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Surface Plasmon Resonance Assay for Chloramphenicol

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We report a rapid and ultrasensitive surface plasmon resonance (SPR) assay of chloramphenicol (CAP) by using large gold nanoparticles (40 nm) for signal enhancement on a mixed self-assembled monolayer (mSAM) sensor surface. After immobilization of the target antibiotic CAP through its ovalbumin (OVA) conjugates with an oligoethylene glycol (OEG) linker on the mSAM surface, sequential binding of anti-CAP antibody and IgG/nanogold (40 nm) onto the sensor surface afforded a rapid (<10 min) and ultrasensitive assay format for CAP. A limit of detection (LOD) for CAP as low as 0.74 fg/mL was achieved in aqueous buffer, and the linear working range was between 1−1000 fg/mL. While the LOD of CAP in a honey spiked-specimen is 17.5 fg/mL, the detection range is 80−5000 fg/mL. The mSAM sensor surface was also shown to be highly stable with over 400 binding/regeneration cycles performed.

The ability to both rapidly and sensitively detect trace amount of small molecules (<1000 Da) is important in the area of food safety screening for drug residues, detection of biological toxins, and environmental applications. For biomedical analysis, it is also critical in patient monitoring for those small molecular weight drugs with rapid pharmacokinetics and narrow therapeutic ranges.

Recently, several novel bioanalytical methods have been developed for ultrasensitive detection of small molecules such as a liposome-polymerase chain reaction (LPCR) assay using liposomes with encapsulated DNA reporters as a detecting reagent.[1] Sensitivity of the LPCR assay for detection of a biotoxin or cholera toxin β subunit (CTBS) was achieved to a limit of detection (LOD) of 0.02 fg/mL.[1] Another highly sensitive approach is a noncompetitive immunoenzymometric assay that can detect small molecules such as 11-deoxycortisol with attomole-range sensitivity by employing single-chain Fv fragments of antibody (scFv)-enzyme fusion protein and anti-idiotype antibodies.[2] Although these two novel methods provided extraordinarily low detection limits for small molecules, the assay procedures were slow and complicated with many reagents and multiple steps involved. In contrast to these ultrasensitive assays, rapid diffusion immunoassay in a T-sensor[3] or in a microchannel using a concentration gradient format[4] can measure small molecules in less than 1 or 10 min but the assay sensitivity can only reach the subnanomolar level, which is far less sensitive than an LOD value possible with the LPCR or a noncompetitive immuno-enzymometric assay.[1,2] It has therefore been a significant challenge to simultaneously achieve both ultrasensitive and rapid immunoassays of small molecules.

Here, we report a surface plasmon resonance (SPR) assay, using large gold nanoparticles (40 nm), which by their proximity to the sensor surface resulted in a significant enhancement of the SPR signal to create a fast (<10 min) but extremely sensitive (LOD of 0.74 fg/mL), yet simple, cheap, and stable SPR assay format for chloramphenicol (CAP), a model analyte of small molecules.

EXPERIMENTAL SECTION

Reagents and Instrumentation. Mouse antichloramphenicol antibody (mAb) was supplied by Biodesign. IgG/nanogold (40 nm) was obtained from British Biocell International (BBI). IgG/nanogold (10 nm), chloramphenicol, and its succinate sodium salt, ovalbumin (OVA), bovine serum albumin (BSA), succinic anhydride, 11-mercaptoundecanol (11-MUOH), 16-mercaptohexadecanoic acid (16-MHA), triethylamine, and trioxadecanediamine were purchased from Sigma (St. Louis, MO). *N*, *N*′-Dicyclohexylcarbodiimide (DCC) was from Lancaster (Morecambe, England). *N*-Hydroxy-succinamide (NHS) was supplied by ICN (Aurora, OH). Polyethylene glycol (PEG)-400 was from Prolabo (Founteray sous bois, France). Anhydrous magnesium sulfate, phosphate-buffered saline with 0.05% Tween 20 (PBS/T, pH7.4) was from Fluka (Buchs, Germany).

