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Colorimetric Bacteria Sensing Using a Supramolecular Enzyme−Nanoparticle Biosensor

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Supporting Information

ABSTRACT: Rapid and sensitive detection of pathogens is a key requirement for both environmental and clinical settings. We report here a colorimetric enzyme−nanoparticle conjugate system for detection of microbial contamination. In this approach, cationic gold nanoparticles (NPs) featuring quaternary amine headgroups are electrostatically bound to an enzyme [β-galactosidase (β-Gal)], inhibiting enzyme activity. Analyte bacteria bind to the NP, which releases the β-Gal and restores its activity, providing an enzyme-amplified colorimetric readout of the binding event. Using this strategy, we have been able to quantify bacteria at concentrations of 1×10^2 bacteria/mL in solution and 1×10^4 bacteria/mL in a field-friendly test strip format.

Bacterial infections cause 300 million cases of severe illness each year,[1] and are estimated to kill over 2 million children every year.[2] The great majority of these deaths occur in emerging nations, where bacteria are widespread in drinking water and food.[3] Several techniques[4,5] are available in laboratories for pathogenic bacteria detection and identification, including (i) plating and culturing,[6−12] (ii) luminescence,[13] (iii) immunological approaches,[7,8] (iv) nucleic acid probe-based methods[9] (PCR, LCR), (v) mass spectrometry,[10] (vi) microarrays,[11] and (vii) biosensors.[12] Each of these systems has its advantages; however the utility of these methods is generally limited by their high cost for use and the requirement of trained operators.

Recent advances in nanotechnology have enabled the development of new diagnostic platforms[14] for sensitive and rapid pathogen detection. For example, Ji et al.[15] used positively charged amine-terminated polyamidoamine dendrimers to capture bacteria, reporting a detection limit of 1×10^4 cells/mL.[16] Functionalized gold nanoparticles (AuNPs) have likewise been used to detect bacteria,[17] viruses,[18] cancer cells,[19] and proteins.[20] In 2005, Murphy and co-workers[21] showed that cetyltrimethylammonium bromide (CTAB)-functionalized gold nanorods or nanospheres can conformally deposit to form a monolayer on *Bacillus cereus* by strong electrostatic interactions. More recently, our group[17a] demonstrated bacteria sensing at 2×10^5 cells/mL using a nanoparticle−fluorescent polymer conjugate system.

Two key issues in the design of effective sensors for pathogen detection in the field can be identified. First, the limit of detection (LOD) required for application in either environmental testing[4a,25,22] or clinical applications[25,23] is 10^4−10^2 cells/mL. Second, the readout should not require expensive instrumentation. To address these issues, we developed a hybrid colorimetric enzymatic nanocomposite biosensor that uses enzyme amplification to provide high sensitivity for the detection of pathogens in aqueous solutions. The efficacy of this system was then demonstrated in both solution and test strip format.

Our colorimetric sensor design features three main components: (a) β-galactosidase (β-Gal),[24] an anionic enzyme (pI 4.6), to provide signal amplification; (b) chlorophenol red β-D-galactopyranoside (CPRG), a chromogenic substrate, to provide a color readout; and (c) a cationic NP that binds reversibly to β-Gal, inhibiting the enzyme without denaturation (Figure 1a). The AuNPs used here were functionalized with quaternary ammonium ligands to provide high stability, biocompatibility, and a headgroup for tuning surface interactions, all of which are critical requirements for stable and sensitive biosensors (Figure 1b). Binding of the anionic surface of analyte bacteria[25] to the cationic particle surface displaces the β-Gal, with concomitant restoration of activity. The active enzyme converts the pale-yellow substrate into the red product, providing a colorimetric readout (Figure 1a).

Prior to our sensing studies, we conducted activity titrations of β-Gal-catalyzed hydrolysis of the CPRG substrate using **NP1**−**NP4** (Figure 2). These studies were performed using 0.5 nM β-Gal, a concentration that provided a reasonable time course (~10 min) for the colorimetric event. In practice, β-Gal in phosphate buffer solution (5 mM, pH 7.4) was incubated with various concentrations of **NP1**−**NP4** for 15 min, and then 1.5 mM CPRG (λ_{max} = 595 nm) was added to the NP−enzyme complexes. The normalized first-order rate of chromogenic substrate hydrolysis (V_{max}) was plotted versus the NP/β-Gal molar ratio and decreased upon addition of NPs, as shown for **NP2** (Figure 2b). After preliminary activity studies, **NP2** was chosen as the highest-affinity enzyme inhibitor (Figure S6 in the Supporting Information), as it inhibited the β-Gal activity at very low concentrations and provided the lowest LOD (Figure S7). The solution containing AuNP−enzyme complexes was freshly prepared before each experiment, and no significant precipitation

