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Scientific Report: Gold nanotriangles as LSPR biosensors - DNA hybridization detection

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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 such as medical diagnostics, considering a range of priorities; selection of suitable materials, design, production and technical use of these biosensors in medical applications. Nanobiosensors are gaining importance because of their ability to provide rapid information on the identification and/or quantification of several types of analyte. 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[2]. Due to that, a biosensor using gold nanotriangles (80-100 nm) is going to be addressed. In order to achieve our goal it would be essentially the use of LSPR spectroscopy together with dark field microscopy, exploiting the refined optical properties of these nanoparticles and their possibility to be functionalized with thiolated oligonucleotides. DNA molecules adsorbed to the particle surface lead to a spectral shift of the LSP resonances[2]. 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.

After a pioneering work by Mirkin et al.[3] and some other groups[4] which were interested in the development of DNA detection systems using metallic nanoparticles, mostly spherical, many more studies are being conducted in this field. Although their good results and widely different methods, the recurrent use of gold nanospheres is becoming limitative. Anisotropic nanoparticles due to their shape configuration and higher aspect ratios lead to stronger dipole moment and acutely influence on the surrounding materials. The advantage of the tips, the so-called 'hot spots' promote a more efficient binding of molecules and consequently higher sensitivities.

Aiming 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. The purposed project was inspired on a similar work described by the host investigation group using commercial 80nm gold nanoparticles. They could discriminate the selective binding of targets regarding the optical properties of the NPs; however the sensitivity can be improved using anisotropic nanoparticles.

Preparatory work

Gold nanotriangles (AuNTs) were previously synthesized according to a photocatalytical method[5]. 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 when activated by a light source (halogen bulb) 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 and a height of 17 nm, evaluated by TEM and AFM. Two thiol-modified oligonucleotides and ssDNA targets were synthesized by Stab-Vida and Eurofins MWG|Operon, respectively.

Regarding the oligonucleotides specifications, according to the size, both have 20 bp and a 5'-thiol group on the C6. However the second oligonucleotide has an additional spacer of 10 adenines. The sequences are the following:

Oligo A:
5'– AGTTCCTTTGAGGCCAGGGA – 3'
Oligo A_10A:
5' - AAAAAAAAAAAGTTCCTTTGAGGCCAGGGA – 3'

The ssDNA targets have 50 bp each, which the complementary has a region that matches with the SH-capture DNA and the non-complementary is totally different *i.e.* none of the bases match.

Complementary Synthetic Target:
5' – TCAAGGAAACTCCGGTCCC**T**TTCCCATCAAGCCCTAGGGCTCCTCGTGGC – 3'
Non-Complementary Synthetic Target:

5’ – TTCCCATCAAGCCCTAGGGCTCCTCGTGGCTGCTGGGAGTTGTAGTCTGA – 3’

The second idea of the project was that after the system is optimized to detect the synthetic targets, it would also be tested with dsDNA targets (DNA fragments / PCR products) including the sequence to detect. In order to do it, DNA was extracted from FTA Cards and a PCR was performed to amplify the region in interest. Therefore, two targets were considered: the complementary target and the non-complementary target, which were amplified from two different genes, LCT and FTO. Each fragment has 345 and 445 bp including the specific sequence that afterwards can detect polymorphisms, one responsible for the lactose intolerance (LCT) and the other for obesity (FTO). Both samples were purified with ExoSAP and quantified by NanoDrop 1000. Their temperature profile (melting temperature) was characterized by a melting curve analysis, important information to the hybridization step.

Work carried out and main results

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.

According to the characteristics of the chemical 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) are typically centred at around 650-750 nm (most intense band) depending on the size and 580-600 nm (almost imperceptible). This resonance band corresponds to the thickness, that is constant regardless of the AuNT length, around 17 nm. In all analysed AuNTs, the scattering spectra after each step highlights an evident alteration in the refractive index of the close environment. A red-shift in the more intense peak is expected when the oligonucleotide bonds to the surface of AuNT and further when the target hybridizes.

