Gold Nanorod Bioconjugates
Hongwei Liao and Jason H. Hafner*
Department of Chemistry and Department of Physics & Astronomy, Rice University, 6100 Main Street, Houston, Texas 77005
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Gold nanorods have been stabilized, conjugated to antibodies, and characterized for biological applications. The stabilizing surfactant bilayer which surrounds gold nanorods was replaced by thiol terminated methoxypoly(ethylene glycol) so that the nanorods are stable in buffer solutions free of surfactant. Nanorod bioconjugation was accomplished with a heterobifunctional cross-linker, with antibody activity confirmed by a strip plate assay. Nanorod bioconjugates were characterized by independent measurements of the nanorod and antibody concentrations. The nanorod molar extinction coefficient was measured from dense yet well separated nanorod films to be 4.4 ± 0.5 × 10⁴ M⁻¹ cm⁻¹, thus allowing quantitative determination of nanorod concentrations. However, a colorimetric protein assay overestimated the antibody concentration and is therefore likely perturbed by the presence of the nanorods electromagnetically and/or chemically.

Introduction

Noble metal nanoparticles exhibit a strong optical extinction at visible and near-infrared (NIR) wavelengths due to a localized surface plasmon resonance (LSPR) of their free electrons upon excitation by an electromagnetic field. The resulting intense color of noble metal nanoparticle solutions was explained theoretically by Mie for spherical gold colloid in 1908.1 Recent advances in the synthesis2,3 and chemical modification4 of noble metal nanoparticles have led to widespread interest in their properties and applications, as thoroughly reviewed by Daniel and Astruc.5 For example, the aggregation of oligonucleotide-functionalized gold nanoparticles, when mixed with a complementary single-strand DNA linker, allows the colormetric detection of as little as 10 fmol of single-stranded DNA.6 Due to the sharp, sequence-dependent melting transition of the DNA-nanoparticle ensemble, single base-pair mismatches between the probe and linker can be detected.7 Another detection platform monitors shifts in the plasmon resonance peak wavelength upon binding of analyte molecules to the nanoparticle surface.5,9 The shift is due to the altered dielectric properties of the nanoparticle’s local environment by the presence of the analyte. With bioconjugated nanoparticles, biomedically relevant, label free analytes can be detected down to 10 nM concentration with this simple technology.10 Individual noble metal nanoparticles are also employed as microscopic labels. While this is traditionally the domain of immunospecific electron microscopy, gold and silver nanoparticles have recently been applied for optical imaging since they have large scattering cross sections and do not photobleach or blink like fluorophores or semiconductor nanoparticles.11–13

Significant biomedical applications become possible when gold nanoparticle resonances are tuned to the near-infrared (NIR), where tissue is relatively transparent.14 Photothermal cancer therapy by tumor ablation has been demonstrated in vivo by NIR laser excitation of tunable gold nanoshells accumulated at a tumor site.15,16 The nanoshells can be designed to have strong NIR absorption,17 resulting in significant localized heating of the surrounding tissue.18 Gold nanoshells can also serve as targeted contrast agents for diagnostic imaging modalities such as optical coherence tomography when they are tuned for strong scattering in the NIR.19 Tunable gold nanoparticles could therefore serve as both a diagnostic and therapeutic technology for cancer treatment.20 The further development of these diagnostic and

several advantages of nanorods for the applications described above. Their sharp, polarized NIR resonances could enable multiplexed biological sensing and biomedical imaging through multispectral and polarization sensitive detection. Biosensor applications should also benefit from the nanorod geometry, as it has been shown that elongated nanoparticles have an inherently higher sensitivity to the local dielectric environment than similar sized spherical nanoparticles. Polarized scattering from nanorods can provide microscopic imaging applications with orientation information at the molecular scale. Photothermal therapies may benefit from the small size of gold nanorods in terms of their ability to permeate tissue and the leaky vasculature of tumors. Apart from these inherent properties, a practical advantage of gold nanorods is that simple procedures have been recently developed for high-yield nanorod synthesis which use cetyltrimethylammonium bromide (CTAB) surfactant as a soft template. However, biological applications have not been widely pursued with gold nanorods in part because nanorod solutions are stabilized by the presence of concentrated CTAB surfactant. Although conjugates have been formed in the presence of CTAB, surfactant solutions are cytotoxic and may interfere with established protein-linking protocols. A recent report explored the cytotoxicity of CTAB stabilized spherical gold nanoparticles. It was found that toxicity was due to free CTAB in solution but not CTAB which is bound to the nanoparticle. However, for gold nanorods the removal of the bulk CTAB will cause aggregation. We have therefore explored the use of commercially available thiol-terminated methoxypoly(ethylene glycol) (mPEG-SH) as a stabilizer for nanorods since it will strongly bind the gold nanorod surface and is biocompatible.