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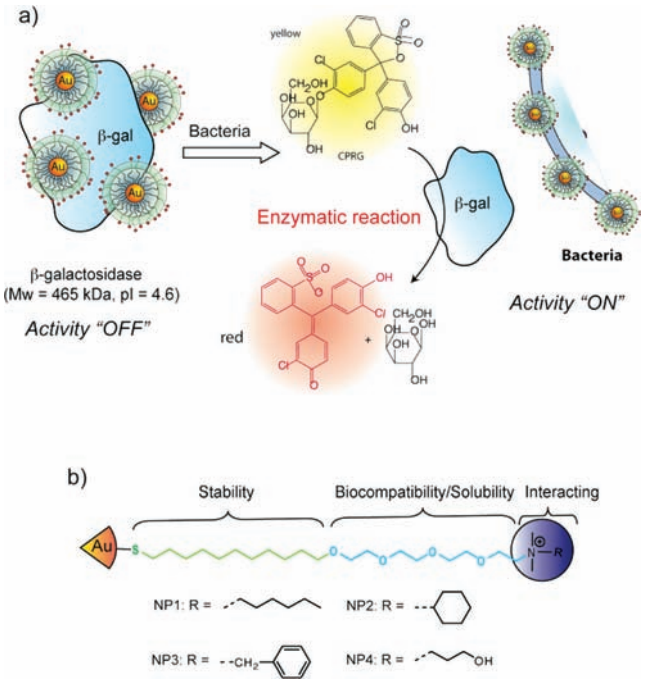

Figure 1. (a) Enzyme-amplified sensing of bacteria, showing the relative sizes of the 2 nm core diameter NPs and β-Gal. (b) Structures of ligands used for sensing studies.

or color change was observed during or after the experimental process. As a control, the enzyme inhibition by neutral tetraethylene glycol- and carboxylate-functionalized NPs ($\mathbf{NP}_{TEG}$ and $\mathbf{NP}_{CO2}$, respectively) was also studied, and no inhibition was observed (Figure S8).

For our initial sensing studies, we used *Escherichia coli* (XL1) as a model analyte (Figure 3). In these studies, we could reproducibly differentiate bacterial levels as low as 100 cells/mL (three replicates were carried out for each sample, and each sample was also replicated three times). Each concentration could be discerned not only by intensity curves and the V_{max} histogram but also by visible color changes; images taken immediately (10 min) after reading by an LCD camera (Figure 3 top) demonstrate this colorimetric effect. Similar changes in V_{max} were observed using the Gram-positive bacteria *Streptomyces griseus* and *Bacillus subtilis* (Figure S11), indicating the generality of the system.

We next investigated the application of our design to a test strip format suitable for potential field use,[26] featuring visual readout of the originated color in comparison to a reference color scale.[27] A key issue in this format is generating rapid and reproducible response times. Rapid bacterial penetration occurs on highly porous papers, while restriction of NP−enzyme conjugates to the surface occurs on less porous materials. Considering these issues, we explored a wide range of available materials to maintain the enzyme activity and the efficiency of the enzyme inhibition and activity recovery processes. Of the materials tested, GF/B binder-free microfiber filter paper was selected as the platform because of its high wet strength, high loading capacity, and rapid response. The formulation of our strip sensor featured 25 mM CPRG and 15 nM β-Gal, providing conversion from yellow to dark-red within 10 min with uninhibited enzyme. Inhibition studies were then carried out to determine the optimal concentrations of cationic **NP2** and β-Gal for formation of the hybrid enzymatic nanocomposite

