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Host Institution
Institute of Photonics and Electronics (IPE)
Academy of Sciences of the Czech Republic

Period: 02-09-2013 to 28-09-2013

Project:
Sviluppo di nuove metodologie ottiche per l'interrogazione di sensori chimici e biochimici basati su Risonanze Plasmoniche di Superficie

"Development of new optical interrogation methods for chemical and biochemical sensors based on Surface Plasmon Resonances"

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Contents

1 Introduction to the project and Motivations

The described experience stems from a collaboration project between two research groups from the National Research Council National Institute of Optics (INO–CNR) (Italy) and from the Institute of Photonics and Electronics (IPE) Academy of Sciences of the Czech Republic. New interrogation methods for Surface Plasmon Resonance (SPR) sensors are investigated with the target of experimentally explore their intrinsic limits [1]. A novel SPR refractive index sensor, based on time domain measurements, was already implemented as a proof of concept at INO Naples [2]. The main potential for the SPR sensors application stands into the high sensitivity selective detection of analytes of chemical and bio–chemical interest, that is realized by the application of surface immobilization techniques. From there was born the purpose of moving the new developed system application toward these detection targets with the aim to evaluate its performance in terms of accuracy and limit of detection. Furthermore the acquisition of experience on SPR most advanced bio–sensing techniques and on the related experimental issues must also be considered as preliminary step to a finalized calibration of the detection system to the specific filed demands. In the present collaboration project the expertise of the INO group in optical systems was combined with the well consolidated expertise in bio-sensing of the IPE group. The motivation of this Short Term Mobility experience was centered on achieving a good learning of bio–sensing fundamental concepts and issues. Typical bio-sensing selective techniques have been studied in details with a particular attention to the sensor functionalization methods, buffers and samples chemical preparation. This study has been conducted in a theoretical and practical approach. The experience at IPE was focused on a proof of principle experiment finalized to the DNA oligonucleotides selective detection. In order to consolidate the basic concepts and the technical expedients related to bio-sensing techniques, during the Short Term Mobility stay, the experiments were realized on a well-established and engineerized SPR sensing system in use at IPE. This setup is based on a spectroscopic readout with a broadband SPR interrogation source (section 2).

1.1 Surface Plasmon Resonance basics and project background

A Surface Plasmon Resonance consists in an electron plasma oscillation that can be excited by polarized electromagnetic radiation, under specific conditions, at the interface between a metal and a dielectric medium. The coupling of the radiation to the SPR strongly depends on the complex permittivity of the involved materials and on the interface constructive parameters. In a SPR based sensor the sample is placed on the sensing surface, to be considered as a dielectric overlayer, and its refractive index (RI) influences the SPR coupling condition. In particular the SPR response depends both on the sample bulk refractive index changes and on the mass of chemical substance deposited on the

surface. An effective refractive index (n_{eff}) can then be defined in order to account for both contributions. Thanks to the very thin penetration depth of the plasma oscillation, of the order of $\sim 50nm \div 100nm$, SPR based sensors are highly suitable for detecting variations of n_{eff} due to chemical effects in proximity of the surface.
In order to simplify the description of our sensor working principle let's restrict our field to the so called *Kretschmann* coupling configuration. A metal thin layer is deposited on a lab–slide realizing the SPR surface interface (SPR–chip). This chip is superimposed on an optical prism and the plasma oscillations are coupled by the electromagnetic evanescent field transmitted through the prism in an Attenuated Total Internal Reflection (ATR) scheme [3] (see figs. 1 and 2).
In a general approach, the sample refractive index change Δn_{eff} can be detected from modulation of the source radiation by the SPR coupling. Depending on the specific setup, different observables can be used for the readout [6], leading to a classification in spectroscopic or interferometric methods. In a spectroscopic method, the wavelength shift of the SPR is measured for a given incidence angle with a broadband source. On the other side, since the incidence angle of the exciting radiation is a conjugated variable of the coupling wavelength, the measurement can be also implemented in an angular domain. In an interferometric method the phase shifts or the polarization changes of the interacting radiation are observed. For all these readout methods, assuming Y be the sensor output change due to a refractive index variation in the sample and R the SPR–chip proper response, the refractometric sensitivity of an SPR sensor can be schematized as follows:

$$S = \frac{\partial Y}{\partial n_{eff}} = \frac{\partial Y}{\partial R} \times \frac{\partial R}{\partial n_{eff}} \tag{1}$$

