Colloidal Au-Enhanced Surface Plasmon Resonance for Ultrasensitive Detection of DNA Hybridization

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Abstract: A new approach to ultrasensitive detection of DNA hybridization based on nanoparticle-amplified surface plasmon resonance (SPR) is described. Use of the Au nanoparticle tags leads to a greater than 10-fold increase in angle shift, corresponding to a more than 1000-fold improvement in sensitivity for the target oligonucleotide as compared to the unamplified binding event. This enhanced shift in SPR reflectivity is a combined result of greatly increased surface mass, high dielectric constant of Au particles, and electromagnetic coupling between Au nanoparticles and the Au film. DNA melting and digestion experiments further supported the feasibility of this approach in DNA hybridization studies. The extremely large angle shifts observed in particle-amplified SPR make it possible to conduct SPR imaging experiments on DNA arrays. In the present work, macroscopic 4×4 arrays were employed, and a $\sim$10 pM limit of quantitation was achieved for 24-mer oligonucleotides (surface density $\leq 8 \times 10^8$ molecules/cm^2). Even without further optimization, the sensitivity of this technique begins to approach that of traditional fluorescence-based methods for DNA hybridization. These results illustrate the potential of particle-amplified SPR for array-based DNA analysis and ultrasensitive detection of oligonucleotides.

Introduction

Surface plasmon resonance (SPR) is a surface-sensitive analytical technique based on the ability to detect dielectric constant changes induced by molecular adsorption at a noble metal film.[1−3] In a typical experiment the reflectivity of a $\sim$50 nm thick Au film is measured as a function of incident angle. The first commercial instrument (BIAcore) to detect these changes by monitoring the formation of a biomolecular complex was introduced in 1990.[4−6] Since then, several systems have been developed, and this technique has been widely used in biomolecular interaction studies, including determination of affinity constants and kinetic binding parameters.[7−12] Unfortunately, the inability of conventional SPR to measure extremely small changes in refractive index hinders its application in ultrasensitive detection. To address this drawback, several approaches have been developed. Among them, substantial interest has been focused on utilizing external labels to enhance the sensitivity of the current technique.[13−26] In these approaches, a "sandwich" geometry is used, in which a surface-bound molecule (e.g. antibody or oligonucleotide) binds the analyte specifically, bringing it to the surface. This event leads to a very small change in SPR response. To increase the magnitude of the response, a second specifically interacting biomolecule is introduced. Often this second, "probe", molecule carries a high molecular weight or high refractive index tag. Consequently, a much larger change in SPR reflectivity can be

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observed. To date, liposomes,[13] latex particles,[14−16] and certain proteins[17] have been tested as amplification tags.

Metallic nanoparticles have also been employed to enhance SPR response.[18−26] These particles offer ease of preparation, high density, large dielectric constant, and biocompatibility.[27] Pollard-Knight and co-workers used 30-nm diameter colloidal Au nanoparticles in an immunoassay to demonstrate the concept of this amplification technique.[19] Particle-enhanced SPR has also been used to develop cellular assays for cholera cells.[20] Natan and co-workers have developed an Au-amplified SPR sandwich immunoassay and achieved a picomolar detection of human immunoglobulin G.[21] The Natan group has also conducted systematic studies of the effect of particle size and surface coverage on SPR response.[22,24,25]

Herein, we further extend the scope of Au nanoparticle-amplified SPR to analysis of DNA hybridization. Detection of DNA hybridization holds great promise for the rapid clinical diagnosis of genetic diseases. SPR has been used in DNA analysis for nearly a decade.[28−34] However, few efforts have been devoted to improving sensitivity, despite intensive interest in kinetic studies of DNA-related interactions. In this report, we describe the use of biocomplexes comprised of oligonucleotide probes and colloidal Au particles for DNA hybridization detection via SPR. Specifically, oligonucleotide probes were conjugated to colloidal Au particles and used to selectively recognize surface-confined target DNA via sequence-specific hybridization with in situ detection. A substantial improvement in the SPR response was achieved compared to the unamplified detection event. To further support the validity of this approach, the melting behavior of the surface-bound DNA duplex was examined. In addition, a restriction enzyme that can recognize a specific DNA duplex sequence was employed to release the particles from the surface. Using a home-built SPR imaging instrument, a quantitation limit of less than 8×10^8 molecules/cm^2 for a 24-mer oligonucleotide and approximately 5 orders of magnitude in dynamic range were observed. This result represents a greater than 1000-fold improvement over the unamplified binding event under similar conditions.[33,35]

To our knowledge, these experiments comprise the first example of Au-amplified SPR for DNA hybridization detection. The sensitivity of this method already approaches that of standard fluorescence techniques for scanning gene chips.[36] Further improvements in the sensitivity of particle-amplified SPR can be achieved by optimizing hybridization conditions and reducing the background caused by nonspecific interactions. In addition, larger particles can be used to greatly increase sensitivity, as previously demonstrated.[24] Thus, particle-amplified SPR offers a potentially powerful new approach to high throughput analysis of DNA arrays, such as those used in DNA sequencing and gene expression investigations, and may be applicable to polymerase chain reaction (PCR)-free DNA detection.

