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Original Covalent Approach for Gold Nanorods Immobilization onto Acid-Terminated-Cysteamine Self-Assembled Monolayers for FT-SPR Biosensor Applications

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Abstract

In this paper, we report an original method to immobilize gold nanorods onto mixed self-assembled monolayers (SAMs) of Mercaptoundecanoic Acid (MUA), Mercaptohexanol (MOH) and cysteamine (CYS) onto planar gold surface. Polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images revealed a remarkable shape and size narrow distribution on well functionalized gold surfaces, as well as a tendency to form linear assemblies after immobilization. The results highlight the good distribution of gold nanorods with an average length of 32.6 ± 0.9 nm and width of 13 ± 1.8 nm, the simplicity of the immobilization procedure of gold nanorods and the interest of using them as labels to enhance the sensitivity of FT-SPR-based sensors. gold nanostructured surface FT-SPR measurements of biorecognition using gold nanorods immobilized onto gold surfaces were performed at various prostatic antigen specific (PSA) concentrations, from 5 mg/L to 0.5 mg/L reaching a system sensitivity of 37 ± 2 cm^{-1}/mgL^{-1}.

Keywords: Gold nanorods; SAMs; FT-SPR; Biosensors

Introduction

Antibody immobilization is an important subject with a variety of purposes such as in diagnostic immunoassays. One of its applications is the immunosensor, a biosensor made by immobilizing an antibody monolayer onto a transducer, a support able to convert signals allowing specific antigen biorecognition, which helps to make the sensor more accurate, precise, and reproducible [1].

The formation of organized monolayers on surface by self-assembling is interesting in an increasing number of fields as surfaces nanostructuration, chemistry, biology and molecular engineering [2,3]. Self-assembling monolayers (SAMs) offer the possibility to modify the terminal functions of thiol-chains to bind any type of ligands by covalent, ionic or hydrogen interactions [4]. Various aspects of surface modification procedures, such as choice of surface material, types of assembling molecules, physical organic properties of the formed layers, have long been studied from pure scientific interest [5]. To date a number of strategies have been adopted to assemble metal/semiconductor nanospheres onto planar surfaces. Some methods include organization of nanospheres via simple solvent evaporation [6], attachment onto SAMs via covalent interactions and by Langmuir-Blodgett (LB) technique [7,8]. Immobilization of nanorods, surprisingly, has not received comparable attention. El Sayed [9] have demonstrated that simple solvent evaporation leads to the organization of gold nanorods into one, two, and three-dimensional structures. Others have recently demonstrated a spontaneous self-assembly of gold nanorods in concentrated solutions to produce liquid crystalline arrays [10]. Most of the above-mentioned methods use either nonspecific interactions [11,12] or specialized techniques for the organization of nanorods onto 2D surfaces. A simple method for programmated assembly of nanorods using covalent interactions is yet to be investigated.

SPR has the potential to be a useful technique due to real-time measurements, simplicity of measurement, and possible on-site testing. One of the most useful applications of AuNPs in SPR sensing is the improved detection of small molecules. The improvement has been shown using both unlabelled and labelled techniques incorporating spherical AuNPs [13] or Au nanorods [14,15]. FT-SPR is an SPR-derivatized technique based on the coupling between the incident light and the gold surface Plasmon waves. Conversely to "classical" SPR, which measures changes in the angle of minimum reflectivity, FT-SPR is operated at constant angle, and measures changes in the wavenumber corresponding to the minimum of reflectivity [16]. The interface of a Fourier Transform infrared (FT-IR) spectrometer to an SPR setup provides the excitation light energy and readout detecting adsorption at the solid-liquid interface. Measuring changes in reflectivity as a function of the wavenumber, in the near-infrared (NIR) spectral region, the Fourier Transform Surface Plasmon resonance (FT-SPR) instruments provide quantitative information on the refractive index variation, and thus on molecular binding on a functionalized gold surface [17].

