Rapid bacterial capture with modified magnetic nanoparticles for sepsis diagnosis

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ABSTRACT

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Sepsis (bacterial infection in the blood) currently requires 24 hrs. to detect the bacteria strain. However, sepsis can prove fatal if correct treatment is not administered sooner. Our solution is to use magnetic nanoparticle (MNP) extraction to complete the detection process within 3 hrs. Successful extraction requires chemically coupling MNPs with molecules that have an affinity to bacteria, such as bis(dipicolylamine) with Zinc (bis-Zn-DPA). With modified MNPs we have extracted bacteria with, on average, 100% accuracy. However, these results are consistent regardless of having the DPA molecule attached to the nanoparticles. Further research will clarify the current results. We continue to improve MNP synthesis and bacteria capture with the goal to implement our technique in clinical settings.

Keywords: magnetic, nanoparticles, bacteria, sepsis, rapid diagnosis, bis-DPA

ACKNOWLEDGMENTS

I would like to thank Dr. Robert Davis and Professor Cheryl Davis for seeing my potential and recruiting me to the research group; as well as Dr. Elisabeth Gates for being my side-by-side mentor, teacher, and friend, and answering all of my questions, day and night. I would also like to acknowledge Dr. William Pit for letting us use his lab and instruments for experiments. I would like to thank my co-workers who trained and helped me in the process. Lastly, I thank my family who provided support in my academic and career efforts.

Contents

Chapter 1

Introduction

1.1 Background of Sepsis

The motivation for this research is focused around the severity and treatment challenges of sepsis. Sepsis is an immune system response to a bacterial infection which leads to inflammation and damage of it's own body tissues and organs [1]. There are three levels of this dysregulated immune system response. First is sepsis, which is the initial systemic manifestation of a pathogenic infection, as just explained in the previous sentences. Sepsis has a hospital mortality rate greater than 10%. Second is severe sepsis, which is when the tissue and organ damage escalates into multiple organ failure and tissue hypoperfusion. Third is septic shock, which involves tissue hypotension as well as circulatory, cellular, and metabolic abnormalities regardless of fluid resuscitation as medical treatment. Septic shock has a hospital mortality rate greater than 40% [2]. For every 3 people that die in hospitals, 1 of them was due to sepsis. Sepsis is contracted by 1.7 million adults and correlates to more than 270,000 fatalities per year in the United States. [3]

1.2 Current Methods and Barriers of Detection and Diagnosis

The challenges of treating sepsis arise from the detection and diagnosis methods. Sepsis can be instigated by various strains of bacteria, therefore, it is essential to characterize the strain before any accurate antibiotics can be delivered. Characterizing bacteria requires culturing samples of the patients blood, urine, saliva, wound secretions, and organ lining surfaces, which takes at least 24 hours. In addition, only 17% of symptomatic patients result in positive blood cultures, even when the infectious bacteria is present. In part, this is because samples may be drawn from a patient when the bacteria is less concentrated. The pathogen needs specific conditions to grow enough for the culture tests to detect it [1].

With such delays and difficulty in identifying correct bacteria strains, caretakers can only empirically administer broad-spectrum antibiotics. This involves beginning with treatment for the most likely bacteria, and reassessing regularly as time passes, in order to shrink the spectrum of drug delivery (de-escalating). Once culture test results return, antimicrobial regimen can be corrected to an exact type. However, empirical antibiotic therapy should be de-escalated as soon as possible, because it can lead to drug resistance and high toxicity [4]. Patients may not benefit in any way from broad-spectrum administration if the correct treatment is absent. They may also suffer from side effects of incorrect antimicrobial activity, leading to increased risk [1].

This is why the International Guidelines for Management of Severe Sepsis and Septic Shock 2012 ask that, "a specific anatomical diagnosis of infection requiring consideration for emergent source control be sought and diagnosed or excluded as rapidly as possible," [4]. This research aims to characterize pathogen strains within 3 hours, rather than 24, in order to more rapidly supply sepsis patients with efficient medications, and lower the mortality rate.

1.3 Introduction of Magnetic Extraction

By using magnetic nanoparticles (MNPs), microorganisms can be extracted from blood samples to be concentrated and identified. The MNPs are modified so that they have a natural affinity to bacteria, and maintain a positive zeta potential. This will allow for two ways to attach bacteria and MNP: chemical binding and/or electrostatic attraction. At this point, a strong external magnet can pull the MNPs, with pathogens attached to them, out of their samples. Once removed from their former environment, the bacteria can be isolated and therefore concentrated enough to be rapidly characterized. This proposed solution is not attempting to remove bacteria from patients bodies using nanoparticles as a remedy; it is removing bacteria from samples of patients blood in order to identify the bacteria type, therefore allowing proper antibiotics to be prescribed.

Chapter 2

Experimental Methods

2.1 Magnetic Nanoparticle Synthesis

For MNPs to extract bacteria, they need to first be modified so that they can chemically bind with bacteria. This is done in three main steps. First, a molecule with a natural affinity to bacteria, bis-dipicolylamine (DPA-PEG-COOH), is attached to the surface of the MNPs through an overnight reaction. This is primarily what enables the MNPs to bind to the bacteria when mixed together in blood samples. Before any modification, the surface of the MNPs is covered with amines. After the first reaction step, although many of the surface amines are now bound to the DPA molecules, there are still a significant remainder of amines with nothing attached. Amines could bind with positive ammonium ions in the blood, which is a reaction that could interfere with the desired binding of MNP to bacteria [5]. Second, to prevent such unwanted binding, a poly(ethylene glycol) PEG-SVA 550 (PEG), is attached to the rest of the amines on the surface. Third, Zinc particles ($Zn(NO_3)_2$, are attached to the ends of the DPA molecules. The bis-Zn-DPA, "forms coordination bonds with anionic phospholipids which are present at high density on the outer membrane of Gram-positive and Gram-negative bacterial cells [5]." (see Fig 2.1).

