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Evaluation of the binding of the enzyme tyrosinase to gold nanoparticle surfaces by localized surface plasmon resonance sensing

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Purpose of visit:

In this work we proposed to use LSPR microscopy to study the adsorption of the enzyme tyrosinase to several types of gold nanoparticle (NP) surfaces. Tyrosinase is an enzyme with vast utilization in phenol detection and detoxification [1] and it's conjugation to gold NPs can lead to the retention or even to an increase of their biological stability/activity. The formation of these nanoparticle/enzyme conjugates can improve the kinetic parameters of the enzyme and expand the range of pH in which it is effective.

LSPR sensing, a photonic technique available at the host institute (IPHT) was used as a method of bioanalytical detection involving metallic nanoparticles. This method makes it possible to measure the scattering spectra of individual nanostructures at high precision. In darkfield excitation the particles are illuminated and measured using a sensitive spectrophotometer, allowing detection of small changes in plasmonic resonances. These changes in the plasmon resonance are due to changes in the refraction index in the vicinity of the NPs that can occur due to protein adsorption.

The work focused on gold NPs divided according to shape and surface modification. By shape we used gold nanotriangles and gold nanospheres. In regard to surface functionalization of the NPs, we used pentapeptides of a general formula CALXX (where X can be N or K). These particular peptide sequences offer significant stability to the NPs and can also provide a biocompatible surface since gold nanoparticles capped with these ligands have been shown to acquire properties similar to proteins (Levy 2006), presenting a favorable environment for the attachment of proteins and the preservation of their bioactivity.[2] As a comparison we will use an alkanethiol possessing terminal polyethylene glycol (PEG) subunits. This type of functionalization with PEG containing ligands is typically used to create "stealth" particles by minimizing protein adsorption to NPs and therefore would be an ideal ligand to compare to the peptide functionalized NPs.[3]

Description of the work carried out during the visit and main results obtained

Particle synthesis:

Gold nanotriangles (NTs) were previously synthesized according to a photocatalytical method.[4,5] The method provides high yields in triangles and most of the spheres produced can be easily removed by centrifugation. It also allows to obtain 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. Gold nanospheres were synthesized according to a seeded growth method reported in [6] allowing for the synthesis of particles with diameters from 45 nm to 250 nm. For this work NPs with an average 80 – 90 nm size were used. Functionalization of the spheres with CALKK peptide was performed using a controlled addition of 10 ml of gold colloid to 100 ml of a 0.1 mM CALKK solution at pH 11 and stirring overnight followed by centrifugation at 6000 rpm for 15 min to wash and concentrate the NPs.

Darkfield measurements and chip preparation:

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 fiber-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 fiber coupled to the spectrometer. The detection spot-size is approximately 2 mm in diameter, allowing real single-particle measurements.

E-LSPR (ensemble LSPR) was performed on glass chips allowing for a precise measurement of the plasmon ressonance of an ensemble of immobilized nanoparticles up to a picometer precision. The glass chip is mounted on a microfluidic chamber allowing for the measurement of the plasmon ressonance in liquid and also allowing the flow of reagents and the consequent plasmon shift.

Chips for nanoparticle deposition were cleaned using the standard procedures used in the host laboratory, involving several washes, plasma etching treatment followed by silanization with 3-aminopropyltriethoxysilane (APTES) in order to facilitate nanoparticle attachment to the chip.

Gold nanotriangles

To measure the LSPRs shifts of individual nanotriangles, a two step procedure was used for each chip

1- Immobilization and functionalization of the NTs. Two different procedures were used:

(A) The functionalization is performed in solution before immobilization - In order to remove the excess of CTAB from the original solution, 500 μL of AuNTs were centrifuged (7500 rpm, 10 min) and ressuspended in 50 μL of milli-Q water. 5 μl of a 0.1 mM CALKK solution was added and allowed to incubate for 10 minutes, followed by deposition of 50 ul in a borosilicate chip and incubation in a shaking platform for 1 hour. The chip is then washed with milli-Q water, dried with N_2 and the first LSPR measurement is performed. This procedure was the same used in previous studies at the host lab.
(B) The nanoparticles are firstly deposited on the chip and the functionalization with the peptide performed on the immobilized NTs. The same procedure of centrifugation and deposition of the previous method was used excluding the addition of the CALKK solution. After the washing and drying of the chip the first LSPR measurement was done. Following this the chip was incubated in a 0.1 mM CALKK solution for a variable amount of time. First experiments followed the plasmon shift for different incubation times in order to determine the time at which the functionalization would be complete (no further variation in the plasmon resonance). In the step of experiments for functionalization versus time control experiments using chips incubated with water were used.

