ANALYSIS AND OPTIMIZATION OF A COLORIMETRIC NANOSENSOR FOR
RAPID DETECTION OF *ESCHERICHIA COLI* IN WATER

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TITLE: Analysis and Optimization of a Colorimetric Nanosensor for Rapid Detection of *Escherichia coli* in Water

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ABSTRACT
Analysis and Optimization of a Colorimetric Nanosensor for Rapid Detection of 
Escherichia coli in Water
Sarah Mae Stabler

Safe drinking water is essential for life, yet at least two billion people around the world consume water contaminated with pathogens among other pollutants. Standard methods like polymerase chain reaction (PCR) and membrane filtration have been developed to detect enteric pathogens in water. However, these methods are limited in their accessibility due to long wait times to obtain results, and the requirements of skilled expertise, electricity, and laboratory equipment. This research has focused on addressing some of these limitations by analyzing the mechanisms of work and optimizing an indirect colorimetric nanosensor developed in previous research. The colorimetric nanosensor investigated herein relies on a competitive binding mechanism. When positively charged gold nanoparticles coated with polyethyleneimine (PEI-AuNPs) are added to a water sample containing negatively charged Escherichia coli (E. coli) and β-galactosidase (β-Gal) enzyme, the PEI-AuNPs will preferably bind to E. coli. This leaves β-Gal free in solution to hydrolyze chlorophenol red β-D-galactopyranoside (CPRG) (a substrate added to the water sample). The hydrolysis reaction of CPRG results in changing the solution color and the magnitude of this color change is a function of the amount of E. coli present in a water sample. It was hypothesized herein that the governing factor for the nanosensor functionality is the surface charge/Coulombic interactions rather than the nanoparticle composition or the type of chemical coating on the nanoparticle surface. To test the research hypotheses, positively charged nanoparticles with different compositions and chemical coatings as well as positively charged polymers were tested herein as potential detection agents for E. coli in water using the competitive
binding assay reported in the literature with some modifications. This study produced three main findings that support the research hypotheses. First, gold nanoparticles (AuNPs) were not critical to the nanosensor functionality – other positively charged nanoparticles of silver and iron oxide coated with branched PEI were able to detect *E. coli* as low as $10^5$ and $10^7$ CFU/mL, respectively. Second, the branched PEI polymer itself (i.e., without a nanomaterial) detected *E. coli* at $10^7$ CFU/mL. Third, in the absence of *E. coli*, (1-Hexadecyl) Trimethylammonium Bromide (CTAB), a positively charged polymer, inhibited the hydrolysis of CPRG by β-Gal. This inhibition suggests that other positively charged polymer types have potential applications in colorimetric detection assays that are based on the competitive binding mechanism. The observed behavior with the aforementioned sensing agents indicated that the positive charge was likely responsible for the detection of microbes using this competitive binding detection approach rather than the type of the chemical coating/agent used. These findings open possibilities for more types of recyclable and cost effective nanomaterials and polymers to be developed for detection of *E. coli* using this competitive binding approach. Furthermore, research is warranted for optimizing the sensing agents tested in this study to lower their detection limit and assess their recyclability.

Keywords: nanomaterials, nanosensor, *E. coli*, colorimetric detection, water quality
ACKNOWLEDGMENTS

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1. INTRODUCTION

1.1 Background

Safe drinking water is an essential component of life. However, it has been reported that 144 million people consume untreated surface water from lakes, ponds, rivers, and streams, while 435 million people consume their water from unprotected wells and springs (WHO, 2019). Contaminated drinking water with feces contains enteric pathogens that can cause deadly diseases such as cholera, salmonellosis, shigellosis, and those caused by pathogenic Escherichia coli (E. coli) (Cabral, 2010). The main symptom of these diseases is diarrhea which can lead to severe dehydration and often death in developing countries where the appropriate care is not accessible. The burden of disease and death translates to financial burden from healthcare and time spent away from work or school. In addition, improved water sources and accessibility will result in less time spent collecting the water and more time spent being productive in school or work.

Access to clean drinking water is the start to a healthy, productive, and striving society for communities around the world.

There are a multitude of enteric pathogens to analyze in water quality testing. Currently, no single method exists to detect all microorganisms in a water sample due to factors such as the physical differences between the major pathogen groups, the presence of inhibitors in the sample, and the determination of the pathogen’s origin (Ramírez-Castillo et al., 2015). Therefore, a microorganism that can indicate whether other enteric pathogens are present would simplify testing to just that microorganism. This microorganism is called a microbial indicator and the most common one is E. coli.
Drinking water standards set a maximum contamination level (MCL) for *E. coli* of less than one colony forming unit (CFU) per 100 mL of water (Martinez, 2020b).

The current methods to detect waterborne microorganisms can be categorized as culture dependent and culture independent/molecular methods. The culture dependent methods are those that require growing the microorganism in a culture medium under controlled laboratory conditions. Culture independent methods target nucleic acids like DNA and RNA to identify the microorganism (Cocolin et al., 2013). Culture independent methods include methods such as polymerase chain reaction (PCR), and culture dependent methods include standard methods like membrane filtration and multiple tube fermentation. Some of these methods require skilled expertise to perform and they can be time consuming (i.e., days of wait time), especially for culture dependent methods. In addition, electricity, and laboratory equipment such as incubators or filter pumps, are often needed to perform these methods. Such factors limit the accessibility of these commonly used detection methods to communities. These communities are often those most exposed to contaminated drinking water due to the lack of water treatment infrastructure.

In recent years, many research investigations have focused on developing nanomaterials-based techniques for waterborne microbial detection. Electrochemical, acoustic, magnetic, and optical biosensors have been developed to track microbial activity in water. Of the most preferred biosensors are optical assays due to their ease of implementation, low cost, and are often equipment free methods, making these sensors accessible to communities with limited resources (Choi et al., 2018). Colorimetric
biosensors are a class of optical assays that result in distinct color change observed with the naked eye when microbes are present in a water sample. These colorimetric-based detection assays can be further categorized into direct and indirect assays. A direct assay tracks the color change that occurs because of the increase in nanoparticle size when microbes are present (Choi et al., 2018). However, nanoparticles can aggregate and increase in size for other reasons including acidic pH and high ionic strength conditions (El Badawy et al., 2010). Thus, direct assay may have limited applicability when testing real environmental samples. On the other hand, an indirect assay tracks color producing reactions that are caused by nanomaterial-bacteria and nanomaterial-enzyme interactions (Thiramanas & Laocharoensuk, 2015; Miranda et al., 2011).

Previous research studies have investigated indirect colorimetric detection assays, including one for detection of *E. coli* based on the competitive binding mechanism of positively charged polyethyleneimine coated gold nanoparticles (PEI-AuNPs) to negatively charged enzymes and bacteria (Thiramanas & Laocharoensuk, 2015). In the absence of microbes and nanoparticles, the hydrolysis reaction between the chromogenic substrate chlorophenol red β-D-galactopyranoside (CPRG) and enzyme β-galactosidase (β-Gal) produces a red color in solution due to the production of chlorophenol red (CPR). When positively charged PEI-AuNPs are added to the CPRG/β-Gal mixture, the enzymatic activity of β-Gal is inhibited, and the solution remains yellow because the negatively charged β-Gal binds to the positively charged polyethyleneimine coated gold nanoparticles (PEI-AuNPs). When PEI-AuNPs, β-Gal, CPRG, and bacteria are all present in solution, the PEI-AuNPs preferentially bind to the bacteria over the β-Gal due to the higher negative surface charge of the *E. coli* compared to that of the β-Gal. This
ultimately leaves β-Gal free in solution (i.e., unbound by the nanoparticles) to react with CPRG and as a result, the solution turns red (Figure 1). The magnitude of color change is dependent on the quantity of bacteria in solution (Thiramanas & Laocharoensuk, 2015). Therefore, for a given concentration of PEI-AuNPs, lower concentrations of bacteria will result in higher quantities of free nanoparticles in solution that can interact with β-Gal and reduce its interactions with CPRG. This results in a more yellow color solution. On the contrary, higher concentration of bacteria binds more nanoparticles, which leads to more interactions between β-Gal and CPRG. This results in a solution with dark red color at the highest concentrations of *E. coli*. This detection assay has the advantages of easily observable results and a rapid detection. However, this PEI-AuNPs needs further optimization to reduce its cost, enhance its recyclability potential and reduce its detection limit. The PEI-AuNPs were used in this nanosensor application due to their high surface area to volume ratio, ease of surface modification, and ease of synthesis (Peng & Chen, 2019).

![Figure 1](image)

**Figure 1.** Competitive binding mechanism for microbial detection using positively charged gold nanoparticles (Miranda et al., 2011).
1.2 Hypothesis and Objectives

The hypothesis of this research investigation is that the surface charge of the nanoparticle, not its composition, is the governing factor in the competitive binding interactions between the nanoparticles, bacteria, and β-Gal. For example, replacing gold nanoparticles with silver or magnetic iron oxide nanoparticles should not affect the efficiency of the biosensor as long as the surface charge of the substitution nanoparticles is positive. If this hypothesis is true, then gold nanoparticles can be replaced with other types of positively charged nanoparticles that are cheaper and/or more recyclable. The other hypothesis tested herein was that PEI could also be replaced with other positively charged polymer coatings assuming that the surface charge produced by it, rather than the polymer itself, is responsible for the competitive interactions previously described. The sensing agents outlined in Table 1 were investigated in this study to test the research hypotheses.
Table 1. Sensing Agents Tested

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<th>Acronym</th>
<th>Hypotheses Tested</th>
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<td>Silver nanoparticles coated with branched polyethyleneimine</td>
<td>BPEI-AgNPs</td>
<td>Nanoparticle type (i.e., Ag or Fe$_2$O$_3$) is not the governing factor for the sensing mechanism. Therefore, any of these nanoparticles can replace gold as a nanosensor for detection of <em>E. coli</em>.</td>
</tr>
<tr>
<td>Cerium (Ce$^{3/4+}$) doped iron oxide nanoparticles coated with branched polyethyleneimine</td>
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<td></td>
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<tr>
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<tr>
<td>Iron oxide nanoparticle suspension synthesized in the laboratory and coated with branched polyethyleneimine</td>
<td>BPEI-S-IONP</td>
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<tr>
<td>Amine functionalized iron oxide nanoparticles</td>
<td>Amine-Fe$_3$O$_4$</td>
<td>The surface charge, not the type of coating, is the governing factor for the sensing mechanism in the competitive binding approach. Therefore, any of these sensing agents can replace the PEI-AuNPs.</td>
</tr>
<tr>
<td>Branched polyethyleneimine</td>
<td>BPEI</td>
<td></td>
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<tr>
<td>Chitosan</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>(1-Hexadecyl) trimethylammonium bromide</td>
<td>CTAB</td>
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The main objectives of this research were to understand the mechanism of work and optimize nanosensors that can rapidly detect low concentrations of *E. coli* in water. Based on the research hypotheses stated previously, positively charged silver and magnetic iron oxide nanoparticles were tested herein for their potential to detect *E. coli* in aqueous solution. These nanoparticles were chosen because of their lower cost compared to gold nanoparticles and the iron oxide nanoparticles’ potential to be magnetically separated from solution after use, reconditioned, and reused in more detection assays.
2. LITERATURE REVIEW

2.1 Bacterial Pathogens in Water

Having access to clean and safe drinking water is a basic human right, yet two and a half billion people globally, about a third of the world’s population, consume drinking water from sources contaminated with feces and have no access to improved sanitation (Fenwick, 2006; WHO, 2019). Consumption of such water can cause bacterial infections and result in death. More than 1.5 million children die each year from diarrheal disease, the main disease caused by contaminated drinking water. Therefore, it is critical to understand these diseases, how they are caused, and how they can be prevented (Fenwick, 2006).

More than fifty percent of the diseases associated with contaminated drinking water are caused by microbial intestinal infections where cholera is the most prominent (Cabral, 2010). Cholera is caused by the bacteria Vibrio cholerae O1 and O139 that results in acute, very intense, and watery diarrhea (Cabral, 2010; Martinez, 2020c). The case of diarrhea results in severe dehydration and can lead to death. The infectious dose of Vibrio cholerae is $10^6 – 10^9$ CFU/mL. Another disease caused by contaminated drinking water by human or animal feces is salmonellosis which is caused by Salmonella enterica (Martinez, 2020d). There are two types of salmonellosis, typhoid and paratyphoid fever, and gastroenteritis (Cabral, 2010). Symptoms can include nausea, vomiting, diarrhea, and a fever with the infectious dose being $10^4$ CFU/mL (Martinez, 2020e). Shigellosis, caused by the genus Shigella found in the intestinal tract of humans and other primates, is another disease related to drinking water (Cabral, 2010). The symptoms include fever, abdominal cramps, and dysentery where the infectious dose is $10^2$ CFU/mL (Martinez,
Escherichia coli is another bacterial species that is found in water and can cause significant illness. The different strains that can be transmitted through contaminated water are enterotoxigenic (ETEC), enterohemorrhagic (EHEC), and enteroinvasive serotypes (EIEC) (Cabral, 2010). ETEC, EHEC (serotype O157:H7), and EIEC result in similar symptoms and diseases: gastroenteritis, diarrhea, dehydration, malnutrition, and abdominal pain (Cabral, 2010). The infectious dose of EHEC is $10^{–100}$ CFU/mL; therefore, it is important to detect these bacteria at low concentrations (Martinez, 2020e).

These disease-causing bacteria are just a few of the ones that can be found in water sources. Testing water for all these bacterial species prior to deeming it safe to drink is not realistic due to the extensive testing times as well as the abundance of tests and materials that would be required. Therefore, narrowing it down to a single bacterial species (i.e., microbial indicator) that will indicate whether other bacteria are present in the water is a key step in the assessment of the water quality. The presence of this microbial indicator in water is a sign of the occurrence of other enteric bacteria. However, the absence of the indicator organism does not always ensure the absence of other enteric pathogens. Bacteria of enteric origin are those coming from human or animal intestines and therefore indicate fecal pollution if they are present. The intestines can contain nonpathogenic bacteria like Escherichia coli and Bacteroides, and pathogens, ones like the pathogenic strains of E. coli, viruses, and parasites (Payment & Locas, 2010). However, the ideal indicator organism should have the following characteristics (Payment & Locas, 2010; Martinez, 2020a):

- Should be present whenever other enteric pathogens are present and absent in unpolluted waters
• Should be useful for all types of water

• Should have a longer survival time than the most resilient enteric pathogen; therefore, it should be present in greater numbers than enteric pathogens

• Should be found in warm-blooded animals’ intestines

• Should not multiply in the environment

• Should respond to water treatment processes and natural environmental conditions similar to the enteric pathogens that are of concern

• Should be easy to isolate, identify, and enumerate, and inexpensive to test

• Should not be a pathogenic organism

In the 1890’s, *E. coli* was proposed as the primary drinking water indicator and the fecal coliform test was soon developed to test for total coliforms, which include *E. coli* (Edberg et al., 2000). This group of total coliforms has the characteristic of fermenting lactose, which results in the production of gas and acid within 48 hours of incubation at 35 °C (Martinez, 2020a). However, some of these organisms in the coliform group are found in the environment, like source waters, soils, and vegetation, and are not associated with fecal contamination (Edberg et al., 2000). Many studies indicate that *E. coli* is the only species that is undoubtedly from the intestinal tract and therefore is deemed a fecal coliform (Edberg et al., 2000). For these reasons, as well as possessing the ideal indicator organism characteristics, *E. coli* is the best biological indicator that is used in water quality testing.