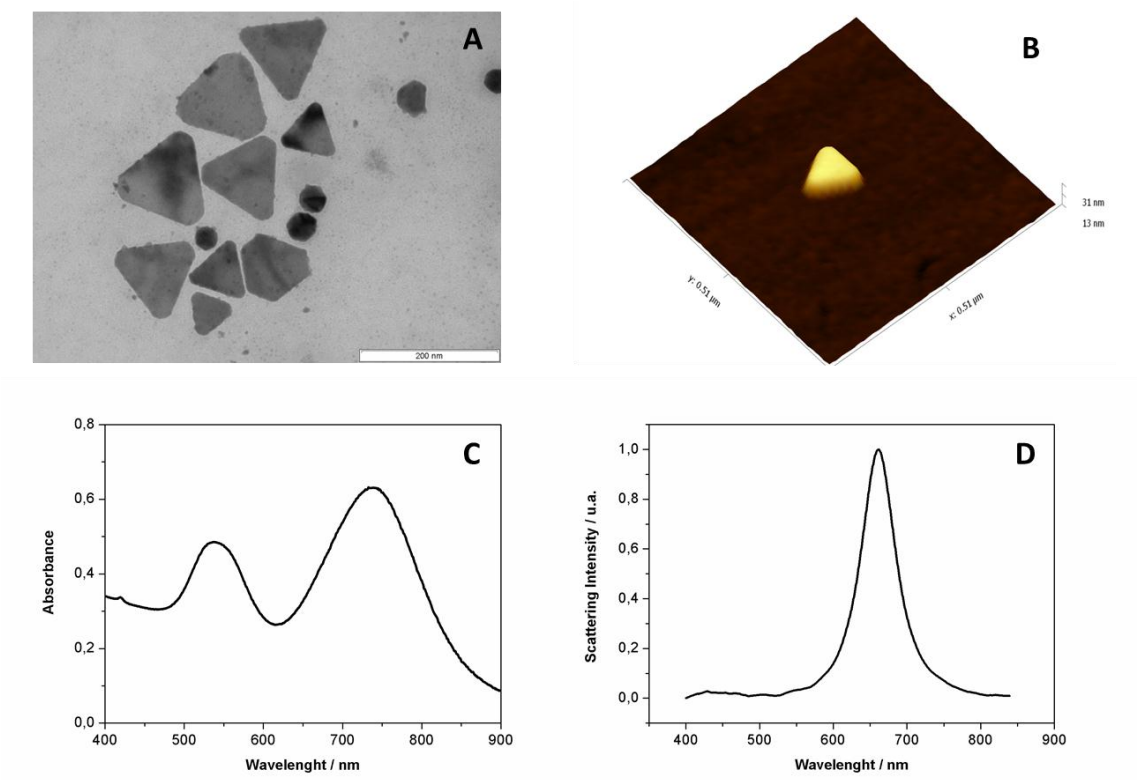

Figure 1: (A) Representative TEM image of CTAB-AuNTs. The bar represents 200 nm* **(B)** Amplitude AFM picture of an AuNT **(C)** UV-Vis spectra of such colloidal solution **(D)** LSPR spectra of the selected AuNT showed represented in AFM picture. *91 ± 17 nm; (N=49 particles) AuNTs were counted from different images of the same sample.

Project 1 – Nanotriangles functionalized with thiol modified oligonucleotides for detection of ssDNA synthetic targets:

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:

1. *Immobilization of the AuNTs*: In order to remove the excess of CTAB from the original solution, 500 µL of AuNTs were centrifuged (7 500 rpm, 10 min) and ressuspended in 50 µL of milli-Q water. This volume of AuNTs was deposited in a borosilicate chip and let it shaking during 1 hour.
2. *Immobilization of the oligonucleotide*: in an eppendorf 1 µM solution of the SH-oligonucleotides (oligo A and oligo A_10A) were prepared using KH_2PO_4 1M solution, as buffer. The chip was dipped in this solution incubating and left stirring for 15 hours (overnight) in a platform shaker, at room temperature. After this period, the chip was moved into a 1 mM 6-mercapto-1-hexanol solution (MCH) and left stirring for 1 hour more. After the immobilization the chips were rinsed with water and dried with N_2.
3. *Hybridization with target ssDNA*: in an eppendorf 1 µM solution of each ssDNA target was prepared with saline-sodium citrate 2x (SSC hybridization buffer). The chip was dipped in this solution for 3 hours at 25°C. Then they were rinsed

with a diluted solution of SSC, in order to slowly decrease the ionic strength at the AuNT surface (first 0.2x SSC and then water) and dried with N_2.

- Scattering spectra of the same particles (10-12 NTs) were acquired.

