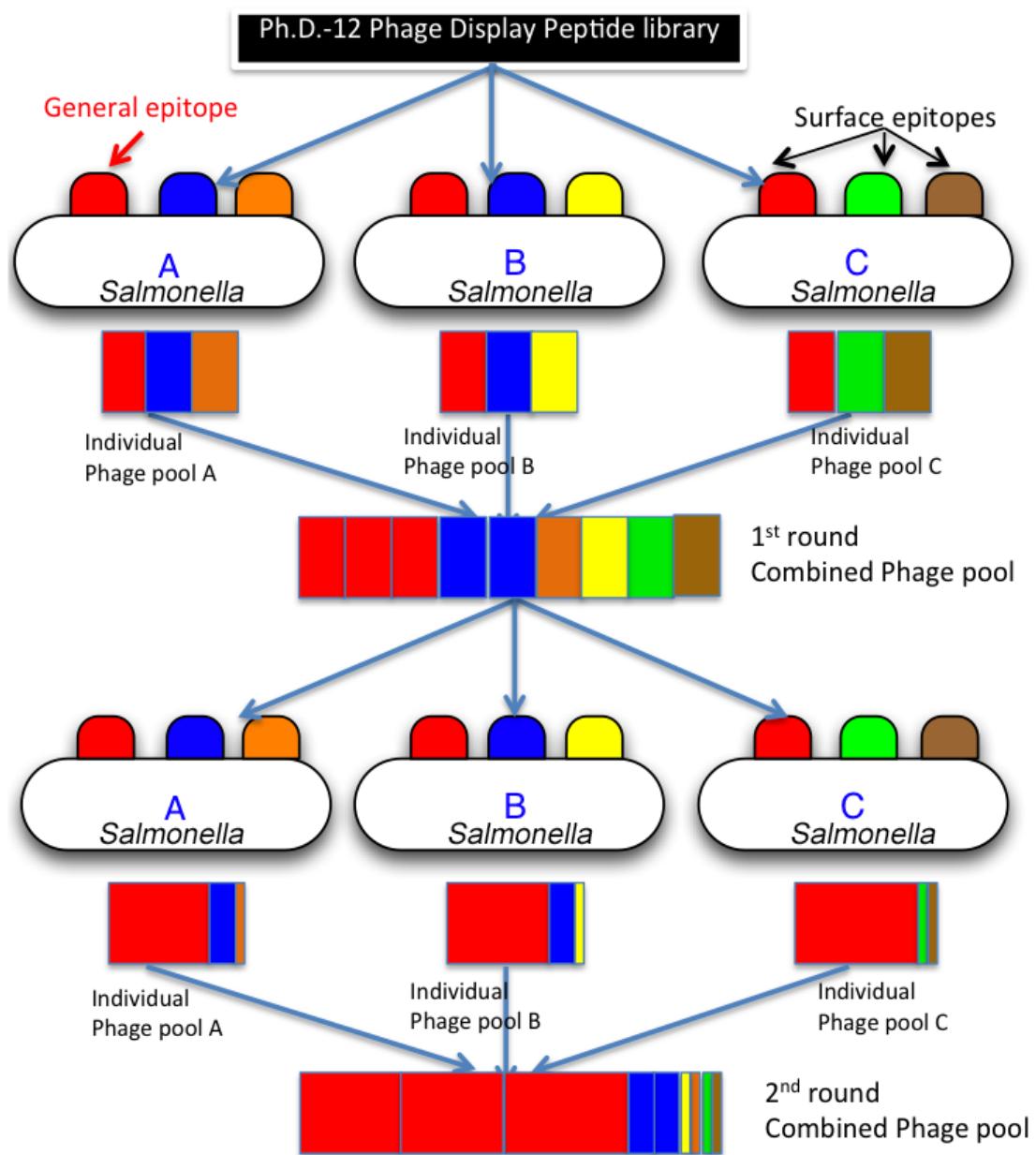
Figure 3.12. Parallel biopanning scheme. In this model, the red epitope represents is the universal surface molecule present in all *S. enterica* while the other colored epitopes represent those molecules present only in some serovars. As demonstrated in the figure, mixing of phage pools from each individual biopanning results in increasing the relative concentration of those phages that bind to the universal epitope. Successive rounds of biopanning should further enrich for those phages binding to the universal epitope.
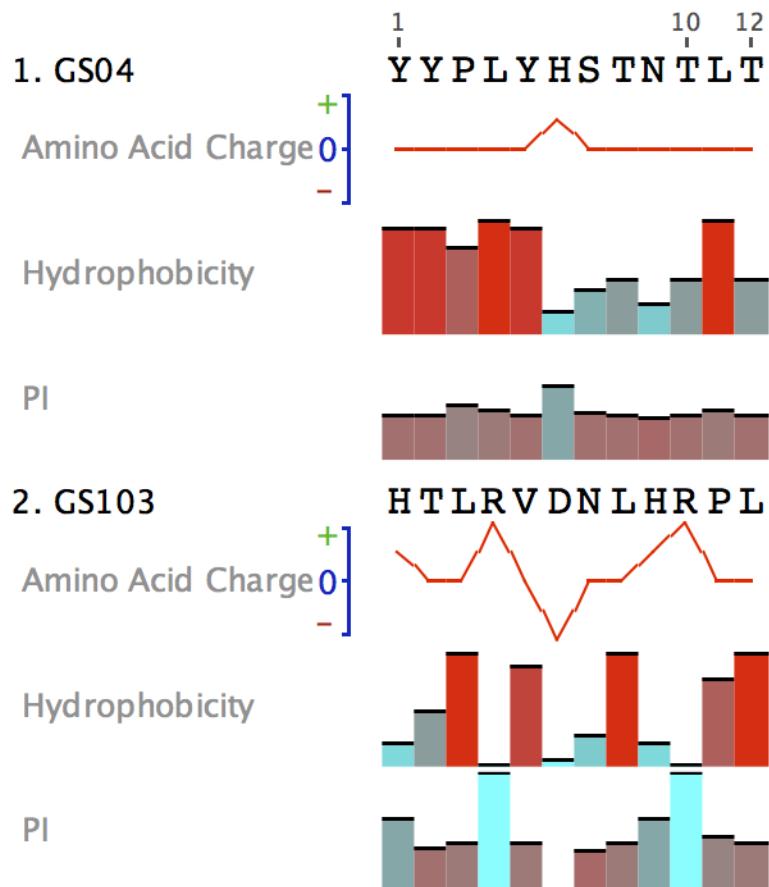
Figure 3.13. Isoelectric point and hydrophobicity analysis of GS04 and GS103. Basic characteristics of twelve amino acids displayed in GS04 and GS103 were analyzed with Geneious 8.0 (http://www.geneious.com). Hydrophobic residues are represented in red and hydrophilic residues are in blue. In isoelectric point analysis, red bar represents low isoelectric point and blue bar represents high isoelectric point.

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[Online.]

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<center>**Chapter 4**</center>

<center>**Optimizing Immobilization of Phage-Displayed Oligopeptide Probes on
Magnetoelastic Particle Platforms**</center>

Abstract

An ideal detection technology should be fast, accurate, durable with a long shelf life, cost effective, and user-friendly without extensive training or elaborate equipment. The magnetoelastic (ME) biosensors using phage-displayed oligopeptides as molecular recognition probes have advantages over other technologies because they are fast, cost effective, easy to use, and require minimal sample manipulations. Presently, phage probes are immobilized to the ME platforms in a random and non-uniform fashion. Unfortunately, this random attachment to the platform may mask the oligopeptide probes and interfere with their function of capturing specific analytes. This is a serious concern for the GpIII phage-display probes which have only five analyte capturing peptides per virion. Thus, in order to further improve the ME biosensor technology, an approach was developed to efficiently immobilize the Ff class of filamentous bacteriophage probes on ME platforms in a uniform monolayer to enhance capturing of analytes. The approach takes advantage of the

GpIX's location on the opposite side of GpIII, a commonly used protein for display, and tagging GpIX with an affinity tag. This allows the phage probes to be immobilized on any sensor platforms coated with the affinity molecule in a uniform monolayer with the GpIII–displayed oligopeptides situated for optimal analyte capture. The phage immobilization approach consists of two major steps. First, the gene IX of the Ff class of phage is mutated via in vivo insertion mutagenesis using the highly efficient genetic recombineering technology. The gIX mutants that are unable to produce phage particles are collected in their replicative plasmid form and transformed into a bacterial cell that produces an affinity-tagged GpIX to allow formation of infective phage particles. These affinity-tagged phage probes are then efficiently immobilized onto ME platforms that have been coated with the molecule for the affinity-tag. The whole process of affinity tagging a phage only takes three days and can be applied to any Ff class of phage-displayed oligopeptide probes to improve their performance on biosensors. As a proof of concept, *S.* Typhimurium phage probe's GpIX was tagged with StrepTag II and immobilized the tagged probes on streptavidin coated ME platforms. This immobilization strategy improved the performance of a *S.* Typhimurium specific biosensor by almost 5-fold compared to the same probe that had not been affinity immobilized on the sensor platform.

Introduction

With recent outbreaks and fear caused by dissemination of pathogens, including multidrug resistant bacteria, bird flu, swine flu, and ebola, the ability to quickly detect and accurately diagnose the causative agent is becoming ever more important to minimize the deleterious effects of these infectious agents on human society. Biosensor technology combines the accuracy and sensitivity of other approaches with improvement in rapidity of detection (1). In the last two decades, efforts on developing highly accurate and easy to use biosensors that can provide near real-time analysis in various environments has grown explosively (2-10). A typical biosensor is composed of molecular recognition probes immobilized on a sensor platform with characteristics that can be easily measured based on the probe:analyte interactions (4). Recent development of biosensors composed of a magnetoelastic particle and phage-displayed oligopeptides show a significant promise because of their sensitivity, low cost, and user-friendliness (11).

The magnetoelastic (ME) particles are a great choice as biosensor platforms because they can be easily manipulated, made at micro- or nano-size, cost-effective, and highly sensitive to mass change for detecting probe:analyte interactions (7). To detect a mass change, a time-varying external magnetic field (driving field) is used to induce vibrational resonance in the particles. The vibrational resonance of the ME particle results in the emission of a magnetic signal that can be remotely detected using a pick-up coil. Recognition and binding of a target agent to a ME particle acts as a mass load causing the

characteristic resonant frequency to shift to lower frequencies. ME particles can be made very small so that the attachment of a single spore, a bacterium or, in near future, a virus can be detected. MEs can be deployed in large numbers to increase the probability of binding, increase the sensitivity of detection, and reduce detection times. ME particles can be simultaneously moved (guided in a given direction) through a liquid using a DC field, driven into resonance with an AC field and interrogated for binding using a pickup coil (7).

Typical molecular probes used in biosensors include enzymes, nucleic acids, antibodies, live cells, and phage-displayed oligopeptides (4). The use of biosensors with immobilized antibodies to detect HIV (12) and *Salmonellae* (13, 14) has been reported since 1990s with the relative selectivity and sensitivity that is comparable to ELISA (14). For *S*. Typhimurium, a biosensor using an antibody as the probe was highly specific and gave a linear response from 10^5–10^9 cells/ml microbial suspension with the limitation of detection at 5×10^3 CFU/ml (15). Although highly valuable due to the specificity, the main disadvantages of antibody probes are poor environmental stability (16) and the cost and time associated with production of specific antibodies. In contrast, phages displaying peptides or proteins can be selected, isolated, and rapidly reproduced at low cost to be used as molecular probes. In addition, the filamentous phage-displayed oligopeptide probes are robust because they are resistant to heat (up to 80°C) (16, 17), organic solvents like acetonitrile (18), urea (up to 6 M), acid, alkali and other chemicals (19, 20). Thus, phage probes are good candidates to be used with ME particles to form viable biosensors.

Ff class of F pilus-specific phage group contains f1, M13 and fd. These phages are very closely related to each other and share almost 99% identity at the genome level

according to the International Committee on Taxonomy of Viruses database (ICTVdB, http://ictvdb.bio-mirror.cn/ICTVdBintro.htm). Although M13 is the best characterized and most utilized phage among the Ff class of phages, fd is the phage that had been developed for the phage-display technology (21). However, because M13 and fd are almost identical at the genome level, they will be treated as equal in this discussion.