The amine coupling kit (0.1 M NHS, 0.4 M EDC, and 1 M ethanolamine), HBS buffer, SIA kit Au, and the Biacore Q were supplied by Biacore AB (Uppsala, Sweden). The NMR spectra were produced on a 400 MHz Bruker Avance DRX400 NMR (Rheinstetten, Germany). Electrospray mass spectra (ES-MS) were generated using a VG Platform II instrument (Beverley). Spectra of matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight (MALDI-TOF) were recorded on a Bruker Daltonics Autoflex spectrometer.

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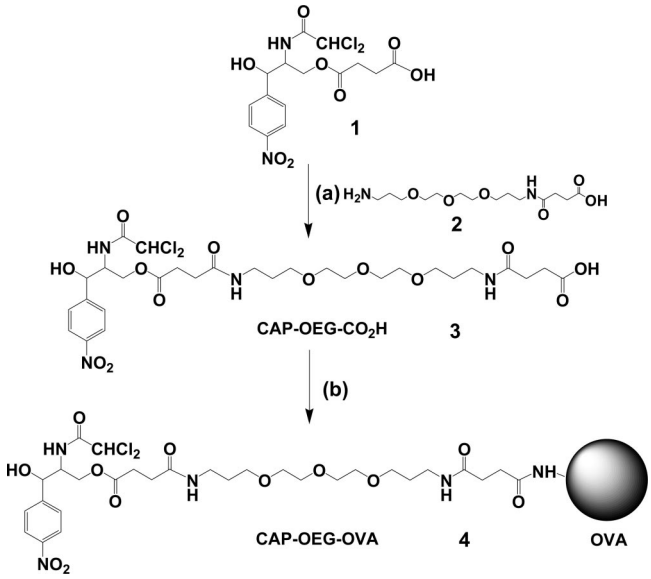
Scheme 1. Synthesis of Chloramphenicol-Olbumin Conjugate (CAP-OEG-OVA)[a]



[a] Conditions: (a) DCC/NHS/DMF, hetero-bifunctional OEG linker **2**, room temperature, overnight; (b) DCC/NHS/DMF, OVA in phosphate buffer, 4 °C, overnight.

Preparation of CAP-OEG-OVA Conjugate (Scheme 1). CAP-OEG-OVA **4** was prepared according to a previous method with some modifications.[5,6] Chloramphenicol succinate sodium salt (200 mg) was dissolved in 5 mL of deionized water. Chloroform (5 mL) was added, and the aqueous phase was acidified with hydrochloric acid (2 M) while stirring. The aqueous phase was then removed, and methanol (1 mL) was added to aid dissolution. The organic phase was dried by passage through anhydrous magnesium sulfate to yield chloramphenicol succinic acid **1** (177 mg, 93%).

The chloramphenicol succinic acid **1** (0.095 mmol) was dissolved in DMF (0.5 mL). A solution of *N*, *N*′-dicyclohexylcarbodiimide (0.143 mmol) and *N*-hydroxy-succinamide (0.143 mmol) in DMF (0.8 mL) was added. The reaction mixture was stirred at room temperature for 7 h, followed by the addition of a heterobifunctional OEG linker **2**[6] (0.12 mmol) in chloroform (1 mL) and triethylamine (0.5 mL). After stirring the reaction overnight, the solvent was removed and the sample redissolved in methanol (1 mL) and water (10 mL). The resulting solution was then acidified with dilute hydrochloric acid and extracted with chloroform (30 mL). After removing solvents, the residue was chromatographed on silica gel using CH_2Cl_2/CH_3OH (8:2) to yield CAP-OEG-CO_2H **3** (35 mg, 50%). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 1.79 (t, $J \approx 6$ Hz, 4H, 2OCH$_2$CH$_2$CH$_2$NHCO), 2.53 and 2.65 (2m, 8H, 2OCCH$_2$CH$_2$CO), 3.32 (m, 4H, 2OCNHCH$_2$), 3.58 (t, $J \approx 6$ Hz, 4H, 2OCNHCH$_2$CH$_2$CH$_2$O), 3.65 and 3.69 (2m, 8H, 2OCH$_2$CH$_2$O), 4.27 and 4.45 (2m, 3H, OCNHCHCH$_2$OCO), 5.14 (d, 1H, HOCH), 6.30 (s, 1H, CHCl$_2$), 7.71 (d, $J \approx 8.4$ Hz, 2H, aromatic), 8.22 (d, $J \approx 8.4$ Hz, 2H, aromatic). ESI-MS (negative mode, m/z) for CAP-OEG-CO_2H **3**: 723 $[M-H]^+$.

