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Attomolar Detection of Protein Biomarkers using Biofunctionalized Gold Nanorods with Surface Plasmon Resonance

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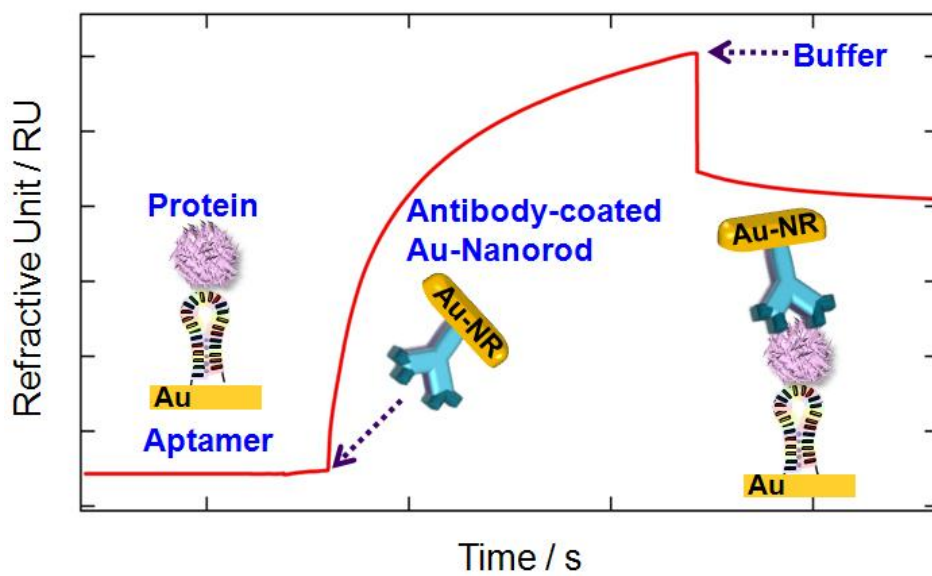
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Abstract: This paper describes an ultrasensitive surface plasmon resonance (SPR) detection method using biofunctionalized gold nanorods for the direct detection of protein biomarkers. Immunoglobulin E, which has separate antibody and DNA aptamer binding sites, was chosen as a model protein for which a sandwich assay platform was designed. Detection was achieved via the specific adsorption of unlabelled IgE proteins onto the surface immobilized aptamer followed by the specific adsorption of anti-IgE coated gold nanorods (Au-NRs). Using the biofunctionalized nanorods in conjunction with SPR, we were able to directly measure IgE proteins at attomolar concentrations. This is a remarkable 10^8 enhancement compared to conventional SPR measurements of the same surface sandwich assay format “anti-IgE / IgE / surface bound IgE-aptamer” in the absence of gold nanorod signal amplification.

Key words : Immunoglobuline E, Surface plasmon resonance (SPR), DNA aptamer, biofuncitonized gold nanoparticle, sandwich assay

Graphical Abstract

A novel detection method utilizing antibody functionalized gold nanorods in conjunction with surface plasmon resonance was developed for the attomolar detection of protein biomarkers.



I. Introduction

There have been extensive efforts in recent years to develop nanoparticle-enhanced analytical technologies for the highly sensitive and selective detection of protein biomarkers that can be powerfully utilized to diagnose various types of diseases.[1-3]

The integration of metallic nanoparticles (NPs) into surface bioaffinity sensors has primarily focused on the functionalisation and application of spherical particles. For example, since their use was first reported by Mirkin *et al*,[4] DNA-modified gold nanoparticles have been incorporated into a variety of solution and surface based sensing formats which often achieve sensitivities significantly greater than established fluorescence-based approaches for nucleic acid detection. However, the transfer of these technologies to protein detection has proven to be more challenging due to the difficulties in biofunctionalizing nanoparticles without losing particle stability as well as preventing non-specific interactions with other sensing surfaces and non-target molecules.