where $\frac{\partial Y}{\partial R}$ stands for the method dependent sensitivity factor and $\frac{\partial R}{\partial n_{eff}}$ represents the chip-dependent term. The sensor *resolution* is defined as the minimum change in the target parameter that produces a detectable output. Considering the state of art of the SPR sensors, the present resolution is of the order of $\Delta n \simeq 2 \times 10^{-7} RIU$ where the main limiting factor has been identified as the amplitude noise of the light source [1].
The setup developed at INO [2] is a possible candidate to overcome the described limitation. The SPR coupling method consists of a typical *Kretschmann* configuration and a single mode narrow linewidth laser source is used. The detection of the sample RI changes is performed through SPR–chip reflectivity variation at the laser wavelength. The chip-prism system is integrated as an intermediate mirror in an optical resonator so that any variation of its reflectivity results in a variation of the resonator optical loss (setup in Fig. 1). The source is locked to a resonator mode by means of the Pound Drever Hall Technique [4]. Considering that the resonator photons storage time is related to the optical losses, acquisitions of the transmitted optical time decay, after switching–off the injection beam, results in a direct loss measurement (Cavity Ring Down technique)[5]. In this sense this technique defines a time domain based loss detection method that is intrinsically immune to the source amplitude noise. The presented setup represents a refractive index change sensor, as a RI change in the sample will produce a SPR wavelength shift that translates in a chip reflectivity change at the interrogation wavelength.

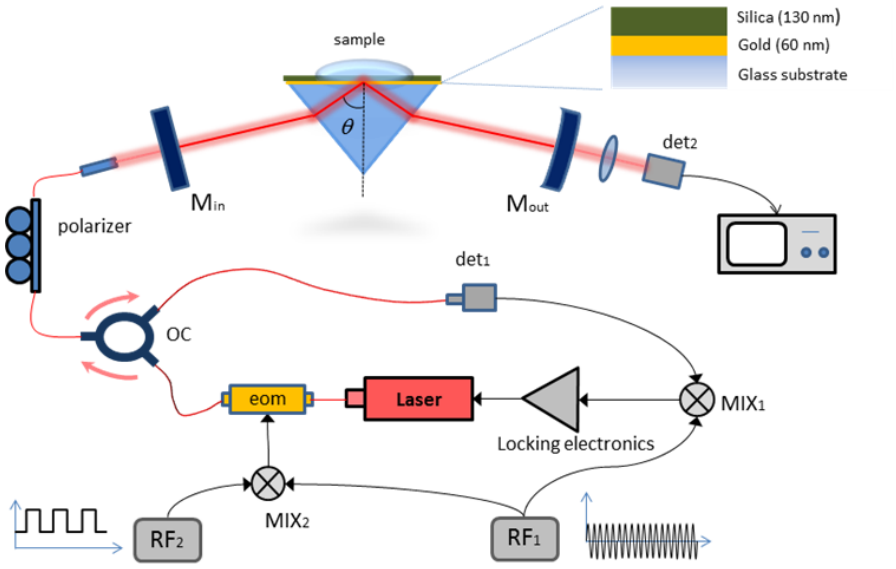

Figure 1: Experimental setup for the optical resonator based SPR sensor [2].

1.2 Surface Plasmon Bio–Sensors, the affinity approach

The high sensitivity of the SPR based sensors to the chemical activity in the region close to the chip surface have triggered the development of many detection methods based on the selective capture of a specific analyte [6, 7, 8, 9]. Specific molecules, classified as *bio-recognition elements* or *receptors* are used, as mediators of the selective detection, to functionalize the SPR sensor surface realizing a sensitive layer. In a basic description model, once the target analyte flows through a buffer solution, it is captured by the *receptors* via chemical bond. As a consequence of this affinity capture the overlayer mass and thickness grows, leading to a local increase of the refractive index Δn_{eff} that depends on the target molecule original concentration in the flow:

$$\Delta n_{eff} = \left(\frac{dn_{eff}}{dc}\right)_{vol} \Delta c \;=\; \left(\frac{dn_{eff}}{dc}\right)_{vol} \quad \frac{\Delta\Gamma}{l} \tag{2}$$

here c is the concentration $(mass/volume)$ of the bound molecule on the sensitive layer, l is the compound layer final thickness and Γ is the analyte surface concentration $(mass/area)$. A linear relation holds between the refractive index change and analyte concentration change on the sensing surface

$$\Delta n_{eff} = K\Delta\Gamma \tag{3}$$

It must be underlined that the surface concentration Γ is different from the original sample target concentration. For this reason a quantitative concentration detection must be supported by a calibration procedure based on the sensing response to well known sample concentrations.

The *limit of detection (LOD)* can be defined for each specific instrument and for the considered immobilization procedure as the minimum detectable concentration of the target. The choice of the bio-recognition elements, their affinity with the analyte and the immobilization procedure play a fundamental role in determining the ultimate LOD. As it will be explained for our experiment, different kinds of chemical bonds occur among the receptor and the surface and between the receptor and the analyte. For this reason, the receptor orientation during the immobilization procedure is very important. Another important feature of the sensing procedure is the reduction of the signal due to non-target analyte captured by the receptors or by the sensing surface via spurious bounds. In the same way also the signal from target molecules detected via non specific bound must be minimized. The main requirements for the receptors and for the immobilization procedures, from which bio-sensing performances depend, can be summarized as follows [8]:

- In order to preserve the accessibility of the receptor to the analyte, the choice of recognition element and its immobilization procedure must be such that the orientation of the receptor respect to the surface is guaranteed.
- The active structure of the bio-recognition element must be preserved after the immobilization procedure.
- The sensing activated recognition layer must present a high affinity to specific bound but also a low capture rate for non-target molecules and for non-specific bounds.