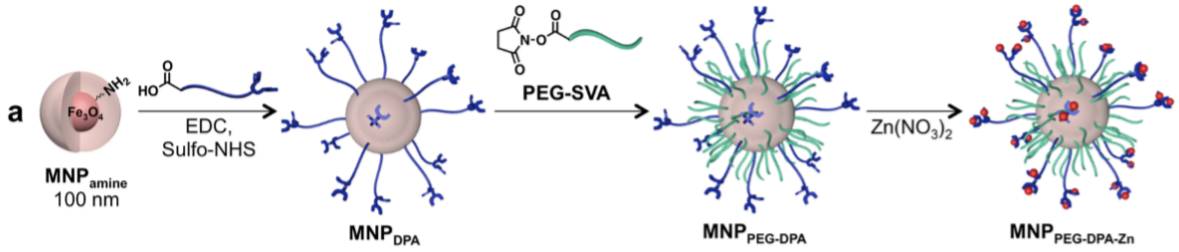

Figure 2.1 [5] MNP synthesis protocol summarized. The first step is to react DPA molecules with the surface of the MNP, the second is to react PEG molecules to the remaining surface area, and the last step is to attach Zinc particles to the ends of the DPA molecules.

The temperature of each reactant and the timing for each reaction is critical in these three steps. Amines on the MNP surface will be deprotonated at pH > 7, and must be deprotonated in order for them to be reactive and capable of binding to DPA. However, the carboxylic acid on the DPA require a pH condition of 4.5-4.75 to successfully react. To compensate for this disparity, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) (EDC) and N-Hydroxysuccinimide (NHS) will be used in the reaction, because EDC and NHS have a wider pH range requirement: pH 4.5-7.2. Thus, the ultimate conjugation of DPA and MNPs can be done in two steps, with the two different pH conditions. First, NHS and EDC can bind with the DPA around pH 4.5 and then the NHS and EDC on the DPA-NHS-EDC conjugation can react with the amines on the surface of the MNPs at pH > 7 [6].

The 200 nm particles are purchased from Ademtech in an unknown buffer. Buffers are used to maintain solutions at a certain pH level. Before synthesis, the nanoparticles are washed in order to remove the unknown buffer and re-suspend them in a known one: 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino] ethanesulfonic acid, N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, TES free acid (TES), of pH 8. TES buffer is used to set the pH of the MNPs at pH > 7, as required in the reaction requirements mentioned above. To wash the sample, it is placed on a magnet for 2 minutes, during which the MNPs are pulled to the side of the microtube. The remaining liquid

(supernatant) is then removed with a pipetter and the MNPs are resuspended in the new buffer.

The reactants EDC, NHS, and DPA are diluted to 10 mg/mL, using Activation buffer at pH 4.5. After all four reactants for step 1 are diluted, 50 μL of DPA, 175 μL of EDC, and 500 μL of NHS are mixed and held on ice for five minutes in order to ensure proper binding [6]. The DPA-EDC-NHS conjugation is then mixed with 500 μL of washed MNPs and placed on a rotater for 24 hours at room temperature to react. After 24 hours, the MNP-DPA-EDC-NHS conjugation (MNP-DPA) is washed again to remove any residue or unattached reactants and to begin step two.

Next, 25 μL of PEG are diluted in TES buffer (pH 8) with a 10 mg/mL concentration are added to the MNP-DPA solution. They are placed on a rotator for 24 hours at room temperature to react. Again, after 24 hours, the MNP-DPA-EDC-NHS-PEG conjugation (MNP-DPA-PEG) is washed to begin step three. Next, 10 μL of Zinc are diluted in water at a 0.25 mg/mL concentration and mixed with the MNP-DPA-NHS-EDC-PEG solution (MNP-DPA-PEG). It is then placed on the rotator at room temperature for 1 hour to react. After one hour, the MNP-DPA-PEG-Zn conjugation is washed again and the MNP-DPA-PEG-Zn batch is ready to be used. The amount of each ingredient was calculated based on the surface area of MNPs.

2.2 Bacteria Capture Protocol

Once the MNPs are modified, they can be used in a bacteria capture experiment. The experiment protocol has twelve steps (see Fig 2.2). First, magnetic nanoparticles, of 200 nm diameter, are synthesized to specifically bind with bacteria, as explained in section 2.1 above. Second, a known pathogen type is grown by the standard ulture plate technique. The bacteria absorbance is measured in order to calculate a starting concentration and dilute (using nutrient broth) to a 10^4 concentration. This ensures more accurate data collection because less colonies will grow, making them easier to count.

