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Improved Localized Surface Plasmon Resonance Immunoassay with Gold Bipyramid Substrates

Seunghyun Lee,[†,‡] Kathryn M. Mayer,[‡,§] and Jason H. Hafner*[,†,‡,§]

Department of Physics and Astronomy, Department of Chemistry, and the Laboratory for Nanophotonics, Rice University, Houston, Texas 77005-1827

Gold nanoparticles bound to substrates exhibit localized surface plasmon resonance (LSPR) in their optical extinction spectra at visible and near-infrared wavelengths. The LSPR wavelength is sensitive to the surrounding refractive index, enabling a simple, label-free immunoassay when capture antibodies are bound to the nanoparticles. Gold bipyramids are nanoparticles with a penta-twinned crystal structure, which have a sharp LSPR because of their high monodispersity. Bipyramid substrates were found to have a refractive index sensitivity ranging from 288 to 381 nm/RIU (−0.62 to −0.68 eV/RIU), increasing with the nanoparticle size and aspect ratio. In an immunoassay, the bipyramid substrates yielded higher sensitivity than nanorods and nanospheres. An immunoassay sensitivity constant which depends on both the optical properties of the nanoparticle and conjugation chemistry was found to be $K_{LSPR} = 0.01$ nm·μm^2 for gold bipyramids.

Localized surface plasmon resonance (LSPR) describes the collective oscillation of free electrons that occurs when light is incident on a noble metal nanoparticle. LSPR results in strong optical extinction which can be tuned throughout the visible and near-infrared wavelengths by adjusting the particle's size and shape. The LSPR spectral extinction peak is sensitive to the surrounding media's refractive index, which allows LSPR-active nanostructures to act as transducers in label-free biosensors. That is, by observing spectral shifts in the resonance wavelength, one can directly measure molecular binding to a nanoparticle surface through minute changes in the particle's dielectric environment.[1−5] LSPR sensing is therefore the nanoparticle analogue of surface plasmon resonance (SPR) sensing with a thin gold film, but requires only simple spectral extinction measurements. LSPR biosensing with gold[6−12] and silver[13−17] nanoparticles has been pursued for the past decade, but most reports describe only the initial and final LSPR peak wavelengths upon exposure to the target, yielding end point assays rather than kinetic measurements of molecular binding at the nanoparticle surface. We recently demonstrated real-time LSPR sensing capabilities by performing a label-free immunoassay based on the LSPR peak wavelength of gold nanorod substrates and determined the rate constants of association and dissociation between primary and secondary antibodies.[18] Here, we extend this result to gold bipyramid[19] substrates, which have a narrower LSPR peak and higher sensitivity to the surrounding refractive index. We also describe the factors which affect sensitivity in these real time immunoassays, and perform an explicit comparison of the LSPR sensing capabilities of gold bipyramids, nanorods, and spheres on a single substrate.

EXPERIMENTAL METHODS

Gold Bipyramid Synthesis. All solutions were prepared fresh for each synthesis using deionized (DI) water, except for the hydrogen tetrachloroaurate(III) (Sigma, No.520918), which was prepared as a 28 mM stock solution from a dry ampule and stored in the dark. First, sodium citrate-stabilized gold seed particles were prepared for the synthesis of gold bipyramids. Typically, a 20 mL solution of 0.125 mM hydrogen tetrachloroaurate(III) and 0.25 mM sodium citrate (Fisher, No. S279) were prepared and mixed briefly. Next, 0.3 mL of a fresh aqueous 10 mM $NaBH_4$ (Acros, No. 18930) solution prepared at room temperature was added, followed by mixing for 2 min. The resulting gold seed solution was kept at room temperature for at least 2 h for complete reaction. Then, the dark pink seed solution was stable and

* To whom correspondence should be addressed. E-mail: hafner@rice.edu.
† Department of Chemistry.
‡ Laboratory for Nanophotonics.
§ Department of Physics and Astronomy.