The application of these rules is finalized to the signal-to-noise ratio maximization for a given selective detection channel. For this reason experimental procedures become more complex compared with the basic *receptor-analyte* scheme described so far. A typical approach consists in the introduction of functional layers between the metal (gold) surface and the receptor stage. In some cases, the introduction of intermediate ligands between the functionalized layer and the receptors, involving different kinds of chemical bond, is also needed to address a signal to noise ratio enhancement. In the described experience a *thiol* based functional layer has been considered and the use of *streptavidin* protein as intermediate ligand was also adopted (section 3.1). In other cases many different signal to noise ratio amplifications strategies for a given target channel may be explored.

2 SPR apparatus for bio-detection at IPE

The apparatus used for the described experience is the "PLASMON IV" system developed and engineered at Institute of Photonics and Electronics (IPE) [8, 10, 11] and based on the wavelength spectroscopy of the Surface Plasmon Resonance. Its working principle is founded on broadband source interrogation of the chip and on the detection of an SPR wavelength shift via spectral analysis of the reflected field. Basic components setup is illustrated in picture 2. The SPR is coupled by TM polarized electromagnetic radiation hitting on the surface of a gold coated chip in the total internal reflection condition. For this purpose a prism in the Kretschmann configuration is used [3]. The chips are realized at IPE by a deposition of $50nm$ gold layer on a $2mm$ thick $BK7$ optical slide. An intermediate $2nm$ Titanium layer is used between the $BK7$ and the gold in order to promote the adhesion. The optical matching between the chip and the prism is realized by a refractive index calibrated optical immersion oil.
The prism with the chip is integrated in a custom made microfluidic setup with 4 independent sample cells and 16 microfluidic channels (2 independent input channels + 2 output channels for each cell), supported by two independent Peristaltic pumps (ISMATEC IPC 12 [14]). The volume of each sample cell is evaluated as $\simeq 50\mu l$. The sample operative temperature is controlled by a Peltier circuit that provides a thermal stability of the order of $0.1K$. Thermal stabilization is realized in the tubing that drive the sample from the pump to the cells before the final injection.
The electromagnetic field source is a fiber coupled OCEAN OPTICS [13] halogen lamp (HL–2000) with an emission range of $580nm \div 990nm$. The light from the source is collimated and polarized. The emitted beam is expanded, slit-shaped and projected as four independent beams on the chip areas corresponding to the four sample sensing cells. The incidence angle is calibrated in order to maximally couple a Surface Plasmon Resonance at $\sim 780nm$ with water as sample overlayer. The SPR-modulated radiation from each sensing cell is collected, by GRIN[1] lenses, in four multimode fibers and sent to a spectrometer , OCEAN OPTICS S2000. This device is realized by a system of four independent single channel fiber coupled sub–spectrometers (1 master+ 3 slaves) [13]. Each subdevice consists of a grating projecting the dispersed light on a CCD array system. The net optical resolution on this setup is in the order of $1nm$. The data acquired by the CCD are processed and sent to a DAQ system with a sampling rate of the order of $3ms$. By averaging over 1000 spectra an overall acquisition rate of $\sim 0,33\ Hz$ is obtained.
In order to evaluate the system response to refractive index changes a few preliminary acquisition sessions have been performed using samples with pre-characterized refractive index. In picture 3 the results of a calibration measurements are reported. The SPR wavelength steps generated by five refractive index samples were acquired. Each sample consists of a solution of $NaCl$ in H_2O at a different concentration (0%, 1%, 2%, 4%, 6%) and thus a different refractive index. The SPR system response to the Refractive Index changes has been estimated in $\frac{d\lambda}{dn} \approx 7 \cdot 10^3 nm/RIU$.