Prostate Specific Antigen (PSA) is prostate cancer marker [18]. In order to early diagnose the prostate cancer in men, studies on development of an ultra-sensitive diagnostic tool are currently performing [19]. Dedicated centralized laboratories use large, automated analyzers, requiring sample transportation, increasing

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waiting times and medical costs for PSA detection. Near-patient or point-of-care testing (POCT) are needed in order to reduce the number of clinic visits, decrease costs to the patient and the healthcare system, increase patient satisfaction and improve clinical outcome. Biosensor development, based on nanoparticles and nanostructures integrating on different devices, have brought POCT for PSA detection closer to reality. A PSA measurement above a cut-off value of 4.0 ng/ml is generally regarded as positive and might indicate the need for a biopsy [20]. Commercial SPR biochip with signal enhancement using a sandwich assay format are currently available and have a limit of detection of 18.1 ng/ml [21].

In this paper, we have evaluated a simple and reproducible method for labelling biomolecules with gold nanorods onto gold surface without utilizing organic solvents, but relying on electrostatic interactions between the positively charged end-groups of Cetyltrimethylammonium bromide (CTAB) and negatively charge of antibody. Gold nanorods synthesis and immobilization were furthermore investigated combining Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). Anti-PSA was then covalently grafted onto well immobilized gold nanorods and the recognition of PSA was essayed by Polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) and Fourier Transform Surface Plasmon Resonance (FT-SPR) measurements.

Matherials and Methods

Reagents

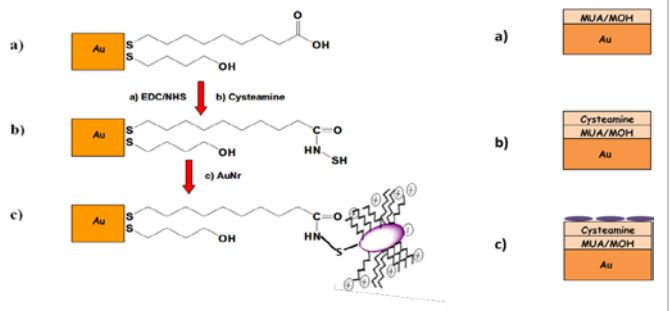
Tetrachloroauric Acid ($HAuCl_4$), Cetyltrimethylammonium bromide (CTAB), sodium borohydride ($NaBH_4$), silver Nitrate ($AgNO_3$), Ascorbic Acid, 1-Mercapto-11-undecanoic acid (MUA), 1-mercapto-6hexanol (MOH), N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-N'-ethylcarbodiimidehydrochloride (EDC), DMF(Dimethylformammide for molecule biology 99%), cysteamine (CYS), ethanol (Normapur 99%), buffer solution (K+phthalate pH: 9; PBS pH: 7), Monoclonal Antibody-Prostatic (anti-PSA), Prostatic Antigen Specific (PSA) and Bovine Serum Albumine (BSA) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). All chemicals were used as such without further purification. Milli Q water was used throughout the experiments. Gold substrates for FT-SPR measurements were deposited at the Institute IMM-CNR in Lecce (Italy).

Synthesis of Au nanorods (AuNr)

Nanorods were made and purified following the well-established seed-mediated procedure previously described [14]. Gold seeds solution stabilized by CTAB, were prepared by reduction of Au_4^+ (0.01 M; 5 mL) ions by ice–cooled $NaBH_4$ (0.01 M; 0.6 mL) in the presence of CTAB (0.20 M; 5 mL). After 4 h seed solution was added to growth solution containing CTAB (5 mL; 0.20 M), HAuCl4 (5 mL; 1×10^{-3} M), $AgNO_3$ (0.25 mL; 4×10^{-3} M) and Ascorbic Acid (70 µl; 8×10^{-3} M). The resultant solution was kept in dark for 24 h. The as-prepared solution was centrifugated at 11.000 rpm for 26 min for three times and then the supernatant was discarded and the residue was redispersed in an equivalent amount of buffer solution (PBS pH: 7). This was repeated twice principally to remove excess of CTAB. Stock solutions were stored at 27-29°C and characterized using UV-Vis spectroscopy and transmission electron microscopy (TEM).