**Experimental Methods**

**Gold Nanorod Synthesis.** Gold nanorods were prepared exactly as described by Sau et al., but scaled up to increase the quantity. Gold seed particles were prepared by adding 250 μL of 10 mM HAuCl₄·3H₂O to 7.5 mL of 100 mM cetyltrimethylammonium bromide (CTAB) with brief, gentle mixing. Next, 600 μL of freshly prepared, ice-cold 10 mM NaBH₄ solution was added followed by mixing for 2 min. The pale brown seed solution was stable and usable for several days. The nanorod growth solution was prepared from the following reagents to a plastic tube in the following order and then gently mixing: 40 mL of 100 mM CTAB, 1.7 mL of 10 mM HAuCl₄·3H₂O, 250 μL of 10 mM AgNO₃. Next, 270 μL of 100 mM ascorbic acid was added, which changed the solution from brown-yellow to colorless. To initiate nanorod growth, 420

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![Figure 1. Gold nanorods. (a) Transmission electron micrographs of the gold nanorods reveal their structure. (b) The absorbance spectrum of the nanorod solution shows both the transverse and the longitudinal plasmon resonance peaks.](image-url)
μL of the seed solution was added to the growth solution, mixed gently, and left still for 3 h.

**Nanorod Characterization.** Extinction measurements on gold nanorod solutions and films were carried out with a fiber optic spectrometer (USB 2000, Ocean Optics) in plastic cuvettes with a 1 cm path length. Extinction spectra of nanorod films were taken by immersing the glass substrate in a cuvette with water. The peak wavelength and absorption were determined by a Gaussian fit between 700 and 900 nm. For transmission electron microscopy, nanorod solutions were dried on a carbon film grid and imaged at 100 kV (JEM 2010, JEOL). Atomic force microscopy (AFM) of strip plates and nanorod films was carried out in tapping mode in air (Nanoscope IV Multimode AFM, Veeco Metrology). Nanorod densities were measured by counting nanorods in 2 μm scans taken in random positions. For Raman spectroscopy, both CTAB-stabilized and PEGylated nanorod solutions were dried onto glass substrates and the films were studied in a microRaman spectrometer (Raman Microscope, Renishaw) at an excitation wavelength and power of 780 nm and 20 mW, respectively. The data presented are an average of five spectra taken in different locations.

**Gold Nanorod PEGylation.** Thiol-terminated methoxy poly-(ethylene glycol) (mPEG-SH) was purchased from Nektar Therapeutics (2M4EOH01). The raw nanorod solution was centrifuged at 7000g for 20 min to pellet the nanorods, decanted, and then resuspended to 1 mL of DI water to reduce the CTAB concentration to 10 mM. One hundred microlitres of 2 mM potassium carbonate and 10 μL of 1 mM mPEG-SH were added to the nanorod solution. The mixture sat overnight at room temperature, then was centrifuged, decanted, and resuspended in DI water several times to remove excess CTAB and mPEG-SH.