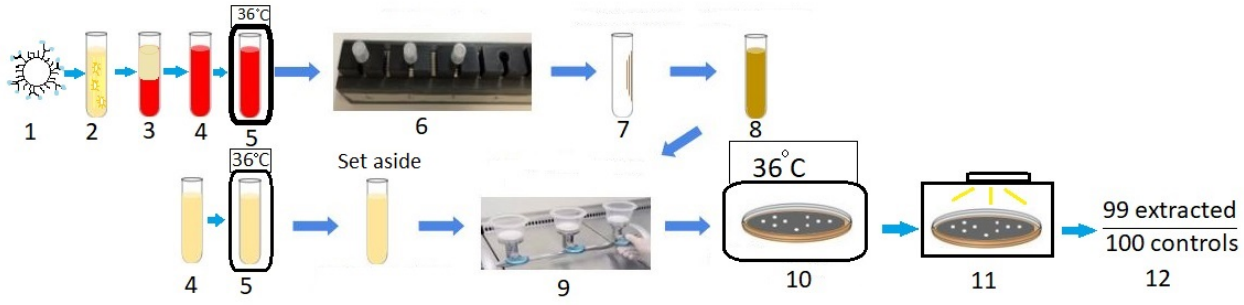

Figure 2.2 The 12 steps of bacteria capture protocol summarized. Steps 4 and 5 are shown twice, once for the samples (top line) and again for the controls (bottom line). On steps 5 and 10, the box represents an incubator. The box in step 11 represents the photo box that takes well lit photos without glare and reflection and the rectangle above it is a cellphone with a camera that captures the image through a hole at the top of the box. Step 12 represents the ratio of colony counts of the samples versus their controls.

Third, human blood samples are centrifuged to separate the plasma from red blood cells (RBCs) and diluted (using the separated plasma) to 10% hematocrit. This is done because whole blood (typically around 50.3-40.7% hematocrit for males and 44.3-36.1% for females [7]) is too viscous for the MNPs to perform well. Fourth, the diluted bacteria, modified MNPs, diluted human blood samples, and Phosphate Buffered Saline (PBS) are vortexed together. The buffer helps maintain the pH level around that of average human blood (7.35-7.45 [8]).

Fifth, the blood/bacteria/MNP samples are incubated for 30 minutes at about 35°C. This allows the MNPs to have enough time to find and bind to the bacteria and is a proper environment for the bacteria to survive. Sixth, the blood/bacteria/MNP samples are placed inside a magnetic rack that was custom designed to perfectly fit the sample test tubes. Each test tube slot has access to the magnet in one quadrant of it's circumference. Samples are left on the magnetic rack for 15 minutes. During this time, MNP-bacteria conjugations (MNPs that have successfully attached to bacteria) should collect on the side of the tube where the magnet is located.

Seventh, the supernatant (the remaining liquid in the tube), which should just be composed of the diluted blood and buffer, is removed using a serological pipette. This leaves the test tubes empty

except for the MNP-bacteria conjugations. Eighth, the MNP-bacteria conjugations are re-suspended in PBS. Doing so enables the MNP-bacteria conjugations to be filtered onto a culture plate using vacuum filtration, which is the ninth step. This step is done in a laminar flow hood, using a vacuum pump that pumps the sample through a 0.45 μm filter onto the plate.

Tenth, the culture plates are incubated at 35°C for about 24 hours, so that the bacteria colonies can grow. Eleventh, the culture plates are photographed by a photo box designed by this research group, specifically for this project. Twelfth, the bacteria colonies are counted using a computer software that was also designed by this research group. Colony counts are recorded for each sample and ready for comparison with the controls.

2.3 Culture Plate Controls and Data Collection

Control samples are prepared with the same amount of bacteria as the samples. These controls are not placed on the magnet. After plating both the samples and controls and incubating for 24 hours, colonies are counted. If the MNPs captured bacteria well, then there should be the same amount of colonies in the samples as there are in the controls. The ratio of sample colonies to control colonies is used as a capture percentage; 100% capture would mean that 50 out of 50 bacteria was captured. Low capture, i.e. 50%, or 25/50 captured bacteria, would mean that the MNPs have low functionality. Colonies are counted by taking high quality photos of the culture plates and using a computer program to identify and keep count of all the colonies. A photo box was designed by this research group to accomplish this task.

2.4 Design of Culture Plate Photo Box

High quality photos of the culture plates requires blocking out ambient lighting and removing glares on the plate lid. A photo box was designed that surrounds the plate completely so as to block out

ambient lighting and remove reflections. Inside the photo box, the plate is placed inside a ring of LED lights that can be tuned to different wavelengths for best quality of imaging. The LED lights directly light the plate without reflections. A hole on the top of the box allows a phone camera to successfully capture the image of the plate.

This research aims to reduce the current amount of time it takes clinicians to characterize bacteria from 24 hours to 3 hours. The reason it takes 24 hours is due to culturing. This research group is still doing preliminary experiments that require culturing, therefore the experimental design lasts 24 hours. Culturing is used to confirm proper functionality of the nanoparticles. Proper functionality means the nanoparticles need to be able to capture approximately 100% of the bacteria that is originally mixed into the samples. Once this is accomplished, the nanoparticles can then be used to extract bacteria within 3 hours rather than 24 because culturing will no longer be required.

Chapter 3

Results and Discussion

3.1 Data Analysis of MNP Synthesis

Promising results from dozens of repeated experiments show that the modified MNPs are capable of capturing bacteria. One way to increase MNP capability is to ensure the DPA molecules are properly attached. After synthesizing the MNPs, three tests are performed to ensure it was done correctly: pH, zeta potential, and zeta size measurements. A measurement of pH reveals the acidity of a solution. The chemical reactions of MNP synthesis are pH sensitive. The pH levels should decrease to around 4.5 with the DPA molecule addition and rise back up again to greater than 7 after. Two methods were used to measure the pH: submerging a pH μmeter into a 5 μL aliquot of the entire solution or the supernatant while the nanoparticles are pulled to the side by a magnet. Figure A.1 shows the pH trends of each synthesis modification.