Chemical immobilization procedure

The schematic representation of the chemical strategy is depicted in Scheme 1.



Scheme 1: Chemical fictionalization of gold nanorods on planar gold surface by lateral view (left) and by frontal view (right): a) SAMs of MUA/MOH; b) activation by EDC/NHS and binding of cysteamine; c) binding of Gold Nanorods.

SAMs formation: Chemistry procedures based on SAMs of thiol (MUA/MOH 1/3) in absolute ethanol have been described previously [22]. Briefly Au slides were immersed in 10^{-3} M ethanolic solution of MUA/MOH 1/3 for a period of 18 h under stirring. After this time, the slides were rinsed in ethanol, dried in flowing nitrogen, and characterized by PM-IRRAS spectroscopy.

EDC/NHS activation and cysteamine linkage onto SAMs film: For immobilization of ammine group of cysteamine (CYS) via carbodiimide binding method, the carboxyl groups of MUA on the surface were activated with a 50 mM NHS and 200 mM EDC solution. After 2 h under stirring the activating solution was removed, the substrates washed and dried under nitrogen and used immediately for CYS immobilization. For this purpose, the gold substrate was immersed in an unstirred 10 mM ethanol solution of CYS at room temperature, in the dark, for 6 h. The gold substrate was then washed with ethanol and milli Q water to remove the excess of thiol and characterized by PM-IRRAS spectroscopy.

Covalent Immobilization of gold nanorods onto cysteamine SAMs films: The SAMs coated gold slides were immersed into the gold nanorods solution for a period of 6 h under stirring. Thereafter, the films were rinsed in deionized water to remove unbound gold nanorods and dried in flowing nitrogen. These nanorods films were used for all further characterization.

Antibody interaction: Antibody anti-PSA (5 mg/L in buffer solution) was deposited on the nanostructured surface. After 1 h, the surface was rinsed with buffer solution then milliQ water for 10 min. To assure the specificity of interaction monitoring, a buffer solution of BSA (5 mg/L) was then used to block the potential residual reacting sites. After 40 min, BSA solution was removed by washing with buffer, pure H_2O and dried under nitrogen. Specific antigen interaction was then evaluated using a buffer solution containing PSA (5 mg/L) during 1 h followed by rinsing and drying with the same procedure. The as prepared nanostructured surfaces were analysed by PM-IRRAS and FT-SPR at each step of interaction and the showed results are the average of at least three measurements. As control test, a gold surface functionalized by SAMs and anti-PSA without step of gold nanorods coating was used for PSA biorecognition monitoring. In order to evaluate the sensitivity of the system, antigen interaction was then evaluated using a buffer solution containing PSA at different concentrations (0.5, 2.5, 5, 10, 20 mg/L) during 1 h followed by rinsing and drying with the same procedure. Each result here represented is the average of at least three measurements.

Instrumentation

UV-Vis spectrophotometer: All the absorption spectra reported in this work have been recorded by using a double-beam Varian Cary 500 UV-Vis spectrophotometer. UV absorption spectra of the solution of gold nanorods (AuNr), as synthesized, and after interaction with proteins in buffer solution, were recorded in the 400-1000 nm spectral range.

Transmission electron microscopy (TEM): TEM measurements were performed with a JEOL JEM 1011 microscope operating at an accelerating voltage of 100 kV. The TEM graphs were taken after separating the surfactant from the metal particles by centrifugation. Typically, 1 mL of the sample was centrifuged for 21 min at a speed of 11000 r/min. The upper part of the colourless solution was removed and the solid fraction was re-dispersed in 1 mL of buffer solution (PBS pH: 7). 2 µL of this re-dispersed particle suspension was placed on a carbon-coated copper grid and dried at room temperature.

Scanning electron microscopy (SEM): SEM images were obtained using a SEM FEG Hitachi SU-70 scanning electron microscope with a low voltage of 1 kV and distance of 1.5-2 mm; the secondary electron detector "in Lens" was used.

Polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS): PM-IRRAS spectra were recorded on a commercial Thermo (Les Ulis- France) Nexus spectrometer. The external beam was focused on the sample with a mirror, at an optimal incident angle of 80°C. A ZnSe grid polarizer and a ZnSe photoelastic modulator, modulating the incident beam between p- and s-polarizations (HINDS Instruments, PEM 90, modulation frequency=37 kHz), were placed prior to the sample. The light reflected at the sample was then focused onto a nitrogen-cooled MCT detector. The presented spectra result from the sum of 128 scans recorded at a 8 cm−1 resolution.

Fourier transform surface plasmon resonance (FT-SPR): SPR substrates were prepared starting from 25 × 25 mm^2 SF10 slabs; then a Ti/Au (2 nm/50 nm) metallic multilayer was deposited on the slabs by e-beam evaporation. The presence of Ti layer was only for adhesion purposes. Then, the substrates were annealed in air at 200°C in order to obtain the optimum conditions for antigen immobilization in terms of the best surface roughness and proper (111) crystallographic orientation. As-prepared gold-coated substrates were first cleaned for 10 min in a boiling solution consisting of H_2O_2 (30%) and milliQ water (1:5 ratio). After cleaning, the chips were thoroughly washed with milliQ water, left in ethanol for 1 h and dried under a stream of nitrogen.

FT-SPR measurements were performed with an SPR 100 module from Thermo equipped with a flow cell mounted on a goniometer. It was inserted in a Thermo-scientific Nexus FT-IR spectrometer using a near-IR tungsten halogen light source. The incidence angle was adjusted to have the minimal reflectivity located at 9000 cm^{-1}, at the beginning of each experiment, so as to be in the best sensitivity region of the InGaAs detector. Immobilization of monoclonal mouse antibodies against rabbit PSA (anti-PSA; 5 mg/L), on the previously functionalized gold surface, was carried out in the test chamber (10 µl/min; T: 27°C).

Results and Discussion

The seed-mediated growth is the most popular method for the synthesis of colloidal AuNr due to the simplicity of the procedure, high quality and yield of nanorods, ease of controlling particle size, and flexibility for further chemical modifications [14,23]. Figure 1 displays the localised surface plasmon resonance (LSPR) bands of AuNr with a strong resonance band at around 708 nm corresponding to the longitudinal plasmon oscillation [24] and a weaker one at ca 510 nm corresponding to the transverse plasmon oscillation band confirming the presence of elongated AuNr isolated from each other. Figure 2 shows TEM investigation that reveals good dispersion in size and shape of nanorods with an average length of 32.6 ± 0.9 nm and width of 13 ± 1.8 nm, estimated from ca.100-350 rods. The immobilization of AuNr onto planar gold surface using by SAMs functionalization, was performed by PM-IRRAS providing to identification of chemical groups onto gold planar surface after each step of functionalization. Figure 3 (spectrum a) was obtained after acid-terminated thiolates MUA/MOH immobilization (scheme 1a) where it is observed the absorption bands of the symmetric υCOO^- and υCH_2 around 1543 cm^{-1} and also the υCOOH at 1673 cm^{-1}. Figure 3 (spectrum b) shows the spectra of the surfaces modified by EDC/NHS (scheme 1b) that reveals a strong absorption at 1760 cm^{-1} due to ν C=O in ester functions confirming the activation of -COOH of MUA molecules. Figure 3 (spectrum c) after CYS modification (scheme 1b) exhibits C-N stretch and NH deformation modes at 1640 cm^{-1}, respectively and an intense NH deformation mode at 1550 cm^{-1}. Figure 3 (spectrum d) was obtained after AuNr immobilization (scheme 1c) highlighting the nature of interaction between AuNr and SAMs coated gold planar surfaces: a $C-N^+$ absorption can be seen at 1230 cm^{-1} close to the one

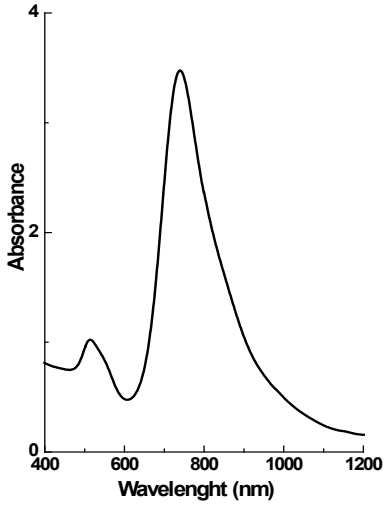


Figure 1: UV-Vis spectra of gold nanorods (λ max=708 nm, black line).