Chart 1. Sequences of Oligonucleotides Used

S1: 5′- H_2N - C_6H_{12} - CGC ATT CAG GAT

S2: 3′ - GCG TAA GTC CTA
AGA GTT GAG CAT - 5′

S3: TCT CAA CTC GTA – C_6H_{12}– SH -3′

S4: 5′- HS - C_6H_{12} - CGC ATT CAG GAT

E1: 5′- TCC TCT CGC GAG TCA
ACA GAA ATA TCC GCG A_7 - biotin -3′

E2: 3′- biotin -A_7 AGG AGA GCG CTC AGT
TGT CTT TAT AGG CGC - 5′

...GANTC...
...CTNAG...

Hinf I recognition site on dsDNA[52]

N: Nucleotide A, T, C, or G.

Experimental Section

Materials. Trisodium citrate dihydrate, poly(ethylene glycol) bis-(3-aminopropyl) terminated (PEG), 16-mercaptohexadecanoic acid (90%) (MHA), 3-mercaptopropionic acid (MPA), ethylenediaminetetraacetic acid (EDTA), and NaOH were obtained from Aldrich. $HAuCl_4 \cdot 3H_2O$ was from Acros. NaH_2PO_4, Na_2HPO_4, sodium dodecyl sulfate (SDS, 95%), NaCl, KCl, concentrated HCl, HNO_3, H_2SO_4, and 30% H_2O_2 were purchased from J. T. Baker Inc. 3-Mercaptopropylmethyldimethoxysilane (MPMDMS) and (3-aminopropyl)trimethoxysilane (APTMS) were purchased from United Chemical Technologies. $MgCl_2$, spectrophotometric grade CH_3COH, and CH_3OCH_3 were obtained from EM Science. CH_3CH_2OH was purchased from Pharmco. 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), and streptavidin were from Pierce. The restriction enzyme *Hin*f I was purchased from Biolabs. HPLC-purified oligonucleotides were purchased from Integrated DNA Technology or from the Pennsylvania State University Nucleic Acid Facility (Chart 1). All reagents were used as received without further purification. H_2O used was distilled and subsequently purified using a Barnstead Nanopure system. Hybridization buffer was prepared with 0.3 M NaCl in a 10 mM phosphate (pH 7.0) buffer, 10 μM noncomplementary 12-mer oligonucleotides was added as the blocking reagent, and 25 mM Tris-HCl (pH 7.8), 100 mM KCl, and 10 mM $MgCl_2$ were mixed and used as the enzyme buffer. Au (99.99%) shots and Cr wires used for evaporation were obtained from Johnson-Matthey Corp. SF11 glass slides (Schott Glass Technologies, $n = 1.78$) were used in the SPR scanning experiments, and Fisher Precleaned microscope slides (BK7, $n = 1.515$) were used in the imaging experiments.

DNA:Au Conjugate Preparation. Colloidal Au (12-nm diameter) was prepared by citrate reduction of $HAuCl_4 \cdot 3H_2O$ as previously described.[37,38] Average particle diameters were determined by transmission electron microscopy (TEM), which showed a standard deviation of less than 10%. Optical spectra of colloidal Au solutions were recorded using a HP 8453 spectrophotometer.

Thiol-labeled DNA:Au conjugates were prepared according to protocols described by Mirkin and co-workers.[39−43] 12-mer (36 μL,

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100 μM) oligonucleotide probe, S3 or S4, was added to a 1-mL Au colloid solution. After standing for 16 h, the solution was aged in a 0.1 M NaCl solution (pH 7, 10 mM phosphate buffer) for 2 days. The DNA:Au conjugate was then washed by centrifugation (45 min, 12500g) to remove excess reagents. The supernatant was disposed of, followed by washing of the red pellet with 0.1 M NaCl. After a second centrifugation, the pellet was brought to the original concentration in a 0.3 M NaCl/phosphate hybridization solution. The conjugates were used freshly as prepared, and an optical spectrum was always taken to ensure consistent solution concentration.

Biotinylated DNA:Au conjugates were prepared from streptavidin:Au conjugate. A solution of Au particles diluted 1:1 in H_2O and adjusted to pH 8.5 with 0.1 M NaOH was added to 40 μL of 1 mg/mL streptavidin stock solution. After incubating on ice for 1 h, the solution was centrifuged (12500*g*, 45 min) to separate unbound protein, and 200 μL of the biotinylated oligo probe (E2, 10 μM) was then incubated with the conjugate for 30 min followed by a second centrifugation with removal of the supernatant and resuspension into hybridization buffer. Again, an optical spectrum was taken to ensure consistent solution concentration as performed with the thiol-labeled DNA conjugates.