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usable for gold bipyramid growth. Next, 0.5 mL of 10 mM hydrogen tetrachloroaurate(III) and 10 mL of 100 mM cetyltrimethylammonium bromide (CTAB) (Sigma, No. H9151) were mixed with 0.1 mL of 10 mM silver nitrate (Acros, No. 19768) for the preparation of the growth solution. Then, 0.2 mL of 1.0 M hydrochloric acid (Hampton Research, No. HR2-581) and 0.08 mL of 100 mM L-ascorbic acid (Fisher, No. A61) were added to the solution in order. Finally, the seed solution was added to the growth solution. The volume of seed solution was varied between 15 and 50 μL to synthesize different sizes of gold bipyramids. These solutions were kept at 28 °C for several hours. During this time, the color changed gradually from almost clear to dark pink, with most of the color change occurring in the first hour. The LSPR spectra of the gold bipyramid solutions were measured using an Ocean Optics USB2000 spectrometer, and the particle images were obtained using a JEOL JEM 2010 transmission electron microscope (TEM).

Gold Bipyramid PEGylation. The CTAB-stabilized gold bipyramids were PEGylated using a process we have described previously.[18] One milliliter of CTAB-stabilized gold bipyramids was centrifuged at 10,000 rpm for 30 min to form a pellet of the gold bipyramids. The CTAB solution was decanted, and the pellet was resuspended in 100 μL of 2 mM potassium carbonate (Fisher, No. P208) and 1 mL DI water. Then, 10 μL of 1 mM thiol-terminated methoxypoly(ethylene glycol) (mPEG-SH, 5000 MW, Nektar Theraputics) was added to the solution, and the centrifuge/decant process was carried out again. The same volume of mPEG-SH and DI water were added to the pellet once more and left overnight to displace the CTAB. The bipyramids were then taken through at least two more centrifuge/decant cycles, resuspending each time in DI water, to further reduce the CTAB concentration.

Substrate Fabrication. Glass microscope slides were cleaned in piranha solution (3:1 H_2SO_4/30% H_2O_2), thoroughly rinsed with DI water, and dried under a stream of nitrogen. *Warning! Piranha solution is very exothermic and corrosive, and reacts violently with organic material. Therefore, handling it requires extreme caution.* The slides were then immersed in a 10% ethanolic solution of aminopropyltriethoxysilane (APTES) (Sigma, No. 440140) overnight, rinsed with ethanol and water in order and dried with nitrogen gas. For bipyramid substrates, the APTES-coated slides were then immersed in a solution of PEGylated gold bipyramids overnight, and then rinsed and dried with the same method. For nanorod-bipyramid hybrid substrates, the APTES-coated slides were immersed in a mixed solution of PEGylated bipyramids and PEGylated nanorods. The nanorods were prepared as described previously.[18,20] Finally, the mPEG-SH layer and other organic contaminants on the substrates were removed using an oxygen plasma cleaner (model PDC-32G, Harrick Scientific) on low power for 30 s with 200 mT of oxygen. The substrates to be used in the immunoassay experiments underwent one additional step: they were immersed in a 1 mM ethanolic solution of mercaptoundecanol (Sigma, No. 447528) and mercaptohexadecanoic acid (Sigma, No. 448303) in a 1:10 ratio for 12 h to form a mixed self-assembled monolayer (SAM). To study the effect of SAM thickness, 1 mM solutions of either mercaptoundecanoic acid or mercaptohexadecanoic acid were used instead.

