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Gold Nanorod Probes for the Detection of Multiple Pathogens**

Chungang Wang and Joseph Irudayaraj*

Foodborne disease has been a serious threat to public health for decades and remains a major public health challenge. An estimated 76 million illnesses, 325 000 hospitalizations, and nearly 5 000 deaths are reported from health hazards in the US per year.^[1] Foodborne diseases are associated with five major pathogens, including *E. coli* O157: H7 (*E. coli*) and *Salmonella* Typhimurium (*S. Typhimurium*), and the costs associated with preventive and curative measures to combat these five major pathogens is estimated to be at least \$6.9 billion annually, according to the Economic Research Service (ERS) in 2000.^[2] Even trace concentrations (≈ 10 – 100 cells) of bacterial pathogens in food, donated blood, or the environment can pose a serious threat to human health. Several detection techniques have been developed, including PCR and ELISA, that are slow, time consuming, and labor intensive, and have limited ability to detect foodborne pathogens in a multiplex format.^[3–11] The challenge here is to develop a rapid, sensitive, and specific method capable of simultaneously detecting multiple pathogens of interest inexpensively. Simultaneous detection of pathogens reduces the number of reactions required to detect the possible presence of individual pathogens, thus saving considerable time and cost. To address this issue, multiplexed PCR, DNA microarray techniques, and array-based immunosensors have been developed to identify two to three foodborne pathogens in one assay.^[12–16] Nanoparticle-based biosensors have also been developed using magnetic nanoparticles,^[17–20] silver nanoparticles,^[21–23] and silver nanoshells^[24] to detect pathogens. However, these methods have a limited ability to detect multiple organisms. More recently, triple-dye-doped fluorescent silica nanoparticles^[25] and quantum dots (QDs)^[26] have been used for detecting multiple bacteria. However, the fluorescent detection modality requires the use of fluorescent probes and is typically susceptible to photo bleaching and careless handling, sometimes requiring specific excitations for sensitive detection, while QDs are difficult to synthesize, expensive, and surface modification is not trivial.

Gold nanorods are elongated nanoparticles with distinct optical properties that depend on their shape.^[27–30] In

particular, they possess two principal plasmon absorption bands, one the transverse plasmon (TP) band, corresponding to light absorption and scattering along the short axis of the particle, and the other the longitudinal plasmon (LP) band, corresponding to light absorption and scattering along the long axis of the particle. The former is located in the visible region of the electromagnetic spectrum at ≈ 520 nm, while the latter is aspect ratio tunable from the visible to the near-IR region of the electromagnetic spectrum. The position and intensity of these bands can be affected by changes in the dielectric constant around the vicinity of these nanoparticles, known as localized surface plasmon resonance (LSPR) or nanoSPR.^[31–33] These properties suggest that gold nanorods have several advantages for applications in biological sensing, imaging, and therapy, which may perhaps benefit from the geometry of these structures.^[34–38] Additionally, the elongated nanoparticles have an inherently higher sensitivity to the local dielectric environment compared to similarly sized spherical nanoparticles. More importantly, gold nanorods with different aspect ratios could be easily fabricated and their unique yet simple “multiplexing” advantage could be harnessed.^[34] However, the as-prepared gold nanorods are stabilized by CTAB bilayers which are very difficult to displace by analytes of interest. It is also known that CTAB is toxic and, in order to develop any valid whole cell detection scheme, requires replacement with biocompatible and stable ligands.

In this paper, we demonstrate a simple and novel method to completely replace the CTAB bilayer on gold nanorods with amine groups provided by a biocompatible and water-soluble chemical, cystamine with a disulfide compound. Further, the amino-terminated gold nanorods can be easily functionalized with antibodies to construct gold nanorod bioprobes. More importantly, we investigated the use of different gold nanorod bioprobes with different aspect ratios, conjugated to anti-*E. coli* and anti-*S. typhimurium* antibodies, as novel optical labels based on changes in SPR band for rapid (less than 30 min) and sensitive detection of two major species of foodborne pathogenic bacteria, *E. coli* O157: H7 and *S. typhimurium*, used as model pathogens in one solution simultaneously at concentrations lower than 10^2 cfu mL⁻¹ (Colony Forming Units mL⁻¹). In principle, the method presented here could be extended to detect multiple bacterial targets simultaneously, using gold nanorod bioprobes of different aspect ratios.

Before applying gold nanorods with various aspect ratios to the simultaneous detection of multiple pathogenic bacteria, gold nanorods with one aspect ratio were used for binding efficiency and specificity studies. Then, simultaneous detection of two species of foodborne pathogenic bacteria, *E. coli* and *S. typhimurium*, was achieved using amine-modified gold-nanorod bioprobes with two aspect ratios, as shown in Figure 1. Gold nanorods with an aspect ratio ≈ 2.6 were fabricated in aqueous solution using a seed-mediated surfactant-directed method and purified by centrifugation and washing as previously described.^[28] The gold nanorods had a cationic surfactant, cetyltrimethylammonium bromide (CTAB), bound to their surfaces in the form of a bilayer. The CTAB renders the nanorods water stable, providing a net positive charge, as shown in Figure 1 (top). To specifically

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Supporting Information is available on the WWW under <http://www.small-journal.com> or from the author.

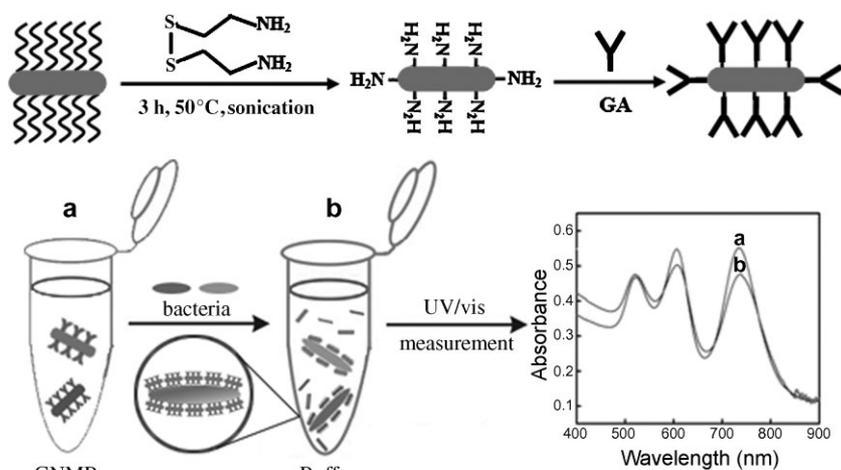


Figure 1. Schematic representation of (top) the synthesis of pathogen-antibody-conjugated gold nanorods and (bottom) the simultaneous detection of two species of pathogens in an assay based on gold nanorod probes (GNPs). CTAB: cetyltrimethylammonium bromide, GA: glutaraldehyde.

target the gold nanorods to the pathogens of interest, anti-*E. coli* and anti-*S. typhimurium* antibodies need to be covalently linked to the surfaces of the nanorods. Covalent attachment of antibodies to nanorods requires appropriate surface modification to replace the tightly packed CTAB bilayer at the side faces of gold nanorods for further biofunctionalization.^[39–41] In this report, a novel and simple procedure is proposed to fully activate the gold nanorod surface with water soluble cystamine, using a disulfide that can access the gold nanorod surface more easily than a single thiol in organothiol compounds, which are insoluble in water. The resulting gold nanorods were fully covered with amine groups provided by cystamine, as shown in Figure 1 (top). Briefly, 10 mL of gold nanorods was removed by centrifuging once at 8000 rpm to remove excess CTAB, and these removed nanorods were dispersed in pure water. Then 1 mL of 30 mM cystamine was added, and the solution was kept at 50 °C under constant sonication for 3 h. This was followed by centrifugation at 8000 rpm for 10 min to remove the excess CTAB and cystamine. In this procedure, control of reaction temperature is the key to the success of the amine activation protocol. Temperature should be increased to drive CTAB molecules to dissociate from the gold nanorod surface; however, if the temperature is increased too much or risen too quickly, the exposed gold nanorods tend to aggregate. The resulting amine modified gold nanorods can then be directly immobilized to antibodies by activating the nanoparticles with glutaraldehyde (Fig. 1[top]). It should be noted that the activation and immobilization steps did not result in any aggregation of the gold nanorods.

The visible/NIR spectra measured for gold nanorods before and after amine modification and further functionalization with antibodies show that the optical properties of the gold nanorods are altered (Fig. 2). The as-prepared gold nanorods have a weak transverse plasmon band at 524 nm and a strong longitudinal plasmon band at 650 nm (Fig. 2A). For amine terminated gold nanorods, obvious redshifts of 7 nm are

observed to present a longitudinal peak at 657 nm (Fig. 2B). Amine modification of the CTAB capping (by replacement) was confirmed by Fourier transform infrared spectroscopy (FTIR) (Fig. S1). FTIR spectra of A) CTAB stabilized gold nanorods and B) amine modified gold nanorods are shown in Figure S1. The spectrum for CTAB in the as-prepared substrates show two intense bands, assigned to asymmetric (2920 cm^{-1}) and symmetric (2850 cm^{-1}) stretching vibrations of C–CH₂ in the methylene chains (Fig. S1A). After amine modification, the intense bands at 2920 and 2850 cm^{-1} become very weak, indicating that the CTAB molecules surrounding the gold nanorods have been replaced with amine groups. (Fig. S1B). When anti-*E. coli* antibody, however, was immobilized onto the amino-terminated gold nanorods through the well-established glutaraldehyde method, the LP peak of the nanorods

redshifted to 671 nm and broadened (Fig. 2C). This redshift could be attributed to an increase in local refractive index due to the antibody, and is the result of an increase in the local refractive index of the medium surrounding the gold nanorods as shown in Figure 2. The absorption peak of the TP resonance is around 520 nm, and is not significantly changed by conjugation because the longitudinal plasmon is more sensitive than the transverse plasmon to the local refractive index of the surrounding medium.

When anti-*E. coli*-antibody-conjugated gold nanorods were mixed with various concentrations of *E. coli*, the LP band intensity of the gold nanorods reduced and always remained less than the LP bands before the addition of *E. coli* (Fig. 3). This is because *E. coli* is much larger in size ($\approx 1\text{--}3\ \mu\text{m}$) than the gold nanorod bioprobes modified by anti-*E. coli* antibody (47 nm), and several antibody conjugated gold nanorods are attached to the *E. coli* surface. It is worth noting that the LP band only decreased in intensity after the recognition event between anti-*E. coli* antibody and *E. coli* attachment. However, the decrease in intensity reduction and

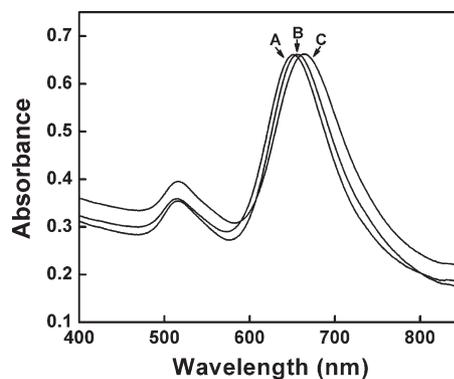


Figure 2. UV/Vis absorbance spectra of A) original gold nanorods after centrifugation, B) amine modified gold nanorods, and C) amine modified gold nanorods and anti-*E. coli* antibody conjugates.

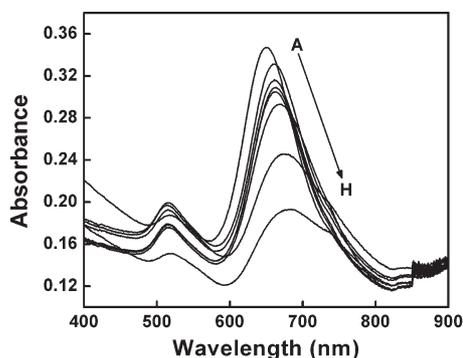


Figure 3. UV/Vis absorbance spectra of A) amine-modified gold nanorods, B) anti-E.coli-antibody conjugates, and C)–H) after the addition of *E. coli* at different concentrations ($1\text{--}10$, 10^3 , 10^2 , 10^4 , 10^5 , 10^6 cfu mL $^{-1}$) to antibody-conjugated amine-modified gold-nanorod bioprobes.

the redshift appear at higher concentrations (10^5 and 10^6 cfu mL $^{-1}$). This redshift is most likely due to the interaction between the gold nanorods attached to the *E. coli* surface. Results indicate that the detection of *E. coli* either in small ($1\text{--}10$ cfu mL $^{-1}$) or large (10^6 cfu mL $^{-1}$) numbers can be detected by changes in the intensity and position of the LP bands. Changes to the corresponding TP bands are not obvious. The detection method demonstrated is very rapid, as the LP-band variation of gold-nanorod bioprobe changes can be observed using a simple optical spectrometer (400–1200 nm range) in less than 30 minutes, even at concentrations as low as $1\text{--}10$ cfu mL $^{-1}$. Similar results were observed for *S. typhimurium* detection (see the Supporting Information, Fig. S2).

Observations of LP band changes can further be confirmed by transmission electron microscopy (TEM) images. Figure 4 presents TEM images of amine-terminated gold nanorods, *E. coli*, and *E. coli* bound to antibody-modified gold nanorods. Figure 4(A and B) shows representative transmission electron microscopic (TEM) images of gold nanorods with aspect ratio 2.6 (47 nm × 18 nm) and *E. coli* ($\approx 1\text{--}3$ μm), respectively. After

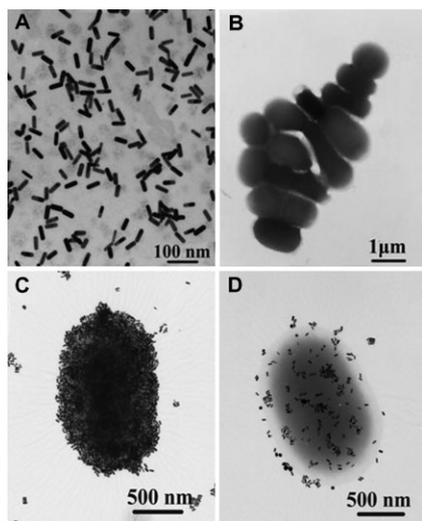


Figure 4. TEM images of A) amino-modified gold nanorods, B) *E. coli*, and C) and D) the specific interaction of *E. coli*-antibody-gold nanorods conjugates with *E. coli*, with different coverage.

the interaction of *E. coli* and anti-*E. coli* antibody modified gold nanorods, many gold nanorod bioprobes bind to the bacteria surfaces (Fig. 4C) and cause a change in the intensity and red-shift of the LP bands. Thus the redshift and the reduction in intensity of LP bands are consistent with the TEM observations. The gold nanorods modified with the antibodies of *E. coli* showed greater coverage of the bacteria. Gold nanorods, modified with antibodies of *E. coli*, attach to the *E. coli* surface at different densities by adjusting its concentration, as shown in Figure 4(C and D).

It should be noted that anti-*E. coli*-antibody interaction is extremely specific, and the presence of a pathogen other than *E. coli* did not perturb the LP band of the *E. coli*-specific gold-nanorod bioprobes. Experiments also indicated that *E. coli* antibody did not cross react with *S. Typhimurium*. *S. Typhimurium* bacteria ($\approx 10^4$ cfu mL $^{-1}$), when mixed with *E. coli*-antibody-conjugated gold nanorods, did not produce any obvious change in the plasmon band in the absorbance spectra. When *E. coli* ($\approx 10^4$ cfu mL $^{-1}$) was added, however, the absorbance spectra redshifted with a corresponding intensity decrease (see the Supporting Information, Fig. S3).

Simultaneous monitoring of two species of pathogens was achieved using gold nanorods with two different antibodies. First, the anti-*E. coli* and *S. typhimurium* antibodies were conjugated to amine-modified gold nanorods with aspect ratios of 2.0 and 3.2, respectively. The two types of antibody-labeled gold nanorods and two species of bacteria were mixed together in phosphate-buffered saline (PBS) and incubated for 30 min. Figure 5 shows the visible/NIR absorbance spectra of samples that contained both *E. coli* and *S. typhimurium* at concentrations in the range of $1\text{--}10$ to 10^8 cfu mL $^{-1}$. As seen from the spectra, the LP bands present changes in intensity when the concentration is less than 10^4 cfu mL $^{-1}$, and both intensity decrease and redshift was observed at higher concentrations ($10^6\text{--}10^8$ cfu mL $^{-1}$). These results indicate that the gold-nanorod bioprobes of each aspect ratio could bind to their respective bacterial target in a mixture of the two species to produce changes in the LP bands of the nanorods. In addition, to address nonspecific binding in the simultaneous detection of two target pathogenic species, gold nanorods of

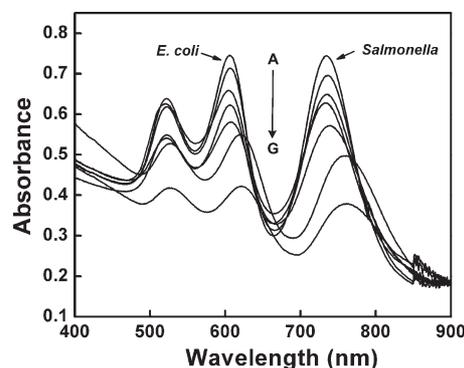


Figure 5. UV/Vis absorbance spectra after the addition of a mixture of *E. coli* and *S. typhimurium* to anti-*E. coli*- and *S. typhimurium*-antibody-conjugated amine-modified gold nanorods with aspect ratios of 2.0 and 3.2, respectively. The concentrations of *E. coli* and *S. typhimurium* were $1\text{--}10$ to 10^8 cfu mL $^{-1}$ (A–G).

two different aspect ratios respectively modified with antibodies of *E. coli* and *S. typhimurium* were mixed with 10^3 cfu mL⁻¹ *E. coli*; the LP band of the bioprobes modified for *E. coli* reduced in intensity, while the LP band of the bioprobes modified for *S. typhimurium* did not change. When 10^4 cfu mL⁻¹ of *E. coli* and *S. typhimurium* was subsequently added to the same mixture, the intensity of the plasmon band corresponding to the two probes decreased simultaneously, indicating the specificity between the antibodies and the corresponding bacteria (see the Supporting Information, Fig. S4).

In summary, in this report we demonstrate the simple synthesis of amine-modified gold nanorods with different aspect ratios and their use in simultaneous detection of *E. coli* and *S. typhimurium* pathogens. This proposed method is simple, rapid, and inexpensive, and simultaneous monitoring of bacterial pathogens at concentrations less than 10^2 cfu mL⁻¹ is possible in less than 30 min, based on changes in LSPR bands of aspect-ratio-tunable nanopropes. The reported detection limits can be achieved without any complex functionalization and fluidic attachments. This work also sets the stage for the first demonstration of LSPR sensors using nanorods for multiplex and sensitive detection of pathogenic agents.

Experimental Section

Materials: Hydrogen tetrachloroaurate (HAuCl₄ 3H₂O, ≥99.99%), sodium borohydride (NaBH₄, ≥99%), silver nitrate (99%), ascorbic acid (AA, ≥99.7%), cetyltrimethylammonium bromide (CTAB, ≥99%), glutaraldehyde (GA), and cystamine dihydrochloride (98%) were purchased from Sigma–Aldrich. Affinity purified goat anti-*E. coli* and goat anti-*Salmonella* antibodies were obtained from Kirkegaard & Perry laboratories. Milli-Q grade water was used in all experiments.

Preparation of CTAB-stabilized gold nanorods with different aspect ratios: CTAB-stabilized gold nanorods were synthesized using the seed-mediated growth method improved by El-Sayed and coworkers.^[28] Briefly, the seed solution was prepared by mixing CTAB (0.2 M, 5 mL) and HAuCl₄ (0.5 mM, 5 mL) with freshly prepared ice-cold (ca. 0 °C) NaBH₄ (10 mM, 0.6 mL). After 5 h, this seed solution was used for the synthesis of gold nanorods. In a flask, 50.0 mL of 0.2 M CTAB was mixed with 1, 1.5, and 3 mL of 4 mM silver nitrate aqueous solution, and 50.0 mL of 1 mM HAuCl₄ was added after gently mixing the solution with 700 μL of ascorbic acid (AA). Then, 120 μL of the seed solution was added into the mixture to initiate the growth of gold nanorods. Our experiments yielded gold nanorods with aspect ratios 2.0, 2.6, and 3.2.

Preparation of Amine Modified Gold Nanorods: After preparation of CTAB-stabilized gold nanorods, excess CTAB molecules on the nanorods (10 mL solution) with different aspect ratios were removed by centrifuging once at 8000 rpm, discarding the supernatant and redispersing the particles in pure water. Then 1 mL of 30 mM cystamine dihydrochloride was added, and the solution was kept at 50 °C under constant sonication for 3 h. This procedure was then followed by centrifugation at 8000 rpm for 10 min to remove excess CTAB and cystamine dihydrochloride.

Covalent immobilization of the antibody onto the amine-modified gold-nanorod surface: The goat anti-*E. coli* and *S.*

typhimurium antibodies were immobilized onto the amine-functionalized gold nanorods with aspect ratios of 2.0 and 3.2, respectively. This was accomplished using the well-established glutaraldehyde spacer method; 10 mL of amine-functionalized nanoparticles were dispersed into 0.01 M PBS (pH 7.4) containing 5% glutaraldehyde for about 1 h. These particles were then collected by centrifugation and redispersed in PBS, and incubated with the antibody for 12 h at 4 °C. The antibody-modified gold nanorods were washed with PBS to remove excess antibody and kept at 4 °C in pH 7.4 PBS.

Preparation of bacteria samples: Bacteria (*E. coli* and *S. typhimurium*) were grown in Luria–Bertini broth at 37 °C and collected with sterile plastic inoculating loops from solid culture plates. The samples were collected after the bacteria were cultured for 13 h with shaking for all experiments. The collected samples were added to 3 mL PBS (pH 7.4), vortexed, and centrifuged for 10 min at 5 800 rpm. The supernatant was discarded. This procedure was repeated three times.

Detection of bacteria (*E. coli* and *S. typhimurium*): Certain amounts of amine-modified gold nanorods with different aspect ratios conjugated with antibodies for *E. coli* and *S. typhimurium* were dispersed in PBS (1 mL), and *E. coli* and *S. typhimurium* cells at varying concentrations were added and gently shaken at room temperature for 30 min, for UV/vis measurement.

Measurement: The aspect ratios of the gold nanorods were determined from transmission electron microscopy (TEM) images, acquired with a Philips CM-100 TEM (Philips, Eindhoven, Netherlands) operating at 100 kV. At least 150–200 nanorods could be counted and measured per grid to calculate the mean aspect ratio of the nanorods after the synthesis step. The samples with *E. coli* cells were also measured using the Philips CM-100 TEM. Absorption spectra of all samples at every step of the experiments were measured with a Jasco V570 UV/visible/NIR spectrophotometer (Jasco, Inc., Easton, MD), in the 400 and 900 nm wavelength range. FTIR spectra were recorded using a Digilab Excalibur FTS 6000 spectrometer fitted with a UMA 600 IR microscope (Digilab, Randolph, MA), with a liquid nitrogen-cooled mercury-cadmiumtelluride detector under a reflection acquisition mode. All samples were measured by FTIR in water.

Keywords:

gold nanorods · medicine · multiplex detection · pathogens · plasmonic properties

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