In this paper, we demonstrate that antibody-functionalized nanorods can be used to greatly enhance the sensitivity of surface plasmon resonance (SPR) measurements for the ultrasensitive detection of protein biomarkers. SPR measurements on a planar gold

film are now well-established as the leading label-free alternative to fluorescence-based methods for the investigation of biomolecular interactions.[5-7] One limitation of SPR is a typical detection limit in the low nanomolar range and efforts to improve sensitivity through the use of spherical gold nanoparticles conjugated to a secondary biomolecular probe as part of a sandwich assay has been reported by several groups. For example, spherical DNA-functionalized gold nanoparticles have been used to create a 100-1000 increase in sensitivity resulting in detection limits in the 1-10 pM range.[8-11]

Gold nanorods are an attractive alternative to spherical particles since the plasmon wavelength can be tuned over a much greater wavelength range opening up new possibilities for enhanced spectroscopic detection.[12] However, due to the use of surface adsorbing surfactant molecules in directing the nanorod growth,[13-14] subsequent biofunctionalisation has proved to be challenging. A number of strategies have been reported to create *localized* SPR sensors either in bulk solution [15] or on individual rods immobilized on a surface.[16] Essential to the successful application of functionalized nanorods as part of a highly sensitive sandwich assay for use in surface bioaffinity sensors is to control both the nanorod and SPR chip surface chemistries in order to control non-specific adsorption on areas where no target molecules are present.

Recently, Sendroiu *et al* demonstrated that rods first coated with a silica layer and then functionalized with DNA could be selectively hybridized-adsorbed onto a two component DNA array gold surface with minimum non-specific adsorption, however no target detection or enhanced sensitivity was reported.[17] To answer the question of whether improved SPR sensitivity can be achieved using nanorods we describe here the creation of a surface sandwich complex (anti-IgE coated gold nanorods / IgE protein / surface-bound IgE specific aptamer) which enables the specific detection of the IgE target at remarkably low concentrations in the attomolar range.

II. Experimental

Chemicals: All chemicals listed below were used as received unless otherwise stated; sodium borohydride (NaBH₄, Sigma-Aldrich), ascorbic acid, silver nitrate (AgNO₃, Sigma-Aldrich), hydrogentetrachloroaurate(III)hydrate (HAuCl₄, Sigma-Aldrich), hexadecyltrimethylammoniumbromide (CTAB, TCI), 11-mercaptoundecanoic acid (MUA; Aldrich), 11-amino-1-undecanethiol hydrochloride (MUAM; Dojindo), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Thermo), *N*-hydroxysulfosuccinimide (NHSS; Thermo), sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC; Pierce), thiol-modified polyethylene glycol, MW 1500 (PEG-SH; Paraon), human immunoglobulin E (IgE, 190kDa, Calbiochem), goat anti-human immunoglobulin E ϵ -chain specific, (anti-IgE, 150kDa Calbiochem), anti-bovine

albumin antibody produced in rabbit (anti-BSA, 155kDa, Aldrich). All rinsing steps were performed using absolute ethanol or Millipore filtered water. DNA aptamer and control DNA sequences were custom-synthesized by Integrated DNA Technologies; the aptamer sequence is 5'-thiol-AAA AAA AAA AAA AAA GGG GCA CGT TTA TCC GTC CCT CCT AGT GGC GTG CCC C-3' (IgE-SH; IDT) and the control DNA sequence is 5'-thiol-(A)₃₀. All biomolecular interactions were performed in phosphate saline buffer (PBS, pH 7.4, Invitrogen) at room temperature unless otherwise stated.

Synthesis of Au nanorods: Colloidal solutions of gold nanorods were prepared according to the well-documented seed-based approach described initially by El-Sayed *et al.*[13] The resulting gold nanorods are stabilized by a bilayer of cationic CTAB molecules. Following washing by centrifuging (6000g, 20min) and resuspending in Millipore water to remove excess reagents from the reaction mixture, stock solutions were stored at 27-29°C and characterized using UV-vis spectroscopy and transmission electron microscopy (TEM). The average length and width of the rods used was 40 (±10) nm and 10 (±2) nm, respectively.