*E. coli* is among the indicator organisms used in the 1989 Total Coliform Rule. This rule requires that all public water systems monitor their system for total coliform bacteria
(Martinez, 2020a). If a test for total coliforms is positive, then fecal coliforms or *E. coli* must be tested and three to four repeat total coliform samples analyzed (Martinez, 2020a). This establishes a maximum contaminant level (MCL) of less than 5% positive tests per month for public water systems that test greater than 40 samples and less than or equal to one positive test per month for public water systems that test less than 40 samples (Martinez, 2020a). In 2013, this rule was revised to include a maximum contaminant level goal (MCLG) and MCL for *E. coli* to protect against fecal contamination (EPA, 2021). The revision also requires a total coliform treatment technique, and if there is a positive test for total coliforms, then an *E. coli* test must be performed, and three repeat total coliform samples taken (EPA, 2021). Because *E. coli* has been one of the major indicator organisms, several methods have been developed and new ones are being developed to detect its presence in water. These detection methods are outlined in the following section.

### 2.2 Detection of Microbes in Water Sources

Several methods exist for detecting microbes in water sources and the selection of the methods for detection depends on a few key factors. These factors include the pathogenic microorganism of interest, the physical differences between the major pathogen groups, the enrichment and concentration of the microbes in the samples, the presence of inhibitors in the sample, and the detection of the host origin of pathogens (Ramírez-Castillo et al., 2015). Due to these many factors, there exists no single method that can analyze a water sample for all pathogenic microorganisms of interest. When determining which method is best to analyze a pathogen in a water sample, it is important to analyze the specificity, sensitivity, reproducibility of results, speed, automation, and cost.
(Ramírez-Castillo et al., 2015). These characteristics can also be optimized to design new methods of detecting microbes in water sources.

There are two main categories of methods to detect microorganisms in water, culture dependent methods and molecular methods (Ramírez-Castillo et al., 2015). Culture dependent methods include growing microorganisms in a culture medium under controlled laboratory conditions. The colonies that grow are then used to determine the type of organism and its abundance in the sample. The traditional approach to determining the type of organism is to look at its appearance or its response to biochemical tests (Kirchman, 2018). The abundance of the microorganism can be determined by plate counting techniques. The main challenge with culture dependent methods is providing the correct conditions for the microbes to grow. Microbes that require extreme growth conditions or are symbiotic and require other partners to grow, are candidates for being unculturable (Kirchman, 2018). Therefore, performing biochemical tests can only be performed for culturable species. Another disadvantage of some of the culture dependent methods is their low sensitivity and the excessive time needed to obtain results (Ramírez-Castillo et al., 2015). On the other hand, molecular methods of detecting microorganisms in water include metagenomic methods that can be sequence-based or function-based (Vester et al., 2014). Sequence-based approaches screen for genes using, for example, polymerase chain reaction (PCR) (Vester et al., 2014). Sequence-based approaches are now the standard method for gene discovery and consist of the sequencing of genes, bioinformatic analyses, and the heterologous expression of identified genes to document activity (Vester et al., 2014). Function-based approaches rely on functional expression of metagenomic libraries to identify genes or
gene clusters (Vester et al., 2014). One disadvantage of these function-based approaches is that they could require amplification if the concentration of the DNA is too low for cloning (Vester et al., 2014). These molecular methods can be specific for particular species and provide phylogenetic information about the pathogens (Ramírez-Castillo et al., 2015). They have been used for health risk assessments, the evaluation of the microbial quality of the water, the efficiency of pathogen removal in water and wastewater treatment, and as a microbial source-tracking tool (Ramírez-Castillo et al., 2015). One of the challenges of molecular methods is ensuring that the DNA of the microorganisms is pure and has the correct fragment size (Vester et al., 2014).

The categories of culture dependent and molecular methods can be broken down further into specific tools and detection methods. The specific detection methods, the water pathogens they can detect, the sample matrix type, and the advantages and disadvantages of these methods are outlined in Table 2. Some of these detection methods can only be conducted under laboratory settings, and therefore cannot be performed in the field. For remote and disadvantaged communities in developing countries that do not have access to a laboratory or electricity in some cases, it is critical that a microbial detection method be developed that can be used in the field with minimal needs for advanced analytical equipment. In addition, standardizing these techniques in the field need to include factors such as sample collection, sample concentration, sample purification, sample processing, analysis, and data collection (Ramírez-Castillo et al., 2015). Some of these factors will be analyzed for the nanomaterial sensor studied in this thesis.
### Table 2. Detection Methods for Microbes in Water (Ramírez-Castillo et al., 2015)

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>Water Pathogens</th>
<th>Sample Matrix</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| PCR (polymerase chain reaction) | E. coli, ETEC, Cryptosporidium, and Giardia | Tap water and environmental water samples | • The most commonly used molecular-based method for detection of waterborne pathogens  
• Rapid analysis | • Necessitates accurate primers and optimal reaction mixtures to avoid the risk of false positive and negative results  
• Inability to discriminate between viable and non-viable cells that both contain DNA  
• Difficult to detect low concentrations of several pathogens such as Cryptosporidium, Giardia, and viruses  
• Lack of data to indicate the real infectious risk to a population  
• Need for water concentration methods  
• Presence of inhibitors in water samples to which PCR is sensitive  
• Result validation required  
• qPCR can only detect and quantify one pathogen in a single reaction  
• qRT-PCR may fail to detect damaged genomes |
| Multiplex PCR | EHEC, Shigella sp., Vibrio parahaemolyticus, P. aeruginosa, and Salmonella sp. | Polluted water and natural water | Allows simultaneous detection of several target organisms | |
| Quantitative PCR (qPCR) | Adenovirus, L. monocytogenes, V. cholerae, V. parahaemolyticus, Pseudogulbenkian a sp., S. typhimurium, S. flexneri, C. perfringens, Campylobacter spp., E. coli O157:H7, Aichi virus, astrovirus, enterovirus, human norovirus, rotavirus, sapovirus, and hepatitis A and E viruses | Wastewater, drinking water, recreational waters, and rivers | • High sensitivity and specificity  
• Faster rate of detection  
• Minimizes the risk of cross-contamination  
• No need for a post-PCR analysis  
• Can detect and quantify pathogens at concentrations as low as one target molecule per reaction  
• qRT-PCR (quantitative reverse-transcriptase PCR) can detect viable cells  
• Can detect VBNC bacteria |
| Microarrays                                                                 | Environmental water and ocean water spiked with pathogens; wastewater; and tap water spiked with multiple organisms. | • Widely utilized to monitor gene expression under different cell growth conditions, detect specific mutations in DNA sequences, and characterize microorganisms in environmental samples |
| • Allows rapid detection of multiple genes of multiple organisms simultaneously in the same sample |
| • Has high throughput capacity |
| • Ability to be automated |
| • Can perform large-scale and data-intensive experiments |
| • Can detect antimicrobial resistance and host origin of contaminants |
| • Can be coupled with PCR for higher sensitivity |
| • DNA microarrays commercially available |
| • Can experience difficulties when distinguishing between viable and non-viable cells |
| • Relatively high cost |
| • May have non-specific hybridization resulting in a lower specificity and low sensitivity |

| Pyrosequencing                                                                 | Milk, bottled water, juice, drinking water of non-chlorinated distribution system | • Provides a large number of sequences read in a single run |
| • Can identify novel pathogens associated with water and address multiple etiologies |
| • The DNA amount in wastewater samples could limit sensitivity |
| • Limited by the cost, complexity of the analysis, the need for increasing availability of massive computing power, and the efficiency of data generation |


- Y. pestis, B. anthracis, Comamonadaceae, Proteobacteria, Bacteroidetes, Planctomycetes, and Elusimicrobia
| Biosensors | C. parvum, *E. coli O157:H7*, *V. cholerae*, *Microcystis* spp. | Oocysts diluted in PBS, drinking water, ground and sea water, and lake water | • Provides selective quantitative or semiquantitative analytical information
• Advantages of automation and miniaturization of biological analytical techniques
• Short analysis times
• Portable
• Real-time measurements
• Do not require sample pre-enrichment | Great sensitivity to pH, change of mass, and temperature |
| --- | --- | --- | --- | --- |
| Fluorescence in situ hybridization (FISH) | *F. psychrophilum*, *E. coli*, and members of the Enterobacteriaceae family | Culture suspension, mixed pure cultures and sludge, activated sludge, drinking water systems, freshwater, and river water | • Allows enumeration of particular microbial cells
• Used for detection and identification of different microorganisms in mixed populations
• Several FISH kits are available in the market
• Viable but non-culturable cells could be detected with direct viable count assay | Low sensitivity
• Pre-enrichment and concentration steps are necessary but can increase inhibitor concentrations and lead to false negatives |
| Immunology-based methods (Serum neutralization tests [SNT], immunofluorescence, enzyme-linked immunosorbent assays [ELISA]) | *E. coli O157:H7*, *S. enterica* typhimurium, *Cryptosporidium* spp., and *Giardia* spp. | Contaminated food | • Can detect VBNC bacteria
• Can detect multiple pathogens | Low sensitivity
• False negative results
• Cross-reactivity with closely related antigens
• Need for pre-enrichment |
In addition to methods for detecting a range of microbial species, there are standard methods for analyzing water samples for bacteria. According to the U.S. EPA, there are two basic methods for analyzing water samples for bacteria: membrane filtration (MF) and multiple-tube fermentation (MTF) (EPA, 2012). Membrane filtration consists of filtering the water sample through a porous disk and placing that disk on a selective nutrient medium in a petri dish. After incubating the plate, the colonies that grow on the filter are counted. A confirmed test can be performed where the colonies that are identified as total coliforms are transferred to *E. coli* 4-methylumbelliferyl-β-D-glucuronide (EC MUG) or nutrient agar with MUG to determine if they contain the enzyme β-glucuronidase (Edberg et al., 2000). The disadvantage of membrane filtration is that coliforms can pass through the filter, depending on pore size, and can be missed in the final coliform count. The advantage is that different nutrient agars can be used for the isolation of different bacteria. For example, KF-Streptococcus agar is used for Enterococcus spp. while the membrane thermotolerant *E. coli* (mTEC) agar is used for the isolation of *E. coli* (Martinez, 2020b). Multiple-tube fermentation consists of adding the water sample to tubes containing Lauryl tryptose broth, incubating the tubes for 24 hours at 35 °C, and observing turbidity or gas production. This test determines a most probable number (MPN). A confirmed test can be performed with the positive liquid being transferred to a tube containing lactose broth with MUG. After incubation, if fluorescence is observed, then MUG hydrolysis occurred, and *E. coli* is present (Edberg et al., 2000). The disadvantage of MTF is the requirement of serial dilutions with many samples. Other confirmed tests for MTF and MF include brilliant green lactose bile broth (BGLB) which is a confirmed test for indicator coliforms, or *E. coli* broth which is a
confirmed test for indicator fecal coliforms. After these confirmed tests, completed tests on MacConkey agar or Eosin Methylene Blue (EMB) agar can be performed to observe any lactose fermentation (Martinez, 2020b).

Another standard method is the defined substrate technology (DST) where a direct color visualization after incubation denotes the presence of total coliforms, and the occurrence of fluorescence indicates that *E. coli* is present (Edberg et al., 2000). An example of this technology is the IDEXX Colilert test which is a presumptive/confirmed growth test of coliform and fecal coliform indicators (Martinez, 2020b). The advantage of DST is that the presumptive test is included with the confirmed test, so it is quicker and less expensive than the MF or MTF tests.

These standard methods do not require skilled expertise to perform but do require laboratory equipment such as an autoclave for sterilization, filter pump, and incubator. Therefore, these methods could be inaccessible to some communities. Some promising methods of microbial detection in water include the use of nanomaterials as sensing agents. The following sections will focus on introducing nanomaterials and reviewing their use as sensing agents.

### 2.3 Nanomaterials

The study and use of nanomaterials is an emerging field in science and technology. Nanomaterials are those that are characterized with at least one dimension in the nanometer range less than about 100 nm (Rao et al., 2004; NIEHS, 2020). Nanomaterials can be made in the form of clusters, particles, quantum dots, nanowires, and nanotubes, and can be structured into arrays and superlattices (Rao et al., 2004). They can be found
in nature as well as engineered from various elements such as carbon and silver (NIEHS, 2020). The focus of this research is on using nanoparticles for detection of microbes. There are two categories for the synthesis methods of nanoparticles: engineered assembly and self-assembly. Engineered assembly consists of manually putting the atoms together to form the nanoparticles where the self-assembly consists of providing the right conditions that allows the atoms to self-assemble to form the nanoparticles. The self-assembly approach can be broken down further into two approaches: the top-down and bottom-up approach. The bottom-up approach starts with atoms and/or molecules and controlling their reaction conditions until the desired size and properties of the nanoparticles are reached (Henry, 2006). This can be achieved using methods such as chemical reduction or chemical precipitation. The top-down approach starts with a macroscopic object and sizes it down (e.g., mechanical milling) until dimensions on the nanometer scale and desired properties are obtained (Henry, 2006).

The structure and properties of nanoparticles differ from macroscopic objects. As the size of a nanoparticle changes, so does its properties. The nanoparticles’ characteristics such as the size and size distribution, shape, crystal structure, and composition govern their behavior and applications. The nanoparticles can be coated with chemical agents to improve their stability (i.e., minimize aggregation) and/or functionality (SCENIHR, 2006). The stability of the nanoparticles can fall into three categories: electrostatic, steric, or electrosteric. Electrostatic stabilization is achieved by creating a common surface charge on the nanoparticles (Cesarano III & Aksay, 1988). The layers of charge that surround the nanoparticles are able to prevent them from aggregating due to the repulsion forces dominating over the attractive van der Waals forces in the suspension. A stronger
stabilization force is steric repulsion. This stabilization mechanism is based on the nanoparticles being coated with an uncharged polymer that creates an osmotic pressure between two coated particles. The high concentration of polymer and low concentration of surrounding water in between the two nanoparticles force water from outside of the particles to flow to the low concentration region in the center (Petsev, 2004). This effectively pushes the nanoparticles apart and prevents aggregation. Lastly, the electrosteric stabilization is based on a charged polymer coating on the nanoparticle surface. This combines the electrostatic and steric forces by providing Coulombic as well as osmotic repulsion forces. Additional surface characteristics that should be considered are the surface functionality and surface area. The surface functionality relates to the functional groups that are adsorbed to the nanoparticles’ surface. These functional groups determine the surface charge of the nanoparticles, provide steric or electrosteric stability, and act as a chemical interface between the surface of the nanoparticles and the surrounding environment (Saei et al., 2017). These physicochemical properties of the nanoparticles should be characterized to understand the interactions that can take place with other components present in solution.

The use of nanomaterials in applications in science and technology is promising but comes with challenges as well. Concerns exist on how nanoparticles affect health, interact with biological systems, and affect the environment (NIEHS, 2020). However, the small size, shape, high reactivity, and other properties make these nanoparticles unique and applicable on a small scale for product development. The opportunities for advancement in technology with nanomaterials merits further research into their potential effects on natural systems.
2.4 Nanomaterials for the Detection of Microbes

The development of accurate, rapid, affordable, and sensitive methods to detect microbes in water is a critical step towards the prevention of waterborne diseases. The use of nanomaterials as biosensors has gained interest due to their unique and advantageous properties that allow for the accurate, rapid, and sensitive detection of microbes (Kumar, 2007). Biosensors are devices that measure biological or chemical reactions by producing signals that are proportional to the concentrations of an analyte (Bhalla et al., 2016).

Many nanomaterials-based biosensors have been developed including electrochemical, acoustic, magnetic, and optical. An electrochemical biosensor utilizes the unique properties of nanomaterials (e.g., high surface area, high mechanical strength, and excellent electrical conductivity) to directly wire an electrode with the biomolecules (Jaiswal et al., 2018). This process tracks the electrochemical reactions that occur within the microbe and biorecognition molecules such as antibodies, enzymes, peptides, whole cells, and nucleic acids (Figure 2) (Gupta et al., 2019). Disadvantages of electrochemical biosensors can be specific to the biorecognition molecules they are tracking. For example, when tracking an enzymatic reaction, the redox active site being buried deep in the enzyme and the inability of the enzyme to orient itself to the electrode, results in inefficient electron transfer between the enzyme and the electrode surface (Putzbach & Ronkainen, 2013). In addition, other disadvantages include non-specific binding, electrode fouling, and poor selectivity for the analyte in complex real-world water samples (Putzbach & Ronkainen, 2013).
Figure 2. Electrochemical biosensor schematic with a transducer tracking the biological sensing element or biorecognition molecule (Ronkainen et al., 2010).