The initial solution is at pH 7 because of the TES buffer in which the MNPs are synthesized. The DPA and EDC molecules are diluted in activation buffer which has a pH of 4.2. Figure 3.1 shows that after the DPA addition, the pH does decrease. The remaining reactions need to occur at pH level greater than 7, therefore the PEG molecule is suspended in TES buffer of pH 7. Figure 3.1

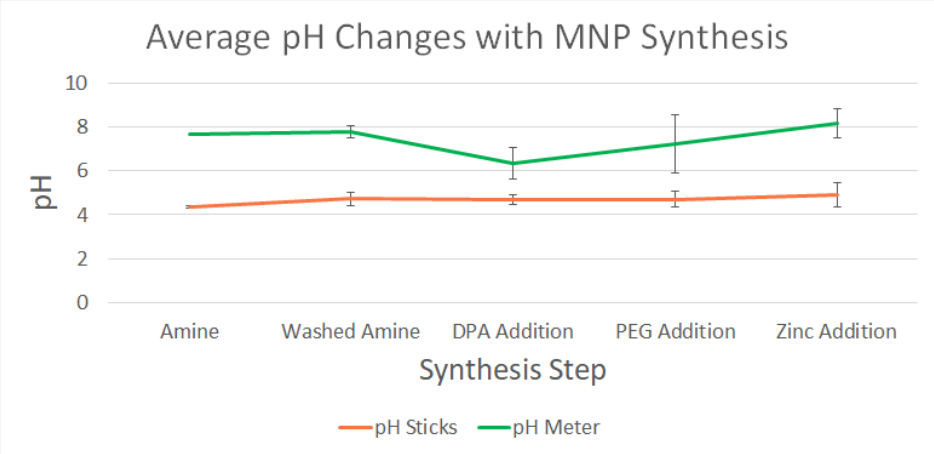

Figure 3.1 A summary of the measurements of pH according to each synthesis step. Each data point is an average of all the batches. A more detailed graph is featured in the appendix (figure A.1). MNP solution aliquots are added to 1 mL of deionized water, otherwise there would not be enough solution for the μmeter to be submerged. Added water could slightly change the pH level. Measurements were taken with two different methods which yield inconsistent results: a pH micrometer and pH sticks. Measurements from the pH meter are assumed to be more accurate because of the apparent sensitivity whereas the pH sticks appear to react less so.

Zeta potential (ζ, mV) of MNPs.

MNP_{amine}	MNP_{DPA}	$MNP_{PEG\text{-}DPA}$	$MNP_{PEG\text{-}DPA\text{-}Zn}$	MNP_{PEG}
39.25 ± 0.67	36.00 ± 1.36	31.70 ± 1.64	33.22 ± 1.14	12.63 ± 0.96

Figure 3.2 Zeta potential should change just slightly with each synthesis step, and decrease by about 3 mV when the DPA is bound to the surface, about 6 mV when the PEG is attached, and increase by about 2 mV when the Zinc is added. This table was made by Dr. Jung-Jae Lee and his research group [5].

shows that after the PEG addition the pH levels do indeed increase, as intended, for all but one sample. Measurement from pH sticks may not properly reflect the pH because they seem to have less accuracy. The results from the pH meter show that pH has been adjusted as intended which enables successful reactions with the MNPs.

Zeta potential is a measurement of the electric potential between the surface of a particle and the material in which it is immersed [9]. With each addition to the magnetic nanoparticle conjugation,