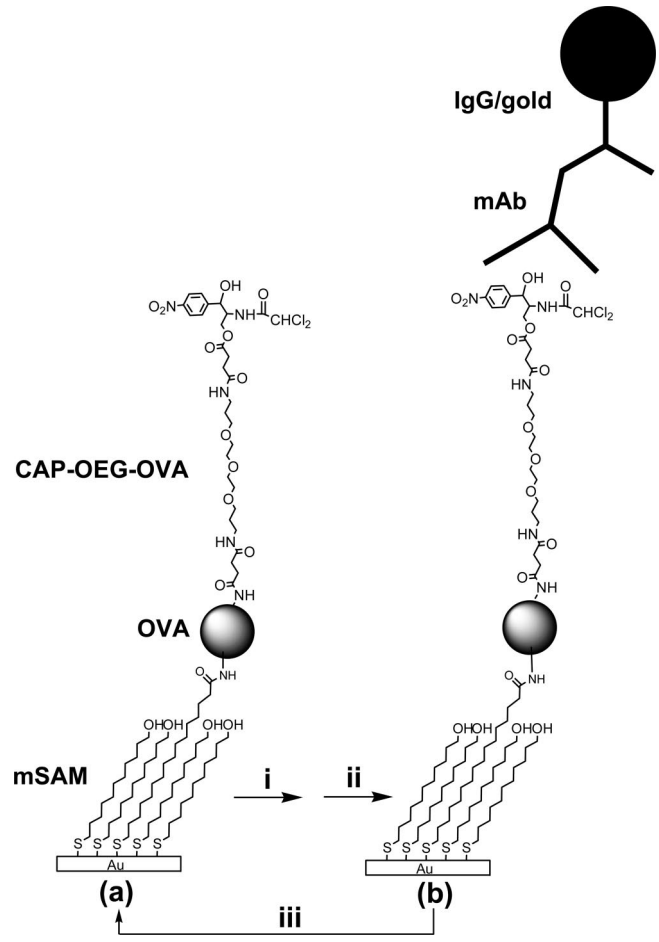


Figure 1. Rapid SPR assay of CAP (i). On CAP-OEG-OVA/mSAM surface (a), mAb binding to the sensor surface (ii). IgG/nanogold (10 or 40 nm) binding on the sensor surface for signal enhancement (b and iii). Au nanoparticles removed from surface (b) to return to (a) after regeneration.

CAP-OEG-CO_2H **3** (0.048 mmol) was dissolved in a solution of DCC (0.1625 mmol) and NHS (0.1625 mmol) in DMF (0.384 mL), and the reaction was stirred at room temperature for 3 h until a white precipitate was formed. The solution was then added to OVA (0.96 μmol) in phosphate buffer (3.84 mL, 0.2 M, pH 7.4) and stirred at 4 °C overnight. The conjugate was dialyzed against deionized water for 2 days (3 changes per day) and PBS/T for 8 h (2 changes) at 4 °C. Finally, the conjugate **4** (2.5 mL) was further purified by passing it through a PD-10 column using PBS/T as the eluent, and the purified conjugate (3.5 mL) was collected. The protein concentration of CAP-OEG-OVA **4** was determined by a bicinchonimic acid protein assay, and its conjugation degree was measured by MALDI-TOF.

