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# Multifunctional Au Nanoparticle Dendrimer-Based Surface Plasmon Resonance Biosensor and Its Application for Improved Insulin Detection

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**Bifunctional hydroxyl/thiol-functionalized fourth-generation polyamidoamine dendrimer (G4-PAMAM)-encapsulated Au nanoparticle (NP) was synthesized and immobilized on a mixed self-assembled monolayer (SAM)-modified gold surface. This modified surface was resistant to nonspecific adsorption of proteins having a wide range of molecular weight and isoelectric points. Part of the dendrimer thiol groups were converted to hydrazide functionalities providing an activated surface available to subsequently immobilize the receptor for developing a sensor surface to immunoaffinity reaction. Herein, the surface plasmon resonance (SPR) detection of insulin was obtained by means of a competitive immunoassay principle. The resulting Au NP dendrimer-modified surface provided an assay with high stability, significantly enhanced sensitivity, and a detection limit for analyzing insulin of 0.5 pM. The SPR detection of insulin was amplified due to the changes in the dielectric properties of the matrixes, occurring upon the biorecognition processes on the sensor surface, through the coupling of the localized plasmon of the NPs with the surface plasmon wave. The developed sensor chip was used to analyze insulin in human serum samples from healthy and diabetic patients. The results showed good correlation to the reference method. The specificity and the improved sensitivity of this biosensing platform could have significant implications for the detection of a wide range of molecules and biomarkers in complex biological media.**

Insulin is a polypeptide hormone which regulates carbohydrate metabolism. Insulin sensing in serum is greatly important for clinical diagnostics and to follow-up patients affected by various types of diabetes.[1] Besides the clinical importance of insulin determination, another aspect to be considered is the detection of this substance in athletes for doping control purposes. In fact, the administration of endogenous insulin positively influences athlete's performances.[2] In the last years, several sensing platforms for the determination of insulin levels were developed. For example, electrochemical oxidations of trace insulin at electrodes modified with different redox mediators,[3] nickel oxide nanoparticles,[4] carbon nanotubes,[5] and ruthenium oxide/carbon nanotube composites[6] were devised. A biochip integrating immunochemical and enzymatic assays within the same microchannel was implemented for the simultaneous measurements of glucose and insulin.[7] Also, different label-free immunoassay methods based on thiol self-assembled monolayers (SAMs) were used for quantitative detection of insulin in serum.[8] The specific antigen-binding activity of antibodies immobilized on these surfaces resulted in high sensitivity, selectivity, and stability. To increase the efficiency and sensitivity of biospecific interactions, much attention was paid to the development of an interfacial layer enabling the immobilization of the biomolecules in a controlled orientation and with minimized lateral steric hindrance.[9] The key step in the development of immunosensors is the immobilization of antibodies onto a support characterized by high density with uniform distribution, retaining their specific antigen binding activities and maintaining accessibility to the antigens.[10]

The binding of antigen molecules to the immobilized antibodies can be monitored by means of several physicochemical transducers. In this field, the surface plasmon resonance (SPR) technique shows singular interesting features. Surface plasmon resonance spectroscopy is a versatile method to probe small changes of refractive index occurring on thin metal films (e.g., Au or Ag) as a consequence of the binding of molecules with a molecular

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receptor such as DNA, enzymes, or antibodies.[11,12] One of the main drawbacks of this analytical technique is related to its inability to detect small changes in refractive index caused from low molecular weight or low concentration compounds at the sensing surface. To address such limitations, several signal amplification approaches were developed.[13]

Specifically, metal nanoparticles (NPs; e.g., Au NPs or Ag NPs), exhibiting a localized surface plasmon (LSP), were extensively used to enhance SPR response.[14] A shift in the SPR energy is observed when the coupling between the LSP associated with the Au NP and the surface plasmon polariton (SPP) associated with the thin metal surface occurred.[15] The enhancement effect in the SPR signal is closely related to the dielectric function of the particles and also to nanoscale phenomena in the near field.[15] Some authors carried out their experiments by means of NPs, especially Au NPs, as amplified labels for biorecognition events,[16] biocatalytic transformations,[17] or sensitive detection of low-molecular-weight compounds.[18] Also, changes in the optical properties of Au NPs due to morphological changes, particles size, and coverage on the SPR response were studied.[19] Furthermore, the steric hindrance is another important issue to be focused.[20] In particular, the sensitivity of the system decreases when the probe size is smaller than the target molecule size. In order to develop stable SPR-based transducers, a careful design of the sensing platform is required. Dendrimer-encapsulated Au nanoparticles could play a central role in each of these aspects.

Dendrimers are highly branched, nearly size monodisperse polymers with several structural properties.[21] These structural features include the incorporation of metal NPs with precise control over the NP size[22] and a defined number of terminal groups for each generation with multiple branch ends, which can be available for consecutive conjugation reactions.[23] Moreover, they are able to form stable, dense, well-organized, and close-packed arrays on surfaces. Indeed, dendrimers are emerging as promising candidates for novel diagnostic platforms.[24] Fourth-generation poly(amidoamine) (G4-PAMAM) dendrimers have been used for DNA and protein immobilization.[25] However, to reduce nonspecific protein adsorption and to use the modified surface in the analysis of complex matrixes, several strategies were attempted.[26−28] In this field, oligo(ethylene glycol) (OEG) and peptide-based self-assembled monolayers are well-known biomaterials providing low specific protein adsorption on a variety of surfaces, mainly due to steric repulsion and excluded volume effects.[26,27] The protein resistance of a SAM-modified surface is dependent on the conformation chain length, mobility, and surface density of OEG-terminated alkanthiol chains.[28] On the basis of these findings,[26,28] this study uses a neutral G4-PAMAM-OH encapsulated Au NP as a biosensing platform for insulin detection.