Successful incorporation of oligonucleotide probes onto the Au surface was verified using a method developed by Mirkin and co-workers.[39−42] In short, we first combined S3:Au and S4:Au conjugate solutions and then monitored flocculation induced by addition of a linking nucleotide. While the optical spectrum of the mixture exhibited the characteristic absorbance of Au colloid at 524 nm, the addition of a linking oligonucleotide (S2, which has sequence complementary to both S3 and S4) led to a red shift of λ_{max}, accompanied by a significant decrease in intensity (Supporting Information). This is due to the formation of nanoparticle aggregates. By gradually increasing the solution temperature above the melting point of the DNA duplex, dehybridization occurred and resulted in the dissociation of the colloidal Au network. Consequently, an increase in the optical intensity at 260 nm, a signature absorbance of free oligonucleotides in solution, was observed. DNA:Au conjugates that did not show reversible flocculation in this test were discarded.

SPR Surface Preparation. Glass substrates were cleaned with aqua regia (3:1 HCl/HNO_3) and Piranha solution (3:2 H_2SO_4/H_2O_2) for 30 min. (**Note:** these are dangerous cleaning solutions, and care must be taken in solution handling.) After thoroughly rinsing with H_2O and CH_3OH, glass slides used in the scanning experiment were derivatized in a 5% MPMDMS/CH_3OH solution for 1 h as previously described.[37,38] Thin Au films (48 nm) were deposited onto one side of the substrates with an Edwards Auto 306 thermal evaporation system. Metal deposition occurred at a pressure of 2×10^{-6} mbar at 0.2 nm/s, monitored by an internal QCM. For imaging experiments, a 2-nm Cr film was evaporated on the substrates prior to Au deposition instead of silane derivatization, to improve the adhesion of the Au film.[23] Substrates were annealed under Ar in a home-built oven at 300 °C for 15 min. The imaging substrates were prepared ex situ, following the same protocol described above. After copious rinsing, the surface was dried under a stream of Ar gas and optically coupled to the imaging prism with microscope immersion oil (Richard-Allan Scientific).

A self-assembled monolayer was affixed to the Au surface through an overnight immersion in a 10 mM MHA/C_2H_5OH solution. An amine-labeled 12-mer oligonucleotide (S1), with a sequence complementary to half of the target, was coupled to the carboxylate groups on the surface via EDC/NHS cross-linking. Amine-labeled PEG (1%) was then introduced to block unreacted sites, followed by rinsing and the incubation of the target analyte, a 24-mer DNA (S2), in hybridization buffer for 2 h. In the unamplified experiment, an untagged DNA probe (S3), with a sequence complementary to the other half of S2, was added and incubated with the surface. In the amplified event, however, the SPR surface was exposed to the Au particle-tagged S3 probes. In both cases, hybridization was carried out for 1 h, followed by extensive rinsing with a 0.2% SDS/0.1 M NaCl buffer.

In the restriction enzyme digestion experiment, an Au surface was derivatized with 10-mM MPA for 30 min. Streptavidin was coupled to the carboxylate groups on the surface using EDC/NHS cross-linking reagents. After incubating biotinylated 37-mer DNA (E1) with the protein-derivatized surface for 1 h, biotinylated oligo (E2):Au conjugates were then incubated for 2 h. Afterward, the DNA-confined surface was incubated with 50 units/150 μL *Hin*f I in an enzyme buffer with or without $MgCl_2$ for 30 min at room temperature prior to taking SPR measurements.

Instrumentation. Surface Plasmon Resonance (SPR). The home-built scanning SPR apparatus used in this investigation has been previously described.[21,22] Briefly, a 1in. diameter hemispherical prism (SF11 glass, EscoProducts) was index-matched to the SPR surface in a Kreschmann geometry setup,[1] with a He−Ne laser (632.8 nm, Melles Griot) used as the excitation source. The reflected laser light was collected by a silicon photodiode detector (Thorlabs). Angular positioning of the sample was accomplished using a $\theta-2\theta$ rotation stage with the prism/sample mounted on the top. A miniature peristaltic pump (Instech Laboratories, Inc.) was used to deliver analyte solutions to the sample cell at 0.1 mL/min. A 5-min H_2O rinse at 1 mL/min was always carried out before collecting SPR measurements. All experiments were completed at room temperature except for the DNA melting experiment, in which the temperature of the sample cell was increased by flowing warm dehybridization buffer (80 °C as measured at the water bath) for 20 min. A much higher temperature, ~27 °C above the melting point of the surface-confined DNA duplex,[41] was applied here due to thermal gradients in the liquid delivery apparatus. The actual temperature at the surface is estimated to be between 60 and 70 °C. The sample cell was cooled to room temperature before the SPR measurement. Kinetic studies were carried out by monitoring SPR reflectivity changes as a function of incubation time at a fixed angle (53.2°).

