Detection of Pathogenic E. coli Using Fluorescence-Based Maltose Coated Gold Nanoparticles

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Abstract

Urinary tract infections (UTIs) result in over 10 million doctor visits each year. 80-90% of these infections are caused by pathogenic *E.coli* bacteria. Current diagnostic methods take at least 2-3 days and are quite labor-intensive. The delay in diagnosis can lead to empirical, rather than evidence-based, management of UTIs, leading to the rise of antibiotic resistant bacteria. Developing a sensitive, specific, and affordable point-of-care biosensor device is imperative in delivering a speedy and accurate diagnosis. Processing clinical samples is one of the most time-consuming steps of diagnosis, and recently, there have been successful attempts to internalize nanoparticles coated in maltoheptaose by *E. coli*, avoiding the sample processing step altogether. This study analyzes the potential of maltose-coated gold nanoparticles attached to a double stranded DNA (dsDNA) probe system to detect complementary 16S rRNA as a biosensor for different strains and species of bacteria.

Introduction

Bacterial pathogens that cause infectious disease are important targets for detection and identification in healthcare and medicine. For example, urinary tract infections (UTIs) result in over 10 million doctor's visits each year, where the uropathogenic bacteria *E. coli* cause 80-90 % of infections (other bacteria include *P. aeruginosa* and *S.epidermidis* among others).[1] As of now, the process from sample collection to diagnosis of these types of infections takes at least 2-3 days using the current "gold standard" methods of culturing, microscopy, and antibiotic susceptibility testing (AST). The absence of a diagnosis in a timely matter results in the over and misuse of antibiotics, which could lead to the emergence of antibiotic resistant bacteria, as well as spread of the infection if left undiagnosed. The development of point-of-care testing with rapid, sensitive, and cost-effective detection methods is essential in evidence-based, rather than empirical, management of UTIs and other infectious diseases as well as the development of a proper patient treatment plan.

Currently, biosensors have shown much promise in the field of pathogen detection due to their portable size, sensitivity, and rapid analysis. They allow for earlier and more sensitive detection, less than 10^3 CFU/ml, without sample processing, significantly expediting the process. This is especially important considering that many bacterial infections are caused by as low as 10 organisms.[2]

Specifically, nanoparticles have important applications in biosensing due to their optoelectronic, magnetic, and size-dependent properties. Gold nanoparticles (GNPs), in particular, possess additional light-scattering and absorption properties that are 4-6 orders higher than that of organic dyes and

fluorophores.[3] Moreover, GNPs are biocompatible and can be easily conjugated to biomolecules, including nucleic acids and carbohydrates.[4]A recent facile and eco-friendly procedure to synthesize maltose, a G2 carbohydrate similar to maltoheptaose, a G7 carbohydrate known to promote nanoparticle internalization by pathogenic *E. coli* [5], coated GNPs has recently been discovered.[6]

Additionally, GNPs conjugated to double stranded DNA (dsDNA) probes have been widely used as optical biosensors to detect targets. Some current methods work via a competitive displacement mechanism. In this mechanism, a single stranded DNA (ssDNA) detector probe (usually 24 base pairs) that could bind to a biological target analyte is first hybridized to a short (usually 12-18 bp) ssDNA synthetic target sequence and then conjugated to the nanoparticle. GNPs, due to their luminescent quality, can effectively quench the fluorescence of these probes when they are in close proximity. When the detector probe is exposed to the biological target, a longer sequence that is more thermodynamically and kinetically favorable, the biological target competes with and eventually displaces the synthetic target to restore fluorescence through release of the GNP and rehybridization of the detector strand with the biological target analyte.[7]

The same strategy can be applied for bacterial detection using 16S rRNA as the target analyte due to its high copy number, highly conserved genetic sequence within a species, and hypervariable regions that differ greatly between species.[8] This study incorporates the competitive displacement mechanisms of dsDNA probes with MGNPs as a quencher into pathogen detection using 16S rRNA. The general experimental scheme can be seen in **Figure 1.** The results from this study will serve as a comparison to current pathogen detection methods.