Biofunctionalization of Au nanorods: Figure 1a outlines the approach used for the covalent attachment of antibodies onto gold nanorods via EDC/NHSS linking chemistry. Following centrifugation and resuspending in Millipore water, 0.1 mL of 20 mM MUA prepared in ethanol was added to 1.5 ml of nanorods. The solution was kept under sonication for 30 mins at 50°C followed by a further 2 h at 25°C while still sonicating. Next, the Au nanorod solution was centrifuged one more time at 6000 g for 20 mins and

the rod pellet resuspended in 0.85 ml of Millipore water.[18] This results in a carboxylic acid terminated alkane thiol monolayer formed on the surface of gold nanorods. Next, 0.1 mL of 10X phosphate buffered saline (PBS, pH 7.4) along with 50 μ l of a mixture of EDC and NHSS (7.5 mM : 1.5 mM) were then added at room temperature to form an amine-reactive NHSS ester terminated rod surface. After 10 mins, 10 μ l of 1 μ M anti-IgE was added to the solution and gently shaken for several seconds and kept for 12 h at 25°C. This allows the random amine functional groups present on the antibody to covalently attach to the rod surface via the formation of an amide bond.[16] Verification that biofunctionalized nanorod conjugates were prepared was confirmed using UV-vis spectroscopy and TEM. Anti-BSA or anti-protein A conjugated nanorods were also synthesized in the same way as described above. The final anti-IgE nanorod solutions used in SPR enhanced measurements was estimated at about 2 nM using an extinction coefficient of $5 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ at 785 nm.[19]

SPR measurements: A Biacore 3000 was utilized for all real-time monitoring of target bimolecular interactions with surface immobilized probe molecules. Phosphate buffered saline (PBS, pH 7.4) and a flow rate of 5 μ l/min was used for all experiments. Scheme 1b shows a schematic of the immobilization of DNA aptamers specific to IgE on a chemically modified gold surface for real-time SPR measurements. A mixed self-assembled monolayer of MUAM and PEG-SH (MW 1500) was first formed by soaking a bare gold chip overnight in an ethanolic solution composed of a 0.5 mM : 0.05 mM ratio of MUAM : PEG-SH. After gentle rinsing with water, the chip was placed in a humidity chamber and covered with 1 mM SSMCC in 100 mM TEA buffer pH 7 for 30 mins to create a thiol-reactive maleimide-terminated surface.[20] Next, the gold chip

was exposed to a 1 mM solution of 5'-thiol modified IgE specific aptamer overnight. Finally, the chip was rinsed with Millipore water and dried under a nitrogen stream prior to use. Regeneration of the IgE aptamer chip surface between measurements was achieved by rinsing with 0.05 M HCl followed by 8 M urea, DI water and PBS buffer each for a minimum of 40 mins before injection of various concentrations of target biomolecules. An extra washing step with PBS buffer (pH 12.5) before the urea washing step was employed to regenerate aptamer chips after measurements involving the injection of nanorod samples. The SPR system used in this work has four sample injection channels. Two of the channels were used to introduce target samples with the remaining two channels utilized to inject reference (negative control) samples.