Imaging SPR. An imaging instrument was constructed as previously reported.[23] Two minor modifications to the optical path and sample cell were made to accommodate multiwavelength excitation and fine angle adjustments. The 5 mW He−Ne laser was replaced with a Coherent I-70 Ar^+/Kr^+ laser that permits excitation at multiple wavelengths. Also, the prism assembly was mounted on a rotation stage to accommodate adjustment in the incident angle. Sample cells were fabricated by punching 16 4.5-mm-diameter holes in a 4×4 geometry in 0.5-mm-thick sheets of "press to seal" silicone (Molecular Probes). The polarizer was repositioned after the beam expander and the CCD was fitted with a Nikon 60 mm/$f = 2.8$ Macro lens. Data were plotted as spatial intensity maps of the SPR substrate surface, where an increase in intensity indicates an increase in SPR response. A commercial software package, NIH Image, was used for image analysis.[44] From the spatial intensity map the integrated intensity from each sample cell was calculated, a baseline intensity was subtracted, and each signal was normalized for the area of the sample cell.

Atomic Force Microscopy (AFM). AFM images of samples were measured after collecting SPR curves. Images were captured in the tapping mode on a Digital Nanoscope IIIa instrument (Digital Instruments, CA). Two 5×5 μm images and three 1×1 μm images were collected for each surface. The surface coverage was calculated by manually counting particles.

UV−Vis Absorbance Spectroscopy. UV−vis spectra were collected using a HP 8453 diode-array spectrophotometer equipped with a HP 89090A Peltier temperature controller. By changing the solution temperature from 25 to 65 °C at 1 °C/step, a DNA melting assay was tested by monitoring the optical intensity changes at 260 nm.[45] Hybridization buffer was used as the blank. To ensure the quality of DNA:Au conjugates, a solution assay was always carried out by following the published protocol.[41]

Results and Discussion

Methods for DNA:Au conjugate formation have been previously reported by Mirkin and co-workers.[39−43] In short, chemically modified oligonucleotide probes are attached to Au

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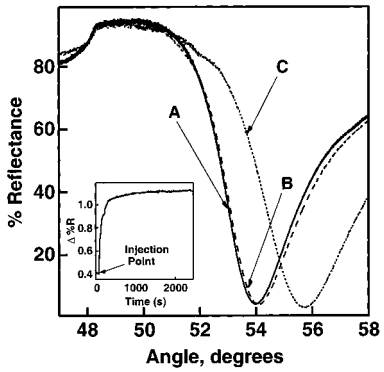
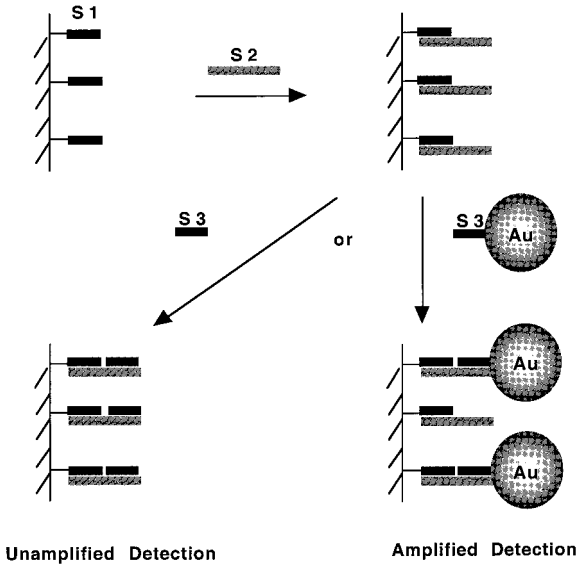


Figure 1. SPR curves of surfaces prepared in sequential steps as illustrated in Scheme 1: a MHA-coated Au film modified with a 12-mer oligonucleotide S1(A), after hybridization with its complementary 24-mer target S2 (B), and followed by introduction of S3:Au conjugate (C) to the surface. Inset: surface plasmon reflectance changes at 53.2° for the oligonucleotide-coated Au film measured during a 60-min exposure to S3:Au conjugates.

Scheme 1. SPR Surface Assembly



nanoparticles via thiol chemisorption. This approach was adapted in our investigations to prepare DNA:Au conjugates. Successful incorporation of oligonucleotide probes onto the Au surface was verified using a DNA melting assay in solution (Supporting Information).

The sandwich hybridization assay format used in the Au-amplified SPR experiment is depicted in Scheme 1. After derivatizing the Au surface with a submonolayer of 12-mer oligonucleotide (S1) with a sequence complementary to half of the target analyte, the target DNA (S2) was introduced, and hybridization led to a very small angle displacement (0.1°) in SPR reflectivity minimum (Figure 1, curve B). Subsequent exposure of the SPR surface to the solution containing Au particle-tagged S3 probes (S3:Au) led to a pronounced angle shift (Figure 1, curve C)—an approximately 18-fold increase in SPR angle shift compared with what was observed in the nonamplified assay.[46] Several factors contribute to this enormous enhancement: (i) each particle binding event increases the mass load on the surface by a factor of 1000 compared to a single strand of 24-mer oligonucleotide. (ii) The bulk refractive index

(46) Adding untagged DNA probes (S3), with sequence complementary to the other half of S2, caused an indiscernible angle shift (data not shown). This is not unexpected since the mass change is relatively small, and biomolecules usually have low dielectric constants.[47]