Nanomaterials have also been used as acoustic biosensors which utilize acoustic waves to gain information about the entity being measured (Lec & Lewin, 1998). Piezoelectric materials are used to fabricate these acoustic biosensors and are where the electromechanical transduction takes place (Lec & Lewin, 1998). Molecules that are encoded with the acoustic biosensors will illuminate when exposed to ultrasounds (Figure 3). The acoustic biosensor will emit acoustic waves when enzymatic activity is present, therefore detecting microbial activity. These biosensors are highly sensitive, small and portable, have fast responses, are highly accurate and inexpensive (Lec & Lewin, 1998). However, the efficiency of these biosensors is dependent on the properties of the solution the compounds are suspended in due to the acoustic waves needing to travel through the liquid (Durmus et al., 2014). In addition, the fabrication and manual handling of acoustic biosensors is complicated and requires professional expertise.

Figure 3. Acoustic biosensor tracking enzymatic activity when exposed to ultrasounds (Lakshmanan et al., 2020).
Magnetic biosensors utilize magnetic nanoparticles for direct application of tagged supports to the sensor (Rocha-Santos, 2014). The magnetic nanoparticles can also be integrated into the transducer material or dispersed throughout the sample (Rocha-Santos, 2014). When dispersed in the sample, they are attracted by an external magnetic field onto the biosensor. Multiple transduction principles can be used such as electrochemical, optical, piezoelectric, and magnetic field (Rocha-Santos, 2014). One method is immunomagnetic separation which coats nanoparticles with antibodies against the target organisms. During incubation, the target organism is attached to the nanoparticle when the target antigen is captured by the antibody (Ramadan & Gijs, 2012). One disadvantage of this system is that the target organism must then be transferred for further experiments, such as PCR, to detect the organism (Ramadan & Gijs, 2012). Fluorescent tags can also be attached to the antibodies to get a fluorescent detection in solution (Figure 4). In addition, washing and concentration steps are required to prevent existing particulates from masking the target organism for detection and quantification (Ramadan & Gijs, 2012).

**Figure 4.** Magnetic nanoparticles coated with antibodies to capture the target organism (Nikoleli et al., 2018).
Optical biosensors composed of nanomaterials is the last major category of microbial detection methods using nanomaterials. Optical biosensors are the most preferred due to their ease of implementation while meeting criteria such as affordability, sensitivity, specificity and being user friendly, robust, rapid, equipment free, and easily deliverable to communities (Choi et al., 2018). Various optical biosensors have been developed based on surface plasmon resonance (SPR), surface-enhanced Raman scattering (SERS), photoluminescence, and colorimetry (Figure 5) (Choi et al., 2018).

**Figure 5.** Optical biosensors: SPR, SERS, photoluminescent, and colorimetric methods (Modified from Cantale, 2011; Bhardwaj et al., 2019).

SPR sensors work by passing a plane-polarized light through a glass prism that is placed next to a transducer surface with recognition probes. When an analyte binds to a bioreceptor, a change in angle occurs for the light departing the prism (Bhardwaj et al., 2019). This sensor has a high sensitivity and rapid detection of pathogenic microorganisms; however, false results can occur due to fluctuations in refractive index with temperature or composition of the water sample, and non-specific interactions from
structurally similar molecules can interfere with the target interaction (Bhardwaj et al., 2019).

SERS utilizes Raman spectroscopy to identify molecular fingerprints that correspond to the chemical composition of a pathogenic species, and SERS-active substrates to amplify the Raman signal of bacteria (Bhardwaj et al., 2019). Nanomaterials act as the SERS-active substrates in this method and are directly conjugated with Raman reporter molecules (organic dyes). The advantages of this method are the high sensitivity, rapid results, low cost, multiplexed detection, and portability (Bhardwaj et al., 2019). However, some sensors utilizing this method require a secondary labelling dye that limits its use for in-situ and high-throughput detection of pathogens due to high reactant volumes, preparation steps, and analytical time (Bhardwaj et al., 2019).

Photoluminescent detection of microbes can use either fluorescence or chemiluminescence. Organic dye-labelled recognition probes, such as amino acids, proteins, and antibodies, are used with fluorescent tags, such as fluorescent nanomaterials (Bhardwaj et al., 2019). The change in fluorescence is then monitored with a photoluminescence spectrophotometer. The advantages of this detection method include ease of handling, flexibility, specificity, and sensitivity (Bhardwaj et al., 2019). However, this method requires sophisticated optics with an excitation source, and a photoluminescence spectrophotometer (Bhardwaj et al., 2019).

Colorimetric biosensors are gaining interest due to their simplicity, practicality, and cost effectiveness. These biosensors work by detecting target molecules that result in a color change reaction that can be observed with the naked eye. There are two approaches to
colorimetric biosensors: direct and indirect assays (Thiramanas & Laocharoensuk, 2015). Direct assays are based on electrostatic aggregation which occurs when the nanoparticles in suspension aggregate and change size in response to chemical and biological reactions (Choi et al., 2018). This sensing approach tracks the size of the nanoparticle as it changes over time resulting in a visual color change. The degree of aggregation is altered by modifying the surface of the nanoparticles with biomolecules such as antibodies, DNA, and proteins (Choi et al., 2018). Indirect assays track enzyme catalyzed color producing reactions based on nanomaterial-bacteria and nanomaterial-enzyme interactions (Thiramanas & Laocharoensuk, 2015; Miranda et al., 2011). Utilizing enzymes in biosensors has the disadvantages of the increased cost, short lifetime, and limited operational conditions (Choi et al., 2018). However, the potential for high specificity and sensitivity merits further research into indirect colorimetric assays.

2.5 Indirect Colorimetric Detection Assays Based on Competitive Binding

A simple and rapid method for the colorimetric detection of gram-positive and negative bacteria in water has been developed by Thiramanas and Laocharoensuk (Thiramanas & Laocharoensuk, 2015). This detection method is an indirect assay that utilizes polyethyleneimine-coated gold nanoparticles (PEI-AuNPs) that are positively charged, the enzyme β-galactosidase (β-Gal) that is negatively charged, and the chromogenic substrate chlorophenol red β-D-galactopyranoside (CPRG). Competitive binding of the positively charged PEI-AuNPs to the negatively charged β-Gal and bacteria results in a color change that could be analyzed qualitatively or quantitatively to determine the microbial concentration in solution. The gram-positive and gram-negative bacteria that were detected using this colorimetric detection method were *Staphylococcus aureus* (*S.*
*aureus* and the enterotoxigenic *Escherichia coli* (ETEC), respectively. The detection limit was as low as 10 CFU/mL, and the linear range extends from $10^6$ to $10^8$ CFU/mL.

The mechanism that results in colorimetric detection of bacteria using the aforementioned approach is competitive binding driven by Coulombic attraction between positively charged particles and negatively charged microbes. The PEI-AuNPs possess a positive charge due to the coating of the positively charged polymer, polyethyleneimine (PEI). This polymer provides a positive charge to play a role in this detection method and to prevent aggregation of the nanoparticles through electrosteric stabilization. On the other hand, β-Gal has a negative charge that is near the anomeric carbon binding position of the galactose site (Huber & Gaunt, 1982). Both gram-positive and negative bacteria are negatively charged. Gram-positive bacteria contain a negative charge due to the presence of negatively charged teichoic acids linked to peptidoglycan or the plasma membrane (Ejaz, 2012). Teichoic acids contain phosphates, which holds a negative charge. Gram-negative bacteria are negatively charged due to an outer layer of phospholipids and lipopolysaccharides (Ejaz, 2012). When the substrate and enzyme are the only components in solution, hydrolysis of CPRG by β-Gal will occur and produce chlorophenol red (CPR). This reaction results in a visible color change from the yellow CPRG substrate to the red CPR product. When the enzyme activity is inhibited, this reaction will not occur, and the solution will remain yellow. When bacteria are not present and only PEI-AuNPs, β-Gal, and CPRG are in solution, the PEI-AuNPs are electrostatically attracted to β-Gal resulting in the two components being bound. This binding results in the inhibition of the enzyme and therefore the solution will have a yellow color. The PEI-AuNPs act as a reversible inhibitor that can bind to and leave the
β-Gal without damaging its activity indefinitely. When bacteria are added to solution, the PEI-AuNPs are electrostatically attracted to both the bacterial surface and β-Gal. When there is a large quantity of bacteria present, the PEI-AuNPs preferentially bind to the bacteria over the β-Gal. This leaves the β-Gal free in solution to bind to CPRG and create a color change to red. This is the competitive binding component of the detection assay and is illustrated in Figure 6. The magnitude of color change is proportional to the quantity of bacteria in solution. Therefore, for a given number of PEI-AuNPs, lower concentrations of bacteria will result in higher quantities of unbound nanoparticles being available to interact with the enzyme and reduce the red color of the solution. Therefore, as the concentration of bacteria decreases, the magnitude of red color decreases as well.

**Figure 6.** Competitive binding mechanism resulting in a red color change when bacteria are present in sufficient quantity (Thiramanas & Laocharoensuk, 2015).

This colorimetric detection of bacteria is dependent on three characteristics: the magnitude of positive charge on the nanoparticles, the relative surface charge of the components in solution, and the stoichiometric point of NPs to enzyme ratio. The highly positive nanoparticles are critical for electrostatically attracting the negatively charged
microbes. Utilizing negatively charged nanoparticles will theoretically result in no nanoparticles binding to the bacterial surface due to similar surface charges. Method 1 tested this hypothesis by using negatively charged Citrate-AuNPs and comparing them to the PEI-AuNPs. Using transmission electron microscopy (TEM), the PEI-AuNPs were seen coating approximately the entire surface of the bacteria while there was a low density of Citrate-AuNPs binding to the surface of the bacteria (Figure 7). Therefore, this binding mechanism is highly dependent on the strong positive charge of the PEI-AuNP. In addition, the relative surface charge, or magnitude of each charge, is critical for this detection method. The relative surface charge is measured by zeta potential of the components (PEI-AuNPs, β-Gal, and bacteria). PEI-AuNPs possess a highly positive charge of around +54 mV, while the β-Gal and bacteria possess negative charges of approximately -13 mV and -40 mV, respectively (Thiramanas & Laocharoensuk, 2015). The higher negative surface charge of the bacteria compared to β-Gal explains the reason that PEI-AuNPs favors the interactions with the bacteria. This is one of the criteria for the competitive binding to occur. Lastly, the stoichiometric point of NPs to enzyme ratio determines the PEI-AuNP concentration with respect to an optimum concentration of β-Gal needed for performing the detection assay.
Figure 7. TEM images of the PEI-AuNPs attached to bacteria (a) and the Citrate-AuNPs attached to bacteria (b) (cite). The small black dots are the nanoparticles (Thiramanas & Laocharoensuk, 2015).

This colorimetric detection method using competitive binding is a promising solution to a simple and rapid method for bacterial detection in water sources. A relatively low detection limit of 10 CFU/mL can be obtained, sample enrichment steps are not required, and a bare-eye readout of results occurs in a short time of three hours. This satisfies the requirements of an effective detection method that is simple and rapid. In addition, this method was successfully applied to an analysis of a drinking water sample, and has the potential application for screening of total bacteria contamination based on an experiment performed in the presence of both ETEC and S. aureus. These benefits merit further research into this detection method.

Areas of improvement for this detection method include the cost, environmental impact, and recyclability. The mining of gold results in mine waste on the levels of approximately 20 tons of toxic waste for every 0.333 ounces of gold (Brilliant Earth, 2021). In addition, AuNPs have been known to cause biotoxicity and necrosis due to being internalized in exposed plants (Agtuca, 2014). They can also bioaccumulate
through trophic transfers in food chains (Agtuca, 2014). Lastly, recyclability of the AuNPs can be improved by using a nanoparticle that is magnetic, like iron oxide. The magnetic properties of iron oxide nanoparticles allow them to be removed from solution after detection of microbes is complete, reconditioned, and then reused for further detection.
3. MATERIALS AND METHODOLOGY

3.1 Escherichia coli Culturing

A non-pathogenic *Escherichia coli* (*E. coli*) strain was used in all experiments. A stock solution containing the *E. coli* was streaked, according to the streak plate method, on a plate of tryptic soy agar (TSA). This process was performed to isolate a single colony of bacteria compared to a line of growth where the inoculating loop was streaked (Figure 8). Approximately two colonies were streaked on a new TSA plate every two days to keep the *E. coli* strain growing throughout the year.

![Figure 8](image).

Figure 8. Streak plate method performed on the *E. coli* plates that were streaked (Laboratory Info, 2021).

To obtain an *E. coli* suspension that can be easily pipetted into a well plate, colonies from the TSA plate were transferred to a tryptic soy broth (TSB) growth media. Approximately two colonies were chosen from the last quadrant on the TSA streak plate to obtain the *E. coli* needed for performing any of the detection assays described in Section 3.4. These two colonies were transferred using an inoculation loop to a 15 mL Falcon tube containing 5 mL of TSB. After the inoculation loop was vigorously swished around in the tube to transfer the colonies from the loop to the TSB media, the tube was sealed using a lid with tape wrapped over it. The tube was then placed in a shaking incubator at 30 °C.
for 24 hours. Figure 9 shows a representative stock suspension of *E. coli* after 24 hours of shaking and incubation.

![Figure 9. E. coli after 24 hours of shaking in an incubator at 30 °C. Growth is seen at the bottom of the cone in the tube.](image)

Serial dilutions were performed to determine the concentration of *E. coli* in CFU/mL in the stock suspension (Figure 10). The stock suspension was initially diluted 10 times using nine parts phosphate buffer solution (PBS) to one part of the stock suspension to obtain a $10^{-1}$ dilution. Serial dilutions were then performed to the $10^{-8}$ dilution, similar to Figure 10. A volume of 0.1 mL from the $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$ dilutions were pipetted onto individual TSA plates. These dilutions were chosen because growth of 30 to 200 colonies on the plate is required to perform the *E. coli* concentration calculations. Once pipetted onto the TSA plate, a plate spreader was used to create an even lawn on the agar. These plates were then incubated for 24 hours at 37 °C. Plates that grew between 30 and 200 colonies were then used to calculate a concentration in CFU/mL using Equation 1.
This method was performed three times on separate days with different stock suspensions of *E. coli* to ensure precision.

![Figure 10](image)

**Figure 10.** Serial dilution and plating method of the *E. coli* to determine the concentration of stock suspensions in CFU/mL (Tankeshwar, 2016).

\[
C = \frac{CFU \times DF}{V}
\]

**Equation 1**

Where

- \(C\) = Concentration of stock solution (CFU/mL)
- \(CFU\) = Number of colonies counted on the plate
- \(DF\) = Dilution factor = \(10^{\text{Number of times diluted (positive)}}\)
- \(V\) = Volume of solution plated (mL)

### 3.2 Nanoparticles Tested for *E. coli* Detection

Five different suspensions of nanoparticles (four commercial and one synthesized in the environmental engineering laboratory) were investigated in this study for the detection of *E. coli*. These nanoparticles were 1) branched polyethyleneimine coated silver nanoparticles (BPEI-AgNPs), 2) amine functionalized iron oxide nanoparticle suspension
(Amine-Fe$_3$O$_4$), 3) cerium (Ce$^{3+}$) doped iron oxide coated nanoparticles coated with BPEI (BPEI-Fe$_2$O$_3$), 4) commercial iron oxide suspension modified in the laboratory with BPEI (BPEI-M-IONP), and 5) iron oxide nanoparticle suspension with BPEI coating synthesized in the environmental engineering laboratory (BPEI-S-IONP). These nanomaterials were selected for this investigation to test the research hypotheses and understand the mechanism of work of the sensor – what is responsible for *E. coli* detection, is it the nanoparticle type, surface charge, or the chemical nature of the coating/surface functionalization agent? The nanomaterials tested were all positively charged because of the amine groups on their surfaces but differed in the nanomaterials type and surface coating or functionalization agent.