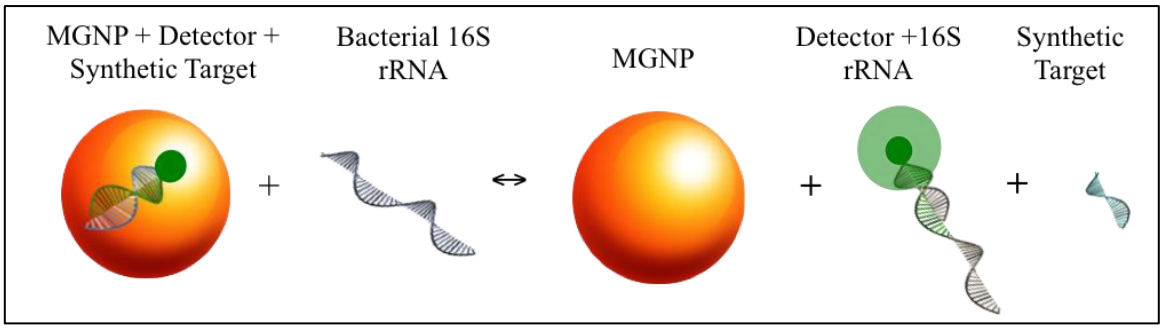


Figure 1: Competitive Displacement of Nanoparticle and Synthetic Target by 16S rRNA

Materials and Methods

Bacterial and clinical urine samples. Uropathogenic clinical isolates, including *E. coli*, *P. aeruginosa*, and *S. epidermidis*, were obtained and collected with the approval from Stanford University and VA Palo Alto Health Care System (VAPAHCS) Institutional Review Board. Identification of microorganisms was performed in the VAPAHCS clinical microbiology laboratory. Both bacteria were inoculated with Luria broth (LB) in a shaker at 37 C and grown until the OD_{600}= 0.5. The bacteria were then mixed with 40% glycerol and stored at -80°C. S. Samples were pelleted by centrifugation for 5 minutes at 7,000 RPM (microfuge), the supernatant was removed, and washed with PBS buffer (0.5 M, pH = 7.4). For the controlled lysis experiments, the bacteria were heated at 90°C for 10 minutes in PBS buffer (0.01M, pH =7.4) and then cooled down for 5 minutes before incubation with MGNPs.

Molecular probe design. Probes were designed using the following workflow. A target sequence was designed by alignment against 16S rDNA sequences from NCBI using GenBank. The detector probe was designed to binid to the loop region of the 16S rRNA. The loop was designed by: folding the target sequence, picking the loop sequence, and checking the loop specificity using *mfold* and NCBI BLAST). Finally, the stem was designed by: picking the stem sequence, folding the molecular beacon (MB), checking the MB self-dimer, and checking MB specificity using IDT OligoAnalyzer 3.0. The sequences were ordered from Integrated DNA Technologies Inc (Coralville, IA, USA). The buffer solution for the dsDNA probe contains 1X TE buffer. A 100 nM concentration of the probe was used.

Probe Name	Label	Sequence	Base Pairs
***E. coli* Detector**	5'-FITC	5'-CTG CGG GTA ACG TCA ATG AGC AAA-3'	24
***E. coli* Target**	Unlabeled	5'-TTT GCT CAT TGA CGT TAC CCG CAG- 3'	24

Synthesis and functionalization of maltose-conjugated GNPs with dsDNA probes. Maltose conjugated gold nanoparticles were synthesized based on the facile and eco-friendly procedure by Katti et al. (2009) using sodium citrate, instead of THPAL, as the reducing agent. Nanoparticles were left to incubate with dsDNA probes for 10 minutes at 37°C and cooled down to room temperature for 5 minutes before incubating with bacteria. The dsDNA probes were previously mixed in a 4:1 target to detector ratio for 10 minutes at 37°C. The final samples were all 200 µl.

Incubation of functionalized MGNPs with Bacteria. The functionalized MGNPs (2% in 0.01M PBS, 100 µl) were added to bacterial samples (100 µl) and incubated at 37°C for 10 minutes.