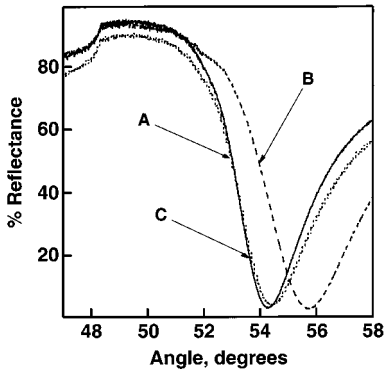


Figure 2. SPR curves of S1/S2-coated Au surface after exposure to the S4:Au conjugates (A) or S3:Au conjugates (B) at room temperature, and followed by heating above T_m with continuous rinsing for 20 min (C).

of the Au particle is significantly higher than that of the biomolecules.[47] (iii) The electromagnetic interaction between metallic nanoparticles and metal surfaces may also help to increase the SPR response by influencing the plasmon mode propagation.[21]

Since SPR is a surface-sensitive technique, kinetic data can be acquired in real time to monitor the progress of the reaction. The inset to Figure 1 shows the kinetic plot of the second hybridization step—the probe:Au conjugate binding to surface-confined oligonucleotides—by monitoring the SPR reflectance changes at a fixed incident angle, 53.2°. As expected, the signal changed dramatically in the first 5 min, with the hybridization process nearly complete after 60 min. Accordingly, 1 h of probe:Au hybridization was chosen for the remaining experiments described here.

To verify that binding of particles to the surface was due to hybridization of complementary strands, a DNA melting analysis was performed. Figure 2 shows SPR spectra collected before and after the heating (dehybridization) process. Note that curve A was obtained after exposing the surface to the noncomplementary conjugate, S4:Au prior to the S3:Au probe solution; this process was aimed at blocking nonspecific adsorption of S3:Au. After introducing S3:Au conjugates, the spectrum obtained at room temperature exhibited the same angle shift as that shown in Figure 1 (Figure 2, curve B). Rinsing the surface with hybridization buffer at high-temperature resulted in a backshift of the plasmon angle toward its initial position due to the dissociation of S3:Au conjugates and S2 oligos from the surface.[49] This result indicates that the shift in SPR angle upon adding S3:Au resulted from a specific hybridization to surface-confined S2, rather than nonspecific adsorption.

The SPR perturbations induced by DNA:Au conjugates were also reversible in a DNA digestion experiment. In this experiment, a restriction enzyme was used to cleave the DNA duplex at a specific sequence,[45] releasing Au particles from the surface. A slightly different surface geometry was used. As illustrated in Scheme 2, instead of using a third strand of oligonucleotide to bring Au particles to the surface, colloidal Au was directly

(47) Refractive index of protein is approximately 1.45−1.46, while bulk Au is $0.167 + 3.44i$.[48]

(48) http://corndog.chem.wisc.edu/fresnel/fcform.html (accessed March 1999).

(49) Note that a broadening in curve C can also be perceived. This may be attributed to a slight perturbation of the alkanethiol monolayer upon heating the surface.[50,51] However, no discernible particle coverage decrease was observed on the AFM image of the control surface (exposed to the conjugates in the absence of the linking oligos) after increasing the surface temperature. Presumably, the loss of Au particles, i.e., the SPR angle shift, caused by surface perturbation from temperature elevation was negligible.

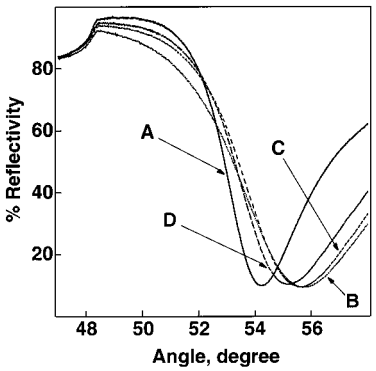
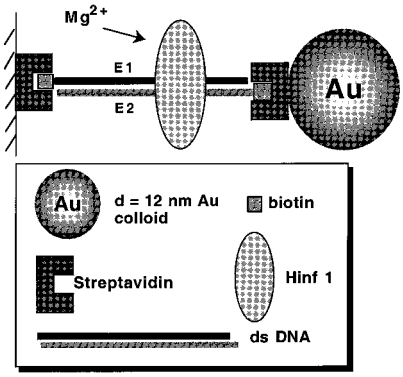


Figure 3. SPR curves of E1-coated Au surface before (A) and after (B) exposure to the E2:Au conjugates, and followed by introduction of the restriction enzyme *Hin*f I without (C) and with (D) the presence of Mg^{2+}.