### 3.2.1 Commercial Nanoparticles

A stable suspension of BPEI-AgNPs in purified water was purchased from nanoComposix (San Diego, CA) with a concentration of 1 mg/mL. The properties of the BPEI-AgNPs, as provided by the manufacturer, are outlined in Table 3. The transmission electron microscopy (TEM) image provided by the manufacturer indicates that the BPEI-AgNPs suspension is monodisperse with a diameter of 40 nm (Figure 11). The BPEI coating used was a 25 kDA polymer that contains a multitude of free amines, which make the BPEI-AgNPs positively charged with zeta potential values ranging from +30 to +95 mV. The BPEI-AgNPs suspension obtained was pure (i.e., free of residual chemicals from the synthesis process) and sterile for use in biological assays (nanoComposix, 2021).
Table 3. Properties of nanoComposix BPEI-AgNPs (nanoComposix, 2021)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass Concentration</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Particle Concentration</td>
<td>$2.8 \times 10^{12}$ particles/mL</td>
</tr>
<tr>
<td>Diameter</td>
<td>40 nm</td>
</tr>
<tr>
<td>Surface Plasmon Resonance Peak Wavelength</td>
<td>410 nm</td>
</tr>
<tr>
<td>Zeta Potential</td>
<td>+30 to 95 mV</td>
</tr>
<tr>
<td>pH of Suspension</td>
<td>7.0 to 8.5</td>
</tr>
</tbody>
</table>

Figure 11. TEM image of the nanoComposix BPEI-AgNPs (nanoComposix, 2021).

An amine functionalized iron oxide (II, III), magnetic nanoparticle suspension in water (Amine-$\text{Fe}_3\text{O}_4$) was purchased from Sigma Aldrich (USA). The properties of this nanoparticle suspension, according to the manufacturer, are outlined in Table 4 and a representative TEM image of these nanoparticles is presented in Figure 12.

Table 4. Amine Functionalized Iron Oxide (II, III) Magnetic Nanoparticle Suspension Properties (Millipore Sigma, 2021a)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Particle Size (TEM)</td>
<td>10 nm (Range: 9 – 11 nm)</td>
</tr>
<tr>
<td>Concentration</td>
<td>1 mg/mL Fe in H$_2$O</td>
</tr>
<tr>
<td>pH</td>
<td>7 – 8</td>
</tr>
<tr>
<td>Magnetization</td>
<td>&gt;45 emu/g (at room temperature; under 4500 Oe)</td>
</tr>
</tbody>
</table>
Figure 12. A representative TEM image for the amine functionalized iron oxide (II, III) magnetic nanoparticle suspension (Millipore Sigma, 2021a).

A stable suspension of core-shell cerium (Ce$^{3/4+}$) doped iron oxide nanoparticles coated with branched polyethyleneimine (BPEI-Fe$_2$O$_3$) was purchased from Sigma Aldrich (USA). The properties of this nanoparticle suspension are outlined in Table 5, as per the manufacturer’s data sheets.

Table 5. Cerium (Ce$^{4+}$) Doped Iron Oxide Nanoparticle Coated with Branched Polyethyleneimine (BPEI-Fe$_2$O$_3$) Properties (Millipore Sigma, 2021b)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM Particle Size</td>
<td>7 – 15 nm</td>
</tr>
<tr>
<td>Hydrodynamic Diameter</td>
<td>81.25 nm</td>
</tr>
<tr>
<td>Suspension Concentration</td>
<td>1.3 mg/mL</td>
</tr>
<tr>
<td>Iron Concentration</td>
<td>0.27 mg/mL</td>
</tr>
<tr>
<td>Cerium Concentration</td>
<td>0.01 mg/mL</td>
</tr>
<tr>
<td>Zeta Potential</td>
<td>29.7 mV</td>
</tr>
</tbody>
</table>
3.2.2 Modified Commercial Nanoparticles (BPEI-M-IONP)

A stable iron oxide nanoparticle (Fe₃O₄) suspension was purchased from US Research Nanomaterials, Inc. (Texas, USA). The properties of this Fe₃O₄ nanoparticles suspension are outlined in Table 6. The nanoparticles had a diameter ranging from 15 – 20 nm (Figure 13).

Table 6. Iron Oxide (Fe₃O₄) Nanoparticle Suspension Properties (US Research Nanomaterials, Inc., 2021)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity</td>
<td>99.5+%</td>
</tr>
<tr>
<td>Actual Particle Size (APS)</td>
<td>15 – 20 nm</td>
</tr>
<tr>
<td>Percent Weight in Water</td>
<td>20 wt%</td>
</tr>
<tr>
<td>pH</td>
<td>7 – 8</td>
</tr>
</tbody>
</table>

Figure 13. TEM image of the iron oxide (Fe₃O₄) nanoparticle obtained from US Research Nanomaterials, Inc. (US Research Nanomaterials, Inc., 2021).

The aforementioned (Fe₃O₄) iron oxide nanoparticles were modified in the laboratory by coating their surfaces with BPEI (MW ~ 1200). The modification with BPEI was
performed according to the method by Arsianti et al. with some modifications (Arsianti et al., 2010). Briefly, the iron oxide nanoparticle suspension (20 wt%) was diluted to a concentration of 3 mg/mL in deionized water. Then, 0.8 g of BPEI was dissolved in 40 mL of deionized water to make a 20 mg/mL BPEI solution. Equal volumes of these two solutions (40 mL each) were combined and sonicated for five minutes with a Qsonica sonicator (Model CL-334). The sonication was performed at 500 watts, 20 kHz, and 50% amp. The purpose of this sonication step was to break any nanoparticle agglomerates so that the BPEI could effectively coat the individual nanoparticles. After sonication, the mixture was placed in a shaking incubator at room temperature for approximately 24 hours to allow time for BPEI to interact with the nanoparticles in suspension. Afterwards, the solution was sonicated for two minutes and then washed in a Beckman Coulter Avanti J-20 XP centrifuge three times; two minutes each wash at 3,000 rpm with the supernatant being removed after each wash. This process was performed to remove residual unreacted chemicals from the mixture. Lastly, the washed nanoparticles were re-suspended in deionized water using sonication for a period of one minute. Before each *E. coli* detection test, the nanoparticle suspensions were sonicated to minimize aggregation and enhance the chances of interactions between the nanoparticles and the microbes when conducting the detection assays.

The BPEI modified iron oxide nanoparticles were characterized using a thermogravimetric analysis (TGA). The characterization with TGA was performed to verify whether the modification with BPEI was successful. The TGA can indicate the presence of (and the percent weight) of organic materials on the surface of the nanoparticles. Before the TGA analysis, the nanoparticles were dried in an oven at 80 °C
overnight to obtain nanoparticle powder. Then, a dried nanoparticle sample was heated in a TGA (TA Instruments SDT650) to 600 °C at a rate of 15 °C/min under nitrogen gas flow.

The concentration of this modified iron oxide nanoparticle suspension was determined using two different methods. In the first method, 10 mL of the suspension was filtered through a 0.22 µm syringe filter. The filter was then dried in an oven overnight at 80 °C. The difference in weight between the clean filter and the dried filter after the suspension was filtered through was used to calculate the concentration of the modified iron nanoparticle suspension. The second method used glassware containing the suspension instead of a filter. The suspension was placed in a glass petri dish and then dried overnight in an oven at 80 °C. The weights of the clean glass petri dish, glass dish with the suspension, and glass dish with the dried nanoparticles were determined. The differences in the aforementioned weights were used to calculate the concentration of the nanoparticles in suspension.

3.2.3 Synthesized Nanoparticles (BPEI-S-IONP)

BPEI coated magnetic iron nanoparticles were synthesized in the laboratory according to the method by Mykhaylyk et al. with slight modifications (Mykhaylyk et al., 2007). Iron (III) chloride hexahydrate (2.25 g) and iron (II) chloride tetrahydrate (0.83 g) were dissolved in 50 mL of DI water. The solution was filtered with a 0.22 µm syringe filter, purged with nitrogen gas to remove dissolved oxygen, and placed on an ice bath to cool down to 2 – 4 °C. A BPEI reagent was prepared by dissolving 4 mL of 29.9% ammonium hydroxide solution, 0.83 g of BPEI, and 0.5 mL of Span 80 (a surfactant to stabilize the nanoparticles after they form) in 12.5 mL of DI water. This BPEI reagent solution was
added rapidly to the aforementioned iron chloride mixture to obtain a primary precipitate. This precipitate was then stirred to keep it in suspension while being heated at 90 °C and purged with nitrogen for two hours (Figure 14). The purpose of heating under nitrogen flow was to convert the primarily formed iron hydroxides to iron oxides.

![Precipitate being purged with nitrogen gas.](image)

Figure 14. Precipitate being purged with nitrogen gas.

After two hours, the nitrogen purging was terminated, and the mixture was cooled to room temperature and kept on a stir plate for approximately 24 hours. The mixture was then sonicated for two minutes using a Qsonica probe sonicator (Model CL-334) and washed in the centrifuge three times, two minutes each wash at 3,000 rpm with the supernatant being replaced with DI water after each wash. After the washing process was completed, the solution was sonicated for one minute to re-suspend the nanoparticles in
DI water. Before conducting the *E. coli* experiments with these nanoparticles, the suspension was sonicated to break apart aggregated nanoparticles.

The BPEI-S-IONP were characterized using TGA (explained in Section 3.2.2) and Fourier-transform infrared spectroscopy (FTIR). FTIR can indicate the type of functional groups on the nanoparticle surface, and therefore the FTIR data was used to verify whether BPEI was successfully coated on the nanoparticle surface. Before the TGA and FTIR analyses, the BPEI-S-IONP were dried in an oven at 80 °C overnight to obtain nanoparticles powder. In the TGA, the dried nanoparticle sample was heated to 600 °C at a rate of 15 °C/min under a nitrogen gas flow. For FTIR analysis, the sample was tested within the range of 400 – 4000 cm⁻¹ wavenumber using a Jasco FT/IR-4600. To determine their concentration, a sample of the BPEI-S-IONP suspension was first dried in a glass beaker at 80 °C in an oven overnight. The weight of the clean glass beaker, glass beaker with the solution, and glass beaker with the dried nanoparticles was determined and the differences in weights were used for calculating the concentration of iron oxide nanoparticles in the suspension.

### 3.3 Cationic Polyelectrolytes and Surfactants

Two cationic polyelectrolytes (BPEI and chitosan) and one cationic surfactant (CTAB) were tested as potential *E. coli* sensing agents. The BPEI (molecular weight = 1,200 g/mol) was purchased from Fisher Scientific. BPEI is a cationic organic polymer that has repeating units of ethylene diamine groups, containing primary, secondary, and tertiary amino groups (Figure 15). BPEI has been used for functionalization of several types of nanoparticles. It has been reported that the primary amines of the BPEI molecules are responsible for covalently linking to carboxyl functionalized nanoparticles.
(nanoComposix, 2021b). BPEI provides the nanoparticles with positively charged surfaces as a result of the protonation of the amine groups of BPEI molecules in solution.

![Chemical base structure of BPEI](image)

**Figure 15.** Chemical base structure of BPEI (Polysciences, 2021).

The second positively charged polyelectrolyte investigated was Chitosan, 85% deacetylated – purchased from Fisher Scientific. Chitosan ($C_{56}H_{103}N_9O_{39}$) is a polysaccharide derived from chitin and contains amino groups, similar to BPEI (Ibrahim & El-Zairy, 2014). The structure is representative of a long-chained copolymer of N-acetyl-D-glucose amine and D-glucose amine (Figure 16) (Ibrahim & El-Zairy, 2014). Chitosan is also biocompatible, and biodegradable which makes it a good alternative to BPEI (Ibrahim & El-Zairy, 2014). The antimicrobial effects of chitosan are a factor to consider in nanosensor functionality, and are dependent on the concentration, molecular weight, pH, ionic strength, temperature, and exposure time (Goy et al., 2016; Atay, 2020).
The third positively charged surfactant (1-Hexadecyl) trimethylammonium bromide (CTAB) was purchased from Fisher Scientific. The CTAB (molecular weight of 364.456 g/mol) consists of a long chain of carbon and hydrogen bonds (Figure 17).

![Chemical structure of CTAB](image)

**Figure 17.** Chemical structure of CTAB (CymitQuimica, 2021).

### 3.4 Overview of the *E. coli* Detection Protocol

Experiments have been conducted to systematically determine the optimal concentrations and amounts of the sensing agents needed as well as the sequence of addition of these agents in the testing wells to achieve the *E. coli* detection goals (Figure 18). The first set of experiments did not include *E. coli* or sensing agents and focused on optimizing the colorimetric reaction between β-Gal and CPRG to result in the hydrolysis of CPRG by β-Gal and therefore turning the solution color to red. Sensing Approach A and Sensing
Approach B included two stages. The first stage was activity assays that were performed without microbes and focused on finding the type, amount, and concentration of sensing agent that has the ability to fully inhibit the activity of $\beta$-Gal leaving the solution color unchanged (i.e., remains yellow). The activity assays were also conducted to determine whether the surface charge or the sensing agent itself is responsible for the interactions with $\beta$-Gal. The second stage of Sensing Approach A and Sensing Approach B focused on the detection of *E. coli* using the optimal mixtures of sensing agents determined based on the results of stage one.

![Diagram](image)

**Figure 18.** Approaches investigated for *E. coli* detection.

The difference between Sensing Approach A and Sensing Approach B was the basis of optimizing the amount of sensing agent used in the detection assay. In Sensing Approach
A, the amount of sensing agent was determined based on adding enough mass of that agent to bind $10^2$ CFU/mL of *E. coli*. The hypothesis for this approach was that as the concentration of *E. coli* in the sample decreases (< $10^2$ CFU/mL), there will be excess chemical sensing agent free in the solution to bind with β-Gal and inhibit its interaction with CPRG (i.e., prevent hydrolysis of CPRG by β-Gal). The lower the *E. coli* concentration in the sample, the more the color of the solution will progress towards yellow and vice versa (i.e., the solution color will progress towards red with higher *E. coli* concentrations).

In Sensing Approach B, on the other hand, the mass of sensing agent required for detection of *E. coli* was determined based on adding enough mass to bind all β-Gal in the sample, which results in a yellow color solution at the lowest detectable *E. coli* concentration. As the concentration of *E. coli* increases, the sensing agent will favor binding *E. coli* than β-Gal because of the stronger Coulombic interactions (i.e., stronger physical attraction) between the positively charged sensing agent and the negatively charged *E. coli*.