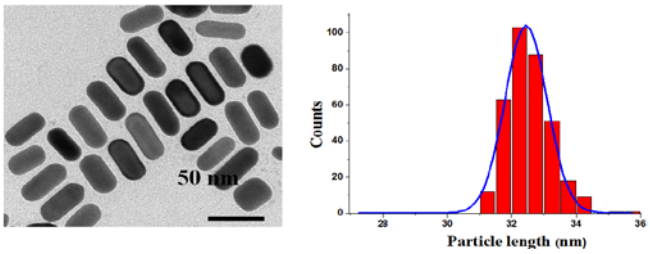


Figure 2: TEM images of gold nanorods (left)and particle size distribution (right).

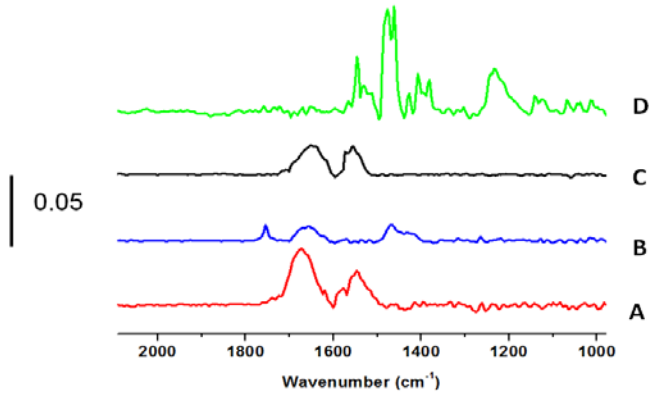


Figure 3: PM IRRAS spectra recorded after MUA/MOH treatment (a), EDC/NHS activation (b), Cys immobilization (c), gold nanorods incubation (d).

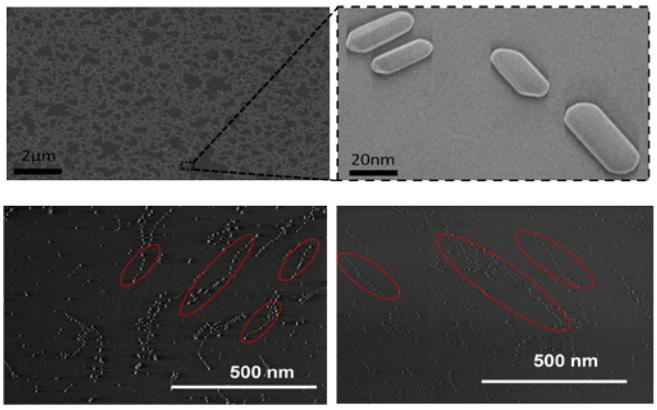


Figure 4: SEM images at three magnifications of gold nanorods, after immobilization on to a planar gold surface by SAMs approach.

reported by Nikobakht and El-Sayed, indicating the presence of CTAB-coated AuNr [25]. New bands centered at 1390 and 1468 cm^{-1} and a very weaker peak at 1532 cm^{-1} appear corresponding to the carboxylate asymmetric stretch modes [26]. These findings indicate formation of a binding between the ammonium head group of CTAB-coated gold nanorods with the cysteamine modified-carboxylic acid groups of the SAMs. Figure 4 shows SEM characterization where it is shown AuNr well aligned on the SAMs surface due to linkage descript above. Thanks to the findings, it is deducible that immobilization of AuNr via covalent interactions by SH of cysteamine layer induce order on nanorods immobilization on gold planar surfaces.