In the present paper, a bifunctional hydroxyl/thiol-functionalized G4-polyamidoamine dendrimer-encapsulated Au NP was synthesized and immobilized on a mixed SAM of alkanethiolates on gold generated from the (2-(2-(2-(11-mercaptoundecyl-oxy)-ethoxy)ethoxy) ethyl alcohol, **a**, ($HS(CH_2)_{11}(OCH_2CH_2)_3OH$) and the (2-(2-(2-(2-(2-(2-(11-mercaptoundecyl-oxy)ethoxy)ethoxy)-ethoxy)ethoxy)ethoxy)ethoxy) acetic acid, **b**, ($HS(CH_2)_{11}$-$(OCH_2CH_2)_6OCH_2CO_2H$). Simultaneously, part of the dendrimer thiol groups were converted to hydrazide functionalities coupled to insulin. SAM-maleimide groups were used to immobilize dendrimers. In this way, dendrimer grafting density, which ultimately reflects protein grafting density, can be modulated by tuning the preparation parameters. Furthermore,


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the dendrimer density on the surface tuned the surface coverage of Au nanoparticles and, thus, the near-field coupling effects.

Here, we investigated the SPR detection of insulin based on an indirect competitive immunoreaction principle. Stability, sensitivity, and regeneration of complete immunoreactivity of the antigen-coat format employed in this study for repeated use in multiple determination of insulin were demonstrated. The sensing platform was used to analyze insulin in human serum samples from healthy and diabetic patients, and the results were compared with those obtained with a radioimmunoassay (RIA) reference method.

## EXPERIMENTAL SECTION

**Materials.** Tris(2-carboxyethyl) phosphine (TCEP), *N*-(3-dimethyl aminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma (Sigma-Aldrich, USA). The different generations of PAMAM dendrimers were purchased from Dendritech Inc. (Midland, MI). Heterobifunctional cross-linking agent succinimidyl 6-(3′-[2-pyridyldithio]propionamido)hexanoate (LC-SPDP), heterobifunctional cross-linker *N*-(ε-maleimidocaproic acid)-hydrazide (EMCH), and 1-hydroxybenzotriazole hydrate (HOBT) were purchased from Pierce Inc. (Rockford, IL). Monoclonal anti-insulin antibody (mouse IgG1 isotype) was purchased from Gentaur (Brussels, Belgium). Human insulin expressed in *E.coli* was purchased from Upstate Biotechnology (NY, USA). Thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were obtained from World Health Organization International Laboratory (Hertfordshire, England). Bovine serum albumin, lysozyme, trypsin, ribonuclease A, aldolase, fibrinogen, ovalbumin, and superoxide dismutase were obtained from Sigma-Aldrich. Lyophilized control serum, prepared from human serum, was obtained from Medidrug (Steinenbronn, Germany). All aqueous solutions were prepared using deionized water (specific resistivity ≥18.2 MΩ cm) obtained from a Direct-Q 3 UV apparatus (Millipore, France).

**Instrumentation.** The ellipsometric measurements were carried out with a vacuum ultraviolet variable angle spectroscopic ellipsometer (VUV-VASE, J.A. Woollam Co., Inc., Lincon, NE). Measurements were performed using a range of wavelengths (400−1700 nm) and multiple angles of incidence (70°, 75°, 80°).[29] To estimate the film thickness, analysis of the VUV data was limited to wavelengths >400 nm, due to the optical transparency of the monolayer organic film in this spectral region. The ellipsometric parameters were evaluated for both the bare clean substrate and the modified surfaces. The WVASE program (version 3.416z, J. A. Woollam) was employed for data analysis and the determination of the thickness values. The refractive index of the film was modeled with a Cauchy dispersion relationship.[30] At least five different locations in each sample were measured and averaged.

Quartz crystal microbalance (QCM) measurements were performed with a Q-sense E4 instrument (Q-Sense, Sweden) using gold "chips" that consisted of a thin quartz crystal coated with a gold layer (Q-Sense, $f = 5$ MHz, $C = 17.7$ ng cm$^{-2}$ Hz$^{1-}$, geometric area of gold working surface = 0.78 cm$^2$). Change in fundamental frequency, $\Delta f$, was converted into a mass change, $\Delta m$ (ng cm$^{-2}$), via the Sauerbrey equation.[31] All experiments were performed at a temperature of 25.00 ± 0.05 °C. Prior to each experiment, chips were cleaned in a 1:1:3 $NH_4OH$ 28%/$H_2O_2$ 30%/$H_2O$ mixture at 70 °C for 15 min, washed several times with water and ethanol, and dried under a stream of nitrogen.

SPR experiments were performed by an Eco Chemie Autolab SPR system (Ecochemie, The Netherlands) that works with a laser diode fixed at a wavelength of 670 nm. The instrument is equipped with a Teflon cuvette (diameter 3 mm). Glass supported gold substrates for SPR spectroscopy, composed of a gold sensing surface (thickness 50 nm) deposited onto a glass microscope slide with a titanium adhesion layer (1.5 nm), were purchased from Xantec Bioanalytics (Muenster, Germany).

High-resolution transmission electron microscopy (HRTEM) was performed using a JEOL 2010 electron microscope (JEOL USA Inc., Peabody, MA). Samples were prepared by placing three drops of sample solutions on carbon-coated copper grids (EM science, Gibbstown, NJ) and allowing the solvent to evaporate in air.