Immobilization of CAP-OEG-OVA onto mSAM Surface (Figure 1). The preparation of the mSAM and the immobilized procedures have been described previously.[6] A mixture of 11-MUOH and 16-MHA (10 mmol, 9:1) was deposited on a bare gold chip (SIA kit Au), and then, the CAP-OEG-OVA **4** (500 μg/mL in 10 mM sodium acetate pH 4.0) was immobilized on the mSAM surface. The immobilization degree of ligands in a Biacore SPR system is reported as refractive units (RU). One RU corresponds to a shift in the resonance angle of approximately 0.1 mdeg and

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the adsorption of 1 pg of material/m^2.[7] A reference flow cell was constructed by immobilization of progesterone-OEG-OVA.[6]

Binding Signal Enhancements and Competitive Assay of CAP (Figure 1). Signal enhancement by IgG/nanogold particles was determined by injecting a fixed amount of mAb solution (60 μL, 20 μL/min) over the immobilized surface, following sequential injection of IgG/nanogold (40 μL, 20 μL/min) at various dilution ratios (see Supporting Information). The IgG/nanogold enhancement curves were produced by varying the concentration of mAb in HBS (0−1 μg/mL) but keeping the Au nanoparticles diluted to a ratio of 1:2 (10 nm IgG/nanogold), and 1:10 (40 nm IgG/nanogold). Regeneration was performed with two pulses of 10 mM glycine (pH 2.0, 20 μL/min).

Honey spiked-CAP samples were prepared according to a literature reference[8] with some modification. In briefly, 5.0 ± 0.02 g of New Zealand honey was weighed into a plastic centrifuge tube and mixed with 9 mL of PBS/T buffer (pH 7.4) until it went into solution. Then the samples were spiked with 1 mL of the CAP working standard solution or HBS buffer for the blank and incubated 5 min at room temperature. A volume of 12 mL of ethyl acetate was added, and the sample was mixed for 30 s with a vortex and then shaken for 30 min on a rolling mixer. Following centrifugation at 3200 rpm for 15 min, 5 mL of the organic layer was transferred to a test tube and evaporated to dryness under nitrogen. The dried sample residue was reconstituted in 1 mL of HBS buffer containing 0.5% methanol and sonicated until it dissolved completely. The samples were ready for the following competitive assays.

Competitive assays were determined by mixing mAb (1 μg/mL for 10 nm nanogold and 0.2 μg/mL for 40 nm nanogold enhancement assays) with a series of standard CAP solutions in HBS buffer or honey spiked-samples (1:1 v/v). After incubation for 30 min, the mixture was then injected (60 μL, 20 μL/min) into the sensor surface, followed by sequential injection of IgG/nanogold particles (40 μL for 2 min). Final regeneration was achieved using the same procedure as above. The results were analyzed statistically using Sigma Plot version 8.0 (SPSS, Chicago, IL). All assay standard curves were fitted to a four-parameter logistic plot.

Determination of Antibody Cross-Reactivity. The cross-reactivity profile of the antibody with thiamphenicol (TAP) and chloramphenicol-base (CAP-base) was determined in buffer using the same assay conditions as described in the competitive assay of CAP. The cross-reactivity value for each drug was calculated by comparing the IC_{50} for CAP with the IC_{50} for TAP and CAP-base according to the following equation:[9]

$$\%\ \text{cross-reactivity} = \frac{IC_{50}\ \text{for CAP}}{IC_{50}\ \text{for TAP or CAP-base}}$$