In order to investigate the ability of the as immobilized AuNr onto gold surface a set of PM-IRRAS data taken after proteins interaction where also acquired (Figure 5). After treatment by anti-PSA solution, the onset of intense amide I and II bands at 1650 and 1550 cm^{-1}, confirms the successful immobilization of antibody (Figure 5 spectrum a). When this antibody-terminated layer was submitted to BSA adsorption, its spectroscopic signature was unchanged (Figure 5 spectrum b) (amide band area equal to 14.8 ± 0.2 a.u. and 14.6 ± 0.2 a.u. for anti-PSA and anti-PSA + BSA, respectively), indicating that no significant amount of BSA molecules were adsorbed. Finally, the interaction with PSA solution led to a considerable increase of amide band area (Figure 5 spectrum c) evidencing the efficient recognition of the target by the sensing layer (amide band area 20 ± 2 a.u for anti-PSA + BSA+ PSA).

Furthermore, PSA biorecognition was investigated using AuNr coated on gold surface by SAMs procedure tested in this work (Figure 6 A black line and B line II) in comparison with a gold surface not coated with AuNr (Figure 6 A red line and B line I), were then monitored by FT-SPR measurements. At time 20 min, a solution of anti-PSA (5 mg/L) was injected for 54 min; then a buffer solution (K+ phthalate pH 9) [14] was flowing to remove any weakly bound antibody yielding a FT-SPR wavenumber value of about 8395 cm^{-1} (primary response). At time 55 min, a solution of bovine serum albumin (BSA; 5 mg/L) was injected

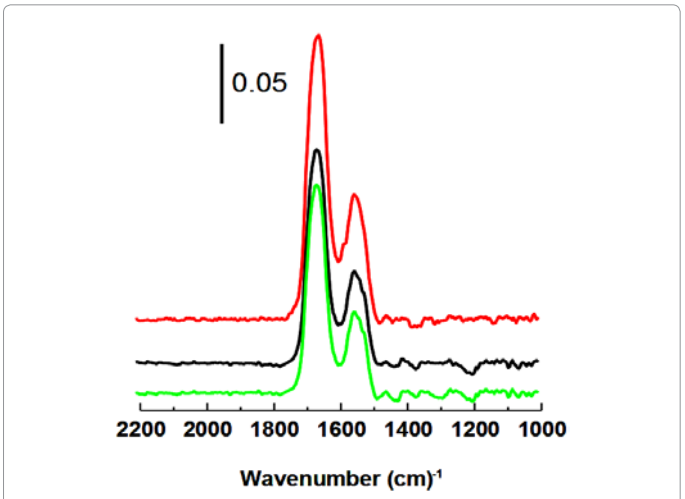


Figure 5: PM-IRRAS spectra obtained after adsorption of anti-PSA (green line), anti-PSA+BSA (black line) and PSA+BSA+anti-PSA (red line).

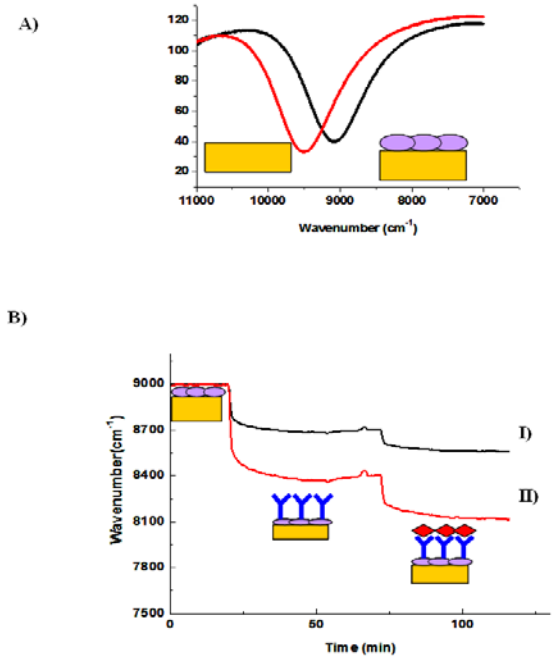


Figure 6: SPR ofAu Nr coated on gold surface by SAMsprocedure (A- black line)in comparison with SPR of gold surface not coated with Au Nr (A red line). Plasmon shift of with gold surface not coated with Au Nr in comparison Au Nr coated on gold surface by SAMs procedure (B line II) during interaction with proteins as function of time.

for 10 min without inducing any wavenumber change. After washing, the wavenumber value slightly decreased, likely due to desorption of weakly bound proteins (second response); then the wavenumber tended to an equilibrium value at 8164 cm^{-1}. These wavenumber shifts indicate that proteins, anti-PSA and PSA, significantly bind the surface. The shifts corresponding to each binding step are reported in Table 1.