Scheme 2. SPR Surface Assembly in the Digestion Experiment



attached to the second oligonucleotide (E2) via a biotin−streptavidin interaction. The sequence of the dsDNA used in the experiment was designed such that the recognition site of the restriction enzyme *Hin*f I[52] was located at the center of the sequence in order to minimize the steric hindrance caused by the adjacent Au film and colloidal Au particles. As expected, a substantial angle shift was observed after introducing E2:Au conjugates (Figure 3, curve B). While no significant change was observed after incubating the surface with *Hin*f I solution in the absence of Mg^{2+}, a necessity for enzyme activity (curve C), the SPR angle backshifted about 50% after introduction of the enzyme with Mg^{2+} (curve D). This result is consistent with the loss of Au particles on the surface via the enzyme-driven cleavage. The incomplete angle shift may be due to nonspecifically bound conjugates (e.g. via streptavidin−Au interactions). Steric hindrance caused by the presence of large Au particles nearby may also be a factor.

Although nonspecific binding of complementary DNA:Au conjugate can be reduced by prior exposure of the surface to noncomplementary conjugate (as was done for the data in Figure 2), the baseline signal due to nonspecific binding lowers the sensitivity of the technique. In a control experiment to examine the extent of nonspecific binding, a SPR surface was prepared such that the step of incubating the surface with the analyte (S2) was skipped. Theoretically, no Au colloid would be expected to bind in the absence of the linking oligos.

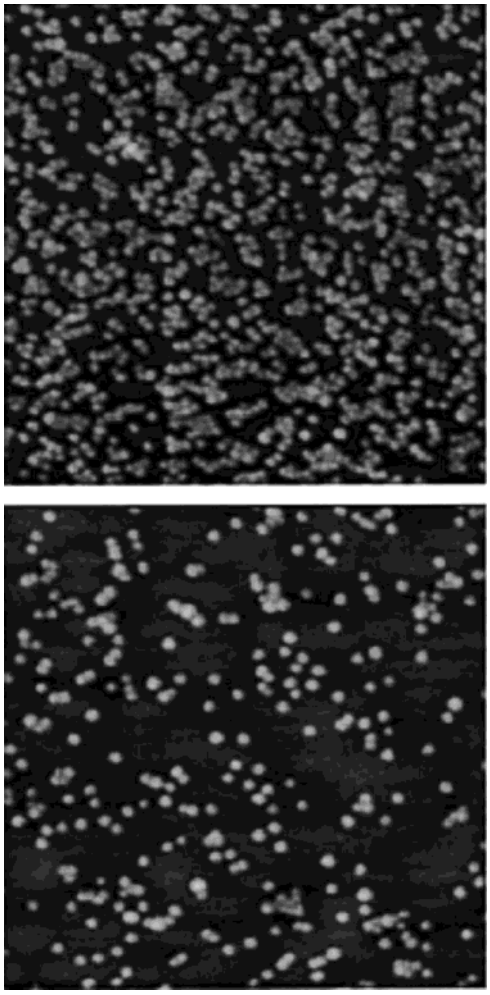


Figure 4. Atomic force microscopic images (1 μm × 1 μm) of S1/S2-coated Au surfaces after exposure to S3:Au conjugates (top) and S4:Au conjugates (bottom). Gray scale: black = 0 nm, white = 50 nm.

However, a substantial amount of surface-bound colloid was still observed. It has been suggested that this phenomenon is mainly due the presence of incompletely covered Au surface that is available to the DNA backbones for nonspecific electrostatic interactions.[53] Initially, this adsorption contributed considerably to the sensor response. To minimize this interaction, a PEG blocking reagent, combined with thorough rinsing of the surface after hybridization to remove the physisorbed particles, was employed.[54−56] As a result, the background was decreased from an initial 40% to about 10% (Figure 4), corresponding to approximately 12% of the observed angle shift (Supporting Information).[57] Note that although the background has been substantially reduced, nonspecific adsorption remains the limiting factor for quantitation of low levels of DNA using Au-amplified SPR. Further efforts to decrease nonspecific binding are underway and will be addressed elsewhere.[58]

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(57) Surface coverage was obtained by manually counting the number of Au particles present on the captured AFM images. The nonspecific adsorption was then calculated as the ratio of the number of nonspecifically adsorbed particles to that of the surface with the linking oligonucleotides.

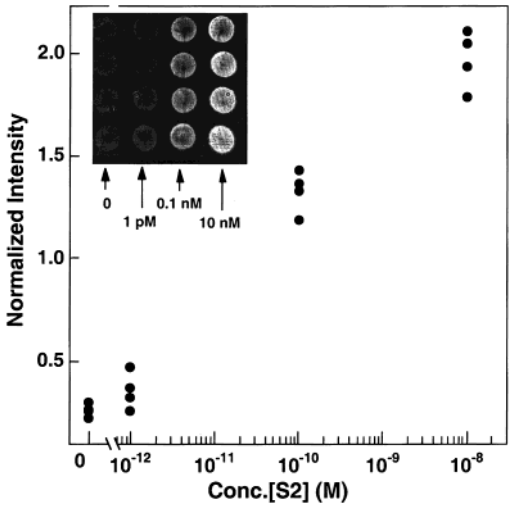

Figure 5. Plot of normalized intensity of SPR reflectance as a function of logarithmic concentration of the analyte 24-mer oligo (S2). Each spot represents one data point at the corresponding concentration. CCD parameters: exposure time = 0.3 s, 16 bit resolution, spot size = 4.5 mm in diameter. Inset: a 2-D SPR image of a Au surface derivatized with 20 μL of buffer blank, 1 pM, 0.1 nM, and 10 nM S2 oligos (from left to right, respectively).