RESULTS AND DISCUSSION

Immobilization of CAP-OEG-OVA Conjugate onto mSAM Surface. Previous work on the design of protein conjugates has shown that the insertion of a water-soluble, 23-atom long OEG linker[10] between the small molecule and the protein can significantly increase the degree of hapten density in the protein conjugate.[6] In the present work, the CAP-OEG-OVA conjugate **4** was prepared by the same procedure[6] and a similar hapten (CAP) number of 11 was achieved (see Supporting Information). There are several advantages in attaching protein conjugates onto the mSAM surface rather than direct attachment of small molecules. These include (1) the surface density of the small molecule can be increased by attachment of small molecules through multiple sites of amino acid residues on the three-dimensional surface structure of the protein and (2) protection and stabilization of the potentially vulnerable mSAM surface by covering the mSAM with the proteins and making the hydrophobic mSAM surface hydrophilic, which also prevents nonspecific binding. In the present study, we have demonstrated an additional benefit whereby the protein conjugates can increase the stability of the chemical bonds between the antigen and the surface. In our work, the CAP is covalently linked to the OEG via an ester linkage, which is easily hydrolyzed under acidic or basic conditions (Scheme 1). However, this vulnerable ester linkage is stable in the CAP-OEG-OVA conjugate **4** on the mSAM surface, on which over 400 regenerations were performed under acidic conditions (pH 2.0) without damaging the antigen surface (Figure 1). Such chemical stability of the protein conjugate is important since many small molecules have no primary amine or carboxylic groups appropriate for a stable amide linked immobilization. Therefore, protein conjugation is an excellent alternative approach in immobilizing these small molecules without the need for a stable amide linkage.

Au Nanoparticle Enhanced Bindings and Sensitivity Improvement. SPR assay has been widely employed for rapid biomolecular interaction studies including detection of small molecules.[11] The label-free format of SPR, however, has limited applications for ultrasensitive bioanalysis because of limited surface refractive index changes for detection particularly for small molecules. For example, current SPR inhibition assays for small molecules have only achieved an LOD of 0.1 ng/mL[12−14] with monoclonal antibodies, or an LOD of 20 pg/mL[15] with a polyclonal antibody, as binding agents. To address this limitation, we have recently developed an SPR assay of small molecules by using 10 nm Au nanoparticles for signal enhancement on a mSAM surface immobilized with analyte−protein conjugates.[6] The assay achieved the lowest reported LOD of 4.9 pg/mL for progesterone and also created a stable mSAM sensor surface as demonstrated by the performance of over 400 binding/regenerations. However, it is

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still a significant challenge to further enhance the sensitivity of SPR analysis of small molecules from an LOD of 4.9 pg/mL to the ultrasensitive level of femtograms per milliter possible with LPCR[1] or the attomole-range achievable with noncompetitive immunoenzymometric assays.[2]

SPR is a quantum optical-electrical phenomenon whereby the plasmon produces an electronic field approximately 300 nm out from the sensor surface. Within this 300 nm range, any SPR response or enhancement effect by metal nanoparticles is collectively a function of substrate metal,[16,17] particle size,[18] and distance[19] between the metallic nanoparticles and metal surface. The electromagnetic coupling between the nanoparticles and the surface, light scattering of the nanoparticles, and energy field modulation by the dielectric spacer layer can all influence the SPR responses. Despite the potential of large Au nanoparticles in SPR signal enhancement for the determination of extremely low quantities of analyte, it has been generally considered that large Au particles are not ideal for SPR analysis because of slow diffusion kinetics and steric hindrance especially for the analysis of small molecules. The large steric bulk of the nanoparticles can block the approach of the antigen to the antibody thereby preventing the approach of the gold nanoparticles sufficiently close to the gold binding surface to allow plasmon resonance in the nanoparticles to occur and the signal to be enhanced. The preparation of a stable surface has also been difficult but is essential to allow repeated surface regenerations to remove large bound nanoparticles. So far, there have been no successes in the literature[5] that use large Au nanoparticles (≥40 nm) for SPR assays of small molecules.

In the present work, we have for the first time to successfully use large Au nanoparticles (40 nm) as an enhancement tag for achieving a SPR assay of small molecules (CAP) at a level of femtogram per milliliter (fg/mL) (Figure 1). Transmission electron microscopy (TEM) results showed that both 40 and 10 nm Au nanoparticles are almost spherical and well separated from each other (see Supporting Information), and therefore the 40 and 10 nm Au nanoparticles for SPR signal enhancement can be compared on their size differences only since both Au nanoparticles-enhanced assays have the same substrate metal and separating distance between the nanoparticles and the surface.