III. Results and Discussion

The preparation of stable biofunctionalized gold nanorods which interact specifically with their intended protein target is essential for enhanced SPR detection. Scheme 1a outlines the surface modification procedure used to create antibody-nanorod conjugates. Briefly, the CTAB layer on the nanorod surface was replaced with a carboxylic acid terminated alkanethiol followed by the formation of NHSS ester functional group available using EDC/NHSS cross linking chemistry. Either anti-IgE, anti-BSA or anti-protein A were then reacted with the NHSS ester to form a covalent link to the nanorod surface (see experimental section for further details). Figure 1 compares UV-vis spectra

of CTAB coated gold nanorods versus anti-IgE coated gold nanorods with both suspended in PBS buffer (pH 7.4). Following antibody conjugation, a red shift of ~8-10 nm was typically observed in the position of the longitudinal surface plasmon resonance peak at 785 nm along with a smaller red shift in the transverse peak at 515 nm; which is indicative of an increase in dielectric constant around each nanorod due to additional molecular layers. This can also be seen in the TEM images shown in the inset of Figure 1 where a surrounding layer with a faint contrast against the background can be seen for the antibody-coated rods compared to the original CTAB-coated rods. In addition, the antibody-conjugated nanorods were stable in solution for at least 5 days at room temperature.

Prior to the utilization of the antibody-rod conjugates for enhanced IgE detection, DNA aptamer SPR chips were prepared. In order to minimize non-specific adsorption of both target protein and nanorod-antibody conjugates a mixed monolayer of thiol-modified polyethylene glycol (PEG-SH) and an amine terminated alkanethiol was first created. The choice of a 10 : 1 stoichiometric ratio of MUAM to PEG-SH in the preparation of the first SAM layer along with the use of 1500 MW PEG was based on a series of preliminary measurements (not shown here) comparing the performance of different

ratios as well as 180 and 230 MW PEG molecules to resist non-specific adsorption. Following SAM formation, the IgE specific DNA aptamer was covalently attached via crosslinking of the 5'-thiol modified end of the nucleic acid sequence to the maleimide terminated chip surface formed on exposing the mixed monolayer to SSMCC (see Scheme 1b).

An outline of the sandwich assay detection format for the enhanced SPR detection of IgE is depicted in Scheme 1b where unlabelled IgE interacts with surface immobilized IgE-specific aptamers before introducing anti-IgE coated nanorods to further amplify the detection signal. Real-time SPR sensorgrams were first obtained for the adsorption of IgE protein only and it was established that 1 nM IgE could be easily measured without a second detection step and a Langmuir adsorption coefficient value of $\sim 2.2 \times 10^8 \text{ M}^{-1}$ also obtained (data not shown). For these measurements at higher concentrations ($> 1 \text{ nM}$), IgE protein solutions were in contact with SPR chip surface for up to 1 hour until the SPR response reached a constant value followed by washing with buffer for 5 mins to remove any non-specifically bound IgE from the chip surface. Additional experiments were also performed using a control aptamer sequence (data not

shown) with no significant change in the SPR signal observed at nanomolar concentrations of IgE.

For measurements at much lower IgE concentrations ranging from 1 aM to 10 fM, target adsorption was allowed to proceed for a minimum of 2 h under the continuous injection of target-containing solution. This initial reaction time is necessary at such low concentrations to ensure that a steady-state surface coverage of IgE has been reached. Note also that the rate of IgE desorption (k_d) from the aptamer surface is in the low nanomolar range[21] ensuring minimal target loss prior to the second nanoparticle amplification step. Figure 2(a) shows a series of SPR curves measured at IgE concentrations ranging from 1 aM to 100 aM. While there is no distinguishable response observed when IgE adsorbs onto the surface, the SPR signal significantly increased when anti-IgE coated gold nanorods were injected, with the nanorod concentration injected at each measurement kept constant throughout.

To ensure that the enhanced IgE detection is specific as well as sensitive, Figure 2(b) shows a series of control measurements alongside the response curve for the detection of 1 aM IgE. These include: (i) injection of anti-IgE coated nanorods onto the aptamer

chip surface in the absence of IgE, and the exposure of nanorods functionalised with a non-interacting antibody (anti-protein A) to the aptamer surface in the presence of (ii) 1 aM IgE and (iii) 100 aM IgE. In each case, following rinsing with buffer, the final SPR signal associated with each control run was comparable or below the response associated with 1 aM IgE detection thus establishing this concentration as the detection limit. Similar control measurements using instead BSA-coated nanorods were also found to result in a similar level of non-specific adsorption both in the presence and absence of IgE.

