Exchange Grant 3846

Scientific Report: *Gold nanotriangles as LSPR biosensors for SNP detection and phenolic compounds.*

Leonor Soares
FCUP, Faculdade de Ciências da Universidade do Porto
FCT/UNL, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Portugal

Purpose of the Visit:

Next generation biosensor platforms will require significant improvements in sensitivity, specificity and parallelism in order to meet the future needs of a variety of fields ranging from medical diagnostics and environmental concerns in the detection of hazardous substances. Nanobiosensors are gaining importance because of their ability to provide rapid information on the identification and/or quantification of several types of analytes. Metallic nanoparticles, such as gold and silver have been widely used as an efficient probe, due to their strongly enhanced surface plasmon resonance at optical frequencies that makes them excellent scatters and absorbers of visible light. The LSPR dependence of these nanoparticles (NPs) on their dielectric environment has been studied over the past few years, being successfully used as a mechanism in the detection of biological molecules. LSPR biosensors are based on sensing changes in the surface refractive index and transducing these changes into wavelength shifts of the LSPR extinction maximum as light is shined onto the patterned sensing platforms. The electric field component of the incident light interacts with the electrons of each metallic particle, causing the collective electron density to oscillate at a frequency that is resonant with the incoming light. This collective oscillation is what we call localized surface plasmon resonance. The versatile nature of LSPRs can be attributed to their strong dependence on a nanoparticle's composition, shape, size, inter-particle spacing, as well as its dielectric properties and those of the local environment[1].

Recently it has been reported that anisotropic nanoparticles, *e.g.* nanoprisms/nanoplates show higher sensitivities in comparison with isotropic nanoparticles. Due to that, two types of biosensors using gold nanotriangles (80-100 nm) are going to be addressed, in which the key point will be essentially the use of LSPR spectroscopy, exploiting the optical properties of the nanoparticles and the possibility to change their surface with proteins, such as DNA and enzymes. Molecules adsorbed to the particle surface lead to a spectral shift of the LSP resonances. The magnitude of the shift is related to the local optical field strength, with strong fields causing strong shifts and thus high sensitivity in sensor applications.

The mushroom tyrosinase (TYR), a copper enzyme that is responsible for the catalysis of oxidation reactions with vast utilization in phenol detection and

detoxification[2]. AuNTs-tyrosinase conjugates have demonstrated a higher efficacy on the enzyme performance[3]. The use of small peptides improves the affinity of the enzyme to the NP, as well as promotes a barrier between the metal and the protein, avoiding its denaturation. The formation of these AuNTs-tyrosinase conjugates was already achieved and confirmed on the first visit (short visit grant 3822) employing the same technique. However, since it was used AuNTs, the detection limit of this biosensor should be ascertained, for further sensitivity comparisons.

In view to develop a biosensor capable to detect single nucleotide polymorphisms (SNPs), it was firstly purposed a label-free method to detect DNA hybridization events using NPs, a system based on a complementary/non-complementary binding to the nanoprobes.

Preparatory work

Gold nanotriangles (AuNTs) were previously synthesized according to a photocatalytical method[4]. The method provides high yields in triangles and most of the spheres produced can be easily removed by centrifugation. Also allows obtaining nanotriangles with controlled size over a large range of sizes. The most interesting aspect in this method is that uses a photocatalyst - a tin (IV) porphyrin, that is responsible for the size control, together with the capping agent, CTAB. The kinetic of the reduction reaction is achieved by the controlled transfer of electrons from the low power reducing agent, triethanolamine (TEA) to the gold cation – Au (III). The nanotriangles have lengths between 80 and 100 nm, evaluated by TEM. The immobilization of the protein and the enzymatic activity of the AuNTs-tyrosinase conjugates were studied in FCT/UNL group, as well as other characterization techniques to ascertain the formation of conjugates, such as UV-Vis spectrophotometry and zeta potential measurements. Both thiol-modified oligonucleotides and target DNA were synthesized by Stab-Vida and Eurofins MWG|Operon, respectively. In terms of size, the thiol-capture DNA has 20 bp each, and their specific sequences are for the **oligo A** 5'- AGTTCCTTTGAGGCCAGGGA -3 and **oligo G** 5'- AGTTCCTTTGAGGCCAGGGG -3, the target DNA has 50 bp each (two of them are complementary to the SH-capture DNA) and the other, the non-complementary, is totally different *i.e.* none of the bases are complementary in all the positions.