**Surface Modification.** *Immobilization of Hydroxyl/LC-PDP-Functionalized G4-PAMAM Dendrimer-Encapsulated Au NP onto Mixed SAM Modified Gold Surface.* The hydroxyl/LC-PDP-functionalized G4-PAMAM dendrimer-encapsulated Au NP (2.2 mg, 98 nmol) was dissolved in 1 mL of phosphate buffered saline (PBS)/ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.4, 0.1 mmol EDTA). To this solution, 0.84 mg of TCEP (2.94 μmol, 30 equiv) was added. The reaction mixture was stirred at room temperature under $N_2$ until all the conjugate was dissolved and converted to free thiolated conjugate (AuNP-G4-OH/SH). Ten microliters of the reaction mixture was diluted to 40 mL with PBS. This solution was added to the SAM-modified gold surface (see Supporting Information for more details). EMCH (100 μL; 446 pmol) in PBS (pH 7.4) was added to the modified surface and, then, was incubated for 3 h at 4 °C under constant stream of $N_2$ flow. The surface was washed with PBS and deionized water, dried under $N_2$, and stored at −20 °C.

*Insulin Immobilization to the Dendrimer-Modified Surface.* Insulin was covalently immobilized onto the AuNP-G4-OH SAM-modified gold surface, after preactivation of the carboxylic groups with a mixture containing 0.5 mM ethyl(dimethylaminopropyl) carbodiimide (EDC) and 0.1 mM *N*-hydroxysuccinimide (NHS) in PBS for 30 min. The sensor surface was incubated for 1 h with insulin at 4 °C. After being rinsed with deionized water, the substrates were blown dry under $N_2$ and used for the SPR measurements or stored at 4 °C.

**Collection of Human Serum Samples.** The performance of the reported assay in the determination of insulin concentration in healthy control subjects and diabetics patients was validated by means of a radioimmunoassay (RIA) modified competitive insulin antibody assay (CIAA).[32] Serum samples were obtained from Mr. Claudio Tiberti, Department of Clinical Science, Sapienza University of Rome. Patients and controls provided written informed consent prior to the collection of the blood samples. The employment of samples for research purposes was approved from

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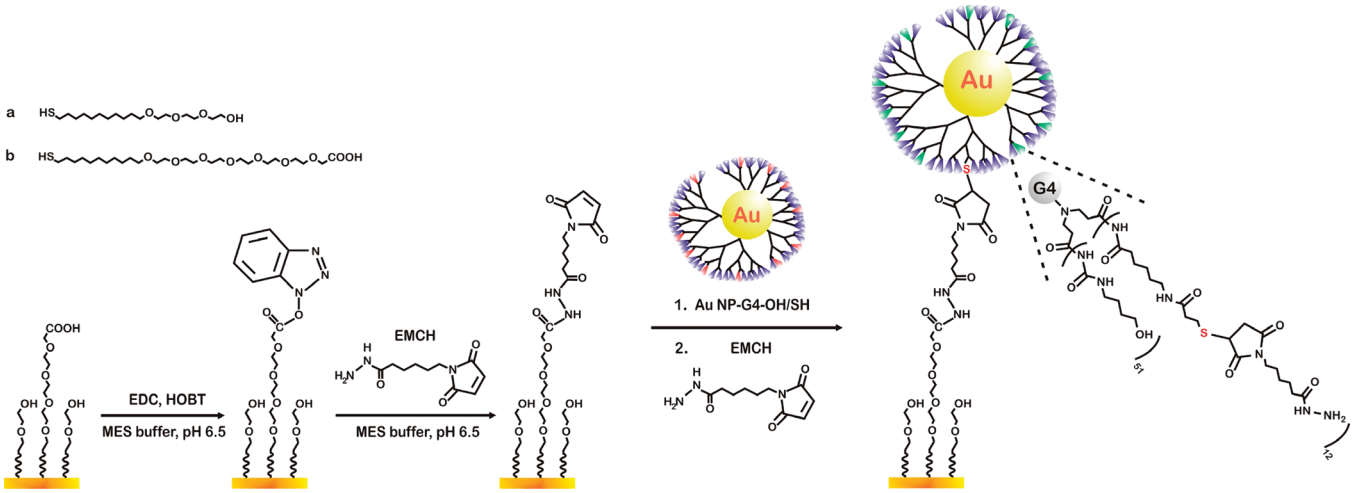
**Scheme 1. Immobilization of Hydroxyl/LC-PDP-Functionalized G4-PAMAM Dendrimer-Encapsulated Au Nanoparticle onto Mixed SAMs of Alkanethiolates on Gold Derived from Tri(ethylene glycol)-Terminated Thiol (a) and the Hexa(ethylene glycol) Carboxylic Acid-Terminated Thiol (b)**




the authority for health. The blood samples were centrifuged at 1300G for 10 min at 4 °C and stored at −70 °C until assayed.

## RESULTS AND DISCUSSION

Poly(amidoamine) G4-PAMAM dendrimers were employed as nanotemplates for the formation of inorganic−organic hybrid colloids in aqueous solution. The addition of $HAuCl_4$ to the neutral G4-PAMAM results in a protonated dendrimer with $AuCl^{4-}$ counterions, which were then reduced to metallic gold. The stable brown-red solution of the resulting colloidal gold formed indicates that the metal colloids were stabilized by the dendrimer. Furthermore, amine terminated G4-PAMAM dendrimers-encapsulated Au NPs were surface-modified with a bioactive functionality, to immobilize proteins, and a bioinert one, to minimize nonspecific adsorption of proteins. The AuNP-G4-$NH_2$ dendrimer was conjugated with the heterobifunctional cross-linking agent succinimidyl 6-(3′-[2-pyridyl-dithio]propionamido)hexanoate (LC-SPDP) through a stable amide bond to provide a protected thiol in the form of a disulfide bond for dendrimer immobilization and antibody conjugation (see Supporting Information for the detailed synthesis). On the basis of a previously reported procedure,[23b] an average of 13 disulfide groups per dendrimer was estimated. Remaining amino groups were converted to hydroxyl groups by reacting with 4-isothiocyanato-1-butanol forming stable thiourea linkages to give hydroxyl/LC-PDP-conjugated G4(Au NP). A stable colloidal solution is formed, as is observed by HR-TEM (Figure S1A, Supporting Information). The size distribution indicates that the average diameter of these particles is 1.7 ± 0.3 nm (Figure S1B, Supporting Information). The disulfide bond of LC-PDP linker was then reduced by TCEP to thiol groups quantitatively.[33]