[1] Gradient–index (GRIN) lens

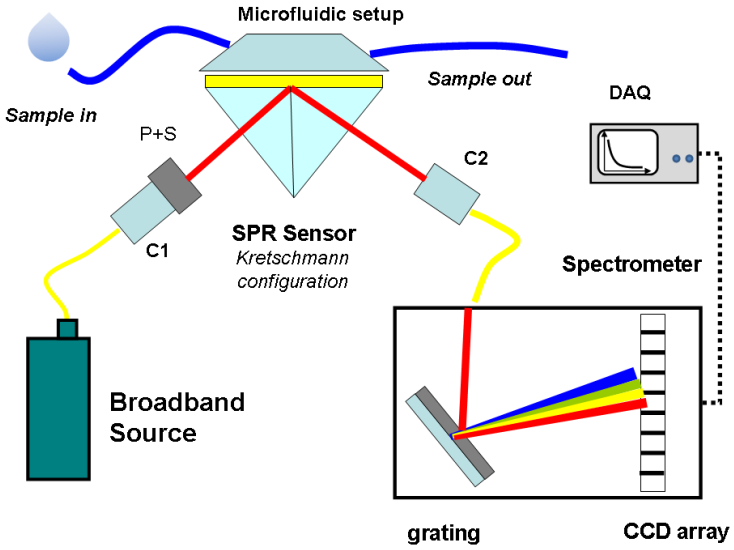

Figure 2: Basic scheme of the apparatus used for the sensing experiment (IPE), ("Plasmon IV")

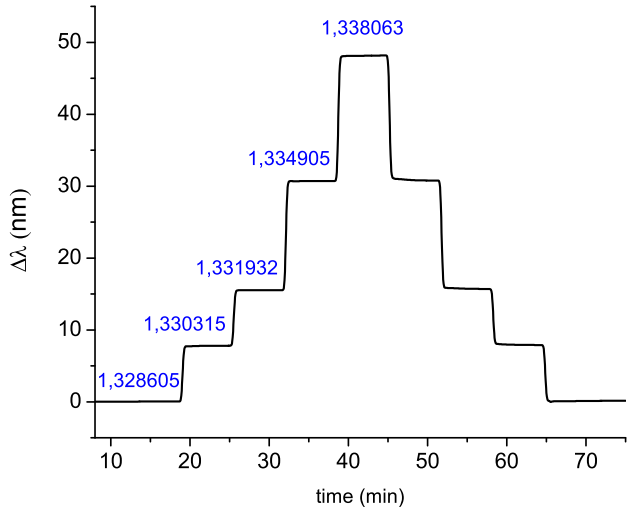

Figure 3: Wavelength shift response to Refractive index changes for the "Plasmon IV" apparatus used in the experience, single channel detection. Calibration with RI pre-measured $NaCl/H_2O$ solutions at different concentrations (blue RI values).

3 Oligonucleotide detection via receptors covalent bound based immobilization

The main experiment realized during the experience of Short Term Mobility at IPE was finalized to the selective detection of DNA oligonucleotides from solutions of well known concentration. Testing the sensor and the immobilization procedure response with known target analyte concentrations represents the procedure leading to a calibration curve that is tipically used for quantitative concentration estimation of an unknown sample. In our case we consider as target species a $20-mer$ DNA oligonucleotide sequence typical of *escherichia coli* bacterium, where the target nucleobases sequence is $C-E4$: $5'-GGAAGGGAGTAAAGTTAATA-3'$. The detection occurs via the immobilization of the conjugate sequence oligonucleotide $B-E4$: $5'-TATTAACTTTACTCCCTTCC-3'$ as receptor. Also a sample with non conjugate sequence has been considered in order to provide a reference signal marking the difference between the non specific bound effect and the specific bond one, $C-E1$: $5'-GCAAGACCAAAGAG\ GGGGAC-3'$. The three involved oligonucleotides sequences are displayed in picture 4, the names "$C-E1$", "$C-E4$", and "$B-E1$" are just conventional labels that we will use in the text to identify them.

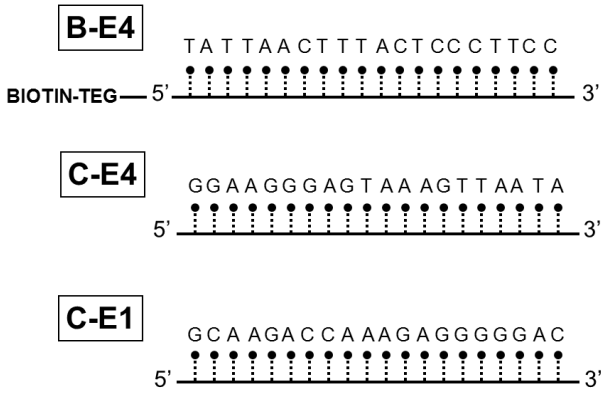

Figure 4: Oligonucleotides sequences considered for the experience, $B-E4$ *biotinylated receptor*, $C-E4$ *target* sequence, $C-E1$ not-conjugated oligonucleotide sequence for reference signal.

The final detection process of the target sequece is based on the hybridization between the two conjugate oligonucleotides, so that starting from two single-stranded DNA molecules it leads to single double-stranded molecule through complementary base pairing. In the following subsections, immobilization steps and target detection session will be analyzed in details.

