Report on STSM -COST Action TD1003

Title: Aptamer biosensors  
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**SPR analysis for aptamer biosensor assembling towards detection of Vascular Endothelial Growth Factor**

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**Introduction**

Using of aptamers as artificial receptors for protein detection has been attained remarkable interest on biosensor research. Comparison of various techniques of detection gives decisive information for finding the optimal conditions in aptasensor assembling. While electrochemical methods allow very good quantitative analysis of the biosensor signal, optical methods such as Surface Plasmon Resonance Imaging (SPRi) provide in contrast, a deeper view of the surface behavior, by looking the surface images provided by CCD detector. For example SPRi may reveal the possible artifacts that could affect the biosensor construction, especially when working with direct probe immobilization onto gold surfaces. Therefore