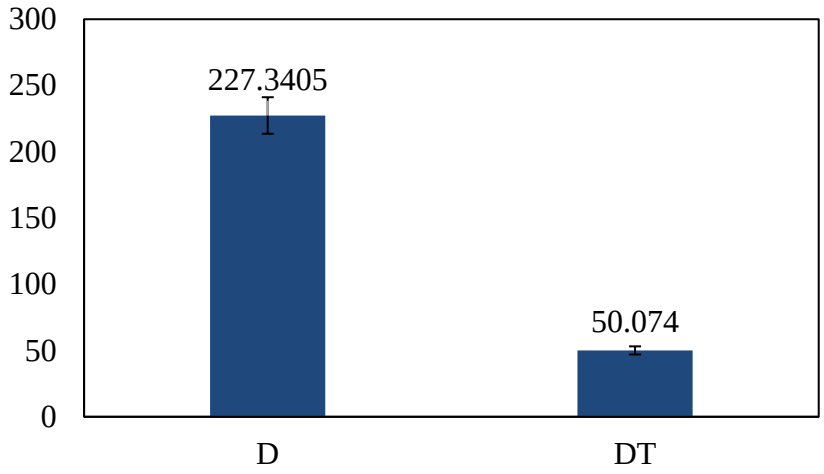
TEM imaging. The thin section samples were prepared as follows. A suspension of MGNP-treated bacterial cells or untreated bacterial cells (1 ml) was centrifuged at 2000 rpm for 5 minutes and the supernatant was removed. The pellet was then resuspended in a solution of glutaraldehyde in PBS (1%, 0.01M). The solution was centrifuged at 1000 rpm for 5 minutes and the supernatant was removed. Fixative was changed and the cells were left on ice for 30 minutes, avoiding any contact with the pellet. Suspension was washed (3 min x 3) with caco (0.1M). The pellet was then incubated in a solution of OsO4 in PBS (1%, 0.2M) at 4°C for 1 hour with one change. The excess OsO4 was washed with caco (0.1M). The solution was then en bloc stained with UA (2%) for 30 minutes. Afterwards, 2 ddH_2O changes were done. The pellet was then dehydrated by incubating the pellet in 50%, 75%, and 90%, and 100% (1 ml, 2 times) ethanol at 4 °C for 7 minutes each. At room temperature, the solution was washed with 100% Acetone (7 min x 2). The embedding resin medium was prepared by mixing Acetone (250 ul) and Spurr's resin (250 µl) for 2 hours at room temperature. The pellet was then incubated (2 hrs x 2) with Spurr's resin (100%). The pellet with embedding resin was allowed to cure overnight at 70°C in a vacuum oven overnight.

Fluorescence measurements. Measurements were run using the following settings for the FITC tag: excitation: 488 nm, emission: 518 nm, and cutoff: 495 nm. The samples were run on a 96-well plate reader three times with the standard deviation as the designated error.

Results and Discussion

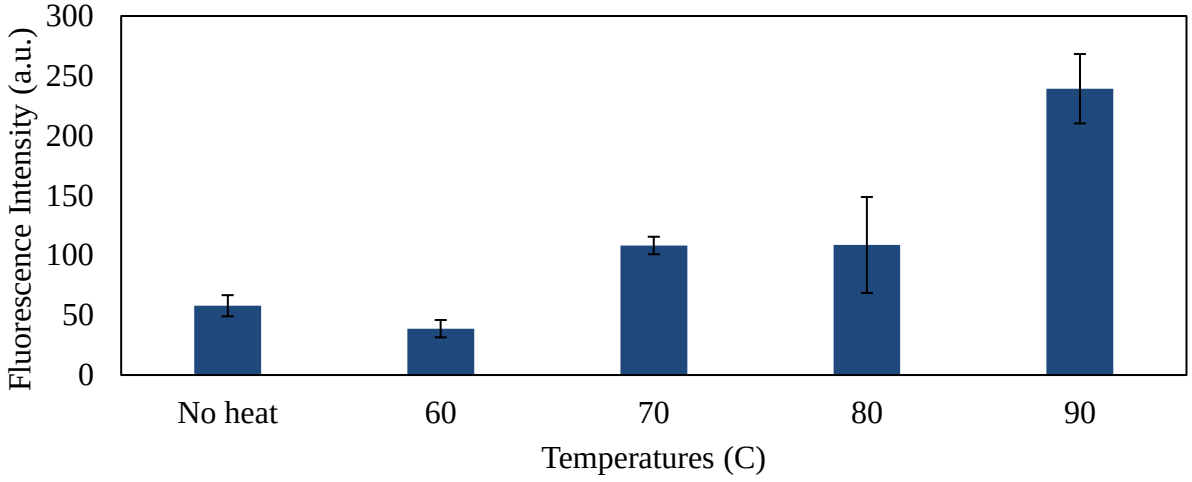
Quenching efficiency. An experiment to determine how effectively the gold nanoparticle can quench the detector fluorophores (DT), to determine background, was done. Specifically, the first experiment tested the detector's fluorophore in the presence of MGNPs, which should exhibit full fluorescence. The second experiment introduced the synthetic target, a strand required for effective hybridization to the MGNP surface. The results showed a 77.9% quenching efficiency, which is acceptable, but a 90% or higher efficiency is most desirable for a marketable biosensor. (**Figure 2**)