3.1 Experimental Procedure

A thiols–based functionalization has been prepared for the gold sensing surface as a preliminary step to the immobilization procedure (see section 1.2). In the specific case a precise concentration ratio of two kind of thiols, that we label as $TH1$ and $TH2$, have been considered, where $TH1$: $HS-(CH_2)_{11}-EG_6-OCH_2-COOH$ and $TH2$: $HS-(CH_2)_{11}-EG_4-OH$ (EG_6 Hexaethylene glycol group, and EG_4 Tetra Ethylene glycol group) [15]. Both are linked to the gold surface at the HS side via covalent bound. The task of the $TH1$ thiol, after its activation, is to prepare the proper base to the receptor immobilization. On the other side the task of $TH2$ thiol is to promote the $TH1$ right orientation and to reduce the capture of target molecules by non specific bound. This is realized by filling empty spaces between the $TH1$ thiols. The optimum concentration ratio between the two species, for the native solution, is $[TH1]:[TH2]=3:7$.
As first step of the experiment run, $TH1$ thiols are activated by a solution of NHS/EDC (NHS: $N-hydroxysuccinimide$ and EDC: $1-Ethyl-3-$ ($3-$ *dimethyl aminopropyl*) *carbodiimide*). This leads to the exchange of the *carboxymethyl* groups ($-CH_2-COOH$) with $N-Hydroxysuccinimide$ esters [8]. *Streptavidin* protein [16] is now introduced as an intermediate ligand towards the detection receptor $B-E4$. *Streptavidin* is going to covalently bind to the $N-Hydroxysuccinimide$ esters on one side and it will be ready to accept *biotinylated* $B-C4$ receptor on the other side. The receptor immobilization is indeed based on the high affinity between *streptavidin* and *biotin* (dissociation constant $K_d \approx 4\times10^{-14}$ [12]) due to shape complementarity, hydrogen bonds, *Van der Waals* and hydrophobic interactions. The optimum native thiols concentration ratio (3 : 7), comes from a calibration study conducted at IPE, and is a parameter strongly dependent on the choice of the intermediate ligand molecule (*streptavidin*) with its characteristics and size. If from one side an increment of $TH1$ thiol concentration increases the specific–bound signal from target molecules, on the other side an incrementof $TH2$ thiol concentration decreases the non-specific bound signal form the target molecules and from the unwanted ones. In this sense, for the given intermediate ligand molecule choice the optimum thiols concentration represents a tradeoff between the two described effects.
After the $B-E4$ immobilization the sensing surface is ready for the target oligonucleotide capture. The experimental procedure described so far, from the preliminary functionalization to the oligonucleotides detection through DNA hybridization, is depicted in Figure 5 and commented in the caption. The involved covalent bounds acts between the activated thiols and *streptavidin* and between *biotin* and the receptor oligonucleotide. In the following section the immobilization steps are analyzed in sequence.

3.2 Immobilization steps

For the practical realization of the immobilization of the molecules on the chip, a preparation procedure is needed before each step of the above presented protocol. The complete immobilization procedure is summarized in the following list. Each step can be recognized from the labels on the single channel signal reported in picture 6.

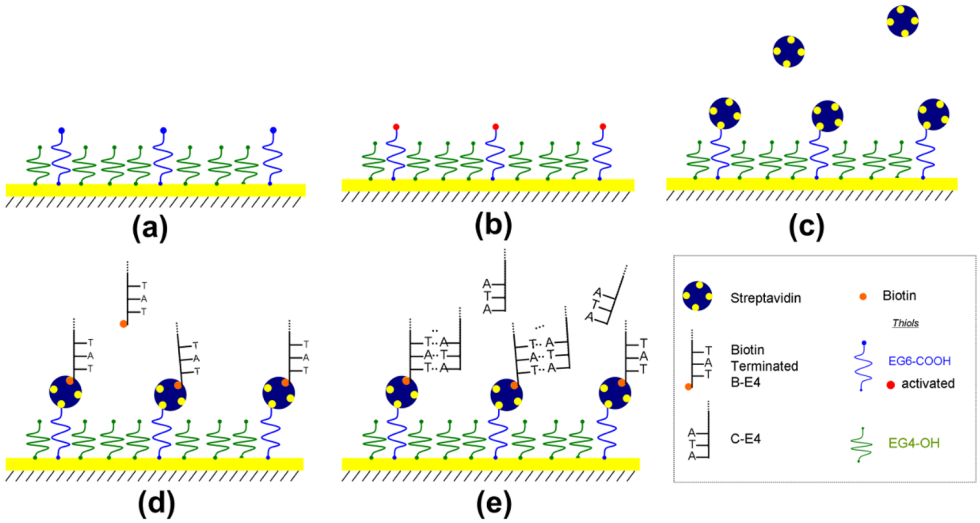

Figure 5: Sequence steps for the selective detection experiment, from the preliminary chip surface functionalization to the analyte detection. (a) preliminary chip functionalization with TH1 and TH2 thiols. (b) TH1 activation through NHS/EDC solution, the *carboxymethyl* groups modified to $N-Hydroxysuccinimide$ esters. (c) Streptavidin (intermediate ligand) bound on activated thiols layer. (d) *Biotinylated* receptor ($B-E4$) immobilization (*streptavidin-biotin* affinity), (e) Conjugate oligonucleotide detection $C-E4$ via DNA hybridization.