2-*Incubation with tyrosinase solution.*

For procedure A the chip is firstly incubated for 2 hours in a 2mM phosphate buffer solution and after this washed, dried and the LSPR measured. After the measurement of the LSPR of the functionalized NTs, the chip was incubated with a tyrosinase solution in 2mM phosphate buffer at pH 7 by depositing a 50 μl drop on top of the chip and left stirring for 2 hours in a platform shaker, at room temperature Different concentrations of tyrosinase between 0 and 1200 U/ml were used. For procedure B only the incubation with tyrosinase solution was used.

First step of experiments using the functionalization of the triangles in solution (method A) was performed using a 600 U/ml concentration of tyrosinase versus a control experiment using only buffer. Typical spectra for a single particle are showed in figure 1.

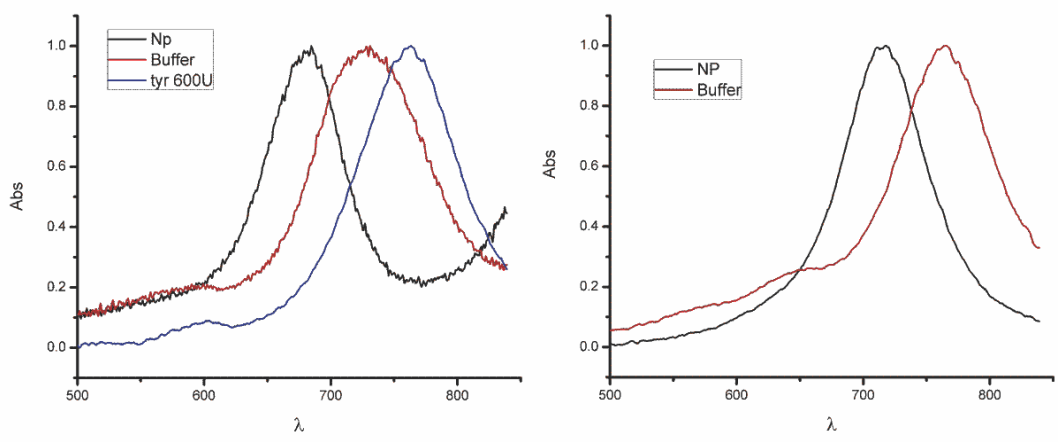

Figure 1 - Single particle spectra obtained through LSPR dark field microscopy for the several incubation steps. Initial measurement of the NP (black), after incubation with buffer (red) and after incubation with tyrosinase solution (blue). Left: spectra for experiment using a 600 U/ml tyrosinase solution; Right: spectra for control experiment using only incubation with buffer.

Although the tyrosinase binding gave a 24.43 ± 6.43 nm shift relative to the first incubation with buffer, large shifts were also observed for this first incubation with buffer (44.03 ± 13.25 nm). Similar large shifts with the incubation in buffer were observed in previous studies and the reasons for this are still unclear. Also there were doubts whether the functionalization protocol used was sufficient for the replacement of the CTAB layer around the nanotriangles. Therefore more fundamental studies on the triangle functionalization were required before studying the tyrosinase binding. For that reason procedure B was used in which the triangles were firstly immobilized on the surface of the chip and then incubated with a more concentrated CALKK solution. This would facilitate ligand exchange of CTAB with the peptide and eliminate the problem of aggregation in solution upon functionalization since the triangles would be already immobilized in the chip. The first experiments consisted on monitoring the LSPR shift of the same set of 12 particles over time while incubating with a CALKK solution. Average shifts over time and the control chip incubated just with water are showed in figure 2.