Figure 2: EC Probe Quenching Efficiency = 77.9%



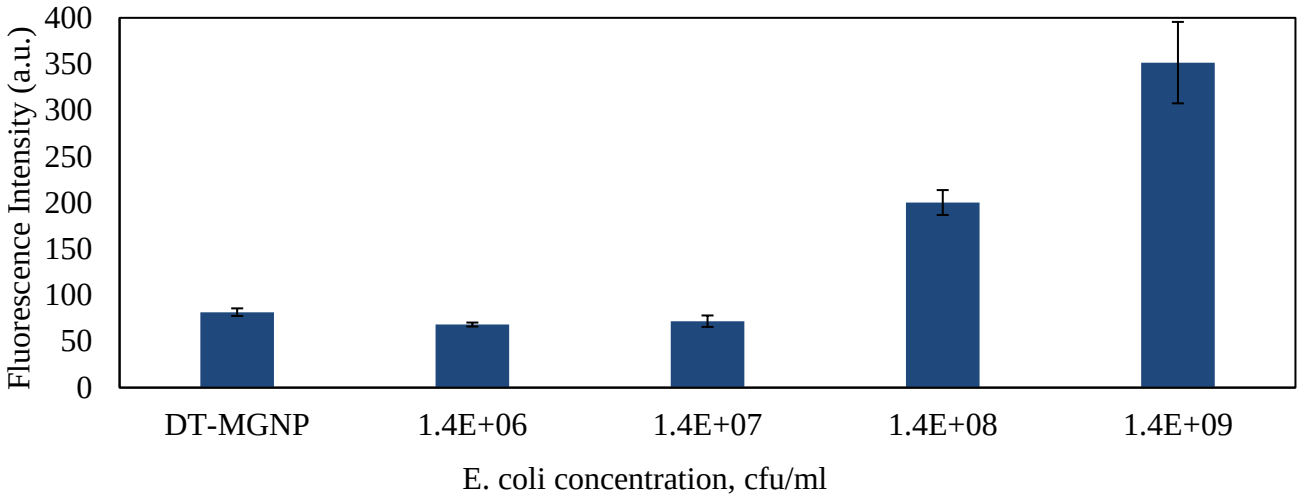
Bacterial lysate preparation. To determine the optimal conditions for thermal lysis, our positive control condition, temperatures ranging from 60°C to 90°C were tested. Specifically, a sample of *E. coli* (strain 137, clinical isolate) with OD_{600}= 0.5, or 10^8 CFU/ml was used heated at each of these temperatures for 10 minutes. 90°C showed the highest increase in fluorescence intensity (**Figure 3**) when compared to the fluorescence of the detector alone (data not shown). The 90°C temperature was chosen and used for the rest of the experiments.

Figure 3: Thermally Lysed *E.coli* + DT-MGNP



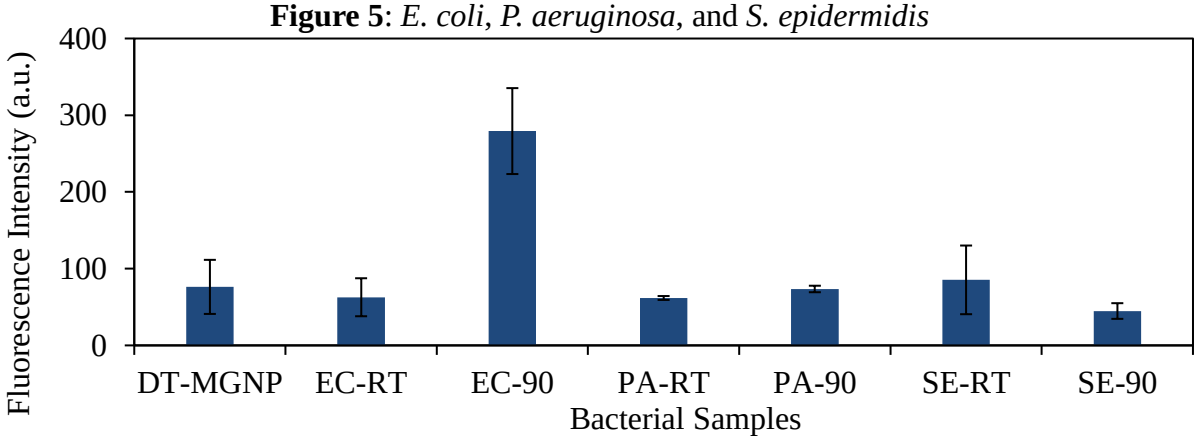
To further characterize the relationship between fluorescence and 16S rRNA release and hybridization, serial dilutions of the thermally lysed *E. coli* were performed and showed the fluorescence intensity increasing with increasing *E. coli* concentrations (**Figure 4**). Unfortunately, this assay does not seem to be sensitive, since the signal does not notably increase until the concentration is at 10^8 CFU/ml. Notably, although heating bacterial cells does effectively release many of its components including nucleic acids, another reason for the positive results arises from the unfolding of the secondary structure of 16S rRNA, facilitating the hybridization process.