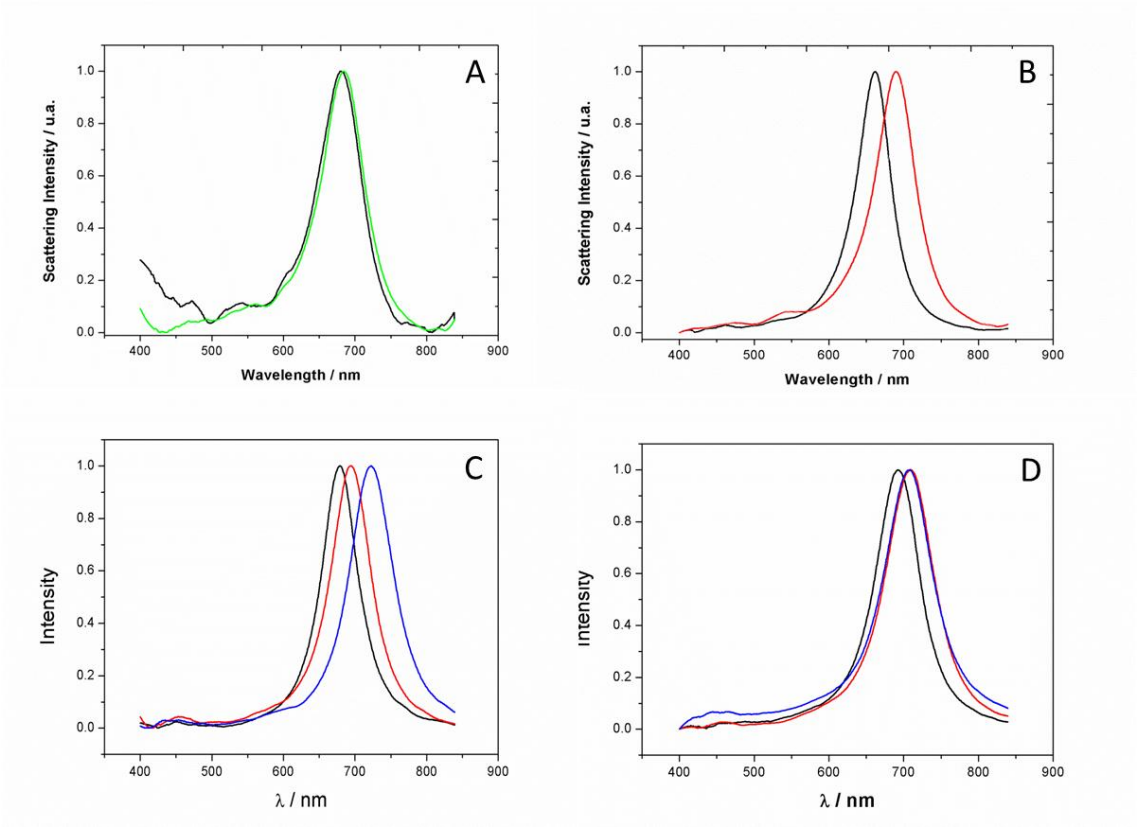

Figure 2: Representative LSPR spectra of the different experimental steps, in which is visible the resonance shifts induced by the characteristics of the chemical environment, *i.e.* by the different refractive index of each element added to the sensing system. For each assay, the shift values are based in the average results of 12 AuNTs: The green line represents the shift promoted by the buffer (1 M KH_2PO_4); The red line represents the shift promoted by the oligo A_10A binding (1 μM) after an overnight incubation period and the blue line represents after hybridization **(A)** 4 nm red-resonance shift **(B)** 24 ± 5.5 nm red-shift **(C)** 33.6 ± 2.5 nm red-shift after hybridization of the complementary target sequence (1 μM) and **(D)** 0.98 ± 0,5 nm shift with the non-complementary target sequence (1 μM).

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 CTAB (n_{CTAB}) to that of the biological layer (n_{DNA}) (Fig. 2B). Previous results showed that although after 2 hours of incubation is enough to have the AuNTs surface all covered, since the resonance shift promoted by the change of incubation time (between 2 hours and overnight) is not significantly different, it is needed at least an overnight period to assure that the DNA is not just adsorbed electrostatically, but effective and covalently bound. This result is very reproducible and constant indicating that all the analyzed NTs have approximately 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[6]. 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.

Specific binding of analyte molecules (complementary target) by the capture molecule layer leads to a further red shift in the LSPR maximum, in about 34 nm (fig. 3C). In the other hand with the non-complementary target, due to the absence of any complementarity shows no shift (fig. 3D). Along a set of experiments, hybridization conditions were applied, such as temperature (85, 37 and 25^{o}C) and SSC concentrations (5x/2x). These parameters were tested to ascertain which one conducted to better results and the conclusion was clear. Higher stringency showed to be the ideal, especially in terms of salt concentration, since it permits only hybridization between nucleic acids sequences that are highly similar.