All five of fd or M13 phage capsid proteins have been used for phage-display. Peptides displayed on GpIII, GpVI, GpVII, and GpIX are represented at five copies per capsid and are located at polar ends of the phage particle. Among the four proteins, GpIII has been utilized the most and has been used to construct commercially available libraries. Some of the commecially available phage display libraries include 7-mer and 12-mer oligopeptide libraries by New England Biolabs (Beverley, MA), short chain fragement variable (ScFv) library by Creative Biolabs (Shirley, NY), and ScFv and Fab libraries by Phage Display (Cambridge, UK). The New England Biolab's Ph.D.-7 and Ph.D.-12 libraries have been used to isolate highly selective probe for detection of *B. anthracis* spores (22, 23) (Chen et al. unpublished data).

The major capsid protein, GpVIII, has also been used to display peptides(24). The major advantage of displaying peptides on GpVIII is that because there are approximately 2800 copies of the protein used to cover the genomic DNA of the phage, the avidity of the probe is significantly increased due to multivalent binding. In addition, due to the close proximity of each GpVIII and displayed peptides to each other on the surface of the phage, they form interactions that result in formation of a "landscape" (24). The landscape phages have been highly successful in yielding probes that specifically recognize *Bacillus anthracis*

spores (25-29) and *S*. Typhimurium (9, 10, 30-34).

The major disadvantage of the landscape phage-display peptide probes is that because their affinity depends on interaction with neighboring peptides, individual peptides cannot be isolated and used apart from the phage capsid. Another disadvantage of GpVIII display is that it is limited to peptides of nine amino acids or less (24). In contrast, much larger peptides or proteins can be displayed on GpIII. Thus, GpIII has been the preferred capsid protein for displaying antibodies or ScFv fragments.

Presently, the phage-displayed oligopeptide probes are immobilized on the ME platforms via physical adsorption method which results in binding of the phages in non-uniform fashion on the sensor. Such immobilization may interfere with optimal performance of the sensor because the phages are not uniformly distributed on the sensor and some of the analyte binding peptides maybe masked. In this study, a novel approach was developed to strongly and uniformly immobilize Ff class of phage probes on abiotic surfaces in a monolayer in order to optimize the performance of ME biosensors.

Materials and methods

Bacteria, plasmids, media and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 4.1. *Salmonella enterica* serovar Typhimurium (ATCC 13311) was grown in nutrient broth (NB) and confirmed for identity, propagated, and maintained on BG Sulfa Agar (Difco 271710). *Escherichia. coli* strains ER2738 and DH10B were grown in Lenox broth (LB) or LB supplemented with appropriate antibiotics at 37°C with aeration, unless otherwise indicated.

Following electroporation, cells were inoculated into SOC medium (35) for antibiotic gene expression. Media were solidified with 1.5% Bacto Agar (Difco, Detroit, MI). The following antibiotic concentrations were used in this study (per milliliter) for selection: tetracycline (Tet), 20 μg; Gentamycin (Gm), 20 μg; and ampicillin (Ap), 100 μg; kanamycin (Km), 50 μg. The chromogenic substrate X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D-thiogalactoside) were used at 50 μg/ml and 40 μg/ml, respectively, for blue/white screens. Anhydrotetracycline (36) was used at 10 ng/ml to induce P_{tet} for expression. Chemicals and antibodies were purchased from Sigma-Aldrich (St. Louis, MO).

E. coli strains DH10B and ER2738 were routinely used as the host strain for cloning and phage infection, respectively. Restriction enzymes EcoRI, HindIII, SanBI, and SmaI, and DNA modification enzyme T4 ligase and Phusion® polymerase were purchased from New England Biolabs (NEB, Beverly, MA). Phage genome in replicative form (RF) and other plasmids were isolated with QIAprep spin miniprep columns Qiagen, Valencia, CA). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction kit (Qiagen) according to the manufacturer's instructions. Molecular manipulations of DNA, including restriction digestions and ligations, were performed via standard techniques (37). Oligonucleotides were designed with the Primer Select of Lasergene suite (DNASTAR, Inc., Madison, WI). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Strains/Plasmids	Genotype and relevant characteristics	Source
E. coli		
DH10B	F⁻ *mcrA* *Δ(mrr-hsdRMS-mcrBC)* □*80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ (ara, leu)7697 galU galK l-rpsL nupG*	Invitrogen
ER2738	F´ *proA⁺B⁺ lacIq Δ(lacZ)M15 zzf*::Tn*10* (Tet^R)/*fhuA2 glnV thi Δ(lac-proAB) Δ(hsdMS-mcrB)*5 (rk⁻ mk⁻ McrBC⁻)	New England Biolabs
SS2660	DH10B/pKD46 Ap^R	This work
SS2691	ER2738/ pZT44	This work
Plasmids		
pASG-IBA4™	P*tet* OmpA signal sequence Strep-tag II® Ap^R	IBA^©
pKD46	*araC* P*araB*::γβ*exo* Ap^R *repA101ts*	(38)
pSU39	Kan^r MCS	(39)
pUCGM	*aacCI* cassette (Gent^R)	(40)
pZT41	pSU39:: pIX	This work
pZT42	pSU39::ΔpIX::*aacC1*	This work
pZT43	M13KE::ΔpIX::*aacC1*	This work
pZT44	pASG-IBA4::pIX	This work
pZT45	pENTRY-IBA51::pIX	This work

Table 4.1. Bacterial strains and plasmids.

Construction of a Strep-tag II::GpIX expression plasmid

M13 pIX was cloned into pASG-IBA4 to generate an inducible N-terminus Strep-tag II affinity tagged GpIX using the StarGate cloning system according to the manufacturer's instructions (IBA, Gottingen, Germany). Briefly, the open reading frame of pIX was PCR amplified with SSO-803 (5'-AGCGGCTCTTCAATGAGTG TTTTAGTGTATTCTTTC-3') and SSO-807 (5'-AGCGGCTCTTCTCCCGCTT GCTTTCGAGGTGAATTTC-3'). The primers were annealed at 55°C and the fragment was extended for 60 seconds for a total of 35 cycles. The PCR fragment was inserted into pENTRY-IBA51 to generate the donor vector, pZT45. The donor vector constructs were verified via restriction endonuclease digestion with XbaI and HindIII. pZT45 was then mixed with the destination vector pASG-IBA4 to generate the inducible Strep-tag II::GpIX construct, pZT44. The plasmid pASG-IBA4 contains the *E. coli* OmpA signal peptide to facilitate the affinity tagged GpIX cross the cell membrane.

Construction of a pIX::aacCI insertion allele

The M13KE pIX gene was amplified by PCR as an EcoRI and HindIII fragment and cloned into respective sites in pSU39 (41) to generate pZT41. The primers used to amplify the M13KE pIX (from +865 to +1649) were as follows: upper primer of the upstream sequence (F1) 5'-CAT*GAATTC*CATCTCAAGCCCAATTTACTACTC-3', lower primer of the downstream (R1) 5'-CCA*AAGCTT*CTTTCAACAGTTTCGGCCGAGTG-3'. The italics indicate EcoRI and HindIII sites, respectively. For amplification of the ~800 basepair

fragment, primers were annealed at 58°C and extension was done at 72°C for 90 seconds. Amplification was done for 35 cycles. The amplified product was ethanol precipitated, sequentially digested with EcoRI and HindIII, separated on a 2% agarose gel, and extracted with Qiaex II DNA gel extraction system (Qiagen). The PCR fragment was ligated into EcoRI/HindIII digested pSU39, electroporated into DH10B, and isolated as Km^R X-gal$^-$ colonies. Twenty four putative clones were screened via PCR and one clone was designated as pZT41.

To construct a pIX::*aacCI* insertion mutant allele, pZT41 was digested with SanBI which cuts once in the middle of pIX, and SmaI digested *aacCI* cassette from pUCGM (40) was inserted to generate pZT42. The orientation of *aacCI* insertion was determined via EcoRI and EcoRV double digest.

Genetic recombineering

E. coli DH10B cells carrying a PKD46 (SS2660) were prepared for genetic recombineering as previously described (38). Briefly, cells were grown in 3 ml of LB supplemented with 100 μg/ml of Ap and grown overnight at 30°C with shaking at 220 rpm/min. One ml of the overnight culture was used to inoculate 100 of SOB supplemented with 100 μg/ml of Ap and grown at 30°C with shaking until OD_{600} ~0.4 was reached. Then L-arabinose was added to a final concentration of 0.1 mM to induce the phage Lambda genes on pKD46 and cells were further grown to OD_{600} of ~0.8. Cells were harvested at 4^oC by centrifugation at 5000 *g*, washed twice with ice-cold 15% glycerol, and resuspended in 2 ml of ice-cold 15% glycerol. The electrocompetent cell concentration was determined

to be 8×10^9 cfu/ml.

The 975 bp linear DNA fragment containing pIX::*aacCI* allele was PCR amplified from pZT42 with the primers F2 (5'-TATTCTTTTGCCTCTTTCGTTTTA-3') and R2 (5'-CGGCTACAGAGGCTTTGAGGAC-3'). F2 corresponds to position 1221 - 1244 and R2 corresponds to 1337 - 1316 on the M13KE genome, respectively. Annealing was performed at 56°C and the fragment was extended for 60 seconds for a total of 35 cycles. The 975 bp PCR fragment was purified with Qiaex II system   (Qiagen, Valencia, CA). In order to recombine the pIX::*aacCI* allele into the phage genome, the linear DNA was mixed with the purified phage RF genome at 3:1 molar ratio, electroporated into recombineering ready electrocompetent SS2660, and incubated at 37°C for 60 minutes to induce recombination and antibiotic resistance expression. 50 μl of the transformants were plated on a LB Gm X-gal plate to select for phage containing cells that had undergone recombination and 50 μl of the transformants were plated on a LB Ap X-gal plate to determine the total number of viable cells. The other 900 μl of the transformants were centrifuged at 8000 *g* for 10 minutes to collect phage lysate. The infection was performed with the collected phage lysate mixed with *E. coli* ER2378 that had been grown to OD_{600} nm of 0.5 at 1:5 volume to volume ratio. The phages were allowed to adsorb for fifteen minutes, the infection mixture was harvested at 8000 *g* for two minutes, resuspended in 1 ml of SOC medium, and transfectants were selected on LB Gm X-gal plates. The validity of the transfectants carrying phage with pIX::*aacCI* were confirmed via the ability of the colonies to grow on LB Gm and LB Tc plates as well as via PCR. Finally, the transfectants was further confirmed by inability of the phage with pIX::*aacCI* in making viable phage

particles. Briefly, putative transfectants were grown overnight in LB Gm. Culture supernatants were collected from 200 ul of the culture, 20 ul of the supernatants were mixed with 180 ul of ER2378 to infect, and plated on LB Gm for selection. The mutant phage with pIX::*aacCI* were unable to assemble, therefore the supernatant have no phage to infect ER2378.

Production of Strep-tag II affinity tagged phage particles

To isolate affinity tagged phage particles, pZT44 carrying anhydrotetracycline (42) inducible Strep-tag II::GpIX allele was electroporated into ER2738 lysogen carrying a phage with pIX::*aacCI* allele, and the transformants were selected on LB plates supplemented with Ap (50 μg/ml) and Gm (10 μg/ml). To produce affinity tagged phage particles, transformants were grown in LB supplemented with Ap (50 μg/ml) and Gm (10 μg/ml), and expression of the tagged pIX was induced with 10 ng/ml of AHT. To scale up the production, after six hours of growth, 1 ml of the culture was inoculated into 100 ml of LB supplemented with Ap (50 μg/ml), Gm (10 μg/ml), and AHT (10 ng/ml) and grown further at 37°C with shaking at 220 rpm for 12 hours. Affinity tagged phages were collected from the culture supernatant.

Phage capture ELISA

Briefly, the wells of a 96-well EIA/RIA plate (Corning 3591, Corning, NY) were coated with 100 μl of 2 μg/ml and 20 μg/ml of lyophilized streptavidin that had been

dissolved in mili-Q water with 0.2% w/v NaN$_3$. The plate was stored overnight at 4°C to promote streptavidin binding to the wells. Following morning, 100 μl of blocking buffer (5 mg/ml BSA) was added to each well and incubated for 1 hour at room temperature and washed three times with PBST (Phosphate buffered saline with 0.1% Tween 20) to remove unbound BSA. A total of 10^9 phage particles in 100 μl was added to each well, incubated for 1 hour at room temperature to promote binding, and washed six times with 100 μl of PBST to remove unbound phage. Bound phages were probed first with 1:3000 diluted anti-Fd phage IgG rabbit antibody (Sigma-Aldrich) followed by 1:5000 diluted anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich). Both reactions were performed at room temperature for 1 hour with gentle shaking. The reaction was developed with 100 μl of 2 mg/ml *p*-NPP (*p*-nitrophenylphosphate) and the kinetic signals were measured on BioTek Cytation 3 plate reader (BioTek, Winooski, VT, USA) for a reaction time of 60 min at 405 nm. The alkaline phosphatase activity was measured as the linear slope of the reaction.