**Nanorod/Antibody Conjugation.** Bioconjugation of gold nanorods to antibodies was carried out with the long chain heterobifunctional cross-linker succinimidyl 6[3′-(2-pyridyl)dithio)propionamido] hexanoate (LC-SPDP, 21651, Pierce) following the procedure provided. Twenty millimolar LC-SPDP was prepared in dimethylformamide (DMF) immediately after equilibrating the vial of LC-SPDP reagent to room temperature. Anti-rabbit IgG (R5506, Sigma) was diluted to 720 g/mL in 100 mM pH 9.3 carbonate/bicarbonate buffer. Wells of an ELISA strip plate (15041, Pierce) were filled with 100 μL of this solution and left overnight at 4 °C. The IgG was exchanged into 1 mL of pure PBS-EDTA (100 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide). Twenty-five microliters of the LC-SPDP solution was added to the 1 mL IgG solution and allowed to incubate for 60 min at room temperature. The IgG was exchanged into 1 mL of pure PBS-EDTA buffer with a desalting column (89889, Pierce) to remove reaction byproducts, excess IgG, and excess LC-SPDP. One millilitre of raw nanorods was centrifuged at 7000g for 20 min, decanted, and then resuspended in 1 mL of the PBS-EDTA. Note that, at this stage, the CTAB concentration has only been reduced to 10 mM, so the nanorods are still stable. Two and a half microliters of the IgG solution with cross-linker was added to the nanorod solution and the mixture was incubated at room temperature overnight. The nanorods were then PEGylated by adding 10 μL of 1 mM mPEG-SH at room temperature overnight as described above. Finally, the bioconjugated nanorods were centrifuged, decanted, and resuspended in PBS-EDTA several times to remove excess CTAB and unreacted mPEG-SH.

**Strip Plate Assay.** Anti-neurofilament rabbit IgG (N4142, Sigma) was diluted to 720 μg/mL in 100 mM pH 9.3 carbonate/bicarbonate buffer. Wells of an ELISA strip plate (15041, Pierce) were filled with 100 μL of this solution and left overnight at 4 °C. The solution was then discarded and the well briefly rinsed. Fifty microliters of the antibody bioconjugated or only PEGylated nanorods were put into the well and left overnight at room temperature. The well was rinsed with DI water three times, dried with a stream of nitrogen, and analyzed by atomic force microscopy.

**Results and Discussion**

For this report we followed the recent method from Murphy’s group on high-yield synthesis of short nanorods33 and found that it scaled up to volumes over 100 mL without modification. Gold nanorods with an aspect ratio of 3.3 (15 nm diameter, 50 nm length) and a longitudinal plasmon resonance at 755 nm were synthesized in 150 mL quantity. TEM images and the extinction spectra of the nanorods are displayed in Figure 1. The CTAB concentrations of original nanorod solutions (raw) and those incubated with mPEG-SH (PEGylated) were sequentially lowered by successive centrifugation, decantation, and resuspension in DI water. The extinction spectra displayed in Figure 2a demonstrate that while the raw nanorod solution is completely lost due to aggregation (a pellet is observed), the PEGylated nanorods remain well-suspended down to CTAB concentrations below 10 μM. Note that the raw nanorod solution will remain well-suspended when the CTAB is diluted to 10 mM, which is often carried out as a first processing step to remove large CTAB crystals. MicroRaman spectra provide further evidence that the CTAB bilayer34 has been displaced by mPEG-SH. The spectra in Figure 2b show that the 180 cm⁻¹ Au—Br peak⁴¹ present in the raw nanorod sample is lost for the PEGylated nanorods, suggesting that CTAB is no longer
present at the nanorod surface. Au–S stretching modes can in principle demonstrate the formation of a thiolate bond, but they are poorly understood and show a wide variation in peak position. The peak at 261 cm\(^{-1}\), which is only present for the PEGylated nanorods, is at a reasonable energy to represent thiolate bond formation between the nanorods and PEG-SH.\(^{42,43}\) Once PEGylated, the nanorods are stable in various buffer solutions.