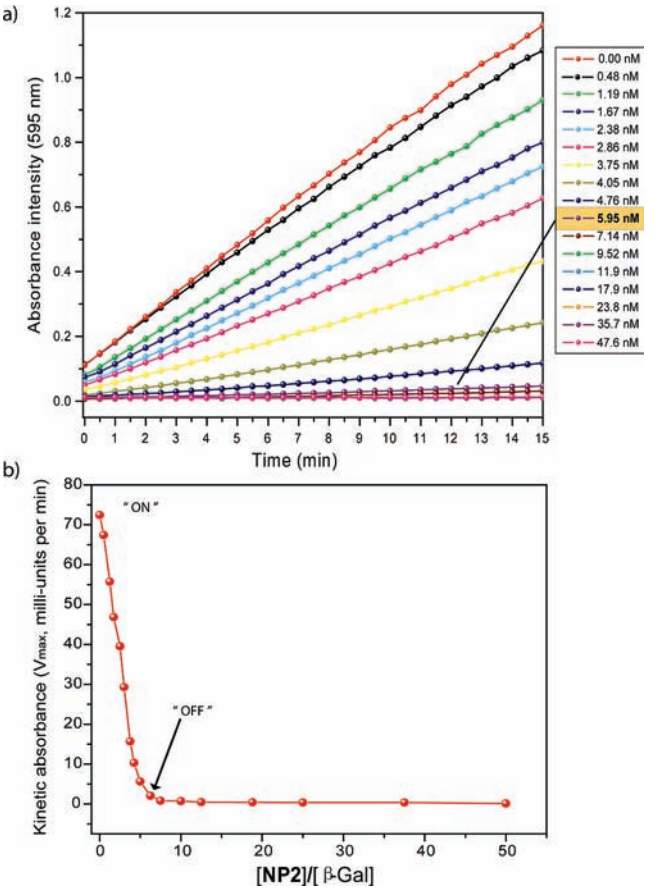

Figure 2. Assay of the inhibition of the activity of β-Gal (0.5 nM) with 1.5 mM CPRG upon addition of **NP2** (5 mM phosphate buffer). (a) Enzyme inhibition upon addition of **NP2.** (b) Inhibition of β-Gal (V_{max}) before (ON) and upon (OFF) addition of **NP2**.

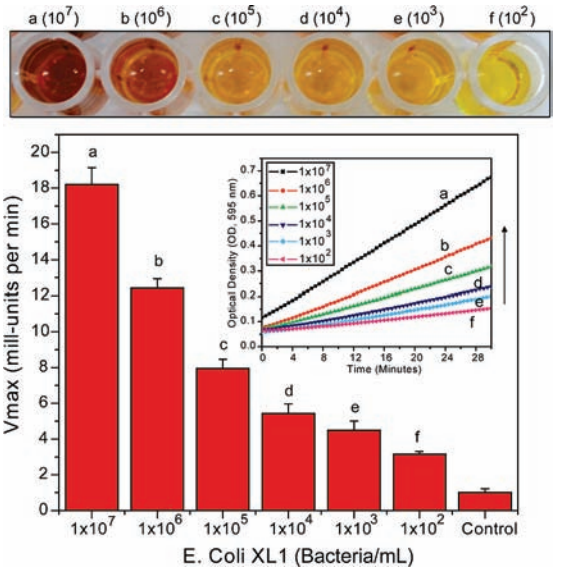

Figure 3. LOD of *E. coli* using the β-Gal−**NP2** nanocomposite. Kinetic absorbance responses upon addition of different bacteria concentrations are shown; the β-Gal−**NP2** nanocomposite without bacteria was used as the control. At the top, microplate wells showing the color change upon variation of the bacteria concentration.

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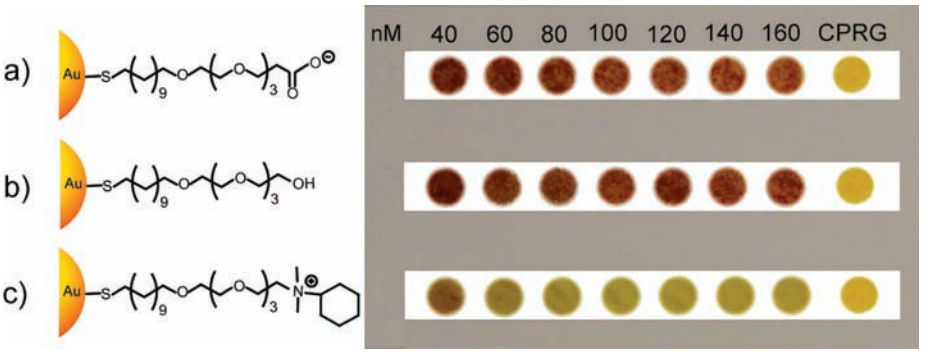

Figure 4. Enzymatic inhibition/colorimetric assay of β-Gal (15 nM) against 25 mM CPRG upon addition of (a) anionic (carboxylate), (b) neutral (hydroxyl), and (c) cationic (quaternary amine) functionalized AuNPs ($\mathbf{NP_{CO2}}$, $\mathbf{NP_{TEG}}$, and **NP2**, respectively) on a platform for testing. The inset shows total inhibition by the cationic **NP2** AuNPs at 80 nM (c), while no inhibition was observed for either the anionic (a) or neutral (b) AuNPs even at 160 nM.