It should be noted that, for identical concentrations, when the proteins are grafted to AuNr immobilized onto gold surface, the wavenumber shift is two times higher than PSA recognition by anti-PSA immobilized on the SAMs layer without AuNr immobilization. In order to determine the sensitivity reached by SAMs-AuNr based nanodevices, FT-SPR measurements of biorecognition were performed at various PSA concentrations, from 5 mg/L to 0.5 mg/L. Figure 7 shows on the left graph the dynamical responses as a function of time for each target concentrations and on the right graph the linearity of SPR shift response when the PSA concentration decreases. The calculated slope of linear response given by this set of experiments is 37 ± 2 cm^{-1}/mgL^{-1} that represents the sensitivity of the system. In conclusion, we successfully developed a novel simple covalent approach using mixed SAMs strategies to link AuNr on gold planar surfaces originating a well ordered active layer. Gold substrates were finally functionalized for interaction monitoring of anti-PSA with PSA. The findings highlighted AuNr coated gold surface as able to doubles the SPR interaction responses. Many authors, proved the assembling importance for biosensing applications: Jena [27] demonstrated that using a nanostructured material as gold nanoparticles integrated with a dehydrogenated enzyme well self-assembled it was originated a stable and fast response for electrochemical biosensing; Politi [15] highlighted that stable gold nanorods based nanobiosystem self-assembled on gold surfaces reveals an efficient detection of lead ions for environmental monitoring applications. On this path, an extraordinary variety of structures, properties, and applications is available for AuNPs motivating many studies and applications in interdisciplinary research involving chemistry, physics, biology, and medicine [28].

Conclusions

In this paper, we described a new concept of functionalization and self-organization of gold nanorods, bearing CTAB positively charged ligands, in order to bind proteins in an anisotropic way, and form linear chains after functionalization. The developed method does not require the use of organic solvent; the easy and versatile way, of forming such hybrid anisotropic nanostructures may have a wide range of applications, particularly using their optical properties to biosensors. Furthermore, ability of gold nanorods to enhance the interaction of SPR substrates with PSA molecules with an effective sensitivity (37 ± 2 cm^{-1}/mgL^{-1}) and specificity (no aspecific responses after BSA interaction were found neither by PM-IRRAS nor FT-SPR techniques) was finally proved. Further work will aim at optimizing the size and shape of gold nanostructures for biosensor signal enhancement.

	Planar SAMs Gold surface (Δcm^{-1})	AuNr linked onto Planar SAMs Gold Surface (Δcm^{-1})
Anti-PSA (5 mg/L)	296	600
PSA (5 mg/L)	110	240

Table 1: FT–SPR shifts observed upon binding of anti-PSA, recognition of PSA.

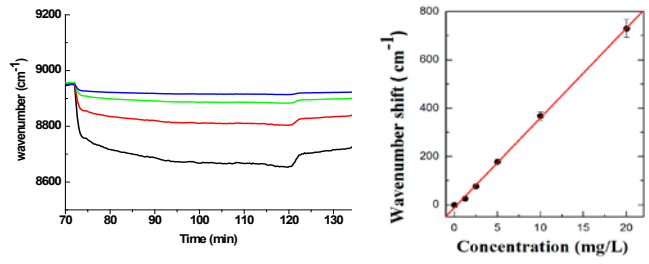


Figure 7: SPR shift of Au Nr coated on gold surface by SAMs procedure during interaction with PSA as function of time at different concentrations (left) and trend of SPR shift by increasing concentration of PSA (B).

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