Mixed SAMs comprising the tri(ethylene glycol)-terminated thiol, **a**, and the hexa(ethylene glycol) carboxylic acid-terminated thiol, **b**, were used for modification of sensor chip, by immersing the gold-coated substrates in solutions of mixtures of **a** and **b** at the stated mole fraction. Carboxylic acid groups of the modified surface were activated by the activation reagents (EDC/HOBT), and the resulting HOBT-activated groups were conjugated to the heterobifunctional cross-linker EMCH (Scheme 1). The maleimide groups of the SAM-modified surface were conjugated to the thiol groups in AuNP-G4-OH/SH to immobilize the dendrimer. The remaining free thiol groups of the immobilized dendrimer were activated by the heterobifunctional cross-linker *N*-(ε-maleimidocaproic acid)-hydrazide (EMCH), which was used to attach the protein. Dendrimer immobilization onto the activated SAM-modified surface was probed in situ by the SPR shift of the surface. SPR experiments evidenced that the amount of immobilized dendrimer is controlled by the composition of the mixed SAM (as the mole fraction between carboxylic acid-terminated thiol, **b**, and hydroxyl-terminated thiol, **a**, used to prepare the SAM). A low density of activated carboxylic acid on the surface induces a small amount of AuNP-G4-OH/SH dendrimer to attach onto the SAM-modified platform, resulting in a small angle shift (Figure S2, Supporting Information). As the mole fraction of carboxyl SAM increases, the change in the angle position of the minimum reflectance becomes larger, attaining the largest plasmon shift for a ratio of carboxylic and hydroxyl SAM close to 0.12, exhibiting an ellipsometric thickness of 24 ± 1 Å. The initial change in plasmon angle evolves linearly with the amount of immobilized AuNP-G4-OH/SH dendrimer; this is consistent with previously reported phenomenon indicating that SPR shift is roughly linear with Au NP coverage.[34] For comparison, the SPR curve upon the immobilization of a G4-OH/SH dendrimer, without the encapsulated Au NP, was recorded (Figure S3, Supporting Information). A significantly higher shift in plasmon angle was observed upon the immobilization of AuNP-G4-OH/SH dendrimer onto a SAM-modified gold surface; in addition, the Au NPs introduced to the surface with the dendrimers resulted in an increase in the minimum reflectance and noticeable broadening of the SPR curve, as a result of the localized coupling. To estimate the average thickness of the modified surface, ellipsometric measurements were performed. The results of theoretical modeling studies, performed with a Cauchy dispersion relationship,[30] to fit raw experimental data suggested that the thickness of the AuNP-G4-OH/SH immobilized onto mixed SAM modified gold surfaces was 29 Å. The 90% confidence limits for the calculated thickness value were determined to be ±1 Å. When compared with different

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**Table 1. Thickness Measurements of Dendrimer-Modified Surfaces, Levels of Immobilized Insulin, and Analytic Performances for Ins-Ab Determination in 0.1 M PBS (pH = 7.2)**

| | ellipsometric thickness (Å ± 1) | insulin surface coverage (ng mm$^{-2}$) | linearity range ($\mu$M) | slope (degrees $\mu$M$^{-1}$) | $r^2$ | LOD (nM) | RSD (%) |
|---|---|---|---|---|---|---|---|
| SAM | 24 | 4.06 ± 0.01 | 0.33 ÷ 4.20 | $(24 \pm 1) \times 10^{-3}$ | 0.9912 | 83 | 4.2 |
| G2-OH SAM | 26 | 6.19 ± 0.02 | 0.16 ÷ 3.30 | $(80 \pm 4) \times 10^{-3}$ | 0.9993 | 58 | 4.8 |
| G4-OH SAM | 28 | 11.45 ± 0.02 | 0.060 ÷ 1.67 | $(20 \pm 1) \times 10^{-2}$ | 0.9901 | 52 | 7.0 |
| G6-OH SAM | 33 | 13.08 ± 0.04 | 0.066 ÷ 2.08 | $(23 \pm 2) \times 10^{-2}$ | 0.9980 | 25 | 6.5 |
| Au NP-G4-OH SAM | 29 | 11.29 ± 0.03 | $(0.042 \div 8.00) \times 10^{-3}$ | 115 ± 5 | 0.9906 | $16 \times 10^{-3}$ | 5.1 |

generations of neutral PAMAM, synthesized with the same procedure reported for the AuNP-G4-OH/SH, without the encapsulated Au NP, the thickness of the modified surface increased with the generation number of the dendrimer (Table 1). The measured thickness was quite smaller than the bulk phase diameter for the G2-OH dendrimer that showed a disk-like three-dimensional structure, as opposed to spherical shapes of G4-OH and G6-OH, that fit the diameters based on modeled geometry.[35] The surface analysis performed in this study clearly indicates the formation of a dendrimer monolayer on SAM-modified gold surface as reported in literature with a different dendrimer-modified surface.[25,35] The measured value of refractive index of 1.38 obtained for the AuNP-G4-OH dendrimer is within the range of refractive index reported previously for a G4-OH dendrimer monolayer on gold ($n = 1.46$).[35]

As a first step in assessing the potential use of the sensing platform for a bioassay in complex biological fluids, the ability of AuNP-G4-OH SAM to minimize nonspecific adsorption of proteins was evaluated. Figure 1 shows the sensorgrams obtained when a solution containing a mixture of eight proteins having a wide range of weight and isoelectric points was injected over a mixed SAM with a ratio of carboxylic acid and hydroxyl-terminated thiol of 0.12, a mixed SAM (with a ratio of 0.12) with covalently immobilized ethanolamine, AuNP-G4-OH SAM, and AuNP-G4-$NH_2$ SAM-modified gold surfaces. Procedures for reaction of the active ester with