After anti-CAP antibody (mAb) binding on the surface, the enhancement performance of the Au nanoparticle/IgG (Figure 2) clearly showed that the large Au nanoparticles (40 nm) dramatically increased the signal of antibody binding by 21.5-fold, while the 10 nm Au nanoparticles only gave 2.3-fold signal amplification. Both signal enhancements lead to a large reduction in the mAb concentration required in a competitive assay of CAP from 6 μg/mL (nonenhanced assay) to 1 (10 nm nanogold enhanced assay) or 0.2 μg/mL (40 nm nanogold enhanced assays) separately. The competition assays were evaluated with a nonenhanced and either a 10 or 40 nm nanogold-enhanced CAP assay

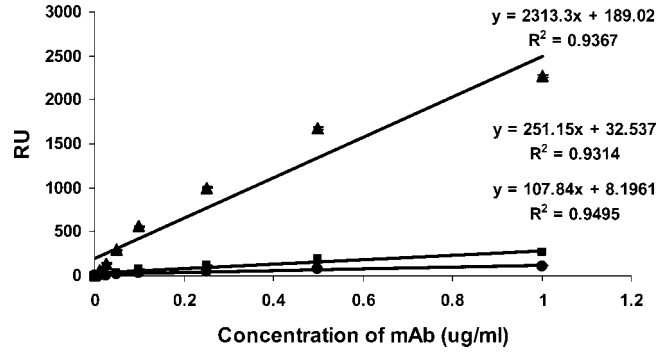

Figure 2. Binding responses in HBS buffer for mAb only (●), IgG/nanogold (10 nm) enhancement (■), and IgG/nanogold (40 nm) enhancement (▲).

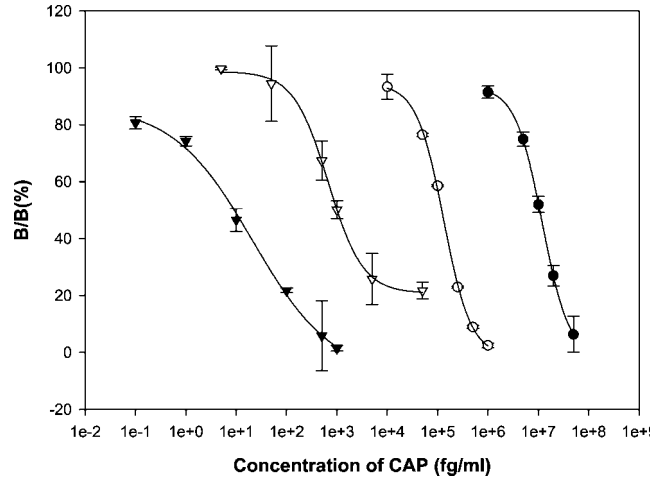

Figure 3. Comparison of four assay standard curves of CAP on CAP-OEG-OVA/mSAM sensor surface with mAb only (●), IgG/nanogold enhancement of 10 nm in HBS buffer (○), and 40 nm in HBS buffer (▼) or for honey spiked-samples (▽).