Figure 4: Lysed *E. coli* Serial Dilutions



Probe specificity. To determine how specific our dsDNA probes were to *E.coli* (EC), other bacteria including *P. aeruginosa* (PA) and *S. epidermidis* (SE) were tested. Bacterial samples at room temperature and at 90°C were used. These experiments showed that the *E. coli* lysate 16S rRNA restored fluorescence of the detector probe as expected from the previous experiment. However, the *E. coli* bacteria alone, along with all other bacterial types (lysed or unlysed) did not restore fluorescence. (**Figure 5**) This most likely means the 16S rRNA target did not hybridize to the detector probe, either because the bacteria did not internalize the MGNPs or the dsDNA could not effectively hybridize to the secondary structure of 16S rRNA.

Figure 5: *E. coli*, *P. aeruginosa*, and *S. epidermidis*



Nanoparticle internalization. To determine whether *E. coli* internalized the MGNPs, TEM images of the bacteria incubated with MGNP (10%, 0.01M PBS). This experiment was done as a proof-of-concept to determine if internalization was possible at all, which is why we chose a 10% MGNP concentration, as opposed to the normally used 2%. The images show what resembles nanoparticles in high electron density granules (black spheres), but further studies are needed to confirm the particles as nanoparticles. (**Figure 6**) Based on morphology and size, it is most likely they are nanoparticles. Additionally, changes in morphology of the bacteria from the process of TEM preparation, as seen in a (*E. coli* is rod-shaped), is normal.

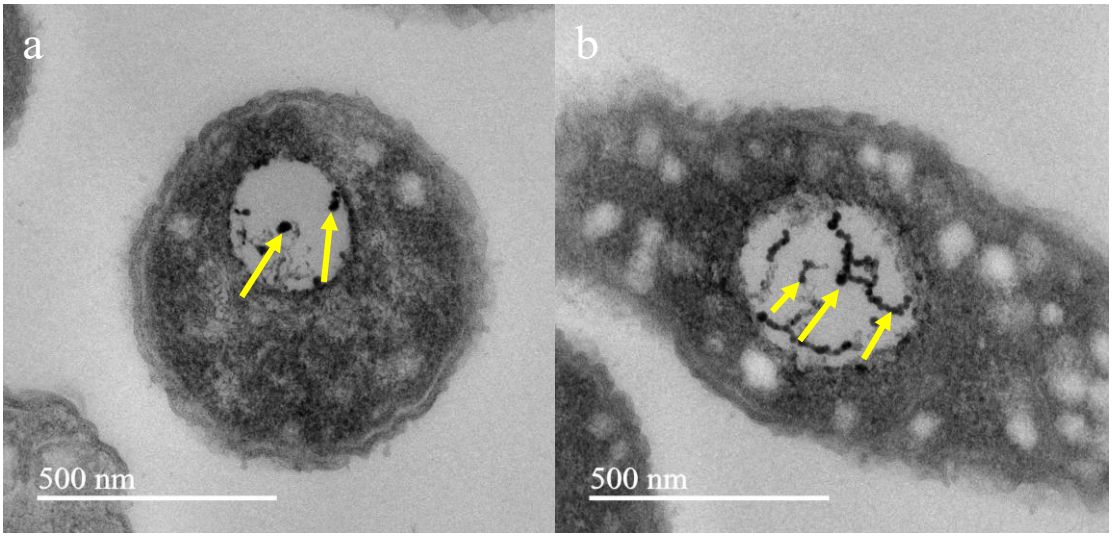


Figure 6: TEM Images of *E. coli* Incubated with MGNPs (a,b)

Conclusion

Overall, the device requires testing a variety of detector and target probe sizes to optimize quenching efficiencies (currently at 77%, data not shown) as well as 16S rRNA and detector hybridization. Further TEM images are required to determine nanoparticle internalization, although it is most likely the case. If it is the case, the reasons behind why these particles are not restoring fluorescence of the detector probe should be further investigated.

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