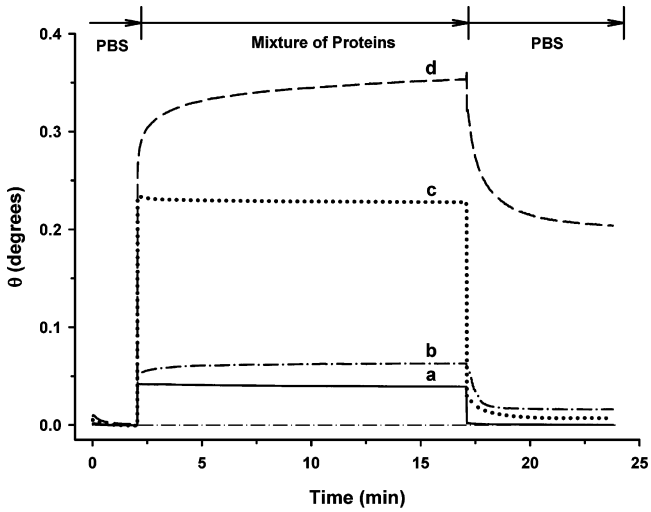


**Figure 1.** SPR sensorgrams for the nonspecific adsorption of a mixture of proteins to different modified surfaces: ethanolamine-derivatized SAM-modified surface (a), underivatized SAM-modified surface (b), AuNP-G4-OH SAM-modified surface (c), and AuNP-G4-$NH_2$ SAM-modified surface (d). The mixture of proteins used in this experiment contained bovine serum albumin, lysozyme, trypsin, ribonuclease A, aldolase, fibrinogen, ovalbumin, and superoxide dismutase, each at a concentration of ~1.0 mg mL$^{-1}$ in PBS.

ethanolamine were similar to those used for proteins and other ligands. For the mixed SAM derivatized with ethanolamine, a fast increase in the angle shift ($\theta$), that reflects differences in bulk refractive index between the solution of proteins and the buffer, was observed (Figure 1, curve a). When the protein solution was replaced by buffer, there was a fast drop in the value of $\theta$ back to its original value (within 30 s), i.e., before injection of proteins. This result suggests that there was no observable nonspecific binding to this neutral SAM. For the underivatized SAM, we observed a slight increase in the SPR sensorgram and a small amount of nonspecific adsorption (c.a. 0.015) after replacing the solution of proteins with buffer (Figure 1, curve b). This nonspecific binding could be ascribed to electrostatic interactions between the positively charged proteins in the mixture (e.g., trypsin and lysozyme) and the carboxylate groups.[36] For the AuNP-G4-OH SAM, the increase in the value of $\theta$ was greater than that observed for the mixed SAM (Figure 1, curve c); after protein injection, the shift in the SPR spectrum is the result of the changes in the dielectric properties adjacent to the Au NPs, perturbing the coupling of the localized plasmon of the NPs with the surface plasmon wave.[14a] After replacing the protein solution with buffer, the net SPR angle shift was very little (ca. 0.007°), indicating a very small nonspecific binding of proteins due to the presence of a few bioactive amine groups on the dendrimer surface. Adsorption of proteins onto AuNP-G4-$NH_2$ SAM revealed that the AuNP-G4-OH SAM surface has at least 30-fold less nonspecific protein adsorption than the amine dendrimer slide (Figure 1, curve d). This finding limits the use of amine-terminated G4-PAMAM-$NH_2$ dendrimer monolayer in protein array applications.

To demonstrate the efficiency of our sensing platform to immobilize biorecognition molecules in the construction of an affinity biosensor, an insulin competitive immunosensor was assayed as a model system. Insulin was covalently immobilized onto the AuNP-G4-OH SAM-modified gold surface. Quartz crystal microbalance (QCM) analysis revealed an insulin binding level of 11.29 ± 0.03 ng mm$^{-2}$. Taking into account that the expected surface coverage for a monolayer of insulin, calculated using the radius of gyration of the protein in solution (1.16 nm),[37] is 6.82 ng mm$^{-2}$, a ~2 fold enhancement of insulin immobilization was obtained using the dendrimer modified surface. This greater protein immobilization efficiency could be ascribed to a net increase in the total surface-accessible area that accompanies the binding of the dendrimers to the sensor surface and the particular three-dimensional steric arrangement of the

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reactive groups available for covalent coupling present on the surface of each dendrimer. The insulin surface coverage does not change appreciably onto the G4-OH dendrimer surface (without the encapsulated Au NP). In addition, different concentrations of surface loading were observed on various generation dendrimers as well as on a SAM control surface (Table 1). The increase in insulin coverage, as evidenced by QCM analysis and SPR sensorgram (Figure S4, Supporting Information), is consistent with the increase in available linker groups on the surface as the dendrimer generation increases.

Figure 2A depicts the sensorgrams, after blank subtraction (nonspecific binding control), for the binding affinity interaction between anti-insulin antibody (Ins-Ab), at various concentrations, and insulin. A rapid increase in the SPR angle shift was observed as soon as the Ins-Ab solution began to flow over the insulin-immobilized AuNP-G4-OH SAM-modified surface. After the equilibration time (about 10 min), the following washing step (with PBS) resulted in an almost stable resonant angle shift; the small decrease of the signal was ascribed to the dissociation of the excess of nonbounded antibody, implying on false response on the sensor. Thus, the sample analysis time of 10 min with a flow rate of 50 $\mu$L min$^{-1}$ was chosen for further immunoassay experiments. The SPR response increased with increasing antibody concentration up to 12.5 nM, with a Ins-Ab detection limit of 16 pM (Figure 2A, inset).