(Figure 3). Different ranges of free CAP detection were determined by mixing mAb with a series of CAP standard solutions in HBS buffer or honey spiked-samples (1:1 v/v). After incubation, the mixture was injected over the sensor surface, followed by sequential injection of 10 or 40 nm nanogold. The linear range of detection was found to be 25−800 pg/mL (IC_{50} of 124.2 pg/mL in HBS buffer) for the 10 nm nanogold enhancement and 1−1000 fg/mL (IC_{50} of 303.6 fg/mL in HBS buffer) or 80−5000 fg/mL (IC_{50} of 664.6 fg/mL in honey spiked-specimen) for the 40 nm nanogold enhancement (Figure 3). The sensitivity was increased by 2.5×10^6-fold after 40 nm Au particle enhancement, achieving an LOD as low as 0.74 fg/mL in HBS buffer or 17.5 fg/mL in the honey spiked-specimen, while 10 nm gold particles only gave 10-fold decrease with an LOD of 34.1 pg/mL (Table 1). The results of 40 nm nanogold enhancements in HBS buffer and honey spiked samples have clearly demonstrated some influence of matrix effects on the CAP assay performance characteristics as shown by different IC_{50} and LOD values in different types of assay matrix. However, such a huge difference between the 40 and 10 nm Au nanoparticles is larger than would be anticipated on the basis of particle mass or volume alone. According to the Mie theory on the radiating plasmons (RPs) model,[20] incident energy is dis-

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Table 1. Summary of Cap Immunoassay Parameters of LOD, IC_{50}, Sensitivity and Signal Enhancement with Change in Signal Enhancement Labeling and Primary Antibody Concentration

assay format	mAb concentration (μg/mL)	LOD	IC_{50}	signal enhancement ratio	assay sensitivity
mAb only (HBS buffer)	6	353.4 ± 66.9 (pg/mL)	13.6 ± 2.7 (ng/mL)	n/a	2.5ng/mL
mAb + IgG/gold (10 nm) (HBS buffer)	1	34.1 ± 4.80 (pg/mL)	124.2 ± 24.8 (pg/mL)	2.3	25pg/mL
mAb + IgG/gold (40 nm) (HBS buffer)	0.2	0.74 ± 0.12 (fg/mL)	303.6 ± 30.3 (fg/mL)	21.5	1 fg/mL
mAb + IgG/gold (40 nm) (honey spiking)	0.2	17.50 ± 4.2 (fg/mL)	664.6 ± 6.7 (fg/mL)		80 fg/mL

sipated by absorption and far-field radiation is created by scattering. Therefore, the huge SPR enhancement effect by 40 nm Au nanoparticles mainly resulted from scattering, which is dominant over absorption for large colloids; while for small colloids (10 nm), absorption dominates over scattering thus producing far less SPR enhancement.

Similar scattering enhancement by large Au nanoparticles (50 nm) has also been recently reported for an ultrasensitive colorimetric detection of methicillin-resistant *Staphylococcus aureus* (MRSA) total genomic DNA (66 ng/mL).[21] Moreover, for ultrasensitive SPR assay, the size of Au nanoparticles (40 nm) for small molecules seems sufficiently large since further increases in the particle size from 45 to 59 nm did not result in any significant change in the SPR response.[18] Such an ultrasensitive SPR assay of small molecules (0.74 fg/mL) is also a result of the proximity of large Au nanoparticles to the Au surface on the chip. For example, the ideal distance, or Au particle−Au surface separation, is 32 ± 5 nm[19] to achieve the maximum SPR angle shift when SiO_2 was used as a dielectric spacer layer.

Finally, the SPR assay showed a small degree of cross reactivity for TAP (3.1%) and no cross reactivity for CAP-base, suggesting that the antibody conjugate is specific for the CAP on the surface.

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CONCLUSIONS

It has been demonstrated, for the first time, that the use of large nanoparticles (40 nm) as a signal enhancement on a mSAM sensor surface creates both a rapid and ultrasensitive SPR assay of small molecules, demonstrated by a rapid CAP assay in less than 10 min and ultrasensitivity (LOD = 0.74 fg/mL). With control of the proximity of the large Au nanoparticles to the mSAM surface through protein conjugation with an optimal OEG linker,[6] the assay design provided a favorable binding and enhancing environment for large Au nanoparticles. Moreover our methods resulted in the formation of a stable sensor surface on which over 400 binding/regeneration cycles were performed without damaging the SAM attachment and immobilized CAP. The assay format is generic and can be applied to many other small molecules for rapid and ultrasensitive detection with various SPR technologies.

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SUPPORTING INFORMATION AVAILABLE

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