Results and Discussion

Strategy for immobilization of phage probes on sensor platforms via affinity binding

In order to optimize immobilization of phage probes on abiotics surfaces including the magnetoelastic sensor platforsm, a genetic strategy utilizing affinity tags was developed. Figure 4.1 illustrates the overall strategy. As a proof of concept, I chose to use Strep-tag II::streptavidin interaction to immobilize the phages on an abiotic surface because the biotin:streptavidin forms one of the strongest non-covalent interactions (43). Strep-tag II is an eight amino acid oligopeptide (WSHPQFEK) tag derived from biotin:streptavidin interaction that has been engineered to strengthen the binding to streptavidin (44). The strategy can be summarized in several simple steps as demonstrated in Figure 4.1. First, GpIX of the Ff class of filamentous phage was mutated in order to affinity tag 100% of the phage particles. Second, I provide Strep-tag II-GpIX in trans to pIX mutants of the phage so that only those particles with the tag are packaged to form infectious phage virions. Finally, I immobilize the Strep-tag II:GpIX tagged phage particles on a sensor platform that has been coated with either streptavidin or a modified StrepTactin. Once appropriate constructs, including pIX::*aacCI* allele to mutate the wildtype gene on the phage genome and a recombinant plasmid with inducible Strep-tag II:GpIX, have been prepared, the whole process of affinity tagging and immobilizing the phages on a sensor platform in a uniform monolayer only takes three days.
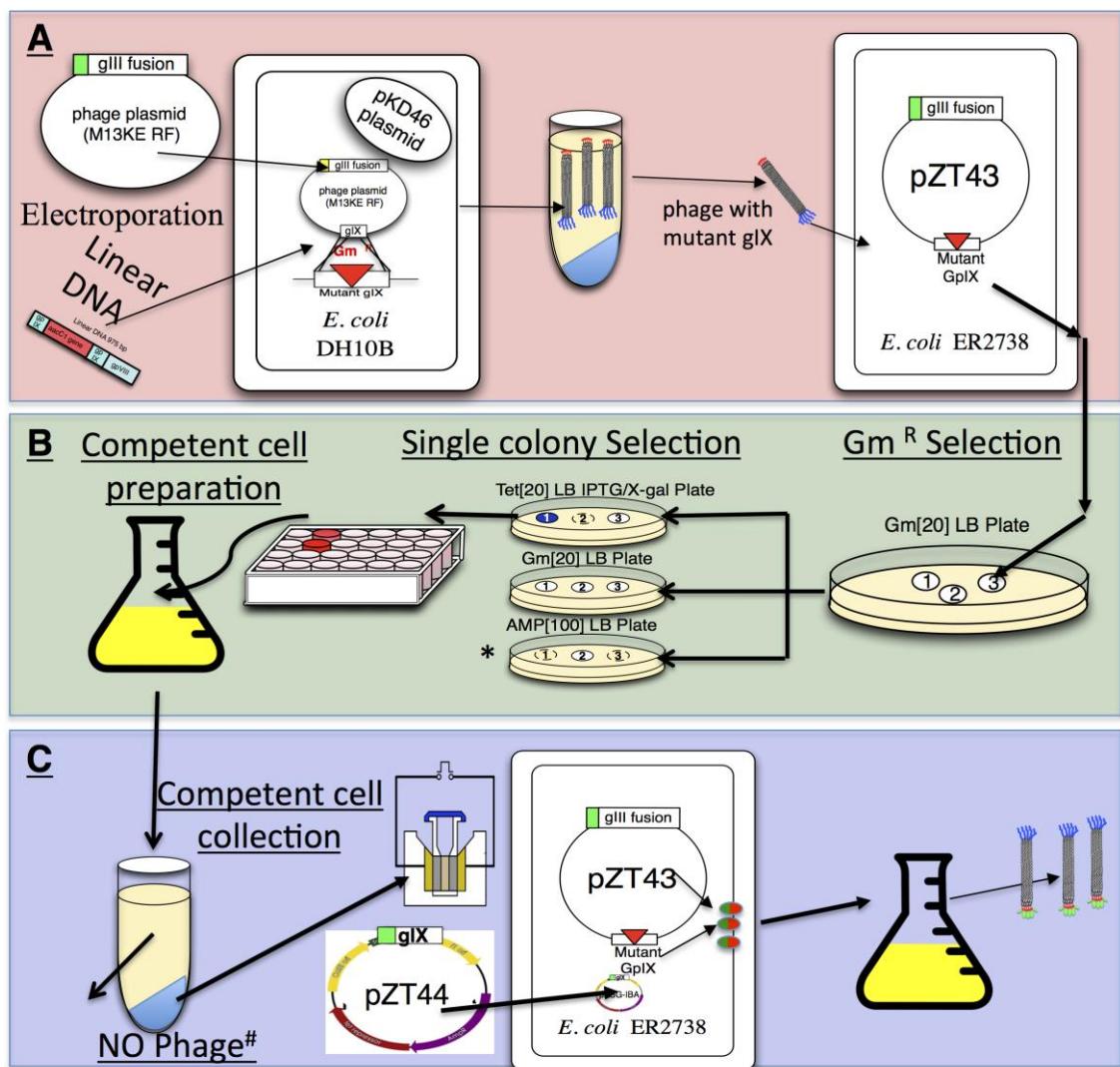
Figure 4.1. A flow chart of genetic approach to tag Ff class of filamentous phages.

A (pink zone): a linear PCR product of pIX::*aacCI* insertion allele and the wildtype phage genome are introduced into the genetic recombineering proficient *E. coli* cell. The mutant allele replaces the wildtype pIX via homologous recombination to yield phages that cannot be packaged into infectious particles.

B (green zone): The pIX::*aacCI* mutant phage carrying cells are selected as Gm[R] colonies, verified for lack of infectious phage production, and phage RF isolated as plasmids.

C (blue zone): The pZT44 carrying inducible Strep-tag II::GpIX allele is electroporated into F[+] *E. coli* carrying phage with pIX::*aacCI* pallele to produce infectious phage particles that are 100% tagged at GpIX.

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Construction of an inducible Strep-Tag::GpIX allele

An inducible Strept-tag II::GpIX fusion protein with the IBA's StarGate cloning system was constructed. This system allows control of fusion expression through utilization of the *tet* promoter that can be induced with AHT. Although, I tried several variations of the Strep-tag II:GpIX fusion, including adding six Glycine residues as a bridge linker between the tag and the protein, none of the constructs were as successful as the final construct in pASG-IBA4 (data not shown). This plasmid contains the *E. coli*'s OmpA signal sequence that facilitates the Strep-tag II:GpIX fusion to cross the cell membrane of the bacterium to be incorporated into the phage virion. Thus, the resulting plasmid, pZT44, was used to affinity tag all of the phages in this study.

Once constructed, I first determined the optimal inducing conditions for OmpA:Strep-tag II:GpIX fusion construct. Because GpIX inserts into the cell membrane before being incorporated into the phage virion (45), I had to find a condition in which the fused GpIX could be expressed at optimal condition without causing deleterious effect on the host bacterial cell. Thus, various concentrations of AHT were tried to induce the fused GpIX without harming the host cell. The results are shown in Figure 4.2. As demonstrated in the figure, I determined that AHT concentration of 10 ng/ml did not cause any deleterious effect on growth of the cell carrying the plasmid pZT44, indicating that *E. coli* was able to cope with the amount of fused GpIX produced under the tested condition.

Construction of a pIX::aacCI insertion mutation allele and genetic recombineering

In order produce GpIX tagged phages, I initially grew phages in an *E. coli* strain overexpressing the Strep-tag II::GpIX fused protein. Unfortunately, this resulted in the production of phage particles that were only partially tagged at GpIX (data not shown). I believe this was due to the competition between the wildtype GpIX encoded by the phage genome and the fused Strep-tag II:GpIX. Thus, it appeared that in order to enhance tagging of the phage particles, the phage genome encoded GpIX should be inactivated. To achieve this goal, I devised a simple genetic approach for efficiently mutating pIX on the phage genome. The method involves utilization of the highly efficient homologous recombination mediated by the phage Lamda's recombination system that had been designated as genetic recombineering (38). This system catalyzes highly efficient recombination between a supercoiled DNA and a linear double-stranded DNA. Thus, it is a simple matter of transforming a genetic recombineering proficient strain with the supercoiled phage genome and a pIX mutant allele as a dsDNA. Once I mutate the phage pIX gene, then I provide the tagged GpIX in trans to the phage to isolate phage particles that are 100% tagged. The scheme to construct a pIX::*aacCI* allele for generating pIX insertion mutants of Ff class of phages via genetic recombineering catalyzed allelic exchange is shown in Figure 4.3.
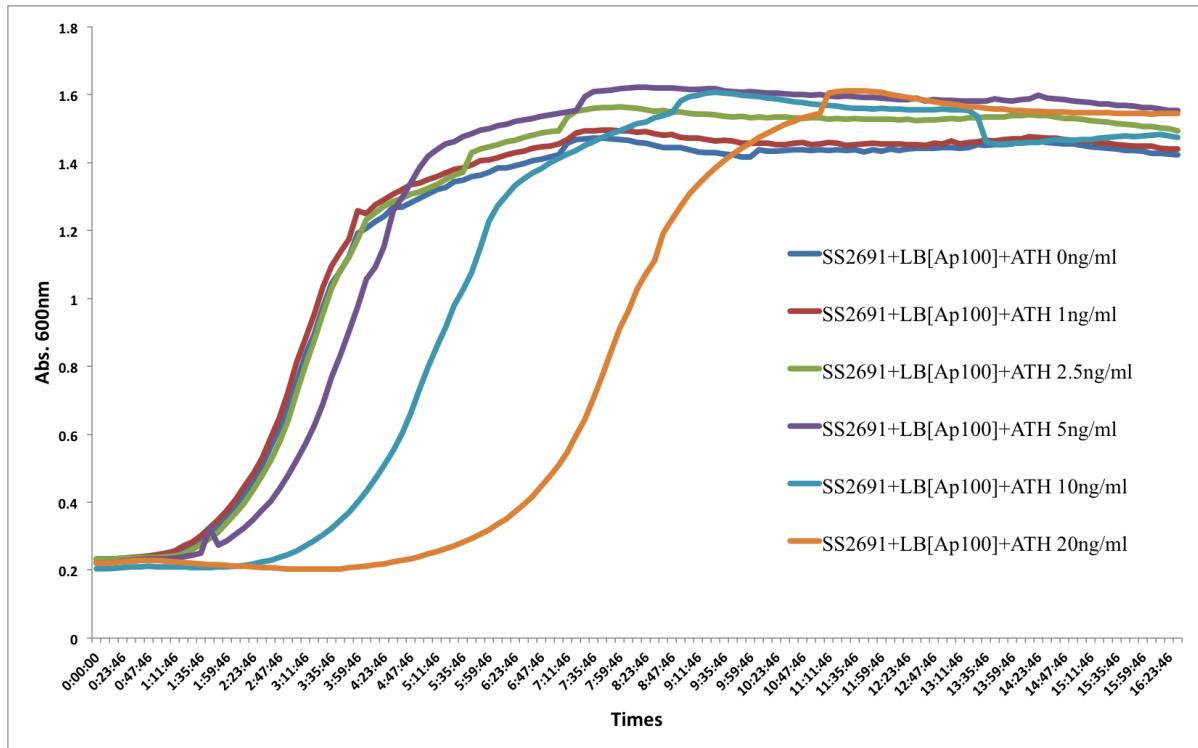
Figure. 4.2. Determination of optimal AHT concentration to induce OmpA:Strep-tag II:GpIX fusion protein from *Ptet*. SS2691, an *E. coli* ER2738 carrying pZT44, was grown at 37°C with shaking in 1 ml of volume in the wells of a 24-well microplate with various concentrations of AHT. The growth curve was performed in a BioTek Cytation 3 microplate reader.