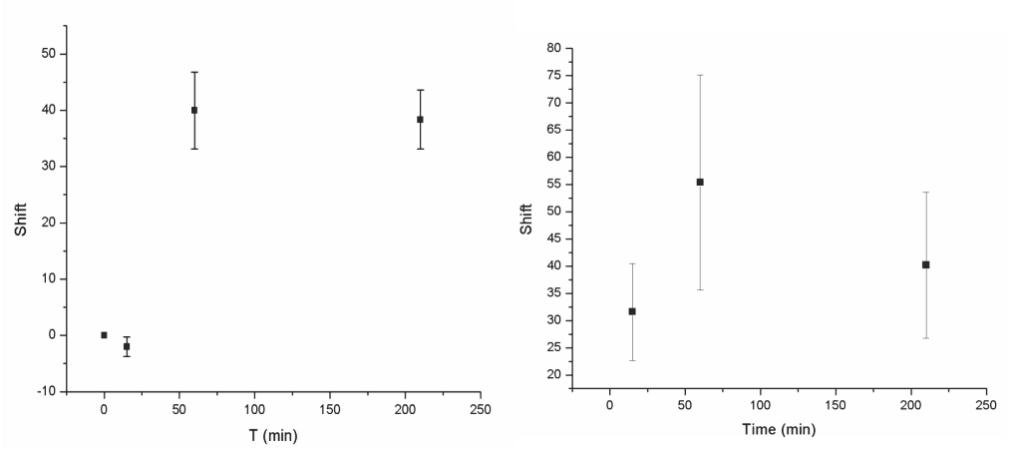

Figure 2 – Graphs representing the average LSPR shift for 12 NPs for incubation with a CALKK solution (left graph) and control experiment incubated with water (right graph) for different incubation times.

Although the NT LSPR shift seems to increase and stabilize after approximately 45mins, the control using water also shows large shifts following an almost random pattern. This experiment was repeated but results showed similar behavior to the previous experiment. Since the results obtained from the control render the experiment inconclusive a long incubation time (48h) with the CALKK solution was chosen in order to guarantee a complete functionalization. The chips containing the functionalized NPs were then incubated for 2h in a 600 U/ml tyrosinase solution (and a control chip in just the 2 mM buffer solution). The average LSPR shift obtained for a set of 12 particles after incubation in the 600 U/ml tyrosinase solution relative to the first measurement after functionalization was 12.3 ± 3.1 nm. However the chip incubated with the buffer showed a similar LSPR shift (11.1 ± 9.8 nm) although with a greater associated error. Once again comparable LSPR shifts were obtained for the case of the buffer relative to the incubation with the protein rendering the experiment inconclusive.

Since the LSPR dark field microscopy experiments with the NTs yielded inconclusive results experiments using e-LSPR (ensemble LSPR) were projected. However these experiments required that a significant amount of nanotriangles could be immobilized to the chip used for the measurements. Attempts to immobilize the NTs using longer incubation times and much more concentrated colloidal dispersions (up to 80X the original concentration) where attempted but resulted in either an insufficient binding to the chip or a complete aggregation on the NTs in solution. It became clear that for this work in the case of the NTs much more comprehensive studies would have to be performed and for reasons of time and logistics no further experiments using the NTs were done.

Gold nanospheres

For the immobilization of the gold spherical NPs (functionalized with CALKK) in the chip, 50 μl of a 1:20 dilution of the colloidal suspension in water was deposited on the surface of the chip, allowed to incubate for 10 mins after which the chip was washed and dried and the LSPR shift of 12 single NPS measured. Following that the chip was incubated in a 2 mM phosphate buffer solution for 2 h, washed/dried and the LSPR shift measured again. Finally the chip was incubated with a tyrosinase solution in 2mM phosphate buffer at pH 7 by depositing a 50 ml drop on top of the chip and left stirring for 2 hours in a platform shaker, at room temperature Different concentrations of tyrosinase between 0 and 1200 U/ml were used. Figure 3 shows the LSPR shift for a representative NP for the maximum tyrosinase concentration tested and the average LSPR shifts obtained for all tested tyrosinase concentrations.