Project 2 – Nanotriangles functionalized with thiol modified oligonucleotides for detection of dsDNA fragments (PCR products):

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:

1. *Immobilization of the AuNTs*: In order to remove the excess of CTAB form the original solution, 500 μL of AuNTs were centrifuged (7 500 rpm, 10 min) and ressuspended in 50 μL of milli-Q water. This volume of AuNTs was deposited in a borosilicate chip and let it shaking during 1 hour.
2. *Immobilization of the oligonucleotide*: in an eppendorf 1 μM solution of the SH-oligonucleotide (oligo A_10A) was prepared using KH_2PO_4 1M solution, as buffer. The chip was dipped in this solution incubating and left stirring for 15 hours (overnight) in a platform shaker, at room temperature. After this period, the chip was moved into a 1 mM 6-mercapto-1-hexanol solution (MCH) and left stirring for 1 hour more. After the immobilization the chips were rinsed with water and dried with N_2.
4. *Hybridization with target dsDNA*: in an eppendorf 1 μM solution of each dsDNA target was prepared with 2x SSC. Since the targets are double stranded the denaturation was performed separately in a thermo shaker at 90^{o}C for 10 minutes. Then the eppendorf is immediately placed in an ice bath for 10 min (with some NaCl!). The chip was then dipped in this solution at 20^{o}C for 3 hours. Then they were rinsed with a diluted solution of SSC, in order to slowly decrease the ionic strength at the AuNT surface (first 0.2x SSC and then water) and dried with N_2.

- Scattering spectra of the same particles (10-12 NTs) were acquired.

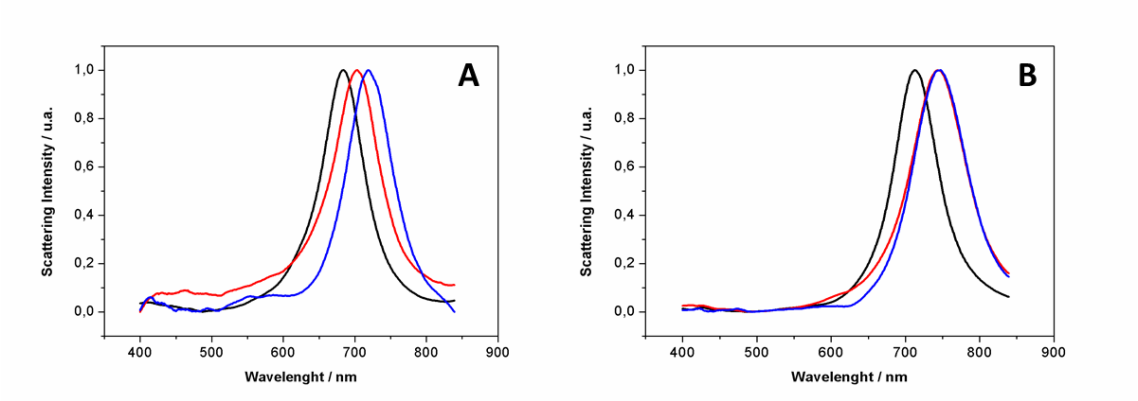

Figure 3: Representative LSPR spectra of the different experimental steps, in which is visible the resonance shifts induced by the characteristics of the chemical environment, *i.e.* by the different refractive index of each element added to the sensing system. For each assay, the shift values are based in the average results of 12 AuNTs: The red line represents the shift promoted by the oligo A_10A binding (24 nm) and the blue line represents after hybridization: **(A)** 21.6 ± 2.9 nm red-shift after hybridization of the complementary target sequence (1 μM) and **(B)** -0.4 ± 1.1 nm shift with the non-complementary target sequence (1 μM).

For the detection of the biological samples, *i. e.*, DNA fragments (dsDNA) the procedure was kept, except in the hybridization step. According with preliminary results, it was observed that under high temperatures – around 85°C (T_m of each target) the AuNTs were being released. Additionally after hybridization, the targets were indiscriminately binding. In order to counteract these facts, the dsDNA denaturation was performed in separate at 90°C to assure that all the DNA gets single stranded. Then, the solution was cooled at -4°C in order to 'freeze' the ssDNA to be maintained in this form. Finally, after 3 hours at 20°C the results were conclusive. It is necessary to highlight that the SSC concentration was kept the same as in the previous experiments (2x SSC). After the hybridization with the complementary target a significant red-shift was observed, around about 22 nm and as expected, an absence of shift for the non-complementary target was displayed.

Future collaboration and projected publications

A publication is already being produced emphasising the potentialities of this DNA biosensor, as AuNTs allow a much greater LSRP shift in comparison with the widely used spherical nanoparticles and has it can be applied either for synthetic (ssDNA) and biological targets (dsDNA).

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.

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