Frist, I cloned the pIX gene and the flanking regions from M13KE phage into pSU39 to generate pZT41. I then inserted a SmaI-cut blunt-ended *aacCI* gentamicin resistance cassette from pUCGM into the unique SanBI site of pIX on pZT41 to generate the plasmid pZT42 that carried the pIX::*aacCI* mutant allele. The validity of the pIX::*aacCI* insertion allele was confirmed via restriction endonuclease digestion as well as by PCR amplification of the allele as shown in Figure 4.4. For genetic recombineering, the 975 bp DNA fragment carrying the pIX::*aacCI* allele was PCR amplified and gel purified.

Then I isolated pIX::*aacCI* mutated phages that had undergone allelic exchange by homologous recombination in vivo. I transformed an *E. coli* strain that was recombineering proficient (SS2660) with 3:1 molar ratio of pIX::*aacCI* PCR amplified DNA and the plasmid RF form of M13KE genome. Transformants that were Gm^R and X-gal$^+$ were selected and grown to collect phage lysate. These phages were then used to infect a F^+ *E. coli* and tranfectants were selected as Gm^R Tc^R and X-gal$^+$ colonies. During optimization of the protocol, I discovered that it was important to catalyze genetic recombineering in a F^- *E. coli* to prevent production and reinfection of the wildtype phage which would lower the yield of recombinant phage genome that had undergone allelic exchange. I also reasoned that even the recombinant phage with the mutated GpIX would be packaged by the presence of the wildtype GpIX from those phage genomes that had not undergone allelic exchange. I expected production of phage particles from these recombineered cells to be sufficiently low, because they are F^- and therefore reinfection is not possible, that upon subsequent infection of the F^+ host, I would be able to isolate those transfectants that were only infected by the recombinant phage with pIX::*aacCI* allele. These recombinant phages are then unable to

form infectious phage particles on their own unless provided with the Strep-tag II:GpIX fusion protein in trans. Validation of the protocol for genetic recombineering to easily isolate those phages that had undergone allelic exchange is demonstrated in Figure 4.5.
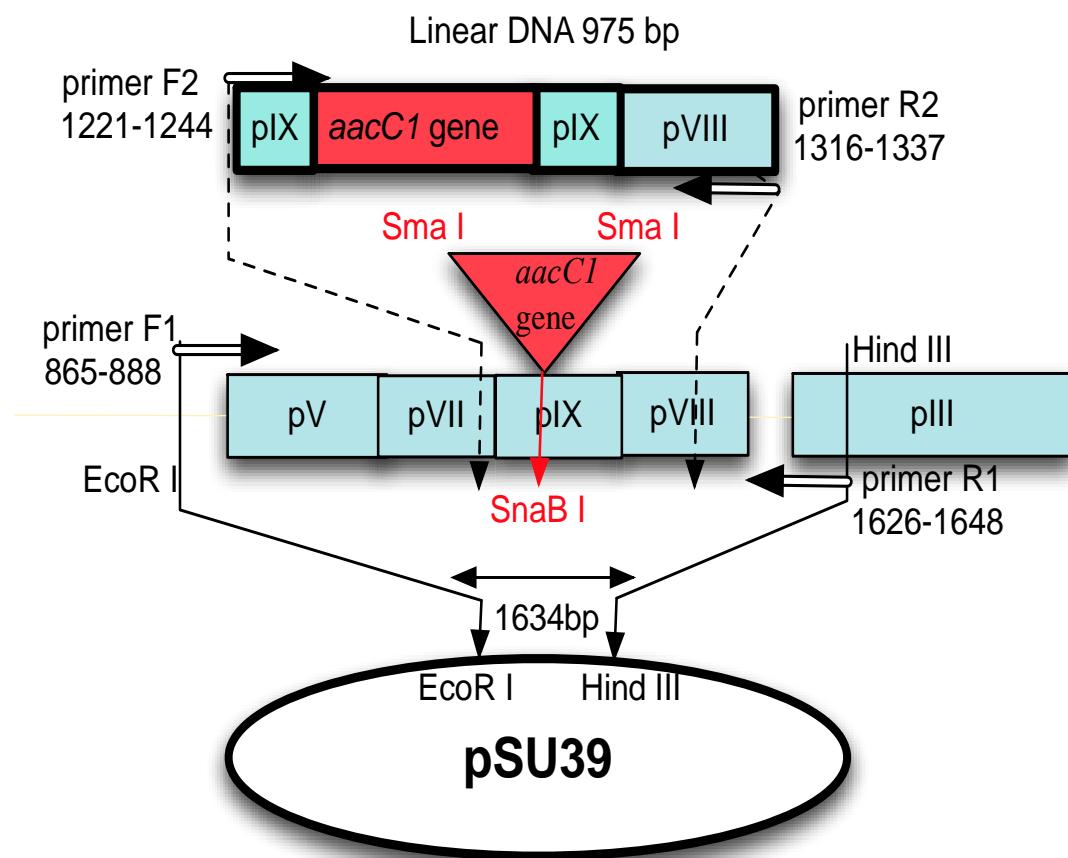
Figure 4.3. Schematic of allelic exchange via genetic recombineering to generate pIX::*aacCI* mutants of Ff class of phages. The M13KE pIX gene was amplified by primer F1/R1 and inserted between the EcoRI and HindIII sites in pSU39. A unique SanBI site in the middle of pIX was used to insert the *aacCI* cassette to generate a pIX::*aacCI* insertion mutation allele. .
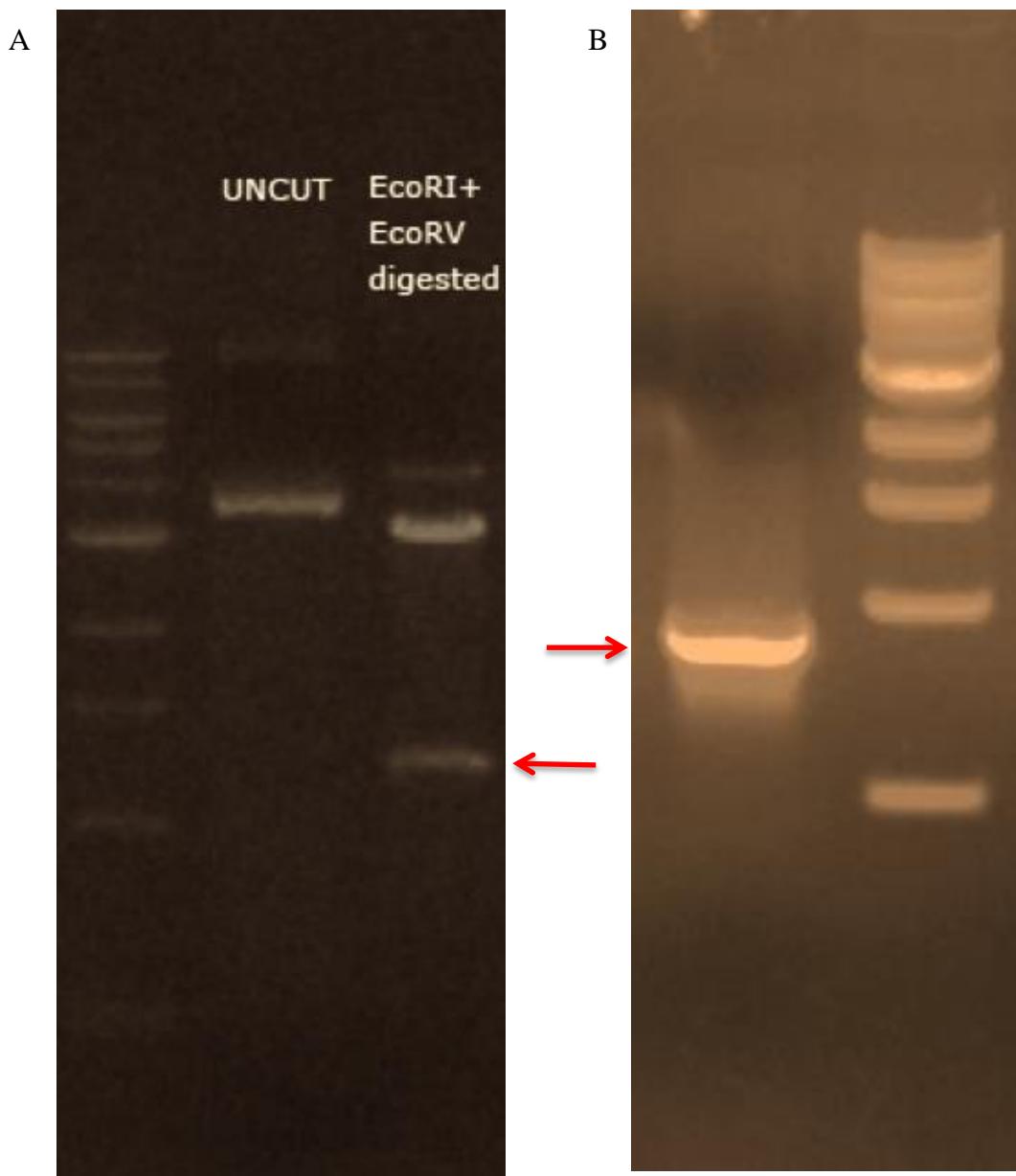
Figure 4.4 Confirmation of a pIX::*aacCI* insertion mutant allele construct. A. pZT44 was digested with EcoRV and EcoRI restriction enzymes to demonstrate the presence of a 1165bp fragment. B. PCR confirmation of the pIX::*aacC1* allele in pZT44 showing amplification of a 975bp fragment corresponding to the expected size of the pIX::*aacCI* fragment.
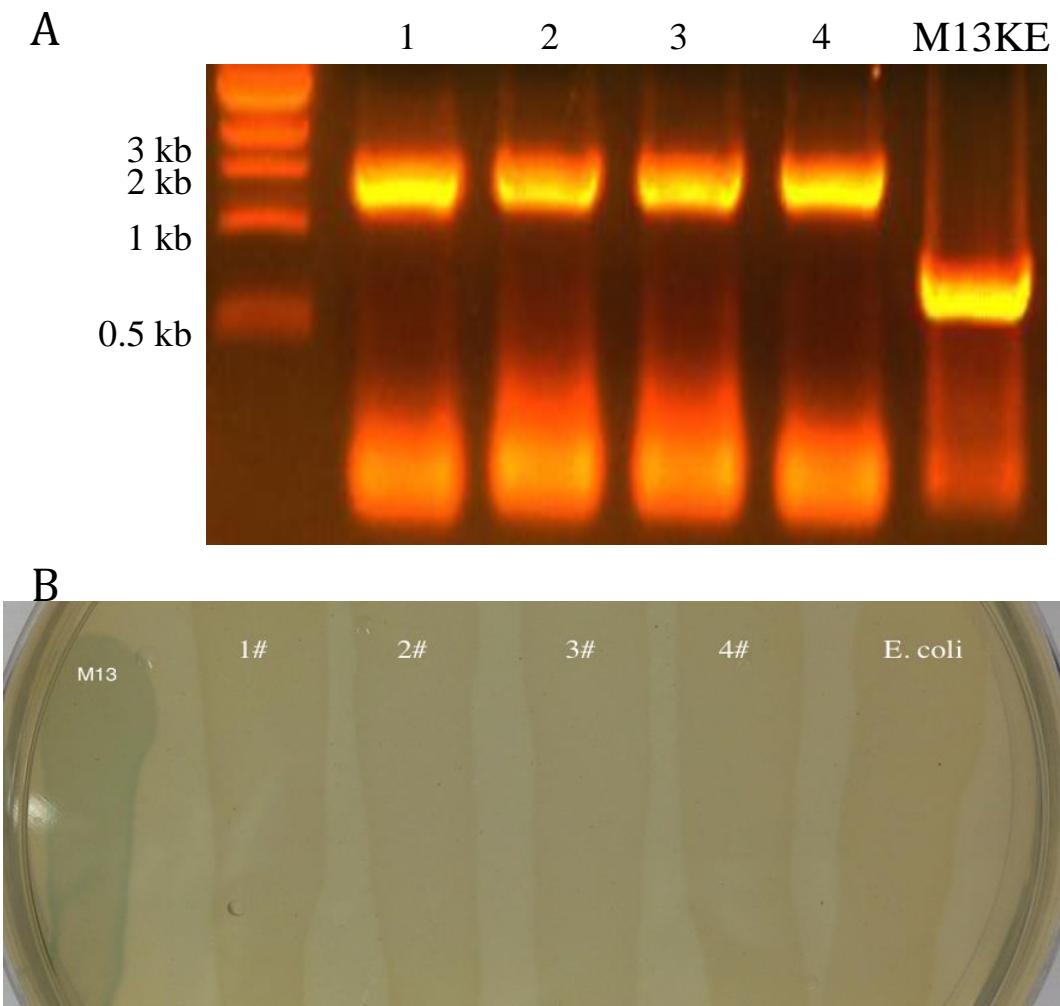
**Figure 4.5. Confirmation of M13KE pIX*::aacCI* mutant.** A. PCR validation of the pIX::*aacCI* allele in the phages 1 through 4 that were isolated following genetic recombineering and infection of the F^+ host as a Gm^R phage. PCR was performed with F1 and R1 primers that yielded ~800 bp fragment for M13KE and ~1600 bp for pIX::*aacCI* mutant. B. Infection of ER2738 with the culture supernatants from putative mutants 1 through 4. M13KE phage lysate was used as a positive control. None of the culture supernatants of 1 through 4 contained infected phage particles as demonstrated by lack of X-gal$^+$ phenotype of blue color on X-gal plates.