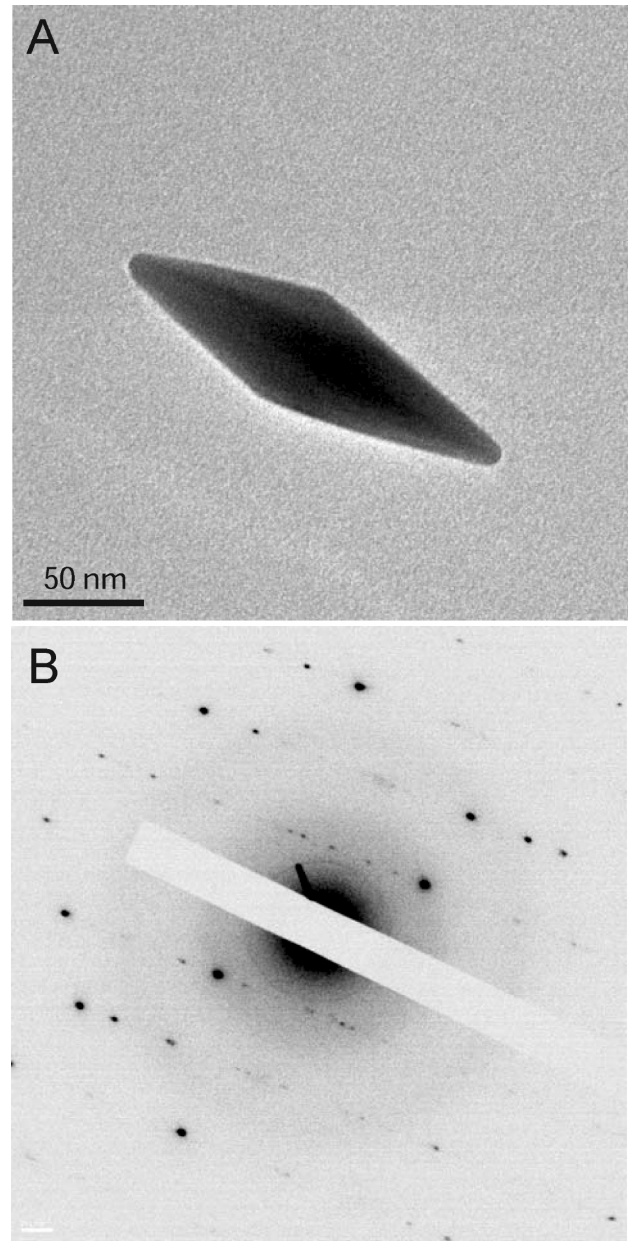

Figure 1. (A) TEM image of a PEGylated gold bipyramid. (B) Electron diffraction pattern of a gold bipyramid, consistent with a pentatwinned crystal structure.

Substrate Bioconjugation and LSPR Sensing Measurements. The bioconjugation procedure for the immunoassay experiments was performed exactly as described previously.[18] The analyte flow rate was controlled by a syringe pump (NE1000, New Era Pump Systems), and the LSPR peak shift was monitored by an Ocean Optics USB4000 spectrometer. During a typical immunoassay experiment, the substrate was first exposed to 0.1 M MES buffer (Sigma, No. M-0164) at pH 6.1 until the LSPR peak wavelength stabilized. Next, it was exposed to a mixed solution of 0.1 M *N*-hydroxysuccinimide (NHS, Sigma No. 130672) and 0.05 M 1-ethyl-[3-dimethylaminopropyl]carbodiimide (EDC, Sigma No. 1769) in the same MES buffer, to activate the carboxyl groups

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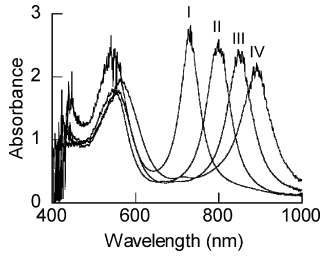


Figure 2. Spectra of four bipyramid samples of varying particle size. Particle aspect ratios from samples I–IV can be found in Table 1.

Table 1. Synthesis Conditions, Product Properties, and Refractive Index Sensitivities of the Gold Bipyramids (BPs) Described in This Paper, As Well As for a Typical Nanorod Synthesis

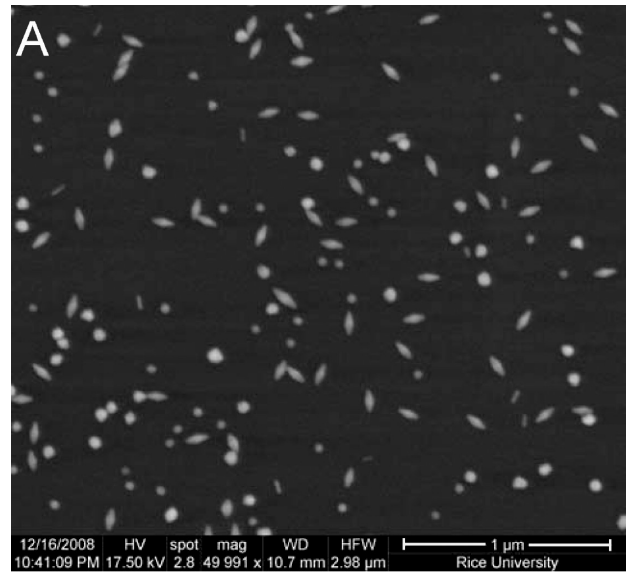
	NR	BP I	BP II	BP III	BP IV
seed volume (μL)		50	35	20	15
BP yield		22%	22%	21%	23%
length/diameter (nm)	50/15	108/44	137/51	162/57	185/58
	3.3	2.5	2.7	2.8	3.2
max. peak LSPR (nm)	760	730	800	850	900
RI sensitivity					
(nm/RIU)	170	289	327	346	381
(eV/RIU)	170	−0.68	−0.66	−0.62	−0.62
fwhm (nm)	125	74	89	100	100
FOM	1.3	3.9	3.7	3.5	3.8