Work carried out and main results

Project 1 – Nanoparticles functionalized with DNA for detection:

In order to modify the AuNTs surface to improve the binding of proteins, one small cationic peptide was used (CALKK). The peptide will also increase the affinity to the surface of the chips that are used for the LSPR measurements, when compared with the original capping. The functionalization of the AuNTs with this specific pentapeptide was based on some preliminary results that show that the enzymatic activity of the BNCs formed with CALKK-AuNTs and tyrosinase is improved in comparison with the performance of the enzyme alone.

At the host institution, the nanoparticles were optically characterized by acquiring single particle scattering spectra. A ZEISS Axio Imager.Z1m microscope in combination with a fibre-optically connected monochromator of type SpectraPro 2300i of Princeton Instruments was used. In this setup, borosilicate substrate with a microstructured chrome grating created by standard lift-off photolithographic process with deposited nanostructures is illuminated from below using a dark field immersion condenser (NA = 1:2 - 1:4), such that only light scattered from the particles on the top side is collected by a microscope objective (100x NA = 0:9). For spectral analysis, the structure plane is imaged on a fibre coupled to the spectrometer. The detection spot-size is approximately 2 μm in diameter, allowing real single-particle measurements. Some previous procedures were carried out before the immobilization and spectral analysis of the nanoparticles, such as the cleansing treatment of the chips, their surface activation (plasma etching) and silanization (wet-chemical method with 3-aminopropyltriethoxysilane - APTES) to ensure a higher adsorption of the particles to the surface of the chip.

In order to investigate the variation of the LSPRs due to changes in the refractive index in the immediate surrounding medium, for each chip, a procedure of three steps was devised:

- The functionalization was carried out in solution; briefly, 500 μL of AuNTs were centrifuged (10 000 rpm, 8 min) and ressuspended in 100 μL of milli-Q water, in order to remove the excess of CTAB. Then 10 μL of 0.1 mM CALKK solution was added to the washed AuNTs and deposited in the glass chip, during 1 hour. However, the modification of NP surface was also performed with particles already immobilized on the chip, dipping it on 500 μL of 0.01 mM CALKK solution (1 hour in a platform shaker).
- The second step was the tyrosinase immobilization, for which chips containing the functionalized AuNTs were dipped in solutions at different enzyme concentrations adjusted with buffer – 2mM phosphate buffer, pH 7 and let it stirring for 2 hours in a platform shaker. A negative control was done, immersing the chip with the NTs in 500 μL of buffer for the same time.
- The same procedure was used in the third step for its corresponding antibody. After every step, the chips were rinsed with water, dried with N_2 and scattering spectra of the same particles (10-12 NTs) were acquired.

According to the characteristics of the synthesis, it is quite likely to have nanospheres in addition to the NTs, as well as some truncated triangles, so it is necessary to assure exactly about the morphology of the nanoparticle, measuring also it size by AFM (between 80-100 nm).

The resonance bands of the AuNTs with those dimensions and characteristics (Fig. 1), is typically centred at around 650-750 nm (most intense band) and 580-600 nm (less intense band).[6] The less intense band corresponds to the thickness, that is constant regardless of the AuNT length, around 15 nm. In all analysed AuNTs, the scattering spectra obtained after each step seems to indicate some variation of the dielectric constant of the close environment. A red-shift of the more intense peak (between 700-800 nm) is observed when the protein is immobilized on the surface of the CALKK capped AuNT and also by binding of the correspondent antibody, however it

was not possible to take most of spectra of the last step, since they were too big and the range was limited, till 850 nm.