Immobilization of Strep-tag II:GpIX affinity tagged phages on streptavidin

In order to produce Strep-tag II:GpIX affinity tagged phages, I transformed ER2738 carrying a TA1 pIX::*aacCI* mutant with pZT44. Phage TA1 is a M13KE derivative that specifically binds to *Salmonella enterica* serovar Typhimurium (Chapter 2). Growing the transformants on LB supplemented with Ap Gm and 10 ng/ml of AHT to induce Strep-tag II:GpIX at 37°C with shaking at 220 rpm for 12 hours yielded 10^{11} pfu/ml. The harvested phages were tested for binding to streptavidin by phage-capture ELISA with plates coated with streptavidin. I tested two different concentrations of streptavidin (2 μg/ml and 20 μg/ml) to coat the wells of a microtiter plate. The data are presented in Figure 4.6. Both non-tagged TA1 and M13KE bound to streptavidin-coated plates minimally. However, the Strep-tag II:GpIX tagged phages bound streptavidin-coated plates approximately four to five-fold better than the non-tagged phages.

In this study, I utilized streptavidin as the affinity molecule to assess the performance of the tag. The data clearly demonstrated the success of the phage tagging system. I believe that since Strep-tag II has almost 70-fold higher affinity to StrepTactin than to streptavidin (46), the tagged phages will bind to any sensor platform coated with StrepTactin even better than to the platforms coated with streptavidin. This phage tagging system is simple and takes only three days to efficiently tag any Ff class of filamentous phage with Strep-tag II:GpIX that can be readily immobilized on a sensor platform coated with StrepTactin or streptavidin.
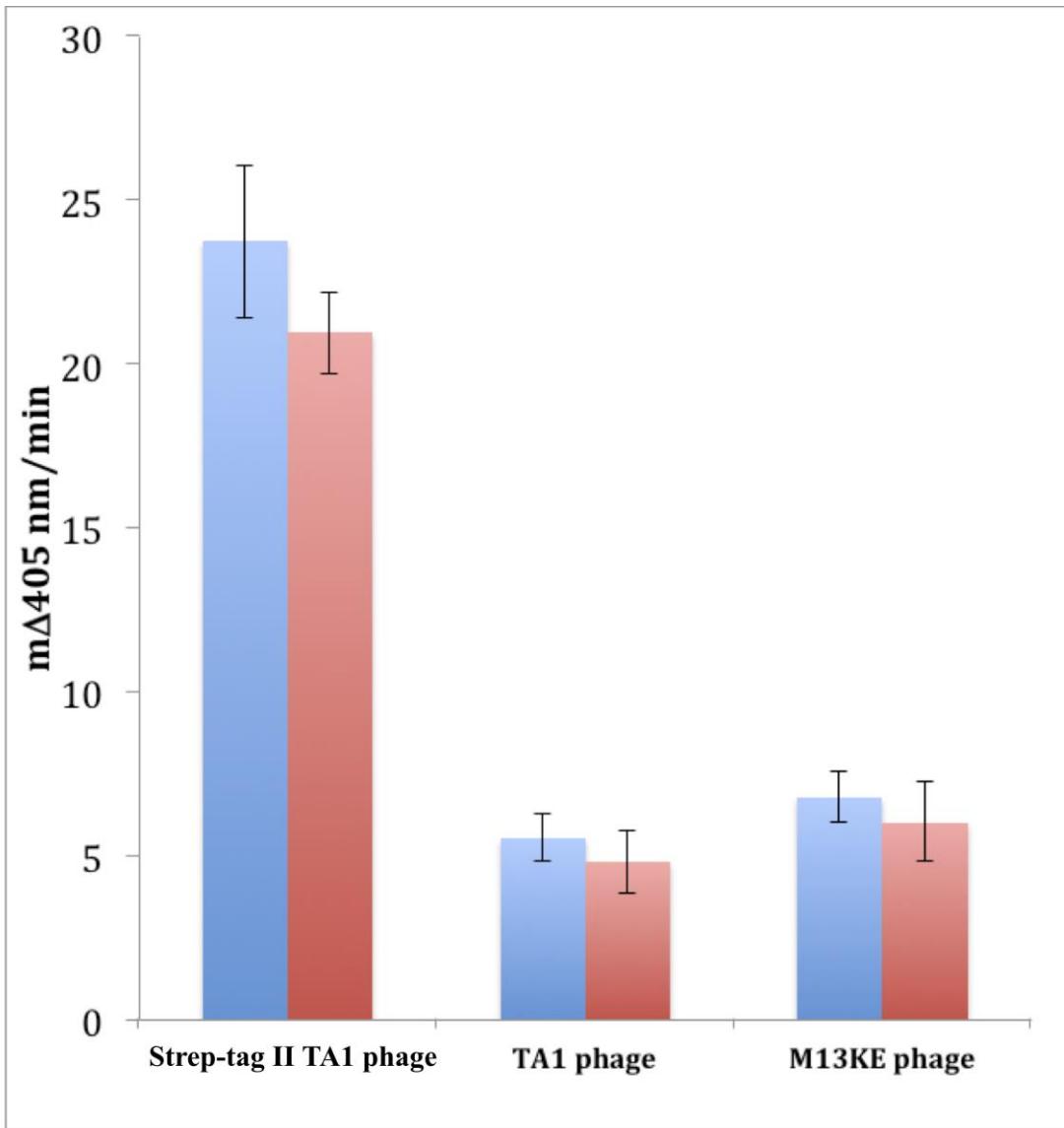
Figure 4.6. Phage-capture ELISA. 10^9 phage particles were added to ELISA plate wells that had been coated with either 2 μg/ml (■) or 20 μg/ml (■) concentrations of streptavidin. Absorbance at 405 nm represents the rate of the reaction of the conjugated alkaline phosphatase and is shown as $m\Delta OD_{405}$ which is ΔOD_{405} x 1000. The data shown are representative of at least two independent experiments with triplicate samples.

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Chapter 5

Validation of improved methods of probe isolation and immobilization on magnetoelastic biosensors for detection of *Salmonella enterica* serovar Typhimurium

Abstract

In previous chapters, I improved the methodologies for isolation of better probes from phage-displayed oligopeptide library and immobilizing the phage probes on magnetoelastic sensor platforms in a uniform monolayer to enhance pathogen detection. In this study, I demonstrate the effectiveness of these developed methodologies. I affinity tagged the *Salmonella enterica* serovar Typhimurium specific phage probe TA1 with Strep-tag II on GpIX and immobilized the phage on a streptavidin coated 0.028 mm x 0.4 mm x 2 mm magnetoelastic (ME) particle. I then used the ME biosensor to capture *S.* Typhimurium. I determined that the linear of range detecting *S.* Typhimurium was from $10^2 - 10^6$ bacterial cells with the 2 mm sensor. The biosensor was highly selective for *S.* Typhimurium and did not capture *S.* Enteritidis, *S.* Newport, or *Escherichia coli* O157:H7. Finally, I made directly compared the TA1 biosensor with a same sized biosensor that had been immobilized with E2, a previously used *S.* Typhimurium phage probe. These results demonstrate that the TA1 biosensor is at least 2 to 4 fold more sensitive than the E2 biosensor for detecting 10^2 to 10^6 cells of *S.* Typhimurium. Thus, the results validate the various improvements I made to enhance the efficacy and performance of the magnetoelastic biosensors immobilized with

phage-displayed oligopeptide probes.

Introduction

Pathogenic serovars of *Salmonella enterica* pose serious public health concern as foodborne and zoonotic pathogens (1-4). This fact was emphasized by several recent outbreaks of *S. enterica* in the United States (5-16). In order to reduce outbreaks of foodborne pathogens, continued surveillance is required. Unfortunately, approximately 2 to 4 weeks are currently needed to confirm identity of the causative agent in an outbreak. Such a long delay in identification of the causative agent inhibits a quick response. Thus, rapid detection of foodborne pathogens is essential to initiate a quick response for public safety. Rapid and reliable identification of pathogens allows for effective disease management decisions including timely application of protective measures such as antibiotic treatment, eradication of infected animals, and recall of contaminated product.

Although traditional methods are highly accurate, the time required (up to several days) to acquire large quantities of the pathogen from foods or clinical samples make them less than ideal for timely prevention of outbreaks. In order to circumvent major problems associated with traditional culture dependent diagnostics, culture-independent diagnostic tests (CIDTs) including immunoassays, nucleic acid assays, and label-free biosensor assays have been developed. These modern assays are fast, sensitive and highly accurate. Unfortunately, they are also costly and require extensive training of personnel.

Biosensor technology combines the accuracy and sensitivity of the other (1-16) approaches with a great enhancement in the rapidity of detection. A typical biosensor is composed of molecular probes immobilized on a platform with characteristics that can be easily measured based on the probe:analyte interactions (17). As long as the molecular

recognition probe is selective, biosensors can be used even in complex matrices (blood, stool, urine or food) with minimum sample pretreatment (18, 19). Based on the method of signal transduction, biosensors can be divided into four basic groups: optical, mass, electrochemical, and thermal sensors (20, 21).

More recently, much effort has been devoted to development of biosensors for detection of foodborne pathogens. These include single-stranded DNA aptamer based noncovalent self-assembly of single-walled carbon nanotubes (SWNTs) for *Salmonella enterica* serovar Paratyphi (22), spectroscopy based Fourier transform (FT) Raman spectroscopy for *Escherichia coli* O157:H7 (23) and optical based two-dimensional angle-resolved light scattering (ALS) for individual rod-shaped bacteria (24). One of the most exciting developments has been the mass perturbance biosensors composed of peptide/protein molecular recognition probe and magnetoelastic particles. These biosensors show a great promise for their sensitivity, low cost, and user-friendliness (25).

Typical molecular probes used in biosensors include enzymes, nucleic acids antibodies, live cells, and phage-displayed oligopeptides (17). The use of biosensors with immobilized antibodies to detect HIV (26) and *Salmonellae* (27, 28) has been reported since 1990s with the relative selectivity and sensitivity that is comparable to ELISA (28, 29). For *S. enterica* serovar Typhimurium, a biosensor using an antibody as the probe was highly specific and gave a linear response from 10^5–10^9 cells/ml microbial suspension with the limitation of detection at 5×10^3 CFU/ml (30). Although highly valuable due to the specificity, the main disadvantages of antibody probes are poor environmental stability (31) and the cost and time associated with production of specific antibodies. In contrast, phage displaying peptides or proteins can be selected, isolated, and rapidly reproduced at low cost to be used as molecular probes. In addition, the filamentous phage-displayed oligopeptide probes are robust because they are resistant to heat (up to 80°C) (31, 32), organic solvents

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like acetonitrile (33), urea (up to 6 M), acid, alkali and other chemicals (34, 35). Thus, phage probes are good candidates to be used with ME particles to form viable biosensors.

Magnetoelastic biosensors using the landscape phages have been highly successful in yielding probes that specifically recognize *Bacillus anthracis* spores (36-41) and *S.* Typhimurium (29, 42-48). A standard label-free biosensor is composed of molecular recognition probes immobilized on a sensor platform. While the sensitivity and ease of measurement are characteristics of the sensor platform, selectivity and avidity are properties of the molecular probe. Magnetoelastic (ME) biosensors composed of phage-displayed peptide probes and ME particles have shown significant promise for their sensitivity, cost effectiveness, and user-friendliness.