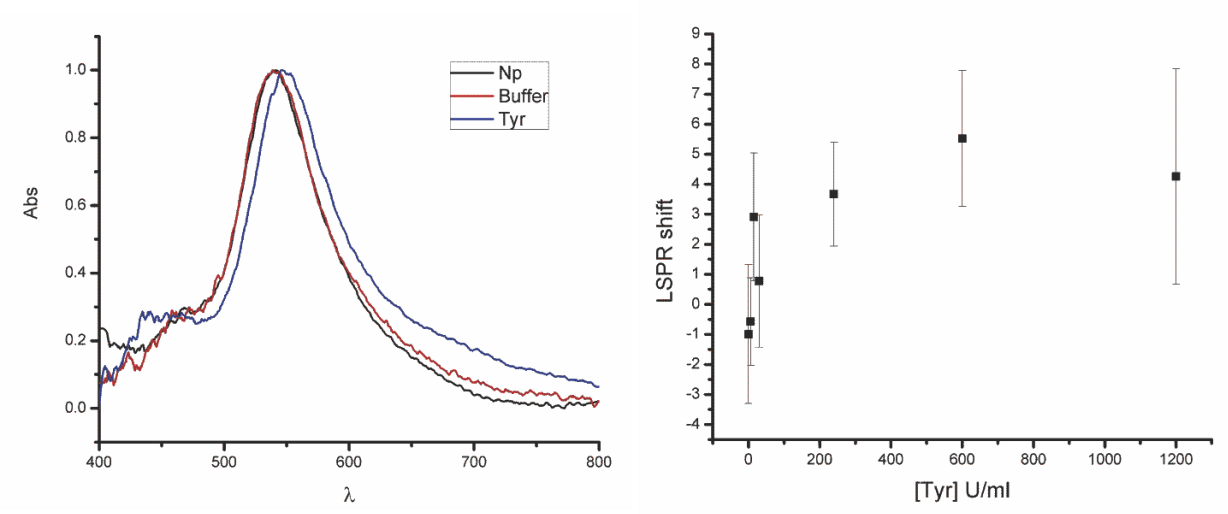

Figure 3 - Left: Representative single particle spectra for the several incubation steps: Initial measurement of the NP (black), after incubation with buffer (red) and after incubation with a 600 U/ml tyrosinase solution (blue). Right: Graph representing the 12 NP average LSPR shift for a 2h incubation with different tyrosinase concentrations.

In contrast with the results obtained with the nanotriangles almost no plasmon shift was detected when the NPs are incubated in phosphate buffer. The average shifts obtained for different tyrosinase concentrations do show a trend with increasing concentration although with some variability between individual particles resulting in large standard deviations for the LSPR shifts. A set of experiments using PEG functionalized NPS was also performed using only the lowest (0U/ml) and highest (1200U/ml) tyrosinase concentration resulting in average plasmon shifts of -5.76 ± 2.72 nm and 2.95 ± 1.46 nm respectively. This is lower than the shift obtained for the CALKK functionalized NPs for the same concentration (1200 U/ml) of tyrosinase (4.26 ± 3.5) indicating that the PEG layer could indeed impair protein adsorption to the NP surface.

However because of the large error associated with the measurement no definite conclusion could be reached by this technique. Therefore e-LSPR measurements were performed that could measure precisely the LSPR of an ensemble of nanoparticles while a tyrosinase solution was injected via a microfluidic system.