on the mixed SAM on the gold nanoparticles.[21] This was followed by another rinse in the MES buffer. Then, the substrate was exposed to rabbit IgG (Pierce, No. 31235) at about 1 μm in the MES buffer, followed by a rinse with 0.05 M phosphate buffered saline (PBS) with 0.25 M NaCl at pH 7.6. Finally, goat anti-rabbit IgG (Pierce, No. 31210) was flowed at the desired concentration in PBS for about 45 min, followed by a final PBS rinse. Absorbance spectra were averaged for 30 s and recorded. Each spectrum was then analyzed in MATLAB with a Gaussian fit to monitor the peak wavelength, height, and width versus time.

RESULTS AND DISCUSSION

The seed-mediated synthesis of gold bipyramids with a pentatwinned crystal structure has recently been described.[19] On the basis of this method, gold bipyramids with varying aspect ratios were synthesized by changing the volume ratio of seed solution to growth solution to find the optimum size for LSPR sensing. A TEM image of a typical gold bipyramid having a tip radius of curvature of less than 5 nm is shown in Figure 1A. The corresponding electron diffraction pattern, shown in Figure 1B, is consistent with a pentatwinned structure as described in the original report. To synthesize different sizes of gold bipyramids, four different volumes of the seed solution (15, 20, 35, and 50 μL) were employed. The extinction spectra of the resulting CTAB-stabilized gold bipyramid solutions are shown in Figure 2. With decreasing seed volume, the bipyramid spectra red shift because of their larger size, and the peak height drops because of the smaller number of particles.

The gold bipyramids were processed into films as previously described for gold nanorods.[18] An ESEM image and extinction



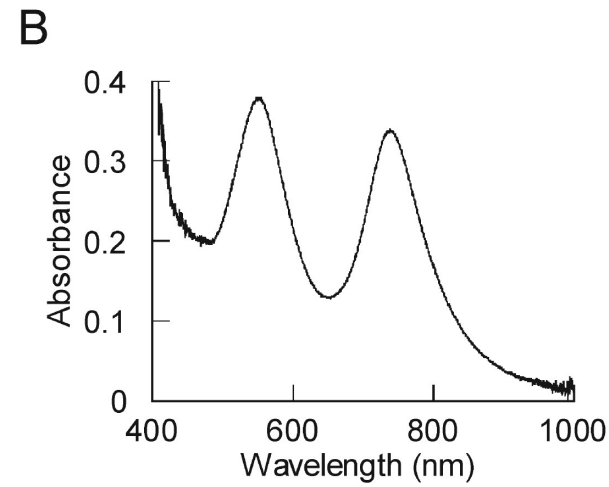


Figure 3. (a) Wet-mode ESEM image of a film of gold bipyramids on glass. (b) Absorbance spectrum of a bipyramid substrate.

spectrum of a typical bipyramid film are shown in Figure 3. The spectrum is sufficiently strong to monitor peak wavelength shifts because of molecular binding onto the nanoparticle surface. The yield of gold bipyramids on the glass surface, defined as the percentage of total particles that are bipyramids rather than spheres, was quantified by atomic force microscopy (AFM, Nanoscope IV), and electron micrographs were obtained using an FEI Quanta 400 environmental scanning electron microscope (ESEM) in wet-mode. Table 1 summarizes the sizes, aspect ratios, yields on the surface, and longitudinal LSPR peak wavelengths and widths of substrates made from each bipyramid sample.