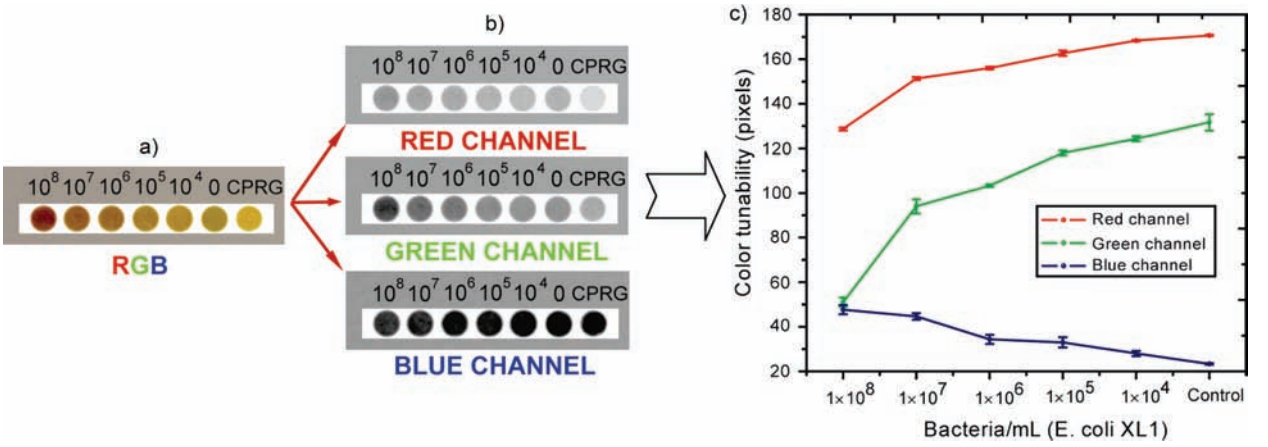

Figure 5. Schematic illustration of the RGB colorimetric analysis for monitoring color changes on the GF/B filter paper spot at pH 7.4. (a) Image of the enzymatic activity response/colorimetric assay of the β-Gal–**NP2** complex upon addition of *E. coli* (XL1) at different concentrations; CPRG substrate was used as the control. (b) Red, green, and blue channels obtained from the original sample in (a) to differentiate among bacteria concentrations. (c) Values for the red, green, and blue channels extracted from the original data in (a). The measurement process was repeated at least three times for each measurement in a series of images.

sensor (β-Gal–**NP2** complex). $\mathbf{NP_{TEG}}$ and $\mathbf{NP_{CO2}}$ were also used as controls with no inhibition observed (Figure 4). The β-Gal–**NP2** complex was ultimately generated by mixing β-Gal (15 nM) and **NP2** (80 nM) and allowing the composite to dry for 15 min.

To test the performance of our system on a paper strip, 3 μL of CPRG (25 mM), complex solution, and solutions of *E. coli* (XL1) containing from 1×10^8 to 1×10^4 bacteria/mL were spotted onto GF/B filter paper at pH 7.4. Images were obtained after 10 min with an LCD digital camera and appropriate lighting. As shown in Figure 5, clear visual differences were observed for concentrations ranging from 10^8–10^4 bacteria/mL. To provide quantitative assessment of the test strips, the RGB profiles of the images were analyzed.[27] The plots of RGB colorimetric channels (all values were taken at least three times) in Figure 5c established the effectiveness of the chromogenic platform, demonstrating that 1×10^4 bacteria/mL can be distinguished using this method.

In summary, we have used enzyme–nanoparticle assemblies to provide rapid and sensitive colorimetric sensing of bacteria. With this system in a solution platform, bacteria concentrations as low as 100 cells/mL could be determined in a matter of minutes. Transfer of this methodology to a test strip format provided a potential tool for field applications with a visual sensitivity of 10^4 bacteria/mL. This work was conducted on the model analyte *E. coli* (XL1), but the detection sensitivity of different bacteria may vary from species to species. Efforts to improve the sensitivitiesy of both formats and to adapt the methodology to dual detection and identification strategies for general applications are ongoing.

■ ASSOCIATED CONTENT

Supporting Information. Complete ref 1a; experimental section; syntheses, ζ potential and dynamic light scattering data, ^{13}C NMR and ^{1}H NMR spectra, and laser desorption/ionization mass spectra of **NP1**–**NP4**; inhibition activity titration; recovery activity; control experiments; and bacteria density images. This material is available free of charge via the Internet at http://pubs.acs.org.

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