It is noted that, along with each well plate tested in Sensing Approach A and Sensing Approach B, control samples were tested. Negative control samples consisted of β-Gal and CPRG only and the microbes and sensing agents were replaced with phosphate buffer solution. These negative control samples were used to confirm that the β-Gal and CPRG were active and interacting properly. The samples containing a sensing agent, β-Gal, and CPRG only served as positive controls.
The *E. coli* detection assay used in the current investigation was conducted according to the method by Thiramanas and Laocharoensuk (referred to herein as Method 1) with modifications related to the volumes and concentrations of sensing reagents used as well as the sequence or the addition of the sensing agents (Thiramanas & Laocharoensuk, 2015). Briefly, Method 1 begins by mixing the chemical sensing agent with beta galactosidase (β-Gal) and *E. coli* in a 96-well plate. A summary of the sensing agents tested herein, and their characteristics are presented in Table 7. This mixture was kept for 1-hour at room temperature to allow for the competitive binding to take place. Then, chlorophenol red-β-D-galactopyranoside (CPRG) was added to the mixture followed by inserting the 96-well plate into a spectrophotometer (SpectraMAX Plus 384 Microplate Reader by Molecular Devices, Serial Number: 126309) to measure the optical density of each well every ten minutes for a period of three hours at a wavelength of 575 nm. The SpectraMAX Plus data was only presented in this thesis for the experiments that had a gradient of color change (which was an indication that the sensor was working as desired).
Table 7. Sensing Agents Tested and Their Characteristics

<table>
<thead>
<tr>
<th>Hypothesis Tested</th>
<th>Nanoparticles</th>
<th>Diameter</th>
<th>Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoparticle type does not make a difference</td>
<td>BPEI-AgNPs</td>
<td>40 nm</td>
<td>+30 to +95 mV</td>
</tr>
<tr>
<td></td>
<td>BPEI-Fe$_2$O$_3$</td>
<td>7 – 15 nm</td>
<td>+29.7 mV</td>
</tr>
<tr>
<td></td>
<td>BPEI-M-IONP</td>
<td>15 – 20 nm*</td>
<td>Not characterized</td>
</tr>
<tr>
<td></td>
<td>BPEI-S-IONP</td>
<td>Not characterized</td>
<td>Not characterized</td>
</tr>
<tr>
<td></td>
<td>Amine-Fe$_3$O$_4$</td>
<td>10 nm</td>
<td>Not provided</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Positively Charged Polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothesis Tested</td>
</tr>
<tr>
<td>Coating type doesn’t make a difference and carrier nanoparticle is not needed</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

3.4.1 Optimization of the Colorimetric Assay

This experimental phase was conducted to determine the optimal quantity and molar concentration of β-Gal and CPRG to allow for the full hydrolysis of CPRG to occur. This hydrolysis reaction results in a change in solution color to dark red. The concentrations tested were 0.5 nM and 0.125 µM for β-Gal and 1.5 mM and 0.75 mM for CPRG. These concentrations were selected based on previous research studies on colorimetric detection of *E. coli* (Thiramanas & Laocharoensuk, 2015; Miranda et al., 2011). The tested volumes of β-Gal ranged from 0.5 to 180 µL, while the volumes of CPRG ranged from 5 to 250 µL. In these colorimetric assay optimization experiments, the volume of the sensing agent solution and the *E. coli* suspensions were replaced with the same volume of phosphate buffer. The optimal volume and molar concentrations of the β-Gal and CPRG were determined to be 30 µL of 0.125 µM β-Gal and 90 µL of 0.75 mM CPRG. At these optimal ratios, the color of the mixtures changed from yellow to red over a three-hour period.
3.4.2 Sensing Approach A: Determination of the Sufficient Amount of Sensing Agent Based on \textit{E. coli} Concentration

\textit{Activity Assays}

Prior to conducting the microbial detection assay experiments, activity assays, (i.e., experiments conducted without microbes) were performed to determine which sensing agent has potential to inhibit the hydrolysis of CPRG by $\beta$-Gal, and whether it is the surface charge or sensing agent that is causing the inhibition. If a sensing agent inhibited the hydrolysis reaction, then it was an indication that it might work for detection of \textit{E. coli} and was thus used in the subsequent microbial detection experiments. In addition, this inhibition would indicate that the surface charge is the governing factor, not the nanoparticle or polymer type. The chemical sensing agents tested in this approach were BPEI-AgNPs, BPEI-Fe$_2$O$_3$, Amine-Fe$_3$O$_4$, BPEI, chitosan, and CTAB. A summary of the experiments conducted in this approach are presented in Table 8.
<table>
<thead>
<tr>
<th>Sensing Agent</th>
<th>Quantity of Sensing Agent</th>
<th>Quantity of β-Gal</th>
<th>Quantity of CPRG</th>
<th>Quantity of PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPEI-AgNPs</td>
<td>2 µL of 100 µM</td>
<td>2 µL of 0.125 µM</td>
<td>30 µL of 0.75 mM</td>
<td>100 µL</td>
</tr>
<tr>
<td></td>
<td>2 µL of 250 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 60 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 100 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 250 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 500 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 750 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 1000 µM</td>
<td>30 µL of 0.125 µM</td>
<td>90 µL of 0.75 mM</td>
<td></td>
</tr>
<tr>
<td>BPEI-Fe₂O₃</td>
<td>5 µL of 1 µM</td>
<td>2 µL of 0.125 µM</td>
<td>30 µL of 0.75 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 50 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amine-Fe₃O₄</td>
<td>5 µL of 1 µM</td>
<td>2 µL of 0.125 µM</td>
<td>30 µL of 0.75 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 50 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPEI</td>
<td>2 µL of 250 µM</td>
<td>2 µL of 0.125 µM</td>
<td>30 µL of 0.75 mM</td>
<td>2 µL</td>
</tr>
<tr>
<td>Chitosan</td>
<td>10 µL of 10 µM</td>
<td>30 µL of 0.125 µM</td>
<td>90 µL of 0.75 mM</td>
<td>100 µL</td>
</tr>
<tr>
<td></td>
<td>30 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 250 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
<td>10 µL of 10 µM</td>
<td>30 µL of 0.125 µM</td>
<td>90 µL of 0.75 mM</td>
<td>100 µL</td>
</tr>
<tr>
<td></td>
<td>30 µL of 10 µM</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 250 µM</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 50 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**E. coli Detection Assays**

The Sensing Approach A E. coli detection assays utilized the sensing agents in the Sensing Approach A activity assays and aimed to determine an E. coli detection limit. Concentrations and volumes of sensing agents were altered to determine the optimal quantities that could detect the highest concentration of microbes ($10^2$ CFU/mL) tested in Sensing Approach A. These optimal concentrations and volumes obtained were then tested across a range of microbial concentrations to determine the E. coli detection limit. The experiments that were performed in this approach are outlined in Tables 9 and 10.
<table>
<thead>
<tr>
<th>Sensing Agent</th>
<th>Quantity of Sensing Agent</th>
<th>Quantity of β-Gal</th>
<th>Quantity of CPRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPEI-AgNPs</td>
<td>5 µL of 10 µM</td>
<td></td>
<td>30 µL of 0.125 µM</td>
</tr>
<tr>
<td></td>
<td>10 µL of 10 µM</td>
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<td></td>
<td>20 µL of 10 µM</td>
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<td></td>
<td>30 µL of 10 µM</td>
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<td></td>
<td>6 µL of 100 µM</td>
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<tr>
<td></td>
<td>10 µL of 100 µM</td>
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<td>20 µL of 100 µM</td>
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<td></td>
<td>30 µL of 100 µM</td>
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<td>5 µL of 1 µM</td>
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<td></td>
<td>10 µL of 1 µM</td>
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<td>20 µL of 1 µM</td>
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<td></td>
<td>5 µL of 5 µM</td>
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<tr>
<td></td>
<td>7 µL of 5 µM</td>
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<tr>
<td></td>
<td>10 µL of 25 µM</td>
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<td></td>
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<tr>
<td></td>
<td>12 µL of 25 µM</td>
<td>30 µL of 0.125 µM</td>
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</tr>
<tr>
<td></td>
<td>15 µL of 25 µM</td>
<td>90 µL of 0.75 mM</td>
<td></td>
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<tr>
<td></td>
<td>18 µL of 25 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 µL of 25 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 µL of 25 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPEI</td>
<td>5 µL of 10 µM</td>
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<td></td>
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<tr>
<td></td>
<td>10 µL of 10 µM</td>
<td></td>
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<td>20 µL of 10 µM</td>
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<td>30 µL of 10 µM</td>
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<td>20 µL of 100 µM</td>
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<tr>
<td></td>
<td>30 µL of 100 µM</td>
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</tr>
</tbody>
</table>
Table 10. Sensing Approach A Experiments Conducted to Determine *E. coli* Detection Limits

<table>
<thead>
<tr>
<th>Sensing Agent</th>
<th>Quantity of Sensing Agent</th>
<th><em>E. coli</em> Concentration in 100 µL of PBS (CFU/mL)</th>
<th>Quantity of β-Gal</th>
<th>Quantity of CPRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPEI-AgNPs</td>
<td>30 µL of 100 µM</td>
<td>10&lt;sup&gt;-5&lt;/sup&gt; – 10&lt;sup&gt;-2&lt;/sup&gt; and 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 250 µM</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>30 µL of 500 µM</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>30 µL of 1000 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 µL of 25 µM</td>
<td>10&lt;sup&gt;-5&lt;/sup&gt; – 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 µL of 25 µM</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18 µL of 25 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPEI-Fe&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>5 µL of 1 µM</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;, 10&lt;sup&gt;0&lt;/sup&gt;, and 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 50 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amine-Fe&lt;sub&gt;3&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>5 µL of 1 µM</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;, 10&lt;sup&gt;0&lt;/sup&gt;, and 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>30 µL of 0.125 µM</td>
<td>90 µL of 0.75 mM</td>
</tr>
<tr>
<td></td>
<td>5 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 50 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPEI</td>
<td>30 µL of 250 µM</td>
<td>10&lt;sup&gt;-4&lt;/sup&gt;, 10&lt;sup&gt;-2&lt;/sup&gt;, and 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µL of 10 µM</td>
<td>10&lt;sup&gt;-5&lt;/sup&gt; – 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 µL of 10 µM</td>
<td>10&lt;sup&gt;-5&lt;/sup&gt;, 10&lt;sup&gt;-3&lt;/sup&gt;, and 10&lt;sup&gt;-1&lt;/sup&gt; – 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>10 µL of 10 µM</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;, 10&lt;sup&gt;0&lt;/sup&gt;, and 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 250 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
<td>10 µL of 10 µM</td>
<td>10&lt;sup&gt;-2&lt;/sup&gt;, 10&lt;sup&gt;0&lt;/sup&gt;, and 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 µL of 250 µM</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
3.4.3 Sensing Approach B: Determination of the Required Amount of Sensing Agent Based on β-Gal Concentration

*Activity Assays*

The Sensing Approach B activity assays were based on determining the required sensing agent quantity to bind all the β-Gal quantity of 30 µL of 0.125 µM. The experimental conditions used in these assays included the use of the optimal volumes and concentrations of β-Gal (30 µL of 0.125 µM) and CPRG (90 µL of 0.75 mM), 100 µL of PBS to represent the *E. coli* volume, and 30 µL of the various concentrations of sensing agents listed in Table 11. The goal of this set of experiments was to determine the sensing agents that could successfully achieve the desired inhibition of the hydrolysis of CPRG by β-Gal for use in the subsequent *E. coli* detection experiments.

**Table 11. Sensing Approach B Activity Assay Experiments**

<table>
<thead>
<tr>
<th>Sensing Agent</th>
<th>Concentration of Sensing Agent (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPEI-AgNPs</td>
<td>10, 25, 50, 100, 200, 500</td>
</tr>
<tr>
<td>BPEI-Fe₂O₃</td>
<td></td>
</tr>
<tr>
<td>Amine-Fe₃O₄</td>
<td></td>
</tr>
<tr>
<td>BPEI-AuNPs</td>
<td></td>
</tr>
<tr>
<td>BPEI-M-IONP</td>
<td></td>
</tr>
<tr>
<td>BPEI-S-IONP</td>
<td></td>
</tr>
<tr>
<td>BPEI</td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>1000</td>
</tr>
<tr>
<td>CTAB</td>
<td>100, 250, 500, 750, 1000, 5000</td>
</tr>
</tbody>
</table>
**E. coli Detection Assays**

The Sensing Approach B *E. coli* detection assays utilized the promising sensing agents determined from the Sensing Approach B activity assays (i.e., the sensing agents that inhibited the hydrolysis of CPRG by β-Gal) to determine the *E. coli* detection limit. The optimal volumes and concentrations of β-Gal (30 µL of 0.125 µM) and CPRG (90 µL of 0.75 mM) were used in this set of experiments. The volume of *E. coli* suspensions in PBS buffer remained constant at 100 µL in these experiments. Various concentrations of 30 µL of sensing agent were tested across a range of microbial concentrations to determine their *E. coli* detection limit. The experiments that were performed in this Sensing Approach B detection assays are outlined in Table 12.

<table>
<thead>
<tr>
<th>Sensing Agent</th>
<th>Concentration of Sensing Agent (µM)</th>
<th><em>E. coli</em> Concentration (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPEI-AgNPs</td>
<td>200</td>
<td>$10^{-5} - 10^{2}$</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>$10^{-5} - 10^{2}$</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>$10^{-1} - 10^{8}$</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>$10^{-1} - 10^{8}$</td>
</tr>
<tr>
<td>BPEI-Fe₂O₃</td>
<td>50</td>
<td>$10^{-5} - 10^{2}$</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>$10^{-5} - 10^{8}$</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>$10^{-1} - 10^{8}$</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>$10^{-1} - 10^{8}$</td>
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<tr>
<td></td>
<td>600</td>
<td>$10^{-1} - 10^{8}$</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>$10^{-1} - 10^{8}$</td>
</tr>
<tr>
<td>BPEI-AuNPs</td>
<td>500</td>
<td>$10^{-1}, 10^{0}$, and $10^{2}$</td>
</tr>
<tr>
<td>BPEI</td>
<td>500</td>
<td>$10^{-1}, 10^{0}$, and $10^{2}$</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>$10^{-1} - 10^{8}$</td>
</tr>
<tr>
<td>CTAB</td>
<td>5000</td>
<td>$10^{-1}, 10^{0}$, and $10^{2}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{6} - 10^{8}$</td>
</tr>
</tbody>
</table>
4. RESULTS AND DISCUSSION

4.1 *Escherichia coli* Concentrations

The results of the serial dilutions experiment to calculate *E. coli* concentration in CFU/mL indicated that the 10^-6 dilution was the only one with 30 to 200 colonies grown on the TSA plate. Therefore, this dilution was used to calculate the *E. coli* concentrations using Equation 1. From the three trials that were performed on separate days, 40 to 130 colonies grew on the plates (Figure 19). These resulted in *E. coli* concentrations ranging from 4.2 x 10^8 CFU/mL to 1.30 x 10^9 CFU/mL. When making *E. coli* stock solutions for the detection assays, the quantity of growth in the tube after the shaking incubator was visually compared to the growth obtained from these microbial concentration experiments. If no comparable growth was obtained, then a stock solution was prepared again. Based on these trials and observations, the 10^9 CFU/mL concentration corresponded well with the stock solution. Table 13 presents the *E. coli* concentrations and their corresponding dilutions. The concentrations of *E. coli* for the Sensing Approach A and Sensing Approach B detection assay experiments were prepared according to Table 13.
Figure 19. TSA plates after 24 hours of incubation at 37 °C. Duplicates of the 10^-6 and 10^-7 dilutions were performed because those were the plates closest in count to the 30 to 200 colony counts used for microbial concentration calculations.

Table 13. E. coli Dilutions from Stock Solution and Their Corresponding Concentrations

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Concentration (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Solution (10^0)</td>
<td>10^0</td>
</tr>
<tr>
<td>10^-1</td>
<td>10^9</td>
</tr>
<tr>
<td>10^-2</td>
<td>10^8</td>
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<tr>
<td>10^-3</td>
<td>10^7</td>
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<tr>
<td>10^-4</td>
<td>10^6</td>
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<tr>
<td>10^-5</td>
<td>10^5</td>
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<td>10^-6</td>
<td>10^4</td>
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<td>10^-7</td>
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<tr>
<td>10^-13</td>
<td>10^-3</td>
</tr>
<tr>
<td>10^-14</td>
<td>10^-4</td>
</tr>
</tbody>
</table>
4.2 Characteristics of the Modified BPEI-M-IONP

The concentration of the BPEI-M-IONP suspension shown in Figure 20 was 870 mg/L (3757 µM) based on the weight of the filter before and after drying, and the volume of BPEI-M-IONP suspension filtered.