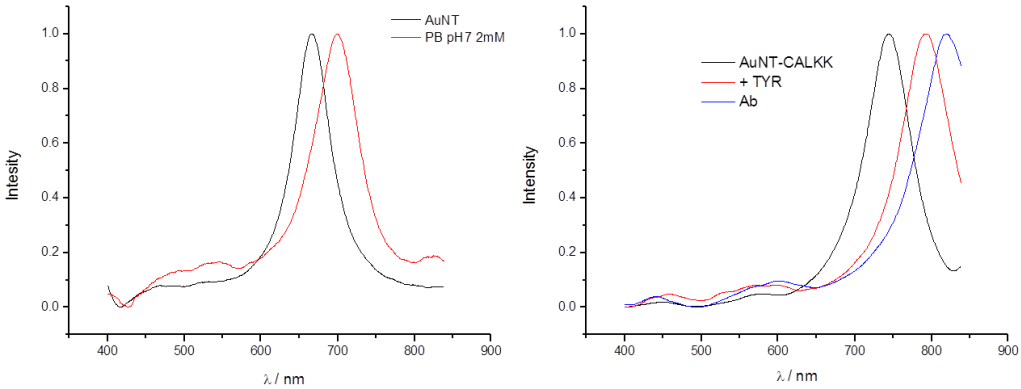

Figure 1: Single nanoparticle scattering spectra of the two selected nanotriangles from different chips: A) NT during 2 hours in phosphate buffer 2 mM, pH 7. NT at each step of the BNCs formation and antibody binding: CALKK capped AuNT (—); TYR immobilized on CALKK-AuNT (—) and Antibody-TYR binding to the BNC (—). [TYR] = 240 U/mL and [Ab-TYR] = 0.01 mg/mL.

The same procedure was carried out with more six concentrations (see table in fig. 2), to investigate the detection limit of this sensor. The average relative shifts of the LSP resonances are highly significant, even considering the buffer influence on the surface of the AuNTs (Fig. 2). The tyrosinase system allowed the detection of very low amounts of adsorbed enzyme, assuming a linear behaviour, since above 15 U/mL enzyme, its detection is possible, according to the 5 nm resonance shift. These results indicate effective adsorption of the tyrosine to the surface of the AuNTs .

[TYR] U/mL	Shift TYR / nm	Real Shift TYR / nm	Deviation /nm
240	55.5	35.1	9
120	40.2	19.8	12.3
80	31.8	11.4	14.3
60	30.9	10.5	8.1
30	27.7	7.3	13.9
15	25.3	4.9	15.6
6	18	-2.4	10.5
Buffer		20.4	3.4

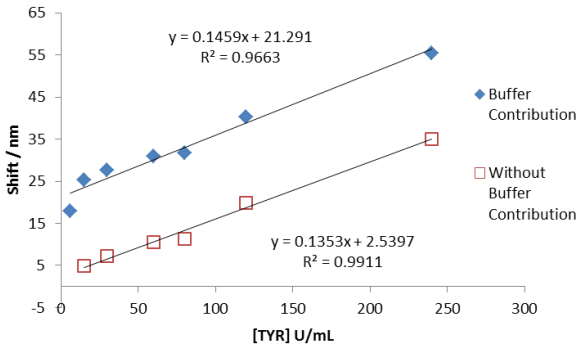

Figure 2: ***Left*** Table indicating tyrosinase concentrations; resonance shifts promoted by the buffer + tyrosinase and the resonance shifts, obtained by subtracting the buffer contribution. All the measurements and values are based on 10-12 NT spectra analysis. ***Right*** The graph represents a linear behaviour, with a very high correlation factor.

Project 2 – Nanoparticles functionalized with DNA for detection:

In order to investigate the variation of the LSPRs due to changes in the refractive index in the immediate surrounding medium, for each chip, a procedure with three steps was devised:

- In order to remove the excess of CTAB form the original solution, 500 μL of AuNTs were centrifuged (10 000 rpm, 8 min) and ressuspended in 100 μL of milli-Q water. This volume of AuNTs was deposited in a borosilicate chip and let it shaking during 1 hour.
- *Immobilization of the oligonucleotide:* in an eppendorf 1 μM solution of two different modified oligonucleotides were prepared using KH_2PO_4 1M solution (buffer). The chip was dipped in this solution and left stirring for 2 hours (also overnight) in a platform shaker, at room temperature. After these 2 hours, the chip was moved in to a 5 mM mercaptohexanol solution (MCH) and left stirring for 1 hour more. After the immobilization of the oligonucleotide, the chips were rinsed with water, dried with N_2.
- *Hybridization with target DNA*: in an eppendorf 1.5 μM solution of each target DNA was prepared with saline-sodium citrate 5x (SSC buffer). The chip was dipped in this solution and putted in an oven, for 2 hours at a specific 'hybridization temperature' determined by the following equation as described by Fotin and co-workers[7], considering the melting temperature (T_m) of each target:

  $$T\,(^{\circ}\mathrm{C}) = 1.2 \times T_{\mathrm{m}}(^{\circ}\mathrm{C}) - 28.2$$