1. *Sodium Acetate* $10mM$ ($SA10$) buffer flow ($pH5$), chip preparation and preliminary chemical adaptation.
2. $N-Hydroxysuccinimide$ / $ethyl(dimethylaminopropyl)carbodiimide$ NDH/EDS flow for $TH1$ thiol activation.
3. $SA10$ buffer flow for chip washing, $pH5$.
4. *Streptavidin* immobilization step ($50\mu g/ml$ in $SA10$ buffer), $pH5$, up to bonds saturation, first baseline level acquisition for intermediate ligand immobilized concentration reference.
5. $SA10$ buffer flow for chip washing and unbound *streptavidin* removal, $pH5$.
6. *Phosphate buffered saline*** (PBS^{**}) washing step [2] , washing step at $pH7.4$,
7. $SA10$ buffer flow for chip washing, $pH5$.

[2] PBS^{**} calibrated PBS concentration, see IPE Internal Note.

8. *Ethanol Ammine* $1M$ flow for unoccupied $TH1$ thiols deactivation. This step eliminates unreacted esters from thiols exchanging them with an inactive *hydroxyethyl amide* [9].

9. $SA10$ buffer flow for chip washing from unbound NHS, $pH5$. Another baseline level measurement can be performed for immobilized ligand (*streptavidin*) concentration reference after washing.

10. *Tris(hydroxymethyl)aminomethane* $(TRIS)$ $10mM$ + $30mM$ Mg^{2+} buffer flow[3] for chip chemical adaptation before receptor immobilization, $pH7.4$.

11. $B-E4$, $100nM$ in $(TRIS)$ $10mM + 30mM$ Mg^{2+} $pH7.4$, flow for target receptor immobilization, up to bounds saturation.

12. $TRIS$ $10mM + 30mM$ Mg^{2+} buffer flow for chip washing, $pH7.4$, baseline acquisition to evaluate the receptor surface concentration level.

13. Short $NaOH$ $(4mM)$ flow for chip deep washing finalized to remove weakly bound oligonucleotides, proteins and other unwanted molecules.

14. $TRIS$ $10mM$ + $30mM$ Mg^{2+} buffer flow for chip washing, $(pH7.4)$ and last baseline evaluation of the receptor surface concentration level before the detection session.

3.3 Oligonucleotides detection

After the receptor $B-E4$ immobilization and after the baseline has reached the surface concentration level, the system is ready for the target oligonucleotide detection. This occurs again with a controlled flow of the target molecule solution on the chip. In picture 6 the results of a sequence of three detection sessions are reported. They are separated each other from regeneration steps realized by a flow of $NaOH$ ($4mM$ solution), resulting in a deep washing, that breaks hydrogen bounds between $B-E4$ and $C-E4$ (dehybridization). $TRIS$ $10mM + 30mM$ Mg^{2+} buffer solution flow steps follow the $NaOH$ solution flow for weak washing and chemical stability restoring on the chip surface.
Equation 3 at page 5 shows a proportional relation between the effective refractive index Δn_{eff} increase and the analyte surface concentration grow $\Delta\Gamma$. Considering a constant sample flow, in this specific experiment the target molecule–to–receptor bound is evidenced by a SPR wavelength shift, i.e. refractive index increase, almost linear in time. Here the analyte binding rate, that depends on the analyte concentration in the target solution, is the parameter to be measured. In picture 7 the results of a detection session where two different concentrations of $C-E4$ ($100nM$ and $50nM$) were pumped in the first two channels at same time while, to get the non specific bound reference signal, the same concentration of non binding target $C-E1$ was flown in the other two channels.