We have investigated the refractive index sensitivities of the bipyramid substrates with different aspect ratios by measuring the LSPR peak wavelength shift in various solvents. The LSPR spectra of the gold bipyramid substrates were measured in water (n = 1.333), acetonitrile (n = 1.3441), ethanol (n = 1.361), dimethylformamide (DMF, n = 1.431), and toluene (n = 1.497). As seen in Figure 4, the LSPR peak red shifts with increasing index. The sensitivities are reported in Table 1. In addition, the figures of merit (FOM) were calculated as sensitivity divided by the LSPR full width at half-maximum (fwhm).[16,22] The bipyramid

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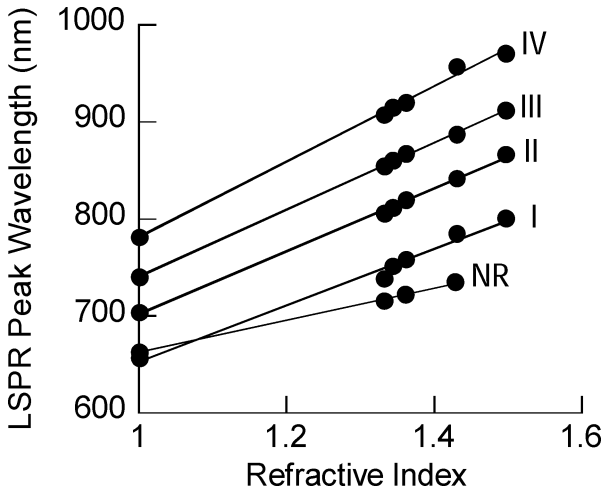

Figure 4. Refractive index sensitivity of gold bipyramids of various sizes on glass substrates. The solutions are water (n = 1.333), acetonitrile (n = 1.3441), ethanol (n = 1.361), dimethylformamide (DMF, n = 1.431), and toluene (n = 1.497).

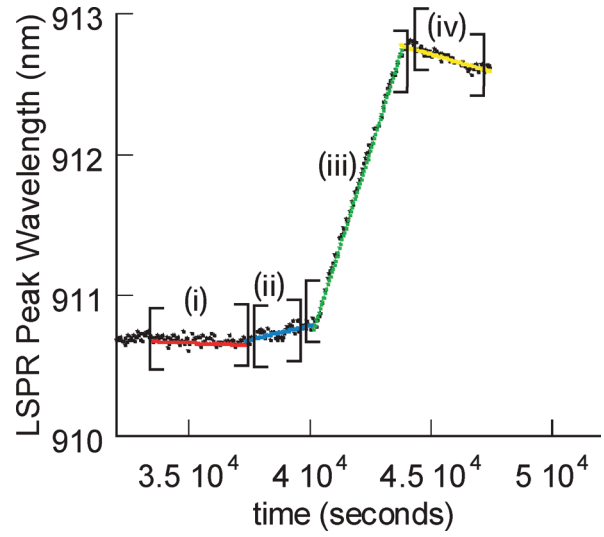

Figure 5. Immunoassay sensorgram. The dose response can be seen as the bipyramid substrate was exposed to target antibodies at 100 pM (i), 1 nM (ii), and 10 nM (iii). Kinetic rates of binding and unbinding were obtained from the 10 nM exposure (iii) and rinse (iv). Segments (i) and (ii) are linear fits, while the (iii) and (iv) are fits to the first-order binding kinetics model. This sensorgram yielded a binding rate of $k_{on} = 3.03 \times 10^3$ M^{-1} s^{-1} and an unbinding rate of $k_{off} = 3.56 \times 10^{-6}$ s^{-1}. The ratio of these gives an equilibrium constant of $K_{eq} = 8.51 \times 10^8$ M^{-1}, which compares favorably with standard literature values for antibody−antigen bonds.

substrates have higher FOM values of 3.5−3.9 compared to gold nanorod substrates because of their monodispersity and sharp tips with potential for strong field enhancement.[23] It can be shown by finite element method simulations that strong local electric fields can contribute to the refractive index sensitivity of nanostructures.[24] As seen in Table 1, the largest bipyramid has the highest sensitivity, but the smallest has the highest figure of merit because of its narrow line width. Our results are in good agreement with a recent report on the refractive index sensitivity of similar gold bipyramid substrates.[25]

To demonstrate the immunosensing capabilities of these substrates, a capture antibody (rabbit IgG) was bound to a carboxy-terminal SAM on the bipyramid surfaces by amide bond formation with a carbodiimide. The functionalized substrates were then exposed to a solution containing the target antibody (goat anti-rabbit IgG), using the methods described previously.[18] By monitoring the optical extinction peak at the bipyramids' LSPR resonance throughout the target exposure and subsequent rinse, we performed a real time immunoassay. A section of the resulting sensorgram is shown in Figure 5. At the lowest concentration of target antibody (100 pM), there was no significant response from the sensor. At 1 nM, the extinction peak began to shift at a rate of 4.0×10^{-5} nm/s, and at 10 nM, the rate increased to 5.5×10^{-4} nm/s. These bipyramid peak shift rates are larger than those from the same experiment carried out on a nanorod substrate (2.1×10^{-5} and 2.1×10^{-4} nm/s, respectively) by a factor of 2, which matches well with the increase in refractive index sensitivity.