Finally, to demonstrate that the nanorod-enhanced SPR response can be quantitative Figure 3 shows a plot where the change in the SPR response at each target concentration is normalized by subtracting the signal increase obtained on injecting anti-protein A functionalized nanorods through the reference channels on the same chip. IgE was introduced to both the sample and reference channels to form surface IgE/aptamer complexes prior to nanorod injection. A linear relationship between the measured SPR signal and analyte concentration was observed below 10 aM IgE, above which the SPR responsivity decreases. For higher IgE concentrations, a reduced nanorod concentration is required to bring back the normalized SPR response back into a linear response range.

This behaviour is similar to previously reported studies involving spherical nanoparticles,[20] albeit at higher starting concentrations. It also appears that a compromise of the higher sensitivity associated with nanorods compared to spheres is a reduced concentration window where a linear response is obtained without having to tailor the nanoparticle concentration. Chip-to-chip variation was also investigated for five different IgE-aptamer chips which showed a $\pm 10\%$ variation in normalized SPR response when compared at the same concentrations of IgE and anti-IgE coated gold nanorods.

IV. Conclusions

We have demonstrated a simple sandwich detection method using biofunctionalized gold nanorods in conjunction with SPR to measure unlabelled IgE at attomolar concentrations (detection limit ≈ 1 aM). An excellent 10^8 enhancement in sensitivity is obtained when directly compared with SPR measurements without any nanoparticle secondary amplification (which has a ~ 0.5 nM detection limit).[22] Furthermore, when compared to previous uses of spherical biofunctionalized gold nanoparticles for signal enhancement,[20, 22] nanorods enable up to a further 10^3 times increase in sensitivity utilizing a similar sandwich assay format. The origin of the additional enhancement

associated with gold nanorods is not yet fully understood and further investigation is currently underway. The typically larger rods will cause a greater refractive index shift per surface binding event than nanospheres. However, since the enhancement is greater than expected, it is likely that plasmonic coupling between the nanorod and gold film substrate is important. Additional experiments exploring both the origin of this enhancement and whether further optimization in sensitivity can be achieved through tuning the wavelength of the excitation light source and resonance properties of the nanorods are necessary. As is the case for all SPR-based detection methods both with and without additional signal amplification, achieving the direct detection of proteins and nucleic acids in biological samples at genomic-level concentrations is a significant challenge; which we envision will be possible as methods for the monodisperse synthesis and successful biofunctionalization of anisotropic metallic nanoparticles and SPR substrates continues to improve.

Figure legends

Scheme 1. Schematics showing (a) antibody functionalization of gold nanorods via the formation of a carboxylic acid terminated alkanethiol on the rod surface followed by antibody conjugation via EDC/NHSS linking chemistry, and (b) sandwich assay detection of IgE with the thiol-modified aptamer probes covalently attached onto the

gold surface of the SPR chip which features a mixed monolayer containing polyethylene glycol.

Figure 1. UV-vis spectra of gold nanorods ($\lambda_{\max} = 785$ nm, bold line) and anti-IgE coated gold nanorods ($\lambda_{\max} = 802$ nm, dotted line). Inset shows TEM images for (a) CTAB coated gold nanorod stock and (b) anti-IgE coated gold nanorods.

Figure 2. (a) Representative SPR sensorgrams of anti-IgE coated gold nanorod-enhanced detection of IgE at concentrations of 1, 10, 50 and 100 aM. (b) SPR response curve for the detection of 1 aM IgE (solid line) is shown alongside control experiments looking at non-specific adsorption of nanorods: (i) anti-IgE coated nanorods onto aptamer surfaces in the absence of IgE, and anti-protein A coated nanorods onto IgE aptamer surfaces in the presence of (ii) 1 aM IgE and (iii) 100 aM IgE.