Figure 20. Modified iron oxide nanoparticles with a BPEI coating before being placed in the oven for drying prior to characterization and concentration determination.

Thermogravimetric analysis (TGA) was performed to validate the coating of BPEI on the surface of the commercial iron oxide nanoparticle (BPEI-M-IONP). Figure 21 presents the TGA curve for the BPEI-M-IONP. This TGA data shows the sample weight loss as a function of temperature as well as the second derivative of the weight loss curve, which indicates the peak temperature at which the maximum weight loss happened. The sample weight loss was observed at 24.2 °C, 57.3 °C, 240.2 °C, and 552.4 °C. The weight loss before 240.2 °C could be attributed to the evaporation of any absorbed solvents remaining from the synthesis or modification processes (Arabi et al., 2016). The weight loss after 240.2 °C could be attributed to the degradation of the BPEI coating present on the surface of the iron oxide nanoparticles (Arabi et al., 2016). This TGA curve is in
agreement with the weight loss profile for PEI coated Fe₃O₄ nanoparticles reported by Arabi et al. (Arabi et al., 2016). The weight loss that occurred in the temperature range of 240.2 – 552.4 °C was approximately 7.7%. This amount of weight loss likely corresponds to the amount of BPEI on the surface of the nanoparticles as well as any other organic chemical coatings that the pre-modified commercial iron oxide nanoparticles possessed. The chemical composition of the coating of this nanoparticle is propriety information and thus, could not be verified.

**Figure 21.** TGA results for the BPEI-M-IONP. The blue line represents the heat flow curve, the green line represents the weight loss curve, and the red line represents the second derivative of the weight loss curve.
4.3 Characteristics of the Synthesized BPEI-S-IONP

Magnetic iron nanoparticles were synthesized and coated with BPEI (Figure 22). The magnetic characteristics were visually observed when the produced nanoparticles attached to a magnetic stir bar used for mixing the suspension. The concentration of the BPEI-S-IONP in this suspension was 7518 mg/L (32,470 µM) based on the weights of the beaker with the BPEI-S-IONP suspension, the beaker after drying in the oven, and the empty beaker, as well as the assumption that the liquid that the nanoparticles were suspended in has the same density as water.

![Synthesized magnetic iron oxide nanoparticles coated in BPEI before being placed in the oven for drying prior to characterization and concentration determination.](image)

**Figure 22.** Synthesized magnetic iron oxide nanoparticles coated in BPEI before being placed in the oven for drying prior to characterization and concentration determination.

After drying a sample of the BPEI-S-IONP suspension in the oven, samples of the dried nanoparticles’ powder were used for the FTIR and TGA analyses, which aimed at verifying the successful coating of the nanoparticles with BPEI. The FTIR spectra obtained herein (Figure 23) was compared to the FTIR spectra of PEI coated Fe₃O₄
nanoparticles found in literature. The FTIR spectra showed a peak around 1471 cm\(^{-1}\) (Figure 23). This peak could potentially be related to the scissoring of CH\(_2\) or C-H bending (Karimzadeh et al., 2017; Millipore Sigma, 2021c). The FTIR spectra also showed a peak around 1600 cm\(^{-1}\), which could be attributed to an amide bond of C=O, NH\(_2\) groups, or N-H bending of an amine (Arabi et al., 2016; Karimzadeh et al., 2017; Millipore Sigma, 2021c). In addition, there was a peak at 1751 cm\(^{-1}\), which could be related to a carboxyl group (Arabi et al., 2016). Lastly, the peaks observed around 2900 cm\(^{-1}\) in the FTIR spectra could be due to the N-H bond stretching (Lee, 2020). In addition, primary and secondary amines reside in the 3300 – 3500 cm\(^{-1}\) range. There were no distinct peaks in this range; however, this could be due to potential components/other factors that could have inhibited the formation of a distinct BPEI peak in the FTIR spectra. These potential components/factors include 1) the Span 80, a surfactant used for synthesis of the BPEI-S-IONP, which contains various functional groups that could appear in the FTIR spectra (Choudhury et al., 2013); 2) water vapor from the air that could have been absorbed when loading nanoparticle powder onto the FTIR, and water vapor can exhibit an FTIR peak in the 3300 – 3500 cm\(^{-1}\) range; and 3) the amount of BPEI that was loaded on the nanoparticles could have been small and thus, did not result in detectable FTIR absorption peaks. Nonetheless, the presence of the other peaks that are characteristic of BPEI in the FTIR spectra could be an indication that BPEI was successfully loaded on the synthesized iron oxide nanoparticles.
Figure 23. FTIR spectrum of the BPEI-S-IONP powder samples.

Thermogravimetric analysis (TGA) was also performed to validate the coating of BPEI on the surface of the synthesized iron oxide nanoparticle (BPEI-S-IONP). Figure 24 presents the TGA curve for the BPEI-S-IONP sample’s weight loss as a function of temperature as well as the second derivative of the weight loss curve. This second derivative data show the temperatures at which the maximum weight losses occurred. The sample weight loss was observed at 28.2 °C, 68.4 °C, 277.6 °C, and 359.4 °C. This is similar to the BPEI-M-IONP TGA curve where the weight loss after 277.6 °C could be attributed to the degradation of the BPEI coating on the surface of the iron oxide nanoparticles (Arabi et al., 2016). This TGA curve is in agreement with the weight loss profile for PEI coated Fe$_3$O$_4$ nanoparticles reported by Arabi et al. (Arabi et al., 2016). The weight loss that occurred in the temperature range of 277.6 – 359.4 °C was approximately 12.2%. This is greater than the coating on the BPEI-M-IONPs which was 7.7%.
Figure 24. TGA results for the BPEI-S-IONP. The blue line represents the heat flow curve, the green line represents the weight loss curve, and the red line represents the second derivative of the weight loss curve.

4.4 Optimization of the Colorimetric Assay

Before the *E. coli* detection assays could be performed, the optimal solution volumes and concentrations of β-Gal and CPRG had to be determined. The optimal amounts of these reagents were defined herein as the β-Gal and CPRG amounts that would result in complete hydrolysis of CPRG. When CPRG is hydrolyzed to CPR, the solution color changes from yellow to red. To determine the optimal solution volumes and
concentrations of β-Gal and CPRG, two main experiments were performed with varying volumes of 0.125 µM β-Gal and 0.75 mM CPRG. The first experiment tested the combination of volumes and concentrations shown in Figure 25. In addition, the two columns on the right of the plate were performed with 102 µL of PBS present in addition to the of β-Gal and CPRG to determine if this dilutes the color change of the solution. This addition of PBS represents the volume that would otherwise be *E. coli* plus sensing reagent in the subsequent detection assays. Since all wells turned red after three hours, the lowest volumes of β-Gal to CPRG that resulted in complete hydrolysis was 30 µL of β-Gal and 90 µL of CPRG. This mixture was chosen for the subsequent experiment because it corresponded to lower amounts of reagents used and thus, would translate into lower cost of the *E. coli* detection assay developed in this study.

![Well plate results for varying CPRG and β-Gal volumes after three hours at room temperature. Column 10 is a duplicate of column 9.](image)

**Figure 25.** Well plate results for varying CPRG and β-Gal volumes after three hours at room temperature. Column 10 is a duplicate of column 9.

Then, another trial was conducted in an attempt to find whether or not the lower amounts of β-Gal to CPRG shown in Figure 26 would result in complete hydrolysis of CPRG. A gradient of color change from red to yellow was obtained when the volumes of reagents were decreased (Figure 26). The 30 µL of 0.125 µM β-Gal and 90 µL of 0.75 mM CPRG resulted in the darkest red color indicating optimal hydrolysis conditions for colorimetric
sensing. Therefore, 30 μL of 0.125 μM β-Gal and 90 μL of 0.75 mM CPRG were the reagent amounts used in the subsequent activity and *E. coli* detection assays.

![Figure 26](image)

**Figure 26.** Second trial for optimizing the amounts of CPRG and β-Gal needed for the colorimetric detection assays.

### 4.4 Results of Sensing Approach A Trials

#### 4.4.1 Activity Assays

The activity assays were conducted to determine which of the following factors is responsible for inhibiting the hydrolysis of CPRG by β-Gal - the surface charge, the nanoparticle type, or the type of chemical coating. These results of the activity assays were also used to determine which of the tested nanomaterials/chemicals had potential for subsequent use in the *E. coli* detection assays. This potential was demonstrated when the nanoparticle/chemical agent was able to bind with β-Gal and inhibit its reaction with CPRG and thus, kept the solution color unchanged (i.e., solution remain yellow). The following Sensing Approach A activity assays were performed using the amounts of assay components presented in Table 8 in Section 3.4.2.
**BPEI Coated Silver Nanoparticles (BPEI-AgNPs)**

Using the volumes and concentrations of β-Gal (2 µL of 0.125 µM) and CPRG (30 µL of 0.75 mM), the first test of BPEI-AgNPs (2 µL of 100 µM) resulted in the solutions remaining yellow. The second experiment utilizing the same volumes and concentrations of β-Gal and CPRG used in the first experiment, and both 2 µL of 100 and 250 µM of BPEI-AgNPs, remained yellow as well. The third experiment resulted in all wells remaining yellow when the optimized concentrations and volumes of β-Gal to CPRG (30 µL of 0.125 µM and 90 µL of 0.75 mM, respectively) were used (Figure 27).

![Image](image_url)

**Figure 27.** Third activity assay with BPEI-AgNPs and the optimized concentrations and volumes of β-Gal (30 µL of 0.125 µM) and CPRG (90 µL of 0.75 mM). The concentrations of BPEI-AgNPs (2 µL aliquots) tested are indicated above.

These results indicated that positively BPEI-AgNPs inhibited the reaction between β-Gal and CPRG and thus, could be a candidate material for use in the subsequent *E. coli* detection assays. Therefore, these results were an indicator that gold nanoparticles originally used in Method 1 could be replaced with other types of nanomaterials since BPEI-AgNPs inhibited the activity of β-Gal as hypothesized herein.
Other Sensing Materials

More experiments were conducted in this study to 1) investigate other nanoparticle types/chemical agents as potential candidates for β-Gal inhibition and 2) determine whether the BPEI is the only coating that causes inhibition. If other positively charged chemicals are also able to prevent the hydrolysis reaction, then, it could be determined that the surface charge rather than the composition of the polymer is responsible for this inhibition.

Next, BPEI-Fe$_2$O$_3$ and amine-Fe$_3$O$_4$ were tested to determine if nanoparticles aside from BPEI-AuNP or BPEI-AgNP have potential at inhibiting the β-Gal and CPRG reaction. Chitosan and CTAB, positively charged polyelectrolytes, were also tested for their ability to inhibit the enzyme activity. The results of these tests indicated that each well turned red after three hours of wait time (Figure 28), which means none of the tested materials resulted in inhibition of the β-Gal activity. Chitosan was hardly soluble in water even after testing various methods to dissolve it such as heating the liquid or purging CO$_2$ into it. This lack of solubility is likely the reason there was not enough chitosan present in solution to cause β-Gal inhibition. Other methods such as dissolving the chitosan in acetic acid were not tested because of the potential for damaging the activity of the β-Gal by the acid. The lack of inhibition of β-Gal’s activity in the cases of CTAB, BPEI-Fe$_2$O$_3$, and amine-Fe$_3$O$_4$ could have been caused by insufficient amount of materials used and thus, higher concentrations of each sensing agent were tested in the Sensing Approach B activity assays.
Figure 28. Results of chitosan (upper left), CTAB (lower left), BPEI-Fe$_2$O$_3$ (upper right), and amine-Fe$_3$O$_4$ (lower right) after three hours. The wells covered by the opaque box were not applicable to this activity assay experiment.

4.4.2 *E. coli* Detection Assays Results

*BPEI Coated Silver Nanoparticles (BPEI-AgNPs)*

Various concentrations of BPEI-AgNPs were tested for the detection of different concentrations of *E. coli* as previously outlined in Table 10. Column six in the well plate in Figure 29 showed that the β-Gal/CPRG control samples (i.e., no *E. coli* or nanoparticles) turned red. This was an indication that these reagents performed as expected. The nanoparticle control samples shown in column seven (i.e., no microbes were present) remained yellow, which was an indication that the BPEI-AgNPs were able to effectively inhibit the reaction of β-Gal and CPRG (Figure 29). The wells with *E. coli*, columns one through five, were expected to turn red (or a color in between orange and red depending on the microbial concentration). However, they remained yellow,
indicating that the BPEI-AgNPs was not able to detect *E. coli* under the conditions tested. It was hypothesized that the lack of detection capability of BPEI-AgNPs could potentially be explained by an abundance of BPEI-AgNPs in the suspension. When all the *E. coli* are bound to BPEI-AgNPs, the rest of the unbound nanoparticles in suspension would bind with the β-Gal and result in a yellow color (i.e., inhibition of the activity). Thus, the next set of BPEI-AgNPs experiments were focused on finding the optimal amount of nanoparticles to be used in the system.

![Figure 29](image)

**Figure 29.** Well plate results after three hours in the SpectraMAX plus. The values across the top are the *E. coli* concentrations in CFU/mL.

Keeping the *E. coli* concentration fixed at $10^2$ CFU/mL and testing volumes ranging from 5 – 30 µL of 100 and 10 µM BPEI-AgNPs produced the results shown in Figure 30. The wells containing 100 µM BPEI-AgNPs remained yellow while wells containing 10 µM BPEI-AgNPs turned red. The goal of this experiment was to determine the volume and concentration of BPEI-AgNPs that would detect the highest concentration of microbes.
and therefore turn the solution red. Since there was a distinct color change from yellow at 6 μL of 100 μM and red at 30 μL of 10 μM, the values used herein needed to be refined to test quantities of BPEI-AgNPs in between, as was performed in the following trial. This would ideally result in a gradient of color from yellow to red.

![Figure 30](image.png)

**Figure 30.** Results of testing volumes of BPEI-AgNP ranging from 5 – 30 μL after incubation for three hours at room temperature.

Another test was conducted using $10^2$ CFU/mL of *E. coli* while changing the volumes and concentrations of BPEI-AgNP, and the results of this test are shown in Figure 31. The red color in the wells was interpreted as an indication of insufficient number of BPEI-AgNPs to bind all the microbes and thus, no inhibition of β-Gal and CPRG would take place. On the other hand, the 25 μM BPEI-AgNP wells resulted in a gradient of color from yellow to red as the volume of BPEI-AgNPs decreased (columns 9 and 10 in
Figure 31. These results likely indicate that once all the microbes were bound by the nanoparticles, the excess nanoparticles left in solution at higher volumes were free to bind with β-Gal and thus, resulted in a color profile that was progressing towards yellow. The volumes that started to turn the solution red were 18, 15, and 12 µL. Therefore, these were the amounts of nanoparticles that were hypothesized to be able to bind $10^2$ CFU/mL \textit{E. coli} tested. Therefore, the next experiment used these amounts across a range of microbial concentrations to determine the detection limit of \textit{E. coli}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{well_plate_results.png}
\caption{Well plate results for varying volumes and concentrations of BPEI-AgNPs after incubation for three hours at room temperature. Columns 6 and 10 are replicates of columns 7 and 9, respectively.}
\end{figure}

When the volumes of 25 µM BPEI-AgNP ranging from 18 to 12 µL were tested, all the wells were yellow across microbial concentrations of $10^{-5}$ – $10^2$ CFU/mL (Figure 32). One potential reason for not obtaining a color gradient from red to yellow as the concentration of microbes decreased could be the potential toxicity of silver nanoparticles and/or silver ions to \textit{E. coli}. Over time, silver nanoparticles dissolve and release silver ions into solution. Both silver nanoparticles and silver ions have been reported to result in
toxic effects to microbes through various mechanisms including causing oxidative stress
or binding to the bacterial cell wall and nuclear membrane resulting in cell distortion and
death (El Badawy et al., 2011; Rai et al., 2009; Navarro et al., 2008). However, it remains
uncertain whether or not the toxicity of silver was the reason the sensor did not function
as desired. This is because two scenarios could happen if toxic effects took place. If the
nanoparticles kill the microbes and then are released again into solution, they would bind
with β-Gal, inhibit its activity, and the solution would turn yellow. On the other hand, if
the nanoparticles killed the microbes and stayed bound to fragments of the cell wall of
the dead microbes, the sensor should still work and the solution should change color.
Therefore, more research was conducted herein on BPEI-AgNPs before ruling it out as
one of the potential sensors for detection of E. coli.