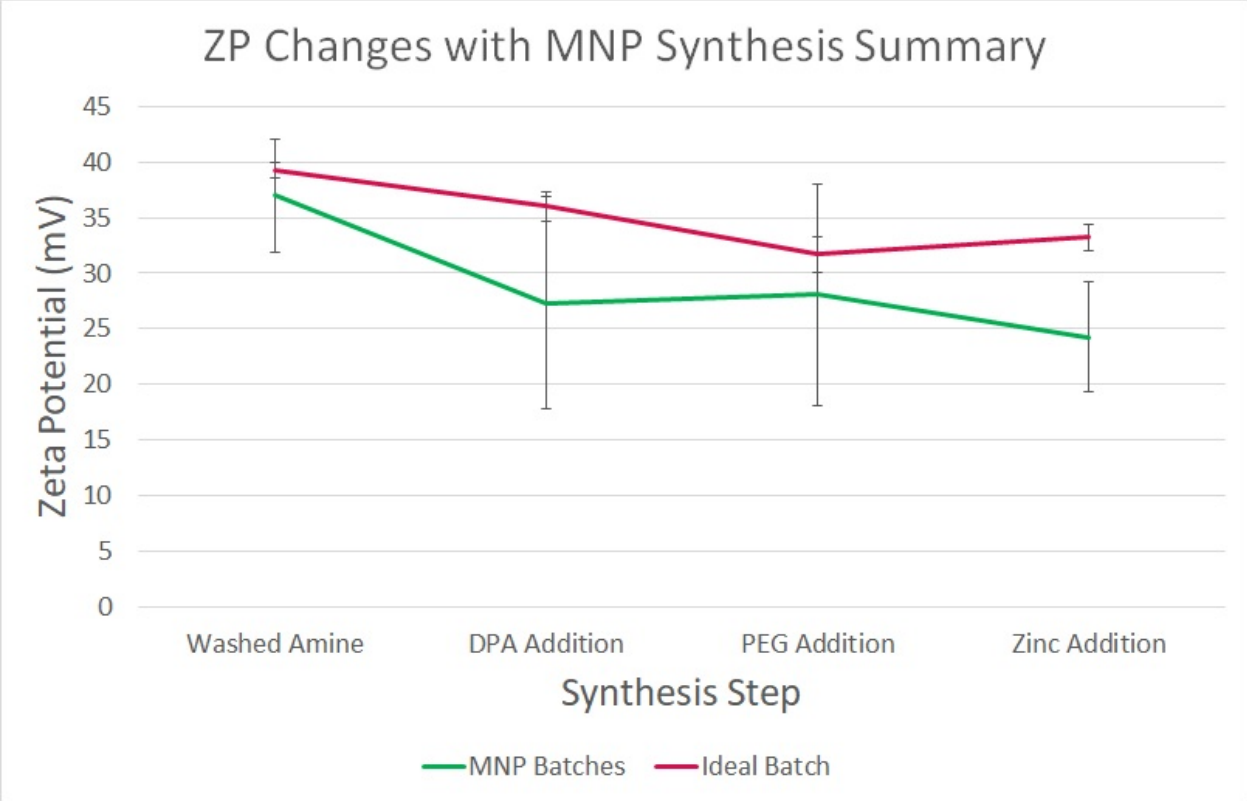

Figure 3.3 A summary of the zeta potential measurements according to each synthesis step. Each data point on the green line is an average of all the MNP batches. The pink line indicates the ideal changes when synthesis steps are successful [5]. A more detailed display of the same data can be found in the appendix (figure A.2).

the zeta potential should change because of the new chemical makeup (See Figure 3.2 [5]). If the zeta potential measurements follow closely with Figure 3.2, it should indicate that the DPA, PEG, and Zinc constituents have bonded successfully to the surface of the MNPs. Each test runs three times and the average is taken for data analysis. Figure 3.3 shows a summary of the results. The green line indicates the zeta potential of each synthesis step as an average of all MNP batches. This pink line shows what the ideal changes should be according to Dr. t 2 mV when the Zinc is added. This table was made by Dr. Jung-Jae Lee [5]. As you can see, the green line doesn't perfectly match the ideal line but it is close. A better correlation is visible in the figure A.2, which has more details about each batch of nanoparticles (not averaged).

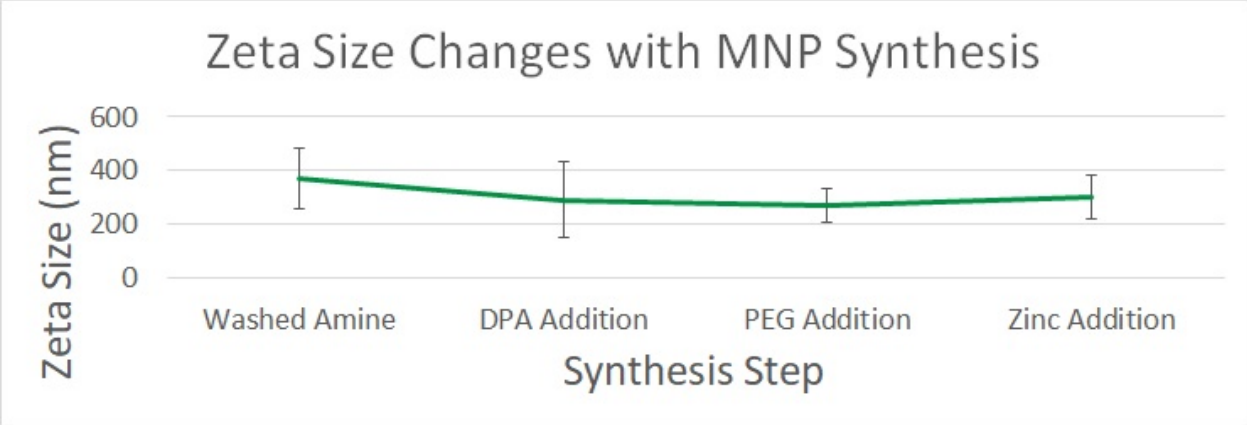

Figure 3.4 A summary of zeta size measurements according to each synthesis step. Each data point is the average of all the MNP batches. A more detailed graph can be found in the appendix (figure A.3). Original size of MNPs is 200 nm.

Zeta size is a measurement of the size of the nanoparticles. The nanoparticles are originally 200 nm. If the size gets much larger than this it can indicate that the individual particles are clumping together. This is undesirable because there will be less free-roaming nanoparticles to capture bacteria, and they will have less mobility in the blood due to larger size.

The average is taken from three runs and displayed in figure 3.4. Most MNP batches remained roughly within the 200-300 nm range, which provides better opportunity for the MNPs to capture bacteria.

3.2 Bacteria Capture Efficiency with Varying Hematocrit

With successful MNP synthesis, capture efficiency in blood is tested through the bacteria capture experiment. Blood has high viscosity due to the red blood cells (RBCs). Hematocrit is the ratio of RBCs to the total volume of blood. Therefore, the higher the hematocrit, the higher the viscosity. This causes more barriers for the MNPs to travel through the blood, find bacteria, and reach the side of the tube with the magnet. In turn, bacteria is less likely to be captured. To see how effective MNPs were at capturing bacteria, experiments were performed with varying degrees of hematocrit.