In previous chapters, I described the efforts to improve the phage:magnetoelastic biosensors. In Chapter 2, I improved the probe isolation strategy and used it to isolate several *S.* Typhimurium specific probes, including TA1. In Chapter 4, I developed a novel affinity-tag mediated immobilization of phage-displayed oligopeptide probes on ME biosensor platforms. In this study, I demonstrate the efficacy of the improved methodologies by capturing *S.* Typhimurium with a ME biosensor that has been immobilized with the affinity-tagged TA1.

Materials and Methods

Bacteria, media, chemicals, and growth conditions

S. Typhimurium (ATCC 13311), *S.* Enteritidis (BAA-708), *S.* Newport j1890, were grown in nutrient broth (NB) and confirmed for identity, propagated, and maintained by MacConkey Agar (ACUmedia, Lansing, MI). *E. coli* O157:H7 (ATCC43888) was grown in Lysogeny broth (49) at 37°C with aeration at 220 rpm unless otherwise indicated. When necessary, media were solidified with 1.5% Difco Bacto Agar (BD, Franklin Lakes, NJ). Antibiotics and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Sigma-Aldrich (St. Louis, MO), and 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was purchased from Gold Biotechnology (St. Louis, MO). The following chemical concentrations were used in this study (per milliliter): tetracycline, 20 μg; X-gal, 50 μg; and IPTG, 40 μg.

All bacteria were grown in an appropriate growth medium at 37°C for overnight in a New Brunswick (Enfield, CT) shaking water bath incubator. 10ml of bacterial cells were collected by centrifugation at 5000 rpm for 10 min, washed twice with 10 ml of 1X TBS (Tris Saline Buffer) and adjusted to OD_{600} 1.0 with 1X TBS.

E. coli ER2738 (New England Biolabs, Beverly, MA) was used as the host strain for phage propagation.

Isolation of Strep-tag II:GpIX tagged phage particles

SS2660, a DH10B derivative carrying pKD46, was made recombineering competent as previously described (50). Then a 975 bp PCR product carrying the pIX::*aacCI* insertion

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allele and the RF plasmid of TA1 genome were electroporated into the recombineering ready SS2660. Recombinant TA1 that have undergone allelic exchange between the pIX::*aacCI* mutant and the wildtype allele were selected as Gm^R and $X\text{-gal}^+$ transformants that were incapable of producing infectious phage particles. Then, pZT44 was introduced into the lysogen that carried the recombinant TA1, the Strep-tag II:GpIX expression was induced with 10 ng/ml of anhydrotetracyclin, and the cells were grown at 37° with shaking at 220 rpm for 12 hours to produce Strep-tag II:GpIX tagged TA1.

Immobilization of Strep-tag II tagged phage probes to streptavidin coated ME platforms

ME sensor platforms (0.03mm × 0.4 mm × 2 mm) were a generous gift from Dr. Bryan A. Chin's laboratory at the Materials Research and Education Center, Auburn University, Auburn, AL. I immobilized untagged phages on ME platforms as previously described using 10^{12} pfu/ml of phage particles (51-53). Briefly, the ME particles were immersed in 200 μl of 10^{12} pfu/ml of phage for 12 hours and washed with a 500 μl of TBS to remove any unbound and loosely-bound phage. To immobilize Strep-tag II tagged phages, I first coated the ME platforms with 200 μl of 1mg/ml streptavidin solution in milli-Q water supplemented with 1mM EDTA and 0.02% NaN_3. The ME platforms were soaked overnight at 4°C and washed twice with in 500 μl of TBS. Then the streptavidin coated ME particle was immersed in 200 μl of 10^{12} pfu/ml of Strep-tag II tagged TA1 phage for four hours at room temperature and washed with TBS to remove unbound phage. Phage bound sensors were stored at 4°C and washed extensively with TBS prior to being used.

To determine the extent of immobilization, ME particles bound with untagged phage and tagged phage were washed 13 times with 100 μl of TBS to collect any phages that came off the sensor. Collected phages were then titered to determine whether the ME particles were

leaching phages with each wash.

Construction of a flow-through analysis system

ME biosensor analysis chamber was constructed to detect the activity of the sensor in a constantly flowing condition. In this system, the sensor is placed in the center of the inner glass chamber which is then inserted into the outer glass chamber coated with a single-layer of solenoid coil. This system is based on the previously published research (47, 52-54) with the modification of having an inner glass tube. The inner glass tube (0.63 mm diameter from Drummond Scientific Co, Broomall, PA), was used for each sample to prevent damage to the outer glass tube that was coated with the solenoid coil. The system was calibrated for 0.1 ml/min flow rate.

Pathogen detection

Resonant frequency measurements of ME biosensors were performed in an electrical circuit that consists of a network analyzer (HP/Agilent 8751A, Agilent Technologies, Inc., Santa Clara, CA, USA), operated in the S11 mode, with a single-layer solenoid coil wound around a outer glass chamber (1mm inner diameter, Drummond Scientific Company, Broomall, PA, USA) with 102 μm copper wire. Static and time-varying magnetic fields were simultaneously applied to an ME biosensor inside the solenoid coil for magneto-mechanical excitation. The resulting response signal was wirelessly detected with the same coil as illustrated in Figure. 5.1. The ME sensor was first moved into the inner glass chamber and then that apparatus was inserted into the outer glass chamber. The pump tube then was connected to the inner glass chamber directly.   The micro-flow pump provided a continuous flow of TBS at 0.1ml/min through the inner glass chamber with the "averaging 16 times"

function of network analyzer turned on and the frequency detection span set as 20KHz. An initial steady-state resonant frequency can be obtained within thirty minutes with this set up. To introduce bacteria, micro-flow pump was turned off and the inner glass tube was connected to a 1 ml syringe containing 1 ml bacterial culture. Pump was turned back on and the frequency was recorded every two minutes. After 10 min, the inner chamber was washed with PBS for 10 minutes and frequency was recorded every two minutes. The frequency difference between the initial (prior to exposure to bacteria) and the final steady state during TBS washing was determined.

Results and Discussion

Comparison of phages bound to ME platforms via physical adsorption vs affinity tag

One of the concerns of working with phage immobilized ME particles is the possibility of removing the bound phages with each washing step. I assayed whether the affinity tag immobilization resulted in stronger binding and thus an advantage over non-affinity bound phages. Untagged and tagged TA1 was prepared and immobilized onto ME particles. Then the ME particles were extensively washed (13 times) and the presence of phage in each wash was determined. The results are shown in Figure 5.1. The phage that was immobilized by the traditional physical adsorption came off with each wash while very few of the affinity-immobilized phages were removed. Thus, the data clearly demonstrate the advantage of immobilizing the phage probes via affinity tag. Because I used Strep-tag II, which has almost 70-fold stronger affinity to StrepTactin (55) than streptavidin I used in this study, I expect that use of StrepTactin will almost completely abolish any phages being removed with wash.

Comparison of affinity-tag immobilized biosensor with physical adsorption biosensor

To test the efficacy of these phage probe improvements, I first determined the advantage of the affinity tag immobilized TA1 biosensor. TA1 phage is a highly selective *S.* Typhimurium probe I isolated in Chapter 2. I tagged TA1 with Strep-tag II:GpIX and immobilized them on a streptavidin coated 0.028 mm x 0.4 mm x 2 mm ME particle to generate the *S.* Typhimurium specific TA1 biosensor.
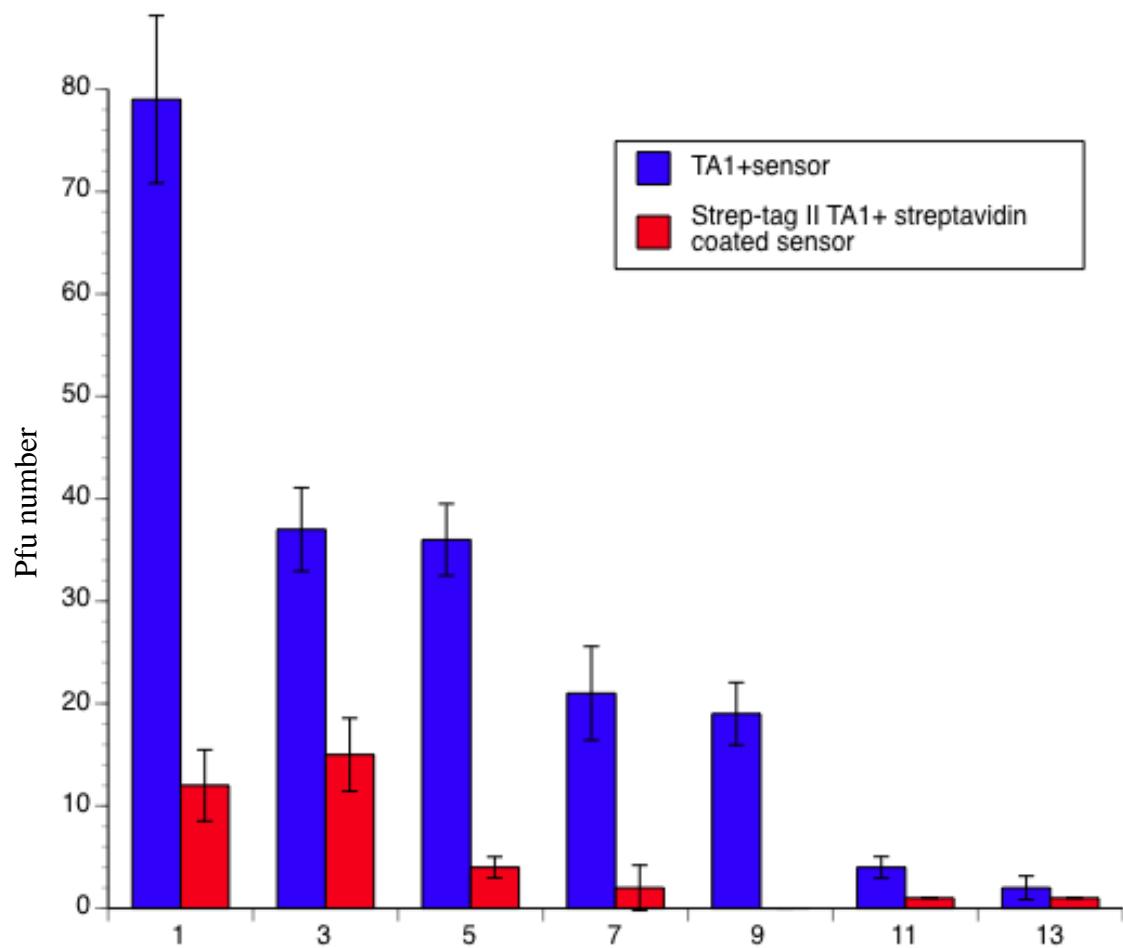
Figure 5.1. Comparison of phages immobilized to ME particles via physical adsorption vs affinity tag. Untagged TA1 was immobilized on a standard ME particle while Strep-tag II tagged TA1 was immobilized on a streptavidin coated ME particle. Each ME particle was washed with 100 μl of TBS for 13 times. The washes were then used to infect ER2738 to determine the presence of phage via counting for pfu on LB plate supplemented with 20 μg/ml of tetracyclin. The data shown are a representative from two independent experiments performed with three sensors each time.
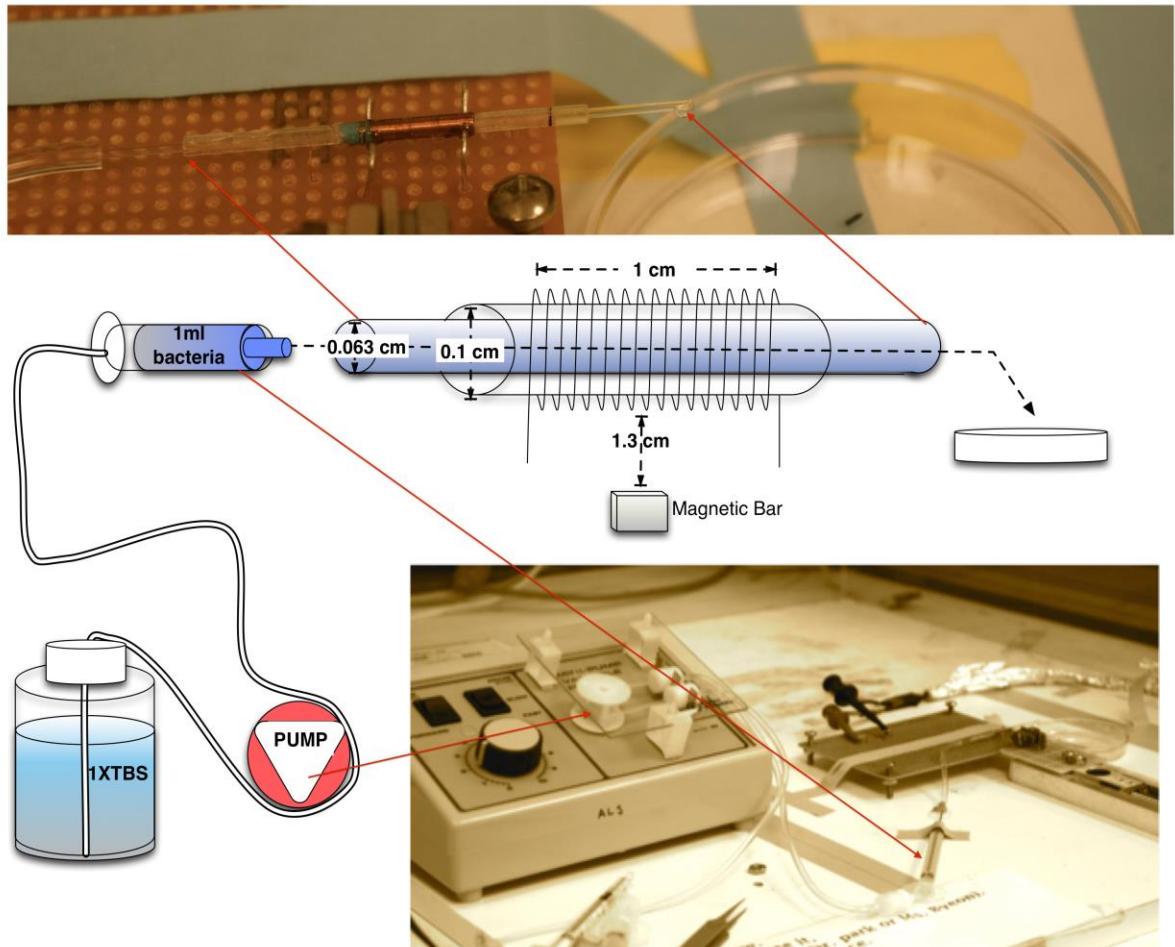
Figure 5.2. Flow-through analysis system for measuring resonant frequency change of a ME particle.