Figure 32. Well plate results after three hours of incubation at room temperature. The
volumes across the top indicate the volumes of the 25 µM BPEI-AgNP solution. The
values to the left of the plate are the E. coli concentrations in CFU/mL.
In summary, the aforementioned BPEI-AgNPs experiments did not lead to a working sensor that can detect the *E. coli* concentrations tested. In addition, there is a possibility that the AgNPs and silver ion toxicity could play a role in the *E. coli* detection. However, the factor of toxicity does not rule out BPEI-AgNPs as a possible detection sensing agent. This is due to multiple reasons including the rate of toxicity being unknown (i.e., time it takes for these nanoparticles/ions to kill the microbes could be longer than the duration of this experiment), and whether the BPEI-AgNPs can leave the microbes once they are dead or if they stay attached to a dead portion of the cell wall. If it takes longer than the three hours required for the detection assay for toxicity to be prevalent, then the BPEI-AgNPs could still be a potential detection sensing agent. In addition, if the nanoparticles can remain attached to dead parts of a microbial cell, then detection is still possible. That is the reason BPEI-AgNPs were tested again in subsequent experiments in Sensing Approach B using a different approach for optimizing their amount.

**Branched Polyethyleneimine (BPEI)**

The first experiment with BPEI (30 µL of 250 µM) and microbes at $10^{-4}$, $10^{-2}$, and $10^2$ CFU/mL resulted in all wells remaining yellow. This indicated that the β-Gal was bound by the BPEI and thus, was unable to react with CPRG and turn red. Therefore, the BPEI did not likely bind to the microbes like BPEI coated nanoparticles have been able to do in past experiments.

The second experiment resulted in a distinct gradient of color from yellow to red for the column with 10 µM BPEI (Figure 33). The higher volumes of this concentration resulted in a yellow solution. One potential reasoning to this is that once all the microbes were
bound by BPEI, the excess BPEI that was free in solution were able to bind with the β-Gal and inhibit the reaction between CPRG and β-Gal. This reasoning assumes that BPEI can act like BPEI coated nanoparticles (i.e., physically bind the β-Gal through Coulombic interactions), and therefore one might conclude that either the charge of the sensing agent or the BPEI itself is the reasoning for the inhibition of the reaction between CPRG and β-Gal. Another possible explanation of this gradient in color change could be that the microbes were not involved in the interactions and the higher the concentration of BPEI, the higher the availability of BPEI to bind more β-Gal and thus, inhibit its reaction with CPRG. Therefore, the color of solution observed was independent of the presence of microbes and was instead dependent on the concentration of BPEI. The 100 µM BPEI column did not exhibit this gradient in color change (Figure 33). This was potentially due to the concentration being too large and therefore all the β-Gal is bound by the BPEI at all amounts tested.
Figure 33. BPEI second experiment well plate results after three hours of incubation at room temperature. The volumes listed are those corresponding to the BPEI concentrations.

The third experiment with BPEI (5 µL of 10 µM) aimed to determine the detection limit by testing *E. coli* concentrations ranging from $10^{-5} – 10^2$ CFU/mL. The 5 µL of 10 µM BPEI was chosen due to the red color wells in the second BPEI experiment described above. In this experiment, each well turned red except for one of the triplicates at $10^{-1}$ CFU/mL, which was considered an outlier (Figure 34). This occurrence of all the wells turning red despite the changing concentrations of *E. coli* could likely be a result of lacking enough BPEI amount to bind all the microbes in solution.
Figure 34. Third BPEI well plate results after three hours of incubation at room temperature. The volume and concentration of BPEI was 5 µL and 10 µM, respectively. Columns 2 and 3 of the well plate are replicates of column 1.

The last experiment with BPEI in Sensing Approach A continued investigations to determine the optimal amount of BPEI for the detection of *E. coli*. The wells that contained 8 µL of 10 µM BPEI turned orange while those with 15 µL of 10 µM BPEI turned gold (Figure 35). This shows similar results to the previous experiment where the color of the solutions in the wells remained the same despite the varying concentration of *E. coli* present in the wells. In the absence of a sensing agent, it is expected that the color of the well would be independent of the amount *E. coli* present because the *E. coli* carries a negative charge like the β-Gal and thus, repulsive forces would prevent their interactions. Therefore, the color change observed in these wells only depended on the volume and concentration of BPEI added. Thus, this color change might be explained by the excess BPEI at higher concentrations that was able to bind with β-Gal to create a less red color (i.e., cause different degrees of inhibition based on its concentration). Toxicity
of BPEI could also be another factor that could explained the observed color gradients shown in Figure 35.

![Figure 35. Fourth BPEI well plate results after three hours incubation at room temperature. The volume and concentration of BPEI as well as the concentration of E. coli present in the wells are listed on the plate photo.](image)

In summary, after testing multiple volumes and concentrations of BPEI, it was determined that a concentration of 10 µM could be used with varying volumes to observe a color gradient. At a concentration of $10^2$ CFU/mL of *E. coli*, a volume of 5 µL of 10 µM BPEI resulted in a red color (no inhibition of the CPRG and β-Gal reaction) while a volume of 15 µL resulted in a yellow color (inhibition of the CPRG and β-Gal reaction). When the range of microbial concentrations was tested, a color gradient was not observed, possibly indicating that the *E. coli* was not involved in the reactions taking place in the well, rather the higher the concentration of BPEI, the more is available to bind with β-Gal and inhibit the reaction to turn more yellow. It appears that the BPEI is
potentially unable to bind to the \textit{E. coli} at low concentrations of microbes and detect their presence or could be killing the microbes at these concentrations that exhibited yellow colors in the wells. It might also be possible that the BPEI requires a carrier material (like a nanoparticle) in order to improve its sensitivity to detect microbes.

\textit{Other Sensing Agents}

Another set of experiments was conducted to test the feasibility of chitosan, CTAB, BPEI-Fe$_2$O$_3$, and Amine-Fe$_3$O$_4$ as potential sensing agents. The chitosan solution was barely soluble in DI water; therefore, the concentration of the chitosan in solution was insufficient to provide any conclusive results as mentioned in Section 4.4.1. For the CTAB, BPEI-Fe$_2$O$_3$, and Amine-Fe$_3$O$_4$, all wells turned red at all concentrations of \textit{E. coli} (Figure 36). The concentration of these materials used in this trial could have been too low resulting in some of the \textit{E. coli} bound by all the sensing agents, leaving excess \textit{E. coli}, $\beta$-Gal, and CPRG free in solution to react and turn the solution to red. Since the \textit{E. coli} does not interact with $\beta$-Gal and CPRG (Figure 37), the $\beta$-Gal and CPRG were free to interact and create a red color regardless of the concentration of \textit{E. coli} tested.
Figure 36. Results of chitosan (upper left), CTAB (lower left), BPEI-Fe₂O₃ (upper right), and amine-Fe₃O₄ (lower right) after three hours of incubation at room temperature. *E. coli* concentrations in CFU/mL are listed across the center of the plate.

Figure 37. Experiment with *E. coli* (concentrations on the left), CPRG, and β-Gal (no sensing agents were added).

In the presence (Section 4.4.2) and absence (Section 4.4.1) of *E. coli* every well turned red. This indicated that there was no inhibition of the reaction between β-Gal and CPRG at the concentrations tested for these sensing agents. In addition, no color gradient was obtained as the microbial concentrations changed. Therefore, further experiments were
conducted using an entirely different approach as presented in Sensing Approach B of this study.

4.5 Results of Sensing Approach B Trials

Sensing Approach B trials were based on finding the amount of sensing agent that would be sufficient to bind the $\beta$-Gal quantity of 30 $\mu$L of 0.125 $\mu$M in the absence of *E. coli*. This differed from Sensing Approach A, which was based on finding the sensing agent quantity that would be enough to bind the highest concentration of *E. coli* ($10^2$ CFU/mL) tested in those experiments. Sensing Approach B approach is more practical because the *E. coli* concentration will be unknown in real-world applications.

4.5.1 Activity Assay Results

This activity assay aimed to determine the sensing agent concentration that was enough to fully inhibit the reaction between CPRG and $\beta$-Gal, and result in a yellow color. Each column on the plate in Figure 38 represents one sensing agent being tested at varying concentrations (with the lowest concentration at the top of the column and the highest at the bottom). This experiment was conducted using the amounts of chemical agents presented in Table 11. The sensing agents that showed potential for inhibiting the reaction between CPRG and $\beta$-Gal were the CTAB, BPEI, BPEI-AgNPs, and BPEI-Fe$_2$O$_3$. The lowest concentrations that resulted in inhibition of the $\beta$-Gal (i.e., resulting in yellow color wells) were 5000 $\mu$M of CTAB, 500 $\mu$M of BPEI, and 200 $\mu$M of BPEI-AgNPs. In addition, an orange color was observed at 100 $\mu$M of BPEI-Fe$_2$O$_3$. Therefore, these materials were determined to be possible candidates for acting as sensing agents for detecting *E. coli* and the optimal concentrations that caused full inhibition in this activity.
assay were used in the subsequent *E. coli* detection assays. The changes in color and how they relate to optical density values can be seen in Figures 39 – 42. For all sensing agents, as the concentration decreased, the optical density increased and the solution became more red.

**Figure 38.** Results of the Sensing Approach B activity assay after three hours of incubation at room temperature.
Figure 39. SpectraMAX Plus optical density results for the CTAB. The 5000 µM has a low optical density (yellow), while the lower concentrations have a high optical density (red).

Figure 40. SpectraMAX Plus optical density results for the BPEI. A gradient of color is seen with varying optical density values.
Figure 41. SpectraMAX Plus optical density results for the BPEI-AgNPs. The 500 and 200 µM have a low optical density (yellow), while the lower concentrations have a high optical density (red).

Figure 42. SpectraMAX Plus optical density results for the BPEI-Fe₂O₃. The 500 and 200 µM have a low optical density (yellow), while the 100 µM has an average optical density (orange), and the lower concentrations have higher optical densities (red).

The results of chitosan were inconclusive because the concentration in solution was not accurately known. Chitosan was barely soluble in water. Attempts were made to increase its solubility by bubbling in CO₂ gas in solution as well as heating the solution to dissolve it further. However, these attempts were not successful, and chitosan was still observed settling in solution. Therefore, wells containing chitosan had unknown concentrations and thus, no conclusions could be made about these results.
The BPEI-M-IONP and BPEI-S-IONP were not very stable, and precipitation was observed in the tubes. The solutions were mixed vigorously prior to adding them to the wells, but the nanoparticles started settling right after the mixing was stopped. Therefore, potential reasoning as to why all wells containing these nanoparticles turned red is that a significant portion of the nanoparticles were not present in suspension during the test and thus, minimal interactions took place between these nanoparticles and the β-Gal. Therefore, the β-Gal was free to interact with CPRG and turn red.

One critical conclusion from this well plate experiment is that the CTAB was able to inhibit the reaction between CPRG and β-Gal. This would prove the research hypothesis that the positive surface charge rather than the sensing agent itself is responsible for the mechanisms of this detection protocol. These Coulombic attractions between the positively charged sensing agents and the negatively charged bacteria or β-Gal are what drives the competitive binding. This finding has promising applications because theoretically other sensing agents that protonate in solution and result in a positive charge could be used for sensing microbes. Thus, this research finding opens doors for future research investigations to optimize this sensing protocol using other positively charged polyelectrolytes.

4.5.2 Results of the *E. coli* Detection Assays

The first *E. coli* detection assay performed in Sensing Approach B was conducted for microbial concentrations ranging from $10^{-5}$ – $10^2$ CFU/mL. In this experiment, the volumes, and concentrations of the sensing agents used were the ones that resulted in full inhibition of the CPRG and β-Gal reaction in the activity assay outlined in Section 4.5.1. The results of this test are presented in Figure 43. Regardless of the concentration of *E.
coli tested, the color of the wells remained unchanged (no color gradient was obtained) and the color was only dependent on the concentration of sensing agent tested. These results likely indicate that the microbes did not interact with the sensing agents at the testing conditions investigated. One potential explanation for this lack in interactions would be that the microbial concentrations tested were too low for this physical competitive binding mechanism to work effectively. It is hypothesized herein that the Coulombic attractions that occur between the sensing agents and microbes may only be dominant over the attraction between the sensing agents and β-Gal when there are enough microbes in solution. This is because β-Gal dissolves uniformly in solution and thus, it is available everywhere around the nanoparticles to interact with. On the other hand, both E. coli and the nanoparticles are suspended in the liquid and their interactions are limited by the random collisions that happen by Brownian motion. Therefore, there is a higher likelihood that a nanoparticle will encounter a uniformly distributed enzyme than a microbe unless there are plenty (dense concentration) of microbes available in suspension. Only in the latter case that the competitive binding mechanism dominates, and the stronger Coulombic attractions will favor the binding of the sensing agent to E. coli over β-Gal. To test this hypothesis, a second experiment was performed at a higher E. coli concentration ranging from $10 - 10^{14}$ as described later in this section.
Figure 43. Results of the first *E. coli* detection assay in Sensing Approach B after three hours in the SpectraMAX Plus. For columns that extend from top to bottom of the plate, the *E. coli* concentrations in CFU/mL along the left side of the plate apply. For columns 7 to 9, the *E. coli* concentrations are $10^{-1}$, $10^{0}$, and $10^{2}$ CFU/mL from top to bottom.

The second *E. coli* detection assay experiment was conducted at a wider range of *E. coli* concentrations ($10^{-5} - 10^{8}$) and utilized the following sensing agents: 200 µM BPEI-Fe$_2$O$_3$, 200 µM BPEI-AgNPs, 5000 µM CTAB, or 500 µM BPEI. A color gradient was visually observed at $10^{7}$ and $10^{8}$ CFU/mL for BPEI-Fe$_2$O$_3$, $10^{5} - 10^{8}$ CFU/mL for BPEI-AgNPs, and $10^{6} - 10^{8}$ CFU/mL for BPEI (Figure 44). The abrupt color change from yellow to red for the BPEI-Fe$_2$O$_3$ could be a result of the lower zeta potential of this nanoparticle type compared to BPEI-AgNPs. This leads to less sensitivity in the nanosensor because the electrostatic interactions would be weaker. To quantitatively analyze the color change observed in this experiment, the optical density results are presented in Figure 45. These optical density results were generally in agreement with the
visual observations of color gradient (i.e., higher optical densities were observed for wells that were darker in color).

Figure 44. Results of the second *E. coli* detection assay for (a) BPEI-Fe$_2$O$_3$, (b) BPEI-AgNPs, and (c) BPEI after three hours of incubation at room temperature.

Figure 45. SpectraMAX Plus optical density results for the second *E. coli* detection assay. The *E. coli* concentrations are represented as exponents (e.g., 8 is equivalent to 10$^8$ CFU/mL).
The results of this trial showed that BPEI alone, BPEI-Fe$_2$O$_3$, and BPEI-AgNPs have the potential for use as nanosensors for detection of *E. coli*. However, more research is needed to lower the detection limit of these sensors. These results also proved the hypothesis of this research that the nanoparticle type is not the governing factor and what matters the most is the chemical coating/surface charge. Furthermore, these results show that the coating itself may be all what is needed to detect the microbes because BPEI alone worked without a carrier nanoparticle (although more work is needed to improve its detection limit). These findings have not been reported in the literature before and open possibilities for a multitude of potential sensing agents that could be used without the need for a nanoparticle carrier. This would reduce the cost of the sensors and enhance their simplicity.