Microarrays have revolutionized the fields of drug discovery and genomics via high-throughput analysis.[59] Corn and coworkers have used imaging SPR to detect in situ DNA hybridization on an oligonucleotide macroarray and further demonstrated the capability to monitor real-time kinetics and discriminate single base mismatches.[17,34,60] Improvements in the sensitivity of imaging analysis using streptavidin or multilayer DNA assemblies yielded 4-fold enhancement compared to unamplified events.[17] Here we explore the potential of utilizing Au-amplified SPR as an efficient detection method for arrays using a home-built imaging SPR instrument to monitor reflectivity changes over the entire Au surface simultaneously.[23] Manually deposited spots, 4.5 mm in diameter, were employed in this investigation. The reproducibility of the Au-amplified SPR technique in DNA analysis was tested first. In this experiment, we employed the same sandwich hybridization assay as described above (Scheme 1). A 16-well (4 × 4) array that contained ss-DNA (S1) tethered to the surface via EDC/NHS cross-linking was designed. After blocking the surface with PEG solution, four different concentrations of the analyte oligonucleotide (S2), 1 fM, 1 pM, 0.1 nM, and 10 nM, were manually delivered to the reaction wells at 20 μL each. Four repeats of each experiment were performed simultaneously. After 2 h incubation followed by rinsing, S3:Au solution (17 nM in particles) was introduced into the reaction wells. The inset of Figure 5 shows an image of the array. The concentration was kept the same in each column. A clear intensity difference can be observed between different columns, with similar intensities recorded within columns. The standard deviation at each concentration was calculated by integrating the spot intensity and normalizing it with respect to the spot area; each data point is plotted in Figure 5. We believe that this error was mainly caused by the liquid delivery technique (manual pipetting).

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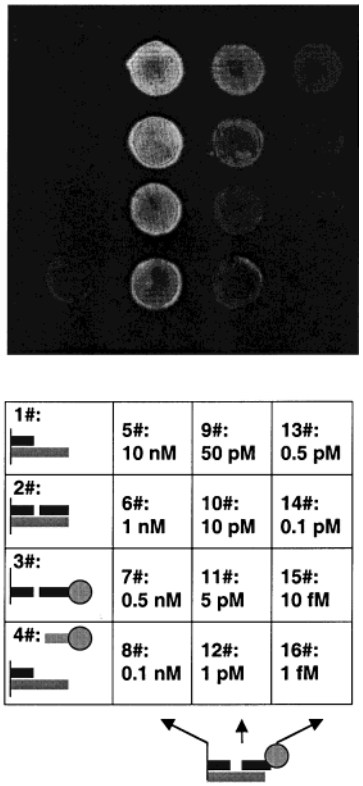

Figure 6. A 2-D SPR image of an Au surface derivatized with 20 μL of the analyte oligonucleotide, S2, with concentrations from 0 to 10 nM as indicated in the table (bottom). CCD parameters were the same as in Figure 5.

The sensitivity of this technique was examined for the same hybridization assay by spotting S2 into 12 wells (Figure 6, no. 5−16) with the concentration varied from 10 nM to 1 fM. The four wells in the first column were used for control experiments (Figure 6, bottom). On a 2-D image (Figure 6, top), no apparent signal can be observed in wells that were not exposed to DNA:Au conjugates (no. 1, and 2), while barely distinguishable response can be observed in the wells that had no linking oligonucleotide present (no. 3) or which were exposed to the noncomplementary probe S4:Au (no. 4). Note that the higher intensity observed in well 4 was probably due to the slightly higher concentration of Au conjugates used, which was evidenced by higher absorbance in its optical spectrum (data not shown). The normalized intensity at each spot (no. 5−16) was plotted as a function of the concentration of the linking oligo (Supporting Information). Since particle-amplified SPR is most sensitive to changes in particle coverage (i.e. DNA hybridization) at low surface densities, the correlation was plotted as a function of logarithmic concentration (Figure 7). While concentrations of oligonucleotides as low as 0.5 pM could be detected, high standard deviations at these low DNA concentrations precluded accurate quantitation. The limit of quantitation in these experiments was ∼10 pM.[61] Again, the high standard deviations are largely due to irregularities in manual pipetting, and it should be possible to substantially decrease them. Considering the 20 μL solution volume used and the spot size (dia. 4.5 mm), 10 pM corresponds to a maximum of 8×10^8 oligonucletides/cm^2, while 0.5 pM corresponds to 4×10^7 oligonucleotides/cm^2 (these coverages are upper limits calculated by assuming 100% of oligonucleotides present in solution hybridized to the surface).