The LSPR sensor response in an immunoassay will depend on several factors in addition to the refractive index sensitivity of the nanoparticle. For example, conjugation chemistry for binding the capture antibody to the nanoparticle will affect the density of capture antibody and therefore target molecules on the nanoparticle surface. Furthermore, the conjugation strategy will affect the distance between the target molecule and the nanoparticle surface and therefore the size of the LSPR shift (as discussed below). To characterize the LSPR sensitivity in a real immunoassay, one can relate the measured LSPR peak shift to the parameters of a simple first-order molecular binding model used to describe the kinetics of the system. According to the model, the concentration of capture-target antibody complexes formed on the surface evolves in time upon target exposure as

$$[C{\cdot}T]_{surf}(t) = \frac{k_{on}[C]_{surf}[T]_{sol}}{(k_{off} + k_{on}[T]_{sol})}[1 - e^{-(k_{off}+k_{on}[T]_{sol})t}] \quad (1)$$

Here, C denotes the capture antibody, T the target antibody; k_{on} and k_{off} are the association and dissociation constants, and *surf* and *sol* denote surface and volume concentrations, respectively. Equation 1 can be expanded for short exposure times to yield the initial linear shift:

$$[C{\cdot}T]_{surf}(t) = (k_{on}[C]_{surf}[T]_{sol})t \quad (2)$$

If one assumes that the observed LSPR peak wavelength shift is proportional to the concentration of capture-target complexes near the nanoparticle surface, then eq 2 can be written

$$\Delta\lambda = K_{LSPR}(k_{on}[C]_{surf}[T]_{sol})t \quad (3)$$

The constant K_{LSPR} describes a nanoparticle substrate's performance in an immunoassay and has units of nm·μm^2 if

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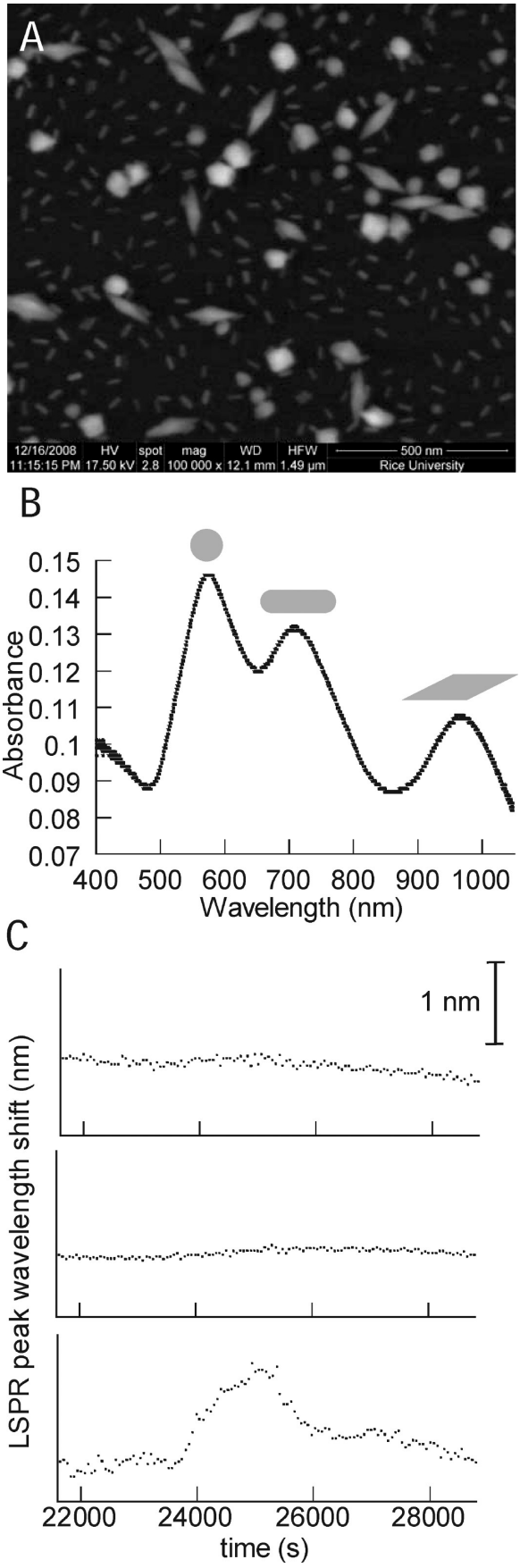

Figure 6. (A) ESEM image of the hybrid substrate containing gold nanospheres, nanorods, and bipyramids. (B) Optical extinction spectrum of the hybrid substrate. (C) Sensorgrams for each of the three particle types in the hybrid substrate immunoassay.