Figure 3. Plot of the anti-IgE coated gold nanorod-enhanced SPR response using IgE aptamer chips for IgE concentrations ranging from 1 aM to 100 aM. The dotted line in the figure is the linear fit of the data. The nanorod enhanced SPR signal for IgE detection was corrected using the SPR signal for non-specific adsorption of anti-protein A coated gold nanorods onto IgE aptamer surface.

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Scheme 1

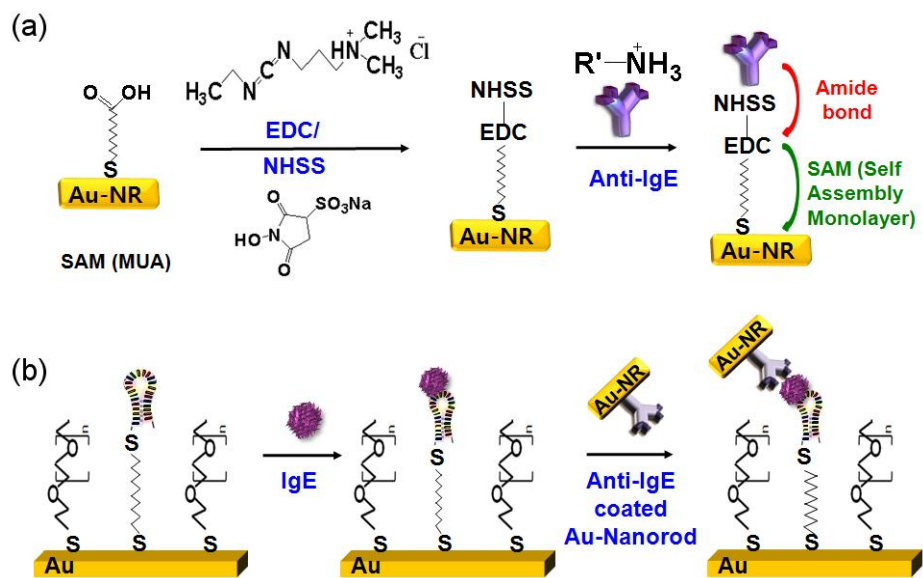


Figure 1

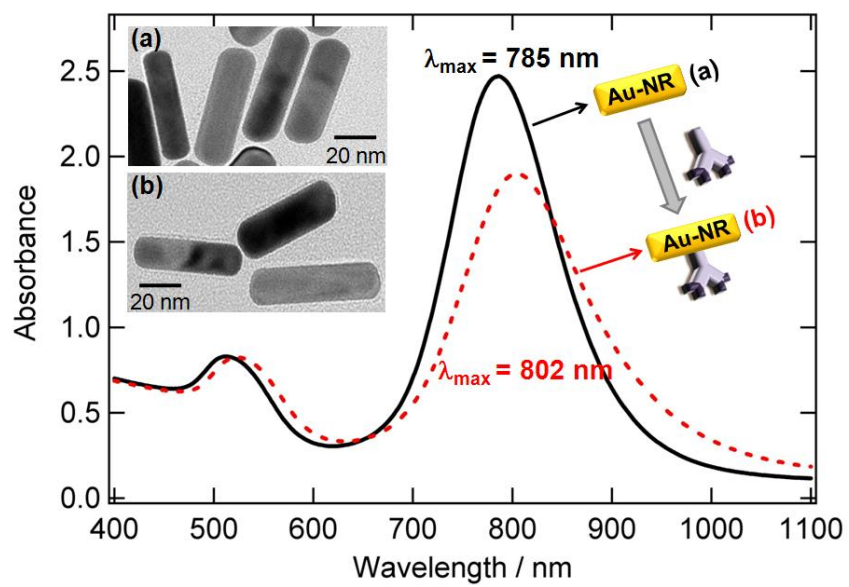


Figure 3