A further aspect relates to the effect of Au NP encapsulated in the dendrimer on the amplified detection of Ins-Ab. Toward this end, the association of Ins-Ab to an insulin-immobilized G4-OH SAM-modified surface was followed by probing the SPR angle shift at different concentrations of Ins-Ab. Clearly, the signal changes observed in the presence of Au NP are significantly higher (Figure 2A, inset). One may realize that the insulin-immobilized G4-OH SAM-surface detects Ins-Ab at a 25 nM concentration, implying a $10^3$ amplification factor of the insulin-immobilized AuNP-G4-OH SAM-modified surface. The analytical performances for the Ins-Ab detection for different dendrimer generation-modified surfaces were evaluated (Table 1). It can be noted from Figure 2B that the sensitivity toward Ins-Ab detection increases for the G2-OH and G4-OH. No difference in measured detection limits observed for different loading densities of immobilized insulin between G4-OH and G6-OH surfaces suggests that the steric hindrance between neighboring insulin molecules on the G6-OH dendrimer limits the interaction with the antibody.

In a control experiment, injection of anti-insulin antibody at different concentrations over the AuNP-G4-OH SAM-modified surface (no insulin immobilized) resulted in a slight increase in the SPR signal with time (Figure S5, Supporting Information). When the solution of antibody was replaced by buffer, a fast drop in the value of the $\Delta\theta$ back to its original value was observed, suggesting that there was no observable nonspecific binding to the dendrimer-modified surface.

Reusability of the same sensor chip is an important aspect for multiple and cost-effective analysis. For this aim, antibody bound to the sensor chip should be removed completely without affecting the insulin-modified surface. The treatment of the insulin-bound sensor with 10 mM glycine−HCl (pH 3.0) and NaCl 0.1 M was demonstrated to be highly effective for this purpose; rinsing with buffer quickly established the original level before the antibody

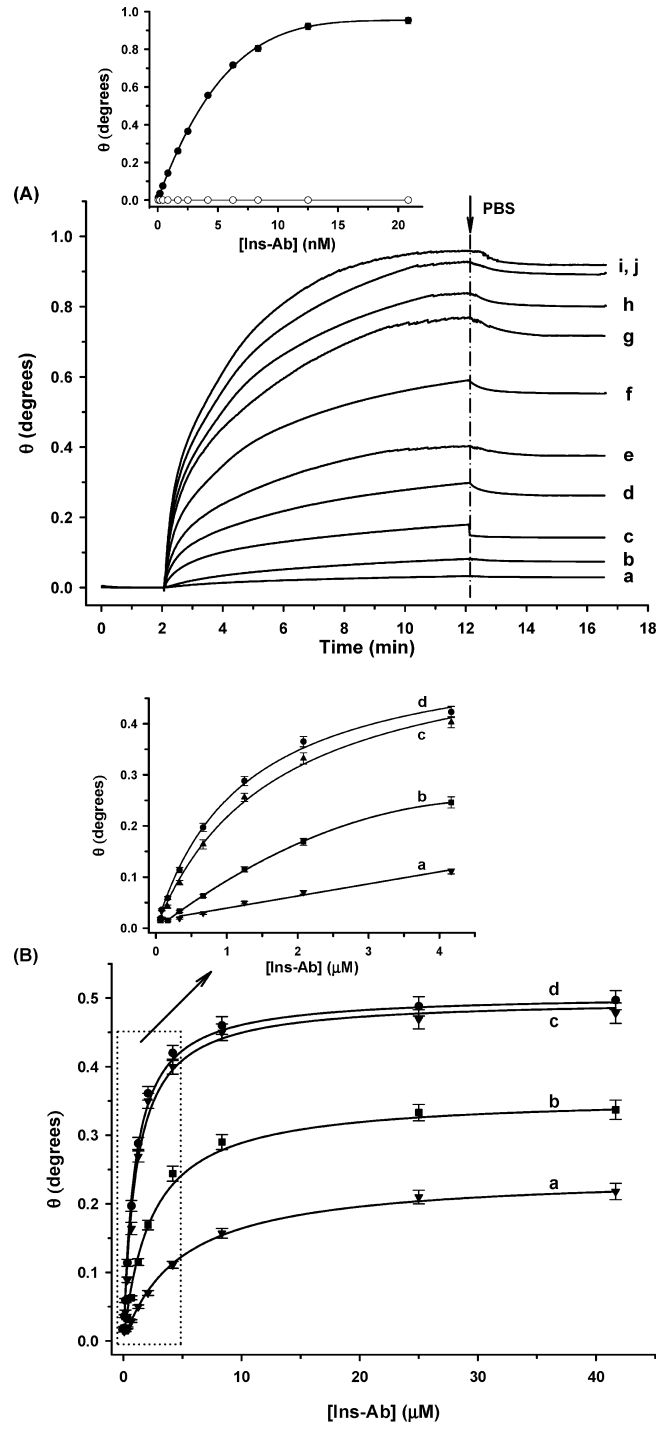


**Figure 2.** (A) SPR sensorgrams obtained after blank subtraction for the detection of the affinity interaction between insulin, immobilized on AuNP-G4-OH and variable concentration of Ins-Ab (nM): 0.2 (a), 0.4 (b), 0.8 (c), 1.6 (d), 2.5 (e), 4.2 (f), 6.2 (g), 8.4 (h), 12.5 (i), and 21.0 (j). Inset: Calibration curves corresponding to the SPR angle shift at different concentrations of added Ins-Ab on the insulin immobilized onto AuNP-G4-OH (close symbols) and G4-OH (open symbols) SAM modified surfaces. (B) Calibration curves corresponding to the SPR angle shift at different concentrations of added Ins-Ab on the insulin immobilized onto SAM-modified surface (a), G2-OH SAM-modified surface (b), G4-OH SAM-modified surface (c), and G6-OH SAM-modified surface (d). Inset: Lower concentration region of the calibration curves. All measurements were performed in 0.1 M phosphate buffer solution (pH = 7.2). Error bars are standard errors of the mean with $N = 5$.

injection, confirming the regeneration of insulin surface (Figure S6, Supporting Information). It was found that a single insulin-immobilized AuNP-G4-OH SAM-modified surface could be effectively used for more than 40 regeneration cycles without a significant loss in sensor performance (less than 5%).