  After hybridization event the chips must cool down during 10 min and the ionic strength at their surface slowly decreased (first with SSC 2x, then SSC 0.2x and water) and dried with N_2. Scattering spectra of the same particles (10-12 NTs) were acquired.

The first recognition layer (oligonucleotide) was attached to the surface of glass-immobilized AuNTs causing a red shift in the LSPR resonance due to refractive index changes from that of air (n_{air}) to that of the biological layer (n_{DNA}) (red spectra in Fig. 3). The resonance shift promoted just by the change of incubation time (between 2 hours and overnight) is not significantly different (fig. 3, on top), meaning that at the end of 2 hours, all the surface is already covered. This result is very reproducible and constant ensuring that all the analyzed NTs have the same amount of oligonucleotide at their surface, considering also that KH_2PO_4 buffer does not alters the resonance of the AuNT. The use of MCH is essential for the efficiency of the hybridization[8]. MCH is responsible for the extension of the capture molecules into the surrounding medium and away from the surface, because MCH molecules are smaller, leading to a much higher packed monolayer formation compared to DNA only, and will also minimize possible DNA backbone adsorption along the substrate surface, thereby decreasing the steric hindrance for further DNA binding. The hybridization procedure was done, after a period of 2 hours incubating the oligonucleotides, however after some experiments, it was noticed that this interval is not enough to guarantee the linkage of the DNA, appearing to be just electrostatically adsorbed. The incubating time was changed to overnight, because after the hybridization at 62°C and further washing procedures, it is

thought that the DNA was being removed from the surface, since it is not covalently bonded.

Specific binding of analyte molecules (complementary target) by the capture molecule layer leads to a further red shift in the LSPR maximum, in about 12 nm (fig. 3 on the bottom). Still, with the non-complementary target although it was not expected to see any shift, due to the absence of complementarity, the obtained result was a blue shift, probably because the temperature is still too high.