Targeted imaging and therapy, as well as immunospecific sensing, require the conjugation of antibodies to gold nanoparticles. While this has been accomplished by electrostatic or hydrophobic interactions for electron microscopy,\(^{28}\) bifunctional cross-linker molecules\(^{44}\) enable the formation of more stable and controlled bioconjugates. The heterobifunctional cross-linker LC-SPDP was used to conjugate secondary antibodies to the nanorod surface before PEGylation, as illustrated in Figure 3a. LC-SPDP consists of a pyridilidithio group that binds to the gold nanorod surface, and an NHS ester which binds primary amines in the antibodies. After PEGylation, excess antibody and PEG-SH were removed by centrifugation, decantation, and resuspension of the nanorods in buffer solution. Activity of the antibodies once conjugated to gold nanorods was tested with strip plates coated with the primary antibody. The plates were exposed to both antibody-conjugated nanorods and nanorods which were only PEGylated, and then analyzed by AFM. Several spots on the plates were checked, revealing a very high selectivity for binding of the antibody-conjugated nanorods to the strip plate, as seen in Figures 3b and 3c. Nanorod densities on the strip plates were measured to be 25 ± 1 µm\(^{-2}\) for the antibody-conjugated nanorods and 0.3 ± 0.1 µm\(^{-2}\) for the PEGylated nanorods. Effective biological applications of gold nanoparticles require well-characterized, reproducible materials. While the strip plate assay demonstrates that at least some fraction of the nanorods has been successfully modified, it provides no quantitative information on the conjugation yield. Quantitative analysis of bioconjugated nanoparticles is challenging since the methods to measure the protein concentration may be affected by the presence of the nanoparticles and vice versa.\(^{45}\) Here we have independently analyzed the nanorod and antibody concentrations to determine their stoichiometry.

The nanorod concentration can simply be measured from the longitudinal plasmon absorbance peak at 755 nm given the nanorod extinction coefficient. While the general shape of nanorod extinction spectra can be reproduced by modifications of Mie theory for spheroids,\(^{30}\) effective medium theory,\(^{29}\) as well as numerical techniques,\(^{36,47}\) quantitative values for nanorod extinction coefficients are not as readily available. El-Sayed’s group has measured nanorod extinction coefficients by using ICP analysis to quantitatively determine the gold concentration in the nanorod solution. In that work the nanorod concentration was calculated from the nanorod volume and gold concentration and then combined with the measured absorbance to yield a nanorod extinction coefficient of \(\epsilon = 1.9 \pm 0.4 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}\) at the longitudinal plasmon resonance of 650 nm.\(^{41}\) In the measurements presented here, nanorod films were deposited on glass substrates by spin coating PEGylated nanorods from an ethanol solution, as seen in Figure 4a. Films were created with sufficient density

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that the characteristic nanorod extinction spectra were observable. Such highly uniform films could only be prepared with PEGylated nanorods, as free CTAB in raw solutions interferes with the deposition. Films of much longer nanorods have been formed by electrostatic immobilization, but extinction spectra were not reported. The film in Figure 4a has a nanorod density of $153 \pm 15 \mu m^{-2}$ determined directly by AFM analysis of several locations. When the extinction spectrum of the film is recorded in water, a peak absorbance of $0.06 \pm 0.001$ is measured at 796 nm. We believe the peak wavelength is red-shifted from the spectra in Figure 1 since the nanorods sit on a glass substrate with a higher dielectric constant than that in solution. The measured absorbance and density yield a nanorod extinction cross section of $\sigma_{50,15} = 8.4 \pm 0.9 \times 10^{-12} \text{ cm}^2$. This corresponds to an extinction efficiency, defined as the extinction cross section divided by the physical cross section, of $1.1 \pm 0.1$ for 15 nm $\times$ 50 nm nanorods.

Although the nanorods are not aligned on the substrate, their long axis is always perpendicular to the propagation vector of the unpolarized incident light from the fiber spectrometer. This extinction geometry conveniently matches calculations of nanorod extinction cross sections. However, to convert this value to a molar extinction coefficient appropriate for determining nanorod solution concentrations, one should consider the random orientation of nanorods with respect to the propagation vector of the incident light, as illustrated in Figure 4c. This factor is accounted for by considering an electric field along the nanorod length, $E_0 \cos \theta$, so the incident intensity is reduced by the average value of $\cos^2 \theta$ from $\theta = 0$ to $\pi/2$. Taking this factor into account, the molar extinction coefficient is $\varepsilon_{50,15} = 4.4 \pm 0.5 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ at the longitudinal plasmon resonance. The value reported here is a factor of 2.3 larger than that determined by El-Sayed’s group using ICP analysis for two possible reasons. First, due to their larger size, the nanorods studied here should have a larger longitudinal extinction coefficient by a factor of about 1.5. Second, based on the strength of the 520 nm peak of the extinction curves in the samples analyzed by ICP, a significant fraction of spherical gold colloid was present in the sample, which would overestimate the number of nanorods, and underestimate the extinction coefficient.