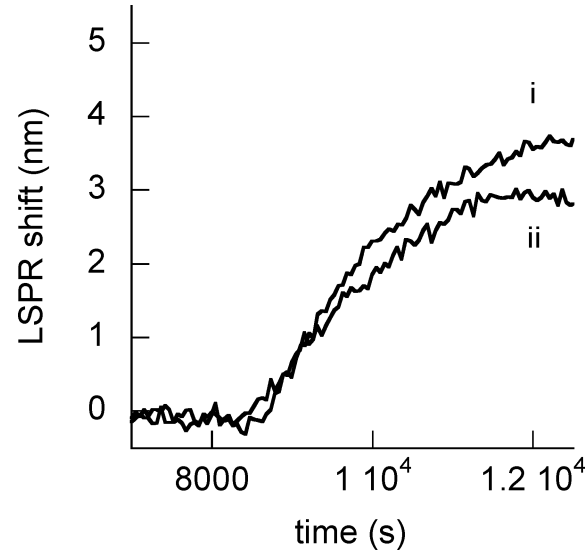

Figure 7. Comparison of the target antibody binding signal from bipyramids coated with (i) mercaptoundecanoic acid and (ii) mercaptohexadecanoic acid.

the shift is given in nm and the capture antibody density on the nanoparticles is given in molecules/μm^2. For the bipyramid results in Figure 5, $K_{LSPR} = 0.01$ nm$\cdot\mu$m^2 if one assumes approximately 20 active antibodies per bipyramid. This assumption is based upon an estimated particle surface area of 10,000 nm^2 and a surface area per active antibody of 100 nm^2, with a binding efficiency of 0.2, similar to results found on gold surfaces.[26] This constant reflects the effect of the nanoparticle refractive index sensitivity and antibody conjugation strategy on the immunoassay sensitivity. The value reported here cannot yet be compared to other LSPR sensor reports since real-time measurements are required.

To explicitly compare the sensing capability of the bipyramids to those of other plasmonic nanoparticles, a substrate was fabricated with a hybrid film containing three particle types: bipyramids, nanorods, and nanospheres. Figure 6A shows an ESEM image of the hybrid film on glass. The optical extinction spectrum of this substrate (Figure 6B) includes three well-separated peaks representing the three particle types, the spheres having an extinction peak near 580 nm, the nanorods near 700 nm, and the bipyramids near 950 nm. By tracking this spectrum in real time during an immunoassay experiment similar to that described above, it is possible to generate sensorgrams for each of the three peaks, seen in Figure 6C. Comparing these, it is clear that the bipyramids are the most sensitive, that is, give the largest extinction peak shift in response to the target binding. The relatively poor signal-to-noise ratio in these sensorgrams, and the apparent lack of signal for the nanorods and spheres, is due to the low density of each type of particle on the hybrid substrate.

Finally, to show that the strength of the LSPR target binding signal decreases with the target's distance from the gold particle surface as discussed above, two substrates from the same fabrication run were coated with carboxy-terminal SAMs of two different lengths: mercaptoundecanoic acid and mercaptohexadecanoic acid. The difference in thickness between these eleven- and sixteen-carbon chains is approximately 7 Å. The same

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immunoassay experiment was then carried out on both substrates. The LSPR shift from the substrate with the shorter SAM was larger by 1 nm as seen in Figure 7.

CONCLUSIONS

Gold bipyramid substrates have a higher refractive index sensitivity and a narrower LSPR line width than gold nanorod substrates. This translates to an improved LSPR immunoassay sensitivity. However, detailed comparisons of different nanoparticles in LSPR immunoassays will require consideration of the chemical strategy for linking targeting agents such as antibodies to their surface.

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SUPPORTING INFORMATION AVAILABLE

Further details are given in Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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