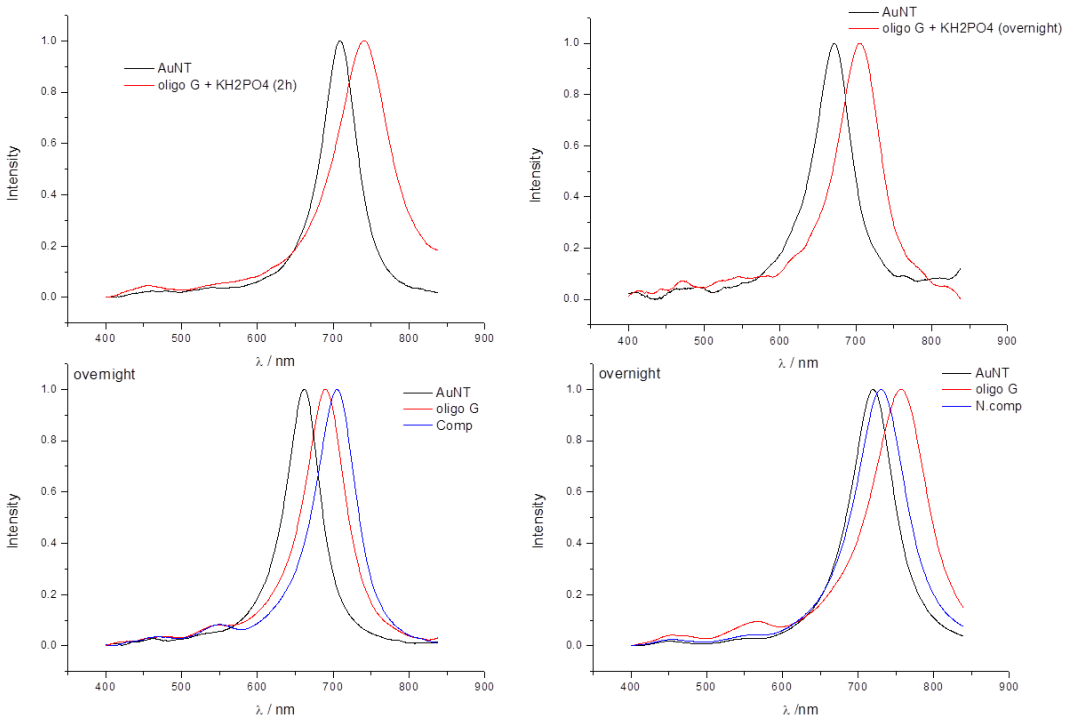

Figure 3: A) LSPR spectra of representative AuNTs after the immobilization of the oligo after 2 hours ($\Delta\lambda_{max}$ (nm) = 29 ± 2) and overnight ($\Delta\lambda_{max}$ (nm) = 34 ± 2). B) LSPR spectra of representative AuNTs after hybridization events (complementary and non-complementary target). Complementary target promoted a red-shift ($\Delta\lambda_{max}$ (nm) = 12 ± 4); Non-Complementary target promoted a blue-shift ($\Delta\lambda_{max}$ (nm) = 5 ± 2).

Additional experiments are needed in order to optimize the results, especially with the non-complementary target.

Future collaboration and projected publications

For Project 2, further studies are required to ascertain the accuracy of the method, namely lower temperatures for the hybridization event. So, this method will have to be optimized in a future visit, in order to produce a publication describing the application of this mew method to SNP detection. A publication is already being produced emphasising the potentialities of this type of biosensors, as AuNTs allow a much greater LSRP shift in comparison with the widely used spherical nanoparticles.

Future collaboration is also being planned with the hosting institution to further investigate on gold coating of silver nanotriangles. It will be interesting also to do a

parallel study of the development of a DNA biosensor with silver nanotriangles, in order to compare the sensitivity and reactivity of the different metals.

References

[1] E. Ringe, J. M. McMahon, K. Sohn, C. Cobley, Y. Xia, J. Huang, G. C. Schatz, L. D. Marks and R. P. Van Duyne; *Unraveling the Effects of Size, Composition, and Substrate on the Localized Surface Plasmon Resonance Frequencies of Gold and Silver Nanocubes: A Systematic Single-Particle Approach*, J. Phys. Chem. C, 2010, **114** (29), pp 12511–12516.

[2] G. Kim, J. Shim, M. Kang, S. Moon; *Optimized coverage of gold nanoparticles at tyrosinase electrode for measurement of a pesticide in various water samples*. J Hazard Mater, 2008, **156**, 141–147.

[3] J. Cortez, E. Vorobieva, D. Gralheira, I. Osório, L. Soares, N. Vale, E. Pereira, P. Gomes and R. Franco; *Bionanoconjugates of tyrosinase and peptide-derivatised gold nanoparticles for biosensing of phenolic compounds.* J. Nanopart. Res., DOI 10.1007/s11051-010-0099-8.

[4] A. Miranda, E. Malheiro, E. Skiba, P. Quaresma, P. Carvalho, P. Eaton, B. Castro, J. A. Shelnutt and E. Pereira; *One-pot synthesis of triangular gold nanoplates allowing broad and fine tuning of edge length*. Nanoscale, 2010, **2**, pp 2209–2216.

[5] I. Kubo, K. Nihei and K. Shimizu; *Oxidation products of quercetin catalyzed by mushroom tyrosinase*, Bioorg. Med. Chem., 2004, **12**, pp 5343–5347.

[6] N. Félidj, J. Grand, G. Laurent,J. Aubard, G. Lévi, A. Hohenau, N. Galler, F. R. Aussenegg and J. R. Krenn; *Multipolar surface plasmon peaks on gold nanotriangles*, J. Chem. Phys., 2008, **128**.

[7] Fotin, A.V., A.L. Drobyshev, D.Y. Proudnikov, A.N. Perov, and A.D. Mirzabekov, Nucleic Acids Research, 1998, **26**, 1515-1521 [8] Levicky, R., T.M. Herne, M.J. Tarlov, and S.K. Satija, Journal of the American Chemical Society, 1998, **120**, 9787-9792.