I then compared the performance of the TA1 biosensor with blank ME particle, Strep-tag II tagged TA1 immobilized on a ME particle, untagged TA1 immobilized on a ME particle, and untagged TA1 immobilized on a streptavidin coated ME particle. The resonant frequency change of the ME particles were then measured with the flow-through analysis system illustrated in Figure 5.2 these results are shown in Figure 5.3. Each biosensor was exposed to four different concentrations of *S*. Typhimurium from 10^3 to 10^6. As clearly demonstrated in Figure 5.3, the TA1 biosensor with Strep-tag II:GpIX TA1 immobilized on streptavidin coated ME particle out performed other biosensors including when TA1 was immobilized on a ME particle via physical adsorption. These data validated the hypothesis that non-uniform binding by physical adsorption may inhibit proper function of the phage probes by masking the analyte-binding domains. Thus, affinity tag mediated uniform immobilization of phage probes increases the capability of the ME biosensors.

Comparison of the TA1 biosensor with the E2 biosensor

E2 is a landscape phage probe that has been extensively used for *S*. Typhimurium detection as a ME biosensor (29, 42-48). Being a landscape phage probe, E2 has a high avidity (56). I hypothesized that although TA1 only has five analyte binding   because phages with exposed analyte binding sites on a ME platform, the TA1 biosensor is at least as sensitive as the E2 biosensor with comparable avidity.   The data are shown in Figure 5.4. Beginning with 10^2 bacterial cells, the TA1 biosensor was clearly shown to be more sensitive with greater resonant frequency changes than the E2 biosensor.   Greater the resonant frequency change for a given number of analytes binding, the more accurate the measurement. Thus, I are confident that the TA1 biosensor is better than the E2 biosensor.   However, based on the data, E2 appears to be a
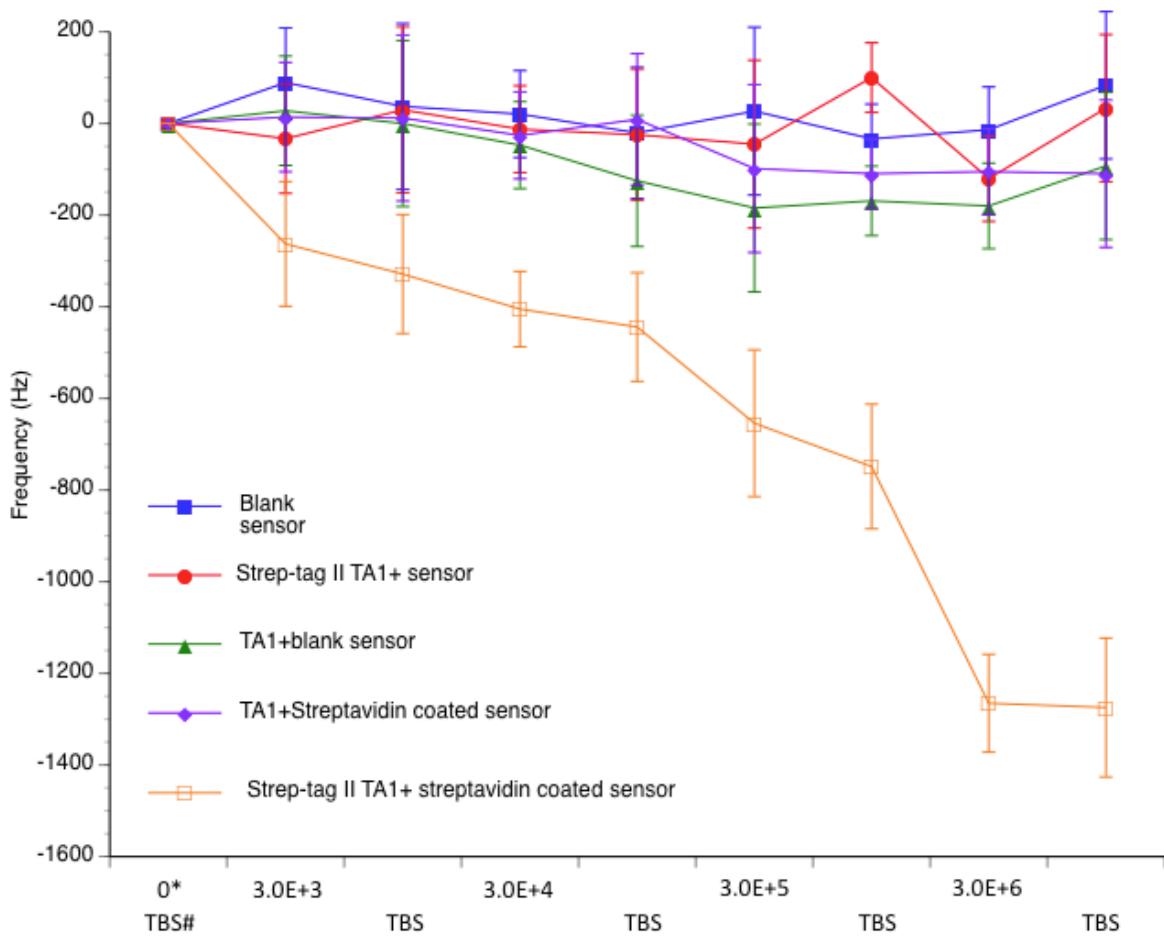
Figure 5.3. Comparison of affinity tag immobilized biosensor versus physical adsorption immobilized biosensor. *S*. Typhimurium was added to the sensor in step-wise increasing concentrations separated by TBS wash. Throughout the experiment, flow rate of 0.1 ml/min was maintained and the resonant frequency change was monitored every two minutes for a total of ten minutes per sample. The resonant frequency shown is an average of five time points per sample. The data shown are a representative of at least three independent experiments with duplicate samples.

better biosensor when the bacterial concentration is greater than 106 whenTA1 biosensor reached the maximum binding. Thus, E2 biosensor still has better avidity than TA1 biosensor. However, TA1 is the better biosensor for detection of low concentrations of S. Typhimurium because it has a lower limit of detection than the E2. These experiments were performed on a relatively large ME particle of 2 mm in length. Thus, utilization of a smaller particle is expected to result in lowering the limit of detection even further to even single cells.

Selectivity of the TA1 biosensor

Selectivity of the TA1 biosensor was determined. I tested the TA1 biosensor detection of *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, and *E. coli* O157:H7. These are all important foodborne pathogens and I wanted to confirm that the TA1 biosensor is highly selective for *S.* typhimurium. As demonstrated in Figure 5.5, the TA1 biosensor only detected *S.* Typhimurium and failed to detect even closely related cousins *S.* Enteritidis and *S.* Newport even at concentrations of greater than 10^7 cells. These data validated the probe selection strategy described in Chapter 2.
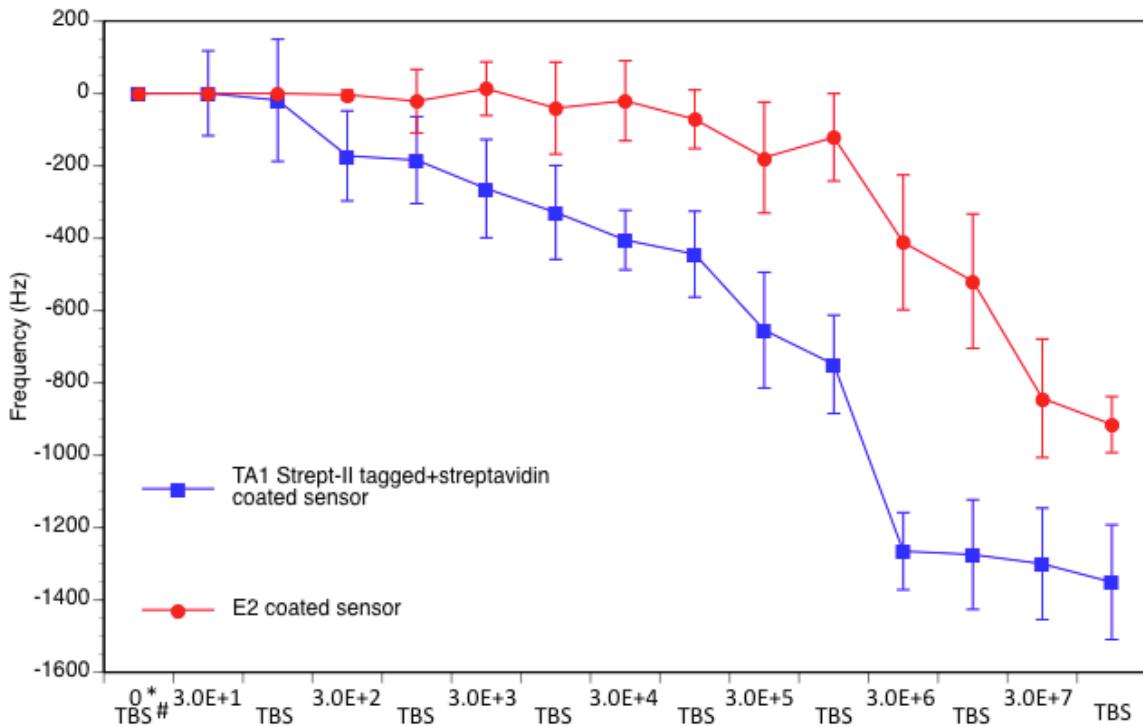
Figure 5.4. Comparison of TA1 and E2 biosensors. *S.* Typhimurium was added to the sensor in step-wise increasing concentrations separated by TBS wash. Throughout the experiment, flow rate of 0.1 ml/min was maintained and the resonant frequency change was monitored every two minutes for a total of ten minutes per sample. The resonant frequency shown is an average of five time points per sample. The data shown are a representative of at least three independent experiments with duplicate samples.
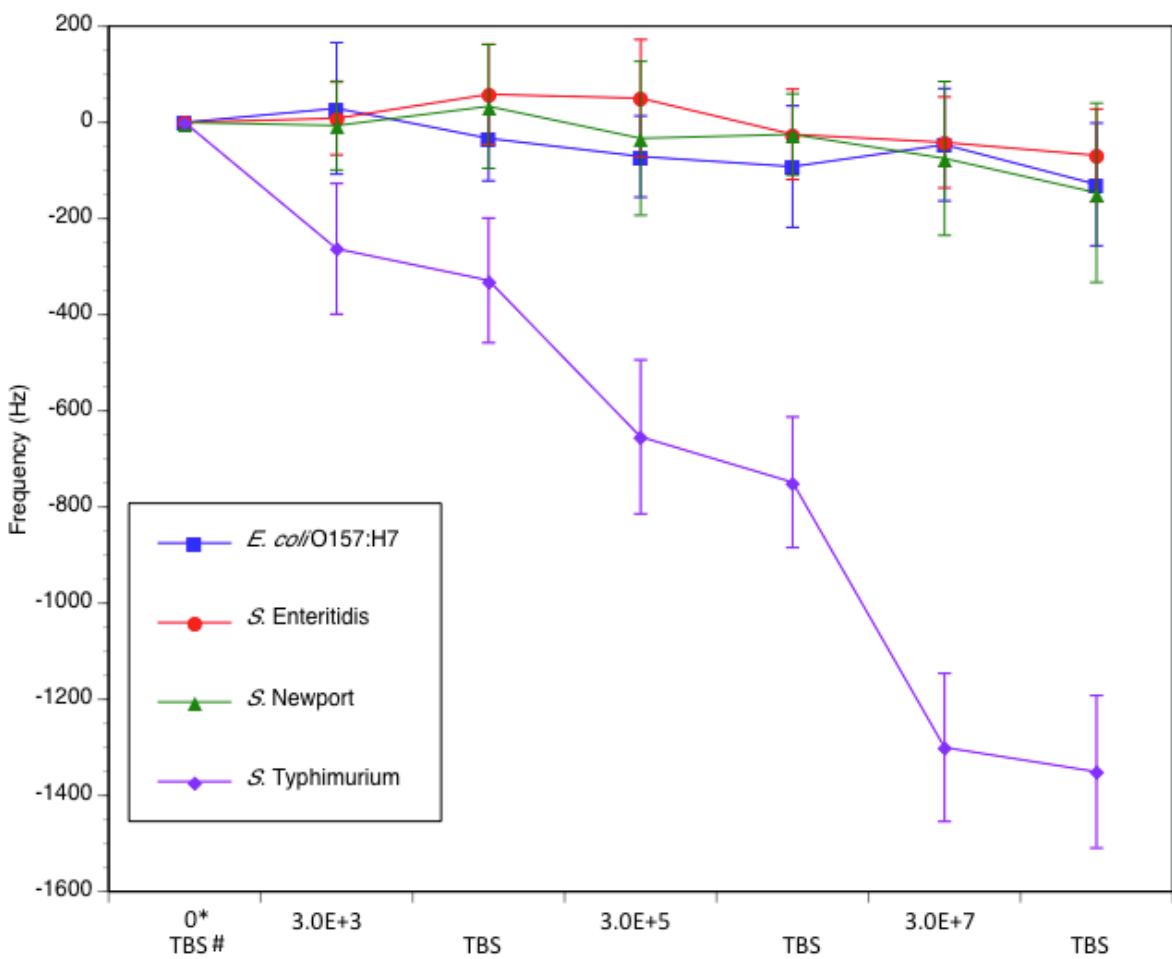
Figure 5.5. Selectivity of the TA1 biosensor. Bacteria were added to the sensor in step-wise increasing concentrations separated by TBS wash. Throughout the experiment, flow rate of 0.1 ml/min was maintained and the resonant frequency change was monitored every two minutes for a total of ten minutes per sample. The resonant frequency shown is an average of five time points per sample. The data shown are a representative of at least three independent experiments with duplicate samples.