[3] *Tris(hydroxymethyl)aminomethane* $(TRIS)$: $(HOCH_2)_3CNH_2$

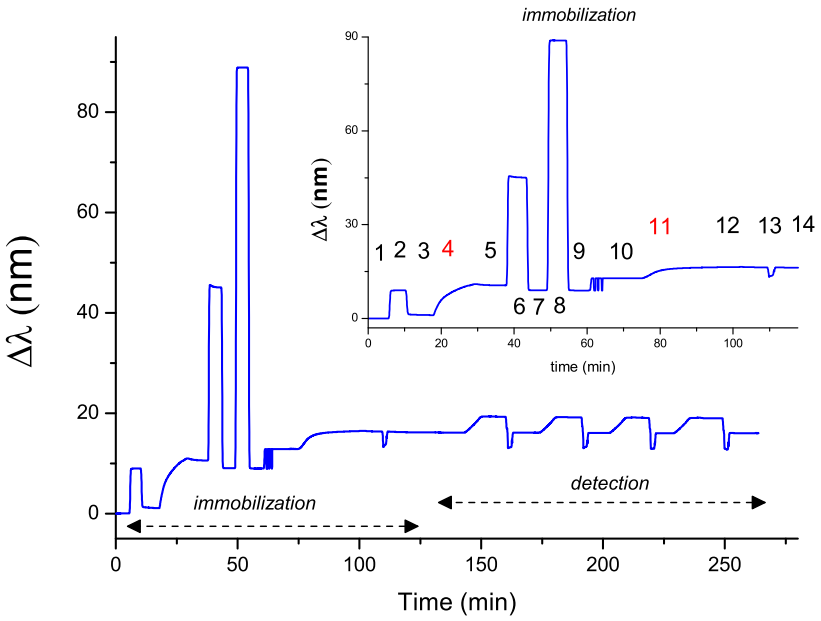

Figure 6: Single channel sensor response to a typical experimental sequence. Inset: detail of the immobilization step, Numeric labels for single procedure steps, red labels mark chemical immobilization processes.

This measurement can be considered as an useful example of the SPR selective detection, as the difference between specific target bound and unspecific signal is evidenced. Two different slopes correspond to the two different $C-E4$ concentrations while no binding grow is evidenced for the $C-E1$ flow. A negligible refractive index step is visible for $C-E1$ channels (see inset), probably due to RI differences between the sample operative solution and the native one. Then, few sessions of simultaneous detection of four different concentrations of $C-E4$ oligonucleotide on separate microfluidic channels were conducted on different chips. The considered concentrations were $100nM$, $50nM$, $10nM$, $5nM$. The typical response signal from the four channels acquisition is reported in the plot in Fig. 8. Again, the different curve slopes evidence different sample concentrations of the target molecule. The measurements were repeated three or four times per acquisition session on the same chip applying the regeneration procedure (see also plot in picture 7). The other detection experiment has been also repeated four times on different chips.

Despite the poor statistic population, the repeated measurements approach have leaded to an estimation of the detection reproducibility on the same chip and on different chips, which is one of the targets of our experience. The resulting calibration curve on different chip is reported in picture 8, where data point represent the averaged values

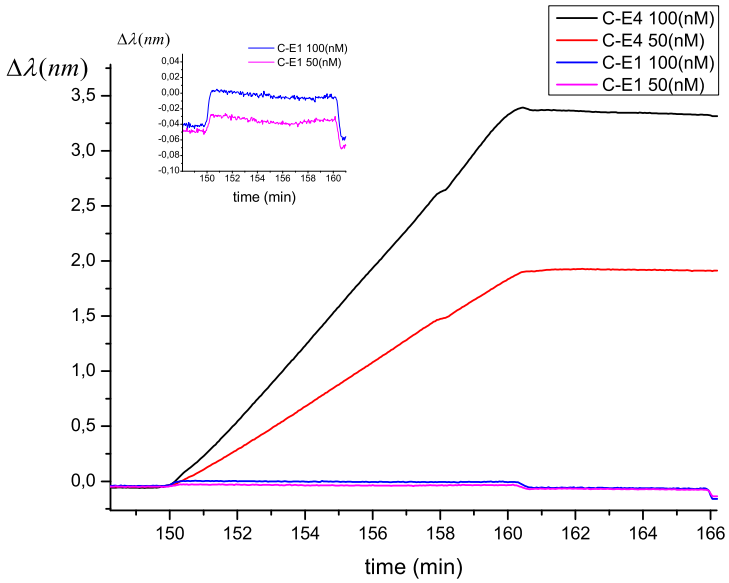

Figure 7: Selective detection of $C-E4$ oligonucleotide at $100nM$ and $50nM$ concentrations. Comparison with reference signal from $C-E1$ at $100nM$ and$50nM$ concentration, from non specific bound.

and the error bars stand for the statistic error. From this plot, a limit of detection (LOD) of the technique of $\approx 2.8nM$ can be estimated. Similar data as in picture 9 are reported in picture 10 for repeated measurements on the same chip separated each other by a deep washing restoring procedure. From a comparison between the error bars in plots 9 and the ones in plot 10 it's easy to note that the measurements reproducibility in a single chip sequence is much higher than the chip-to-chip reproducibility. In both cases a measurements reproducibility $> 91\%$ has been observed.