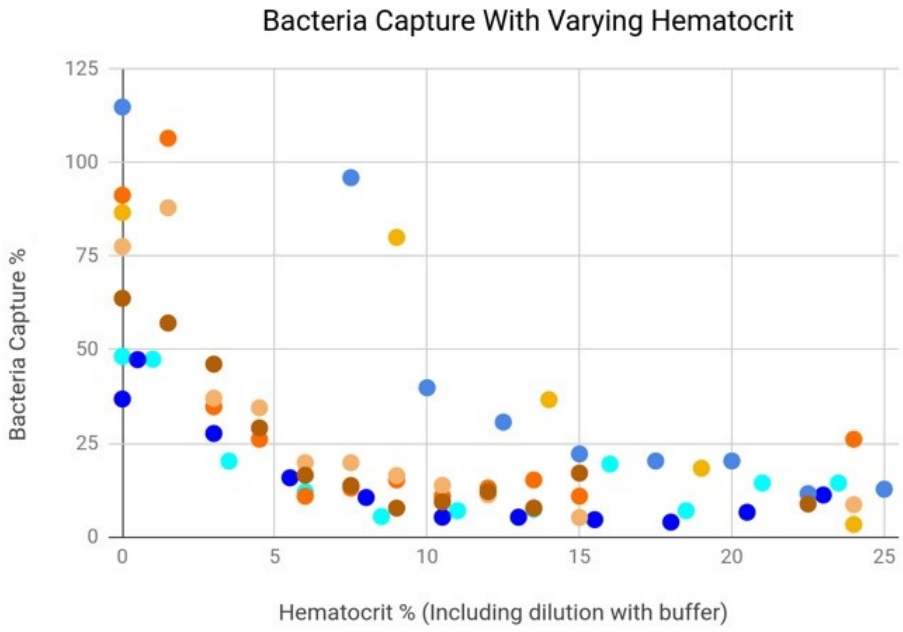

Figure 3.5 Bacteria Capture with Varying Hematocrit.

Multiple tubes of blood were prepared, each with a different hematocrit percentage. Blood samples were diluted using plasma from the same donor.

Figure A.3 shows that with zero hematocrit, meaning no RBCs, the capture efficiency was at it's peak. Zero hematocrit was accomplished by using plasma only. As the hematocrit increased, the capture efficiency decreased. Whole blood has 50.3-40.7% hematocrit for males and 44.3-36.1% for females [7], which means that so far, MNPs will not be able to capture bacteria in non-diluted human blood. However, capturing bacteria from blood samples is still possible as long as the samples are diluted first.

3.3 MNP Efficiency in Bacteria Capture

In preliminary experiments, MNPs had low bacteria capture even with diluted blood, ranging from 30-50% as shown in figure 3.6. Originally, Phosphate Buffered Saline (PBS) was used in the

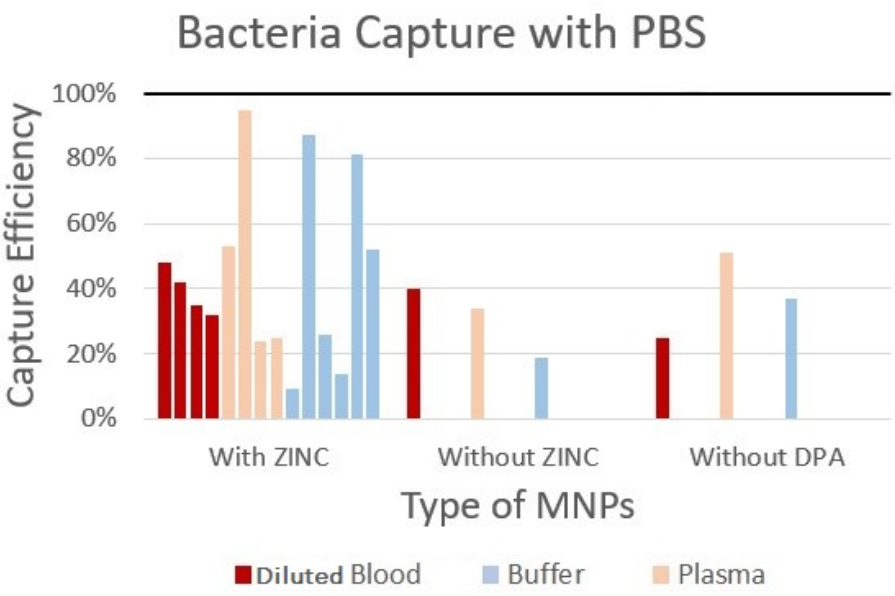

Figure 3.6 Bacteria Capture with Phosphate Buffered Saline (PBS). Various attempts included either blood, plasma, or buffer only; others did not include Zinc or DPA.

samples, until it was discovered that phosphate can interfere with MNP and bacteria binding [10]. After switching the PBS to a new buffer, Tris Buffered Saline (TBS), capture efficiency increased to 100% on average, as shown in figure 3.7.

Figure 3.7 shows that three types of MNPs were used: MNPs with zinc, without zinc, and without DPA. Regardless of the type of MNP, they performed roughly the same. The 100% capture was seen with all three types of MNPs. This was not expected because DPA is the theoretical cause of MNPs binding with bacteria; without DPA, the MNPs shouldn't theoretically attach to bacteria. Therefore, although 100% capture indicates successful extraction of bacteria, it is unclear how it was possible, since the MNPs that didn't have DPA still extracted bacteria. One hypothesis is that the positive zeta potential of the MNPs (ranging from 10-35 mV), allowed for electrostatic attraction between the MNPs and the negatively charged bacteria. Further research is required to confirm or deny this hypothesis and explore other possibilities.