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Chapter 6

Conclusions and Future Directions

Conclusions

As demonstrated by frequent outbreaks of salmonellosis and recalls of *Salmonella* contaminated foods, reducing contamination by this pathogen is important for health of the consumers as well as for the food industry in the United States of America. The magnetoelastic (ME) biosensors using phage-displayed oligopeptides as probes have the advantage over other technologies because they are fast, cost effective and require minimal sample manipulations. Overall, the research efforts improved the efficacy of magnetoelastic biosensors for rapid detection of important pathogens. Specifically, I improved the isolation and attachment of phage-displayed oligopeptide probes to the ME sensor platforms. These optimizations significantly improved the selectivity and sensitivity of the ME biosensor.

Optimizing the phage-displayed oligopeptide probe from isolation to immobilization

The traditional biopanning procedures can frequently yield probes with cross-reactivity. I developed a biopanning procedure that yields more selective probes. A major focus of this procedure is on extensive negative selection with closely related analytes. Several biopanning with closely related analytes successfully depleted potential probes with

cross-reactivity as described in Chapters 2 and 3 of this dissertation. Specifically for the research, I used a total of fourteen serovars of *Salmonella enterica*. Thus, when I isolated probes for detecting *S*. Typhimurium, I performed extensive negative selections with the other thirteen serovars of *S. enterica* as well as with *Escherichia coli* O157:H7 and *Shigella sonnei* and *S. flexneri*. In addition to negative selection, I determined that liquid-phase biopanning eliminated potential bias of analyte binding to the plastic and yielded more selective probes. Thus, combining both the extensive negative selections and liquid-phase biopanning, I isolated and characterized several highly selective *S*. Typhimurium phage-displayed oligopeptide probes (Chapter 2).

During these efforts to optimize probe isolation, I developed a more accurate way to do ELISA when using live bacterial cells as the ligand. This approach is based on liquid-phase biopanning and, again, eliminates the potential bias of the ligand binding to the plastic variable affinity.

It has become obvious in recent years that multiple serovars of *S. enterica* are capable of causing outbreaks. Therefore, it is desirable to be able to quickly detect whether the causative agent of an outbreak is *S. enterica* even before identifying the serovar. To meet this demand, I isolated several phage-displayed oligopeptide probes that recognize all of the serovars I used in this study (Chapter 3). These efforts succeeded only because I developed a novel biopanning strategy that enhanced the chance or isolating probes that recognized "universal epitopes" present in all of the serovars used in the study.

In addition I developed a novel approach to immobilize the phage-displayed probes on the sensor platform (Chapter 4). Presently, phage probes are immobilized via physical

adsorption method that does not elicit formation of a uniform monolayer on the sensor. I hypothesized this non-uniform immobilization may interfere with optimal performance of the biosensor by masking some analyte binding domains of the probe. Thus, I developed a novel affinity tag mediated immobilization technology. To make this technology user-friendly, I developed an easy method for ensuring the tagging of 100% of the phage particles using a genetic approach. As a proof of concept, I tagged the *S*. Typhimurium specific phage probe with Strep-tag II on GpIX of the phage and immobilized it on a streptavidin coated ME particle (Chapter 5). The *S*. Typhimurium biosensor was highly sensitive, selective, and superior to the previously used *S*. Typhimurium ME biosensor for accurately detecting *S*. Typhimurium from 10^2 to 10^6 cfu/ml. I expect the sensitivity of the biosensors to increase as I use smaller ME particles.

In summary, I developed several novel methodologies to improve the efficacy of the ME biosensors couple with phage-displayed oligopeptide probes. I validated these methodologies by constructing a *S*. Typhimurium specific biosensor and demonstrating its superior capability to the existing biosensor. The methodologies I developed can be utilized to isolate probes against any organism of interest and the phage immobilization approach can be adapted for any phage probe in three short days.

Future Directions

One of the objectives to be performed in furture is to identify the ligands or the epitopes that are recognized by these isolated probes. *S.* Typhimurium probes, including TA1, are highly selective for that serovar. The question remains what surface molecule is specific to *S.* Typhimurium and missing in the other serovars? It is also important to ask the reverse question to elucidate identity of the "universal epitope" recognized by two multiserovar recognizing probes, GA04 and GS103 (Chapter 3). One possible way to identify the ligands that are recognized by the phage probes is to do an immunoblot analysis. The ligands could be any of the *S. enterica* surface molecules including saccharides and proteins. Thus, one would have to do careful extractions of various surface components, separate them by electrophoresis, transfer to membrane, probe with the phage probe, and detect with anti-phage antibodies. Alternatively, one could immobilize the phage probes on StrepTactin beads and add the beads to the various extracts containing the *S. enterica* cell envelope that includes O-antigens, core-oligosaccharides, and outer membrane proteins. Then elute the molecules that bound to the probes and identify them via mass spectrometry or nuclear magnetic resonance. Understanding the surface epitopes and the interactions between these epitopes and the cognate oligopeptide probes will help us to design even better probes with higher selectivity.

Another objectives to be pursued include manipulating the oligopeptides to find a consensus sequence among a set of probes to engineer better selectivity. To achieve this, one

could first do an in silico experiment to identify putative consensus sequence, then do an alanine scanning to identify the actual residues that are important, and genetically engineer a consensus sequence peptide. This second goal can be or should be pursued simultaneously with the first objective.

Finally, in order for any biosensor to become practical for everyday use, it is essential that the detection technology be improved. In this study, I detected mass perturbation of the biosensor in a liquid flow through system. While accurate, this system is highly fragile and difficult to use on a daily basis. Thus, a stand-off detection system like the magnetic detector in stores, is needed for biosensors. Such technology has already been developed and is currently being improved. Figure 6.1 demonstrates a prototype detection system that can monitor ME biosensors directly on a food surface (1). Currently, the remote system has to be within a couple of millimeters. I hope this system can be developed to read resonant frequency changes of ME biosensors from several feet in distance in near future.

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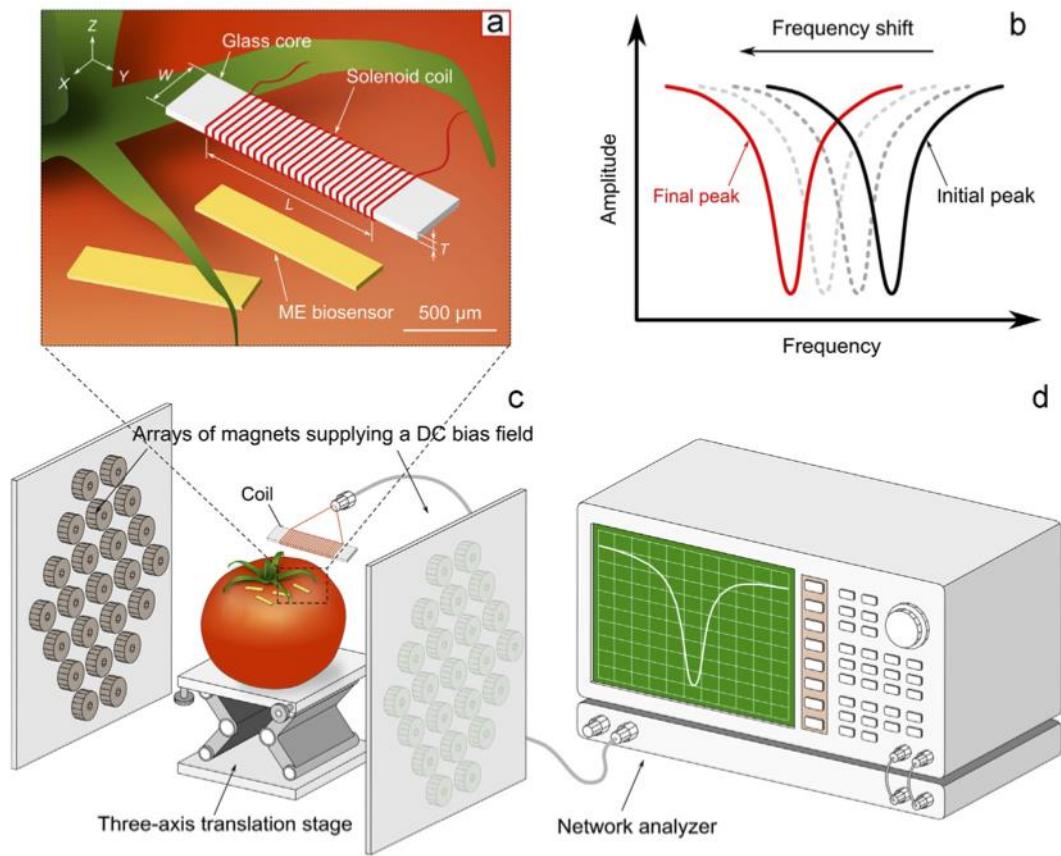
Figure. 6.1 A surface-scanning coil detector for real-time, in-situ detection of resonant frequency of ME biosensors (adapted from (1))

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