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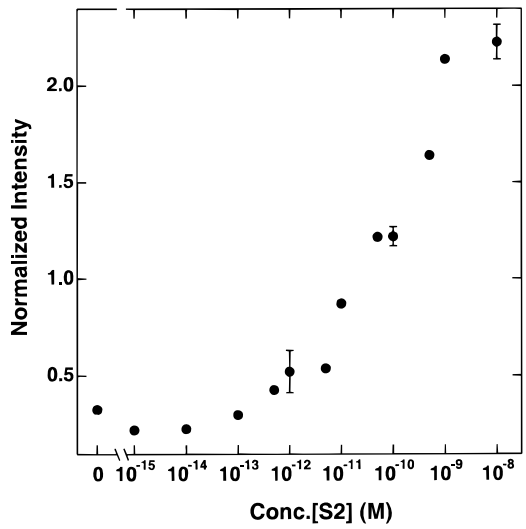

Figure 7. Plot of normalized intensity of SPR reflectance as a function of logarithmic concentration of the analyte 24-mer oligo (S2) from the image shown in Figure 6. The error bars are standard deviations from the data in Figure 5.

Table 1. Comparison of Sensitivity for Au−Amplified SPR in DNA Analysis with Other Techniques

detection method	detection limit of target DNA	refs
radiolabeling	100 fg	71
fluorescence	1.2×10^7 probes/cm^2	36
unamplified scanning SPR	100 fg/100 μm^2 for 10-mer oligos, 150 nM ~120 bp DNA	33, 35
unamplified imaging SPR	10 nM 16-mer oligos	63
Au-amplified scanning SPR	lower than 10 pM 24-mer oligos[a]	this work
Au-amplified imaging SPR	10 pM 24-mer oligos, ≤12 pg/cm^2 ($\leq 8 \times 10^8$ oligonucleotides/cm^2)[b]	this work

[a] The spots on SPR imaging surface can be detected by scanning SPR with ease, which demonstrated a lower detection limit can be achieved with the scanning instrument. Considering an instrumental angle resolution limit of 0.005°, a theoretical detection limit of 2×10^7 particles/cm^2 can be realized.[21] [b] The oligonucleotide surface coverage reported for these experiments is an upper limit, determined by assuming 100% of the molecules in solution hybridized to the surface.

This result demonstrates the advantage of using Au nanoparticle amplification; under identical conditions, but without amplification, even the highest concentration of S2 (10 nM) was undetectable.[62] Table 1 compares literature values for the sensitivity of unamplified SPR, fluorescence, and radiolabeling with particle-amplified SPR. We observe a 2−4 orders of magnitude improvement in sensitivity as compared to literature values for unamplified scanning SPR.[33,35] Importantly, our data were obtained on an imaging instrument. Imaging SPR, while compatible with high-throughput analysis, is less sensitive than its scanning counterpart. Indeed, the SPR response of spot 2 was undetectable using our imaging instrument, whereas the same spot yielded an approximately 0.12° angle shift using our scanning SPR instrument, which has an angle resolution of ~0.005° (Supporting Information). Nevertheless, the sensitivity of the Au-amplified imaging SPR already approaches that of fluorescence.[36] In addition, a dynamic range of greater than 5 orders of magnitude was observed in this experiment.

Note that only 2 h hybridization was employed for the experiment. This is by no means optimal for maximizing hybridization for such low analyte concentrations.[64] The time scale used here was chosen mainly for experimental convenience. Further improvements in the detection limit by optimizing experimental conditions, accompanied with improvement in colloidal Au particle selection (i.e. size, shape, and compositions), are underway. It should also be noted that while it is quite common to attach multiple fluorescence probes to a single target sequence, it is difficult to do so in Au-amplified SPR due to steric hindrance. Nevertheless, assuming a spot size of 50 μm in diameter, which can be easily reached using current microarray techniques,[65,66] our data suggest that the theoretical detection limit for Au-amplified SPR can still be expected to be as low as 60 copies of 24-mer oligonucleotides.[67] This result opens up new opportunities for PCR-free hybridization biosensors and for DNA diagnostics in general.

Conclusions

In conclusion, this work demonstrates the successful application of nanoparticle-amplified SPR in DNA hybridization detection. A quantitation limit of 10 pM ($\leq 8 \times 10^8$ oligonucleotides/cm^2) was obtained for 24-mer oligonucleotides. By showing the dramatically enhanced response of Au-amplified SPR in oligonucleotide detection, this work demonstrates the potential for future applications in ultrasensitive DNA detection and DNA microarray analysis. However, the field of nanoparticle amplified-SPR is still very much in its infancy. Current efforts are focused on further improvements in sensitivity, including optimizing experimental conditions and reducing background, as well as other avenues. Investigations of nanoparticle-amplified SPR for analysis of gene expression and DNA sequencing are underway.

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Supporting Information Available: Optical spectra of DNA:Au conjugates, DNA melting assay of DNA:Au conjugates, SPR data of control surface in nonspecific adsorption studies, plot of normalized intensity of SPR reflectance as a function of S2, and SPR data of unamplified DNA analysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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