The third *E. coli* detection assay aimed to increase the concentration of sensing agents to increase the likelihood of detecting lower concentrations of *E. coli* in solution. The *E. coli* concentrations that ranged from $10^{-1} - 10^5$ CFU/mL remained yellow for BPEI-Fe$_2$O$_3$, BPEI-AgNPs, and the BPEI (Figure 46). Color change was observed at $10^7$ and $10^8$ CFU/mL for most sensing agent concentrations.
Figure 46. Results of the third *E. coli* detection assay after three hours of incubation at room temperature. The *E. coli* concentrations in the first two columns repeat for the other concentrations of sensing agent on the plate.

The optical density results for both the concentrations of BPEI-Fe$_2$O$_3$ and BPEI-AgNPs are graphed in Figures 47 and 48. These are the results from the third hour of measurements. *E. coli* concentrations of $10^7$ and $10^8$ CFU/mL showed a high optical density due to the dark red colors. The abrupt change in optical density for BPEI-AgNPs shows a low sensitivity of the nanosensor. In addition, the peak at 300 µM and $10^5$ CFU/mL for BPEI-AgNPs is assumed to be an outlier. For BPEI-Fe$_2$O$_3$ and BPEI-AgNPs, as the concentration of the nanoparticles increased, the well containing $10^7$ CFU/mL became more yellow. This could be due to an excess of nanoparticles being in solution. When there are excess nanoparticles in solution, after all *E. coli* are bound, they
start to bind with the β-Gal and turn the solution yellow. This idea can be visualized in the hypothetical behavior presented in Figure 49. The point where the sensing agent has bound all the *E. coli* and begins to bind with β-Gal is at the peak of the graph.

**Figure 47.** SpectraMAX Plus optical density results for the BPEI-Fe$_2$O$_3$ concentrations for the third *E. coli* detection assay. The *E. coli* concentrations are represented as exponents (e.g., 8 is equivalent to 10$^8$ CFU/mL).

**Figure 48.** SpectraMAX Plus optical density results for the BPEI-AgNPs concentrations for the third *E. coli* detection assay. The *E. coli* concentrations are represented as exponents (e.g., 8 is equivalent to 10$^8$ CFU/mL).
Figure 49. Hypothetical representation of optimal sensing agent concentration. Left of the peak represents the sensing agent binding with *E. coli*. Right of the peak represents the sensing agent completely bound to *E. coli* and the excess chemical binding with the β-Gal.

The results of Sensing Approach B experiments show that there is potential for some of the materials tested in this study to work as sensors for detection of *E. coli*. However, the detection limit for these sensors need to be enhanced drastically to allow for their use in practical applications. The future recommendations section (Section 5.0) outlines some suggestions for investigations that need to be conducted to gain more understanding of how these nanosensors work and to lower their detection limits.

4.6 Cost Analysis

A preliminary cost analysis was performed on the nanosensors that detected *E. coli* in this study. The costs of these sensors was also compared to those of standard microbial detection methods. The cost analysis of the nanosensors was performed per sample, which is represented by one well in the well plate. One well would contain CPRG, β-Gal, and the sensing agent. The volume and concentration of sensing agent used for this cost analysis were those that resulted in successful detection of *E. coli* (i.e., the cases where a gradient of color change was obtained with varying *E. coli* concentrations). The standard
microbial detection methods include only the cost of the materials itself (i.e., P/A bottles or IDEXX tray). Table 14 displays the results of the cost analysis. It is evident that the nanosensors are a low-cost alternative to standard microbial detection methods. In addition, the BPEI alone was the most cost effective nanosensor compared to BPEI-AgNPs and BPEI-Fe$_2$O$_3$. This cost analysis is a preliminary high-level estimate that only includes material costs. Factors such as labor, equipment, location, supplier, taxes, and shipping fees were not accounted for in this analysis.

<table>
<thead>
<tr>
<th>Table 14. Cost Analysis of Nanosensors and Standard Microbial Detection Methods</th>
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<thead>
<tr>
<th>Components of Nanosensors</th>
<th>Individual Components</th>
<th>Cost per Sample ($)</th>
<th>Quantity Used</th>
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<td>CPRG</td>
<td>0.05</td>
<td>90 µL of 0.75 mM</td>
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<td>β-Gal</td>
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<td>30 µL of 0.125 µM</td>
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<table>
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<td>Hach P/A Test (Hach, 2021)</td>
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<tr>
<td>IDEXX Quanti-Tray 2000 (Analytics Shop, 2021)</td>
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5. CONCLUSION AND FUTURE RECOMMENDATIONS

Clean and safe drinking water is essential to human life, but it is often contaminated with enteric pathogens that can cause deadly diseases such as cholera, salmonellosis, and those caused by *E. coli*. Detection methods to analyze drinking water samples for these enteric pathogens are necessary to help minimize the occurrence of these deadly diseases. Many methods have been developed to detect a microbial indicator, such as *E. coli*, that indicates whether other enteric pathogens are present in a water sample. These methods can include molecular methods such as PCR, or other standard methods like membrane filtration, multiple tube fermentation, among many others. Additional alternative detection methods are needed to overcome some of the limitations of the currently used methods. Such limitations include the requirement of skilled expertise, long wait times to obtain results, need for electricity, and the requirement of laboratory equipment that would make those testing methods inaccessible for certain communities. In recent years, research has focused on developing innovative approaches to rapidly detect waterborne microbes including the use of nanomaterials for producing electrochemical, acoustic, magnetic, and optical biosensors.

This research study aimed to analyze and optimize a previously developed colorimetric nanosensor for the detection of *E. coli* to make the sensor recyclable and more cost-effective. That nanosensor utilizes an indirect colorimetric assay that functions based on the competitive binding of positively charged PEI-AuNPs to the negatively charged β-Gal enzyme and *E. coli*. The stronger electrostatic attraction between PEI-AuNPs and *E. coli* leaves the β-Gal enzyme free in solution to hydrolyze CPRG to CPR. This hydrolysis
reaction changes the solution color from yellow to red and the intensity of the color change increases with the increase in *E. coli* concentration. This nanosensor has easily observable results and achieves a relatively rapid detection. The current research investigation aimed to understand the underlying mechanism of work of this nanosensor to improve its recyclability and cost. This research hypothesized that the governing factor for this nanosensor was the surface charge rather than the nanomaterial or the coating type. If this hypothesis is true, then gold nanomaterials used for making the original sensor could be replaced with other nanomaterials that are more cost-effective and recyclable (e.g., magnetic iron nanoparticles that could be recovered after use by a magnetic field). To test the research hypothesis, positively charged silver and iron nanoparticles as well as positively charged polymers were tested for their ability to 1) inhibit the hydrolysis reaction between β-Gal and CPRG in the absence of *E. coli* and 2) demonstrate competitive binding in the presence of *E. coli*. The experimental investigations conducted herein elucidated which factor (i.e., nanomaterial type, chemical coating type, or surface charge) drives the function of this nanosensor.

This research had three major findings. First, the AuNPs were not critical for the nanosensor functionality. Both BPEI-AgNPs and BPEI-Fe$_2$O$_3$ (which had the same coating type as the AuNPs) inhibited the hydrolysis reaction in the absence of *E. coli* and when the competitive binding protocol was implemented, they were able to detect high concentrations of *E. coli*. Second, it was determined that the BPEI was not the only positively charged chemical coating that inhibited the hydrolysis reaction. CTAB, a positively charged polymer, inhibited the hydrolysis reaction in the absence of *E. coli*. This might indicate that the surface charge rather than the composition of chemical
coating is the governing factor for the Coulombic interactions between the positively charged sensing agents and the negatively charged β-Gal and bacteria. Third, the BPEI itself (i.e., without a nanomaterial carrier) was able to detect microbes at high concentrations and inhibited the hydrolysis reaction in the absence of *E. coli*. These findings provide a foundation for future development of alternative competitive binding-based sensing agents (e.g., nanomaterials and positively charged polymers) that are more recyclable, environmentally friendly, and cost-effective.

One of the main limitations of the sensing agents tested in this study was the high detection limit of these sensors. Future investigation should consider optimizing the experimental testing conditions as well as the properties of the sensing agents to achieve lower detection limits. Specific suggestions for future investigation and optimization include:

- Producing stable magnetic nanomaterials that do not aggregate in the test wells. This could be achieved by modifying the synthesis protocols to produce particles with smaller size and higher mount of surface coatings. The size of nanomaterials could impact the effectiveness of the nanosensors, and those impacts should be evaluated. A high percent coating of charged polymer is required for prevention of aggregation and providing strong surface charge to achieve the desired competitive binding mechanism.

- Optimizing the pH of the aqueous testing media to achieve stronger protonation of the polymers/polymer coatings, which will lead to stronger positive charges and
thus, more favorable competitive binding between the microbes and the sensing agents.

- Investigating more types of positively charged polymers and nanomaterials in addition to the ones tested herein (e.g., EDTA).

- Understanding the limitation of the Coulombic interactions that occur between the sensing agents and the microbes. This research demonstrated that at low *E. coli* concentrations, the competitive binding and the Coulombic forces stop being the governing factor for the mechanism of work of the sensor. Under these conditions (i.e., low concentration of microbes), there is a probability that a sensing agent would favor binding to dissolved β-Gal over physical colloidal particles (i.e., bacteria in this case) in solution because β-Gal would be more readily available for interactions. In other words, the interactions between the sensing agents and low amounts of *E. coli* would be limited by the random collisions. Future research could learn from and build on theories of coagulation of colloidal suspensions (microbes in liquid make a colloidal suspension) to understand and optimize a system to detect *E. coli* when their concentrations are low in solution.

- Evaluating the potential toxic impacts of the sensing agents on *E. coli*. Toxicity could be playing a role in the lack of detection at low *E. coli* concentrations and thus, it needs to be systematically evaluated.

- Evaluating the potential impacts of β-Gal naturally produced by *E. coli* on the performance on the detection assay. This could be tested by preparing well plates that include sensing agents, CPRG and *E. coli*, while relying on the β-Gal produced by the microbes rather than adding it to the testing matrix.
Once lower detection limits are achieved under laboratory conditions, real environmental water samples should be tested to optimize the nanosensor function in real-world situations. Methods for recycling and reusing the nanosensor should also be studied in future research.
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APPENDICES

A. Acronyms

Amine-Fe$_3$O$_4$ = Amine functionalized iron oxide nanoparticle suspension

β-Gal = β-galactosidase

BGLB = brilliant green lactose bile broth

BPEI-AgNPs = branched polytheyleneimine coated silver nanoparticles

BPEI-Fe$_2$O$_3$ = cerium (Ce$_{3/4}^+$) doped iron oxide coated nanoparticles coated with BPEI

BPEI-M-IONP = commercial iron oxide suspension modified in the lab with BPEI

BPEI-S-IONP = iron oxide nanoparticle suspension synthesized in the lab with BPEI

C = concentration

°C = degrees Celsius

CFU = Colony forming unit (number of colonies counted on the plate)

CFU/mL = Colony forming units per milliliter

cm$^{-1}$ = centimeter$^{-1}$

CPR = chlorophenol red

CPRG = chlorophenol red β-D-galactopyranoside

CTAB = (1-Hexadecyl) Trimethylammonium Bromide

DF = dilution factor

DI = deionized

DST = Defined Substrate Technology

EC MUG = E. coli 4-methylumbelliferyl- β-D-glucuronide

E. coli = Escherichia coli

EDTA = Ethylenediaminetetraacetic acid
EHEC = enterohemorrhagic *E. coli*

EIEC = enteroinvasive *E. coli*

EMB = Eosin Methylene Blue

emu = electromagnetic unit

ETEC = enterotoxigenic *E. coli*

FTIR = Fourier-transform infrared spectroscopy

g = gram

\( \text{g/mol} = \text{grams per mole} \)

kDa = kilodalton

kHz = kilohertz

MCL = Maximum contaminant level

MCLG = Maximum contaminant level goal

MF = membrane filtration

mg = milligram

\( \mu L = \text{microliter} \)

\( \mu m = \text{micrometer} \)

\( \mu M = \text{micromolar} \)

min = minute

mL = milliliter

MPN = most probable number

mTEC = membrane thermotolerant *E. coli*

MTF = multiple-tube fermentation

MUG = 4-methylumbelliferyl- \( \beta \)-D-glucuronide
mV = millivolt
mM = millimolar
MW = molecular weight
N/A = not applicable
nm = nanometer
nM = nanomolar
NPs = nanoparticles
Oe = Oersted
PBS = phosphate buffer solution
PCR = polymerase chain reaction
PEI = polyethyleneimine
PEI-AuNP = polyethyleneimine-coated gold nanoparticles
rpm = revolutions per minute
S. aureus = Staphylococcus aureus
SERS = surface-enhanced Raman scattering
SPR = surface plasmon resonance
TEM = transmission electron microscopy
TGA = thermogravimetric analysis
TSA = tryptic soy agar
TSB = tryptic soy broth
UCNP = Up-conversion nanoparticles
V = volume of solution plated
wt% = percent weight
B. SpectraMAX Plus Graphs

Figure B1. Optical density results for BPEI-AgNPs corresponding to Figure 29.

Figure B2. Optical density results for BPEI corresponding to Figure 29.
**Figure B3.** Optical density results for 5 µL of 10 µM BPEI corresponding to Figure 34.

**Figure B4.** Optical density results for BPEI-AgNPs at $10^8$ CFU/mL of *E. coli*, columns six and seven in Figure 31.

**Figure B5.** Optical density results for 25 µM BPEI-AgNPs at $10^8$ CFU/mL of *E. coli*, columns nine and ten in Figure 31.
Figure B6. Optical density results for 25 µM BPEI-AgNPs corresponding to Figure 32.

Figure B7. Optical density results for BPEI corresponding to Figure 35.
Figure B8. Optical density results for chitosan corresponding to Figures 28 and 36.

Figure B9. Optical density results for CTAB corresponding to Figures 28 and 36.
Figure B10. Optical density results for BPEI-Fe₂O₃ corresponding to Figures 28 and 36.

Figure B11. Optical density results for Amine-Fe₃O₄ corresponding to Figures 28 and 36.
Figure B12. Optical density results for chitosan corresponding to Figure 37.

Figure B13. Optical density results for BPEI-AuNPs corresponding to Figure 37.

Figure B14. Optical density results for Amine-Fe$_3$O$_4$ corresponding to Figure 37.
Figure B15. Optical density results for BPEI-M-IONP corresponding to Figure 37.

Figure B16. Optical density results for BPEI-S-IONP corresponding to Figure 42.

Figure B17. Optical density results for 50 µM BPEI-Fe$_2$O$_3$ corresponding to Figure 42.
Figure B18. Optical density results for 100 μM BPEI-Fe$_2$O$_3$ corresponding to Figure 42.

Figure B19. Optical density results for 200 μM BPEI-Fe$_2$O$_3$ corresponding to Figure 42.

Figure B20. Optical density results for 500 μM BPEI-AuNPs corresponding to Figure 42.
Figure B21. Optical density results for 5000 µM CTAB corresponding to Figure 42.

Figure B22. Optical density results for 500 µM BPEI corresponding to Figure 42.

Figure B23. Optical density results for 200 µM BPEI-AgNPs corresponding to Figure 42.
Figure B24. Optical density results for BPEI-Fe$_2$O$_3$ corresponding to Figure 43.

Figure B25. Optical density results for BPEI-AgNPs corresponding to Figure 43.
**Figure B26.** Optical density results for CTAB corresponding to Figure 43.

**Figure B27.** Optical density results for BPEI corresponding to Figure 43.

**Figure B28.** Optical density results for BPEI corresponding to Figure 45.