For the indirect competitive immunoassay of insulin, standard insulin solutions were mixed with an optimal concentration of Ins-Ab and the mixtures were allowed to interact with an insulin-immobilized AuNP-G4-OH SAM-modified surface. The SPR angle shift, which corresponds to the binding of a fixed concentration of Ins-Ab (0.8 nM) on the sensor surface, decreases with increasing concentration of insulin (0.5−86 pM). The low-detection-limit for analyzing insulin corresponded to 0.5 pM, and the determination range was 2−43 pM (Figure S7, Supporting Information). For comparison, the direct immunoassay of insulin was evaluated. SPR sensorgrams of Ins-Ab immobilized AuNP-G4-OH SAM-modified surface upon the interaction with variable concentration of insulin were recorded (Figure S8, Supporting Information). A comprehensive fitting analysis for the interaction of insulin with Ins-Ab was performed with different kinetic models: 1:1 binding, bivalent analyte binding, and two state reaction with conformational change. The standard deviation of the residuals of the global fitting analysis suggested that the best fitting model is the one assuming a 1:1 binding model with mass transport limitation (see Table S2, Supporting Information). The dissociation binding constant ($K_D$) and the forward rate constant ($k_a$), obtained from the kinetic analysis of the association phase, performed according to a predefined model (see Supporting Information for more details), were 1.6 $\mu$M and $5.8 \times 10^3$ L mol$^{-1}$ s$^{-1}$, respectively. The obtained dissociation constant falls very close to the value reported in literature for the interaction of human insulin with Ins-Ab evaluated from the microfluidic binding assay ($K_D = 0.8$ $\mu$M).[38]

The calibration curve, corresponding to the SPR angle shifts for the direct binding affinity between insulin, at various concentrations, and Ins-Ab, gave a detection limit of 8.5 nM (Figure S9, Supporting Information). The Ins-Ab immobilized G4-OH SAM-modified surface provided a detection limit for analyzing insulin corresponded to 0.85 $\mu$M. The higher sensitivity obtained for the AuNP-G4-OH SAM-modified surface, demonstrated that the detection of insulin was amplified as a consequence of the changes in the dielectric properties of the matrixes, occurring upon the biorecognition processes on the sensor surface, through the coupling of the localized plasmon of the NPs with the surface plasmon wave. Nonspecific binding control experiments, performed by injection of insulin at different concentrations over the AuNP-G4-OH SAM-modified surface, showed almost no response to added insulin.

In order to assess the accuracy and applicability of the present method for clinical analysis, a control human serum sample was spiked with a high concentration of insulin standard solution. The high sensitivity of the AuNP-G4-OH SAM-modified surface and the blood levels of insulin in patients affected with diabetes, insulinoma, and hypoglycemia[1] (85−500 pM) suggest the possibility to perform a preliminary dilution of the human serum samples, in order to minimize the nonspecific adsorption phenomena. Accordingly, the control human serum sample was 10-fold diluted in phosphate buffer solution; no remarkable SPR angle change was observed by adding it on the insulin-immobilized AuNP-G4-OH SAM-modified surface (Figure S10, Supporting Information). In addition, nonspecific binding control experiments, obtained by injection of different concentration of insulin in 10-fold diluted serum over the dendrimer-modified surface, showed only a slight increase in the SPR signal (Figure S11, Supporting Information). The control human serum sample is treated as a blank serum for insulin detection. In fact, complementary internal standard addition experiments performed by means of competitive immunoassay confirmed that the insulin concentration in the control human serum was below the detection limit of the present method. Treatment of insulin-modified surface with 10-fold dilute serum samples containing different concentrations of insulin (1−86 pM) in the presence of fixed concentration of Ins-Ab (0.8 nM) yielded the SPR angle response depicted in the sensorgrams reported in Figure 3. After the flow of serum samples, the buffer solution was allowed to flow and the SPR angle shift obtained at the end of the buffer injection was analyzed. From the respective calibration curve, we realize that an insulin concentration as low as 0.8 pM is detectable in the diluted human serum sample and the response observed for insulin in these serum samples was nearly equivalent to that observed for insulin in PBS, with a linear range of 2−43 pM (Figure 3, left inset). The biological level for insulin in the diabetic patients is 85−500 pM, a value that corresponds to a concentration of 8.5−50 pM in a 10-fold diluted sample. Thus, our sensor system reveals the sensitivity to detect the insulin in diabetic patients as well as lower levels of insulin. Also, it should be noted that the analysis of variable concentrations of insulin revealed good precision with relative standard deviation (RSD) of 5.6%. The intra-assay variability was determined by calculating the coefficient of variation (CV) between the duplicates within each run, for each dilution concentration. The obtained values ranged between 2.6 and 6.1% in each of five independent assay runs. The interassay variability was determined by calculating the CV between values obtained in five independent assay runs. The obtained CVs ranged between 3.5 and 4.9%, indicating a good reproducibility. Consequently, the sensor was evaluated on real serum samples, and the results were compared with those obtained with RIA. The insulin levels of nine serum samples from diabetic patients and healthy control subjects were tested (Table S3, Supporting Information). The data obtained with our assay are in good agreement with those obtained with the reference method. The scatter of the data gives a good correlation of $r = 0.995$ (Figure S12, Supporting Information). Therefore, the multifunctional Au NP dendrimer-based SPR immunosensor can provide a sensitive assay for diagnostics in clinically relevant samples.