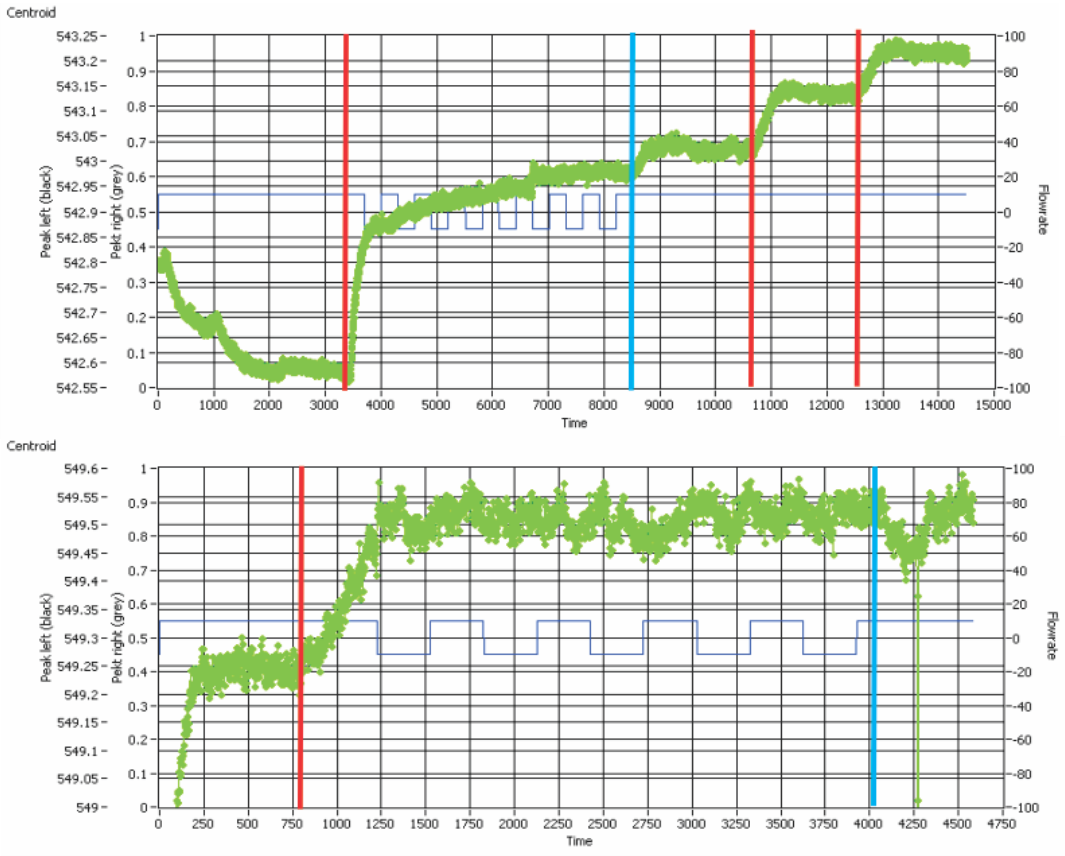

Figure 4 – Graphs showing the LSPR peak position over time by e-LSPR measurement. Red bars in the graph indicate the time of injection of a 1200 U/ml tyrosinase solution and blue bars show washing of the chip with phosphate buffer. Injection of the tyrosinase solution was performed in a loop so that the protein solution is continuously run over the deposited nanoparticles. The top graph shows LSPR peak position for a chip with CALKK functionalized NPS and the bottom graph for PEG functionalized NPs.

In figure 4 we can verify a sharp rise in the LSPR shift upon injection of the tyrosinase solution for both types of NP functionalization. However after about 750 seconds after injection the LSPR shift for the PEG functionalized NPs seams to stabilize after shifting just 0.25 nm relative to the original peak position. In the case of the CALKK functionalized NPs only after about 4000s does the plasmon shift start to stabilize with a LSP shift of about 0.4 nm after the first tyrosinase injection. Also further injections resulted in further rises in the LSPR shift although of lesser magnitude. Since both types of NP have essentially the same LSPR sensitivity (they resulted from the same batch of NPs) and the concentration of tyrosinase solution was the same, differences in the LSPR shift can be due only to different patterns of tyrosinase

adsorption to the NP surface. This allows us to conclude that the PEG functionalized NPs do impair protein adsorption allowing only a limited adsorption in the initial time of injection while for the CALKK functionalized NPs there is a greater and continuous protein adsorption over a wider time frame. However more studies will be needed for a complete characterization of the system.

Future collaboration with host institution

Future collaboration with the host laboratory could involve further studies using firstly e-LSPR that seems to a be a more appropriate technique for the studies of the protein adsorption over time since it allows for direct monitoring of the LSPR shift in liquid phase. For the NTs the use of a layer of polyelectrolytes to facilitate adsorption to the chip could be used. In alternative the functionalization if the chip using a thiol bearing silane could be performed in order to achieve a sufficient surface density of NTs for the measurements. Following the functionalization of the NTs the response of the system to the tyrosinase could be properly characterized. Regarding publications this work still requires further studies in order to yield publishable results.

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