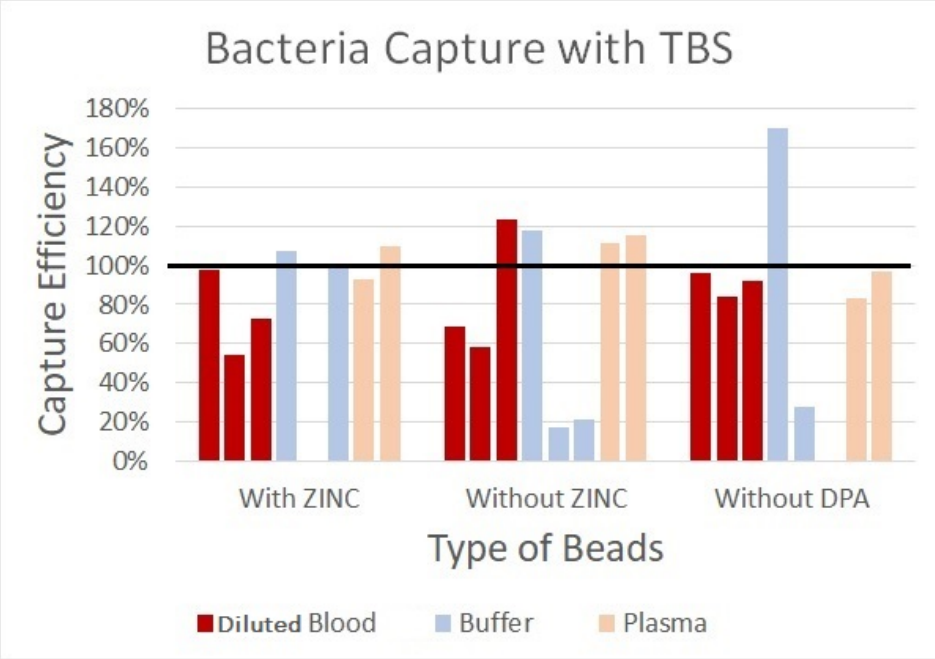

Figure 3.7 Bacteria Capture with Tris Buffered Saline (TBS). Various attempts included either blood, plasma, or buffer only; others did not include Zinc or DPA.

3.4 Discussion and Conclusion

While the cause of 100% capture is unknown and MNPs cannot capture bacteria in whole blood, they have proven highly capable with 10% hematocrit. These are promising results seeing as diluting blood before using MNPs in clinical settings is a simple task. Additionally, this is an improvement from past attempts which resulted in 30-50% capture.

With additional research and confirmation of MNPs ability to capture bacteria with 100% accuracy on average, they will allow for extracting, isolating, and condensing bacteria. This is a step towards using them in clinical settings because with isolated and condensed bacteria, characterization will be possible without 24 hours of culturing. Once this can be achieved, bacteria can be identified within 3 hours and sepsis patients could receive proper antibiotics sooner.

Appendix A

Appendix Title

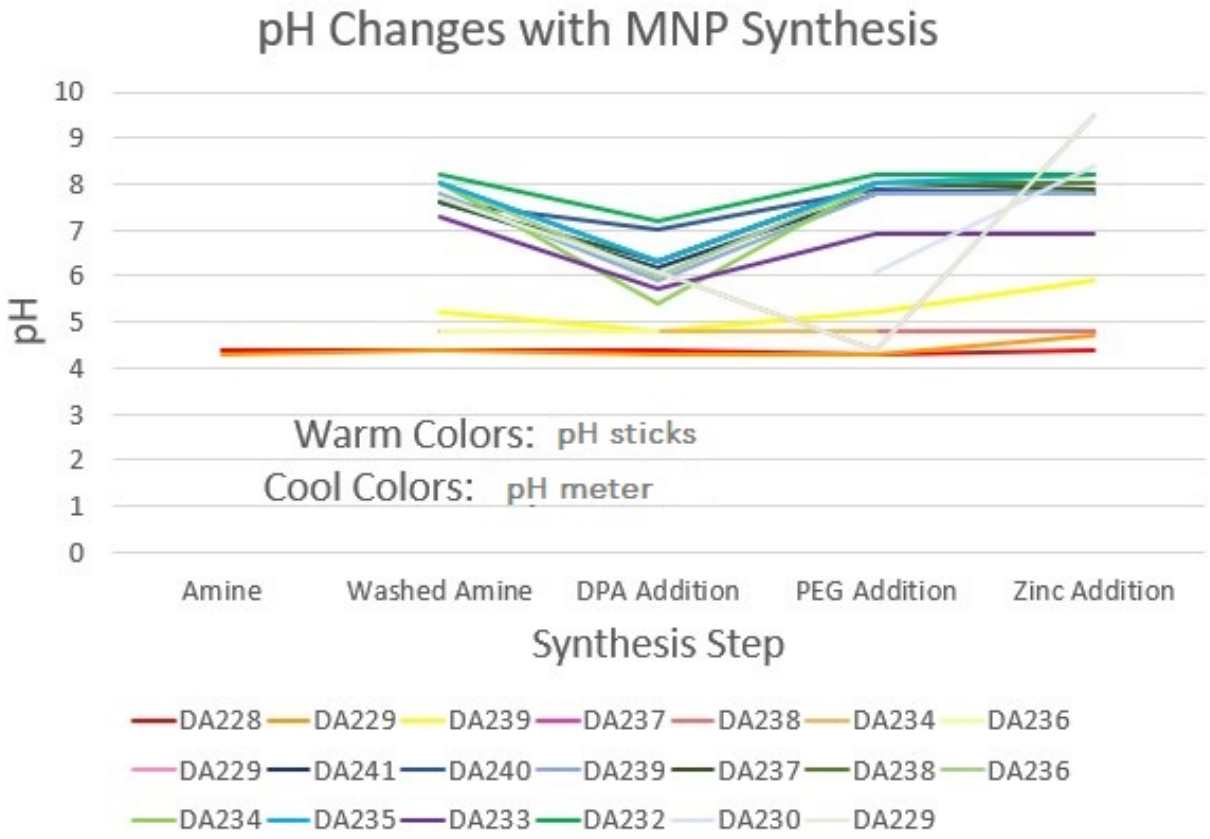

Figure A.1 Measurements of pH according to each synthesis step. This is another graph of the same data in figure 3.1, however, it is more detailed. Figure 3.1 has the average of all the MNP batches. This figure shows every data point. MNP solution aliquots are added to 1 mL of deionized water, otherwise there would not be enough solution for the μmeter to be submerged. Added water could slightly change the pH level. Measurements were taken with two different methods which yield inconsistent results: a pH micrometer and pH sticks. Measurements from the pH meter are assumed to be more accurate because of the apparent sensitivity whereas the pH sticks appear to react less so.