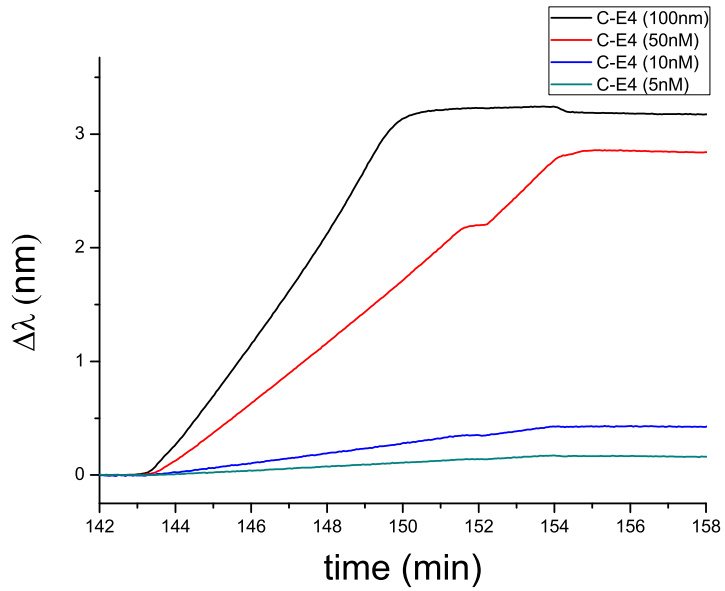

Figure 8: Selective detection of $C-E4$ oligonucleotide at $100nM$, $50nM$, $10nM$ and $5nM$concentrations.

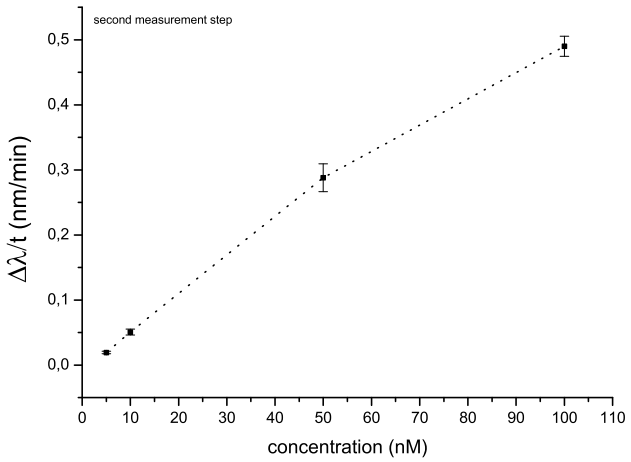

Figure 9: Target oligonucleotides detection calibration curve, wavelength shift response VS target concentration in $TRIS$ $10mM$ + $30mM$ Mg^{2+} buffer solution. Statistic on repeated measurements on different chips

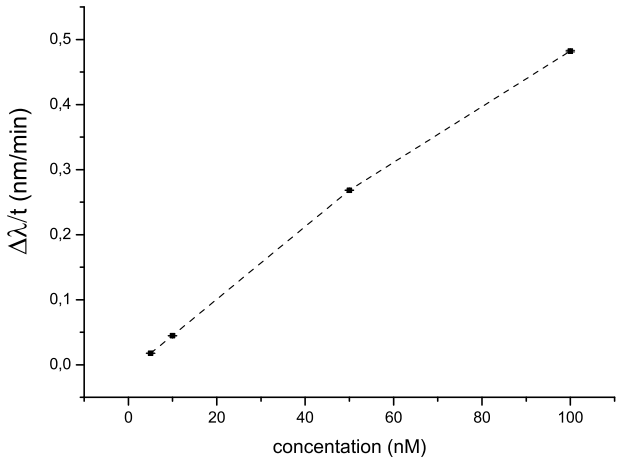

Figure 10: Target oligonucleotides detection calibration curve, wavelength shift response VS target concentration in $TRIS$ $10mM$ + $30mM$ Mg^{2+} buffer solution. Statistic on repeated measurements on the same chip after regeneration.

Conclusions

As illustrated in the presented report, during the Short Term Mobility activity the basic concepts related to the Surface Plasmon Resonance based bio–sensing techniques have been treated. The scientific trial at IPE has been performed using an engineerized SPR sensing setup whose performances represents a state–of the art reference. A proof–of–principle experiment for oligonucleotides detection, based on covalent bound of receptor, was successfully addressed. Thanks to this trial it was possible to look further into all the typical issues related to such specific kind of experiment, ranging from the surface immobilization of the receptors to the final detection of the target molecule. A deep analysis of the surface immobilization chemical protocols has been carried out. The criticality of the detection final results, due to protocols calibration, has been considered as a fundamental point. Themes related to the bio–detection uncertainty and methods–dependent Limit Of Detection of a specific target have also emerged from the experimental activity. The acquired expertise in the bio–sensing field will be essential for the future research activity related to Surface Plasmon Resonance sensors development at INO Naples. This has contributed to mature a more complete approach at the specific scientific research line and also to pave the way to new applications for the developing systems. The Scientific activity carried during the Short Term Mobility has played a fundamental role in consolidating the pre-existent collaboration between the two involved institutions laying the basis for future projects development.

Acknowledgements

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List of Figures

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