The specifity of the designed insulin immunosensor was evaluated by cross-reactions of the immunosensor with other kinds of nonspecific peptide hormones. In our research, TSH, FSH, and LH solution were individually added into the human serum samples. From the SPR angle responses of insulin-immobilized AuNP-G4-OH SAM-modified surface upon analysis of insulin, 8.5 pM (Figure 3, right inset, column a), and upon addition of different concentrations of TSH, FSH, or LH to the insulin solution, any significant effect of the nonspecific hormones on the signal was observed.

## CONCLUSIONS

In the present paper, we have presented an alternative SPR biosensor with high stability and improved sensitivity based on Au NPs encapsulated in hydroxyl/thiol-functionalized G4-PAMAM

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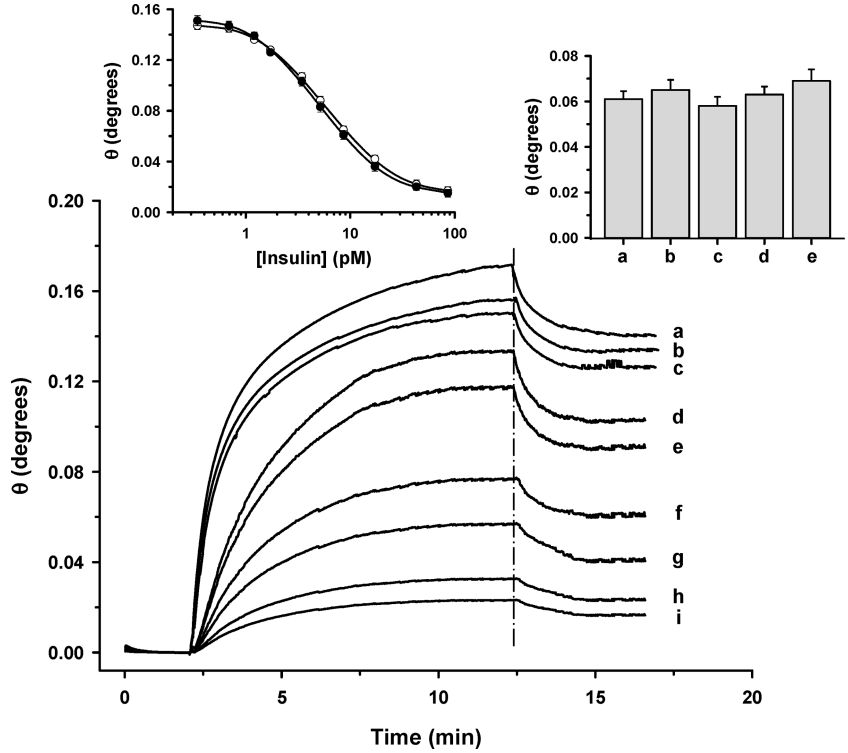


**Figure 3.** (A) SPR responses to the binding of Ins-Ab at 0.8 nM over the insulin-immobilized AuNP-G4-OH SAM modified surface, in the indirect competitive immunoassay, in human serum sample diluted 10-fold with 0.1 M phosphate buffer solution (pH = 7.2) and containing various concentrations of insulin (pM): 0.0 (a), 1.0 (b), 2.0 (c), 3.5 (d), 5.0 (e), 8.5 (f), 17.0 (g), 43.0 (h), and 86.0 (i). The SPR sensorgrams were obtained after blank subtraction. Left Inset: Comparison of SPR angle shift versus insulin concentration in the indirect competitive immunoassay experiments performed in 0.1 M phosphate buffer solution (pH = 7.2) (open symbol) and in human serum sample (closed symbol). The human serum sample was diluted 10-fold in 0.1 M phosphate buffer solution (pH = 7.2). Right Inset: SPR angle shift to the binding of Ins-Ab at 0.8 nM over the insulin-immobilized AuNP-G4-OH SAM modified surface, in human serum sample diluted 10-fold with 0.1 M phosphate buffer solution (pH = 7.2) and containing (a) 8.5 pM insulin, (b) 8.5 pM insulin and 85 pM TSH, (c) 8.5 pM insulin and 85 pM FSH, (d) 8.5 pM insulin and 85 pM LH, and (e) 8.5 pM insulin, 85 pM TSH, 85 pM FSH, and 85 pM LH. Error bars are standard errors of the mean with $N = 5$.

dendrimers immobilized onto maleimide-terminated OEG SAMs. The resulting Au NPs dendrimer-modified surface is a good support for insulin immobilization and enhanced surface density, and an excellent binding affinity toward the antibody was observed. The immunosensor system based on the indirect competitive immunoassay principle showed high sensitivity and specifity for detection of insulin in human serum sample. The assays were validated using human serum samples from healthy control and diabetic patients, and the results, compared with a radioimmunoassy reference method, confirmed the efficiency of the proposed SPR-based immunosensor in real sample analysis. Comparing different sensor systems for insulin detection, the proposed multifunctional Au NPs dendrimer-based SPR immunosensor presents a higher sensitivity for analyzing insulin (Table S4, Supporting Information). The sensor with comparable sensitivity involves a modified electrode and an electrochemical detection. Although the modified electrodes have been successfully employed for monitoring the insulin, they usually have many disadvantages such as low reproducibility and stability under physiological condition. The efficacy of our sensor surface to reduce nonspecific interactions caused by bulk proteins in complex analytical matrixes was also demonstrated. In conclusion, our approach opens up a new route to develop SPR biosensing platforms with high-density functional sites with low nonspecific protein adsorption and capturing of analytes from complex biological media for label-free SPR biosensors.

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## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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