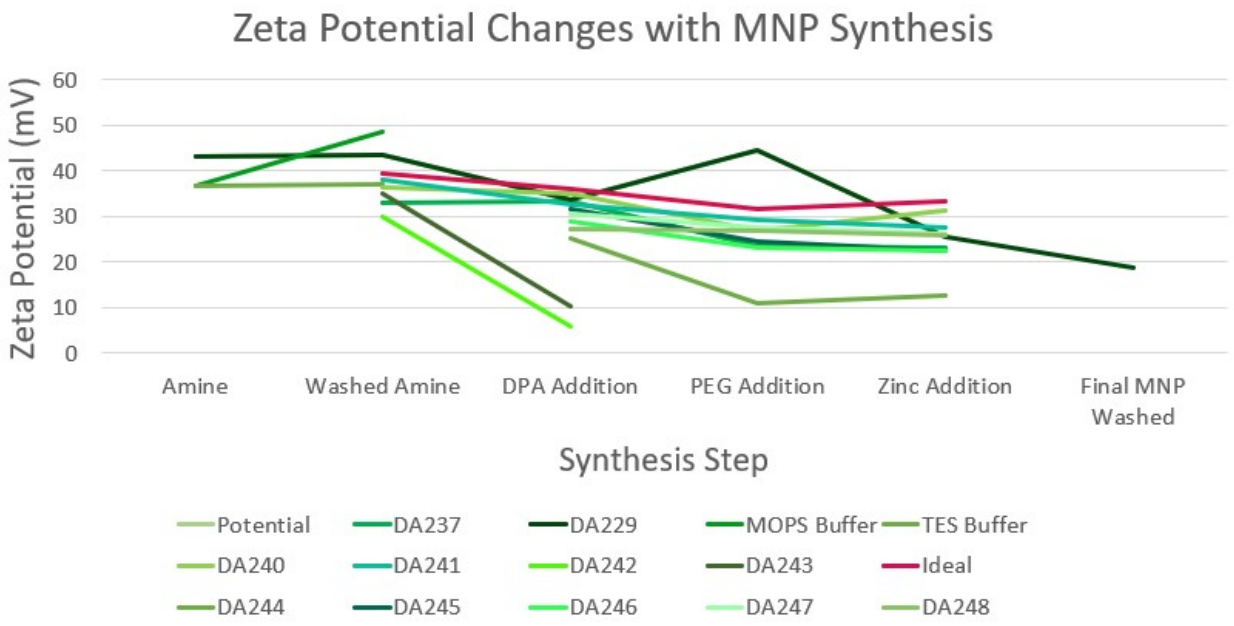

Figure A.2 Zeta potential measurements according to each synthesis step. This data is a more detailed display of the same data in figure 3.3. Figure 3.3 has the average of each batch. Cool colored data points are measured values. The pink line indicates the ideal changes when synthesis steps are successful [5].

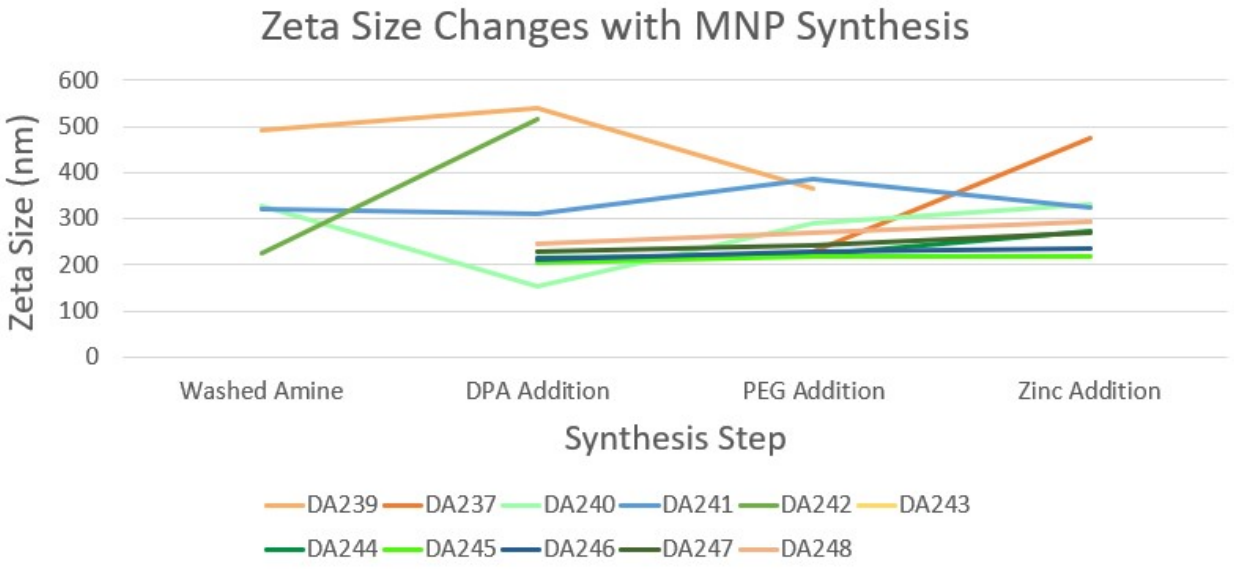

Figure A.3 Zeta size measurements according to each synthesis step. This data is the same that is displayed in figure 3.4 but in more detail. Original size of MNPs is 200 nm.

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