Extinction coefficients are often reported with a subscript which denotes the relevant wavelength, usually chosen to match an absorption due to an electronic transition. However, for plasmon resonances of nanorods and other metallic nanoparticles, the peak wavelength shifts both with the refractive index of the dielectric environment and with the nanoparticle aspect ratio. We therefore note the nanorod dimensions with the extinction parameters rather than the peak wavelength. Extinction measurements of the film of Figure 4b in air and water reveal that, while the peak wavelength shifts over 50 nm, the peak absorbance changes very little, so the reported values are not highly sensitive to refractive index. The nanorod dimensions are denoted rather than the aspect ratio since larger nanoparticles will have much larger cross sections as the particle size approaches the wavelength and scattering becomes significant.

Given $\sigma_{50,15}$ calculated above and the absorbance at the longitudinal plasmon resonance, the bioconjugated nanorod solution of Figure 3 has a nanorod concentration of $4.0 \pm 0.5 \times 10^{10} \text{ mL}^{-3}$. The presence of protein will likely have little affect on the large NIR extinction of the nanorod longitudinal plasmon mode, so nanorod concentrations can be assumed accurate even after conjugation. Due to the low concentration of protein in the bioconjugate solutions, the highly sensitive bicinchoninic acid (BCA) total protein assay was chosen to determine the concentration of secondary IgG. In this colorimetric assay, proteins reduce Cu$^{2+}$ to Cu$^+$ under alkaline conditions. Chelation of the BCA and Cu$^+$ results in an absorption at 562 nm, whose absorbance is proportional to the protein concentration. This assay will certainly be perturbed since the nanorods have significant extinction at 562 nm. We have removed the nanorod contribution by simply subtracting a spectrum of pure nanorods at the same wavelength from that of the bioconjugate.

concentration as the bioconjugated nanorods. Note that this subtraction ignores potential optical near field enhancements of the BCA spectrum and possible interference of the gold surface with the Cu$^{2+}$ reduction or BCA chelation. These issues aside, the assay yields a peak at the expected value of 562 nm (see Supporting Information). The resulting antibody concentration of 5.5 $\mu$g/mL implies that there are several hundred antibody molecules per nanorod. This value is certainly an overestimate considering that the input antibody concentration for conjugation was only 5 $\mu$g/mL and that the nanorod surface area is too small to hold so many antibody molecules. The nanorod does therefore interfere chemically and/or electromagnetically with the BCA assay. However, the large measured stoichiometry suggests that a significant portion of the nanorods contain antibodies and should be useful for reproducibly evaluating bioconjugated nanorod samples. Improved analytical methods are under development.

**Conclusion**

The structural and optical properties of gold nanorods suggest their use for several biomedical sensing, imaging, and therapeutic applications. Gold nanorods were stabilized in the absence of saturated CTAB surfactant and conjugated to IgG antibodies. Their selective binding was demonstrated with a strip plate assay, and the antibody/nanorod stoichiometry was determined by independently characterizing their concentrations in solution. The nanorod molar extinction coefficient was measured from thin nanorod films, and a BCA assay was used to determine protein concentration. Although the nanorods appear to perturb the BCA assay, the results support the strip plate assay, suggesting that nanorod/antibody conjugates have been formed. During the revision of this manuscript, Takahasi et al. published a report describing the bioconjugation of gold nanorods with phosphatidylcholine for the controlled release of plasmid DNA by NIR radiation.\(^{50}\)

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**Supporting Information Available:** Figure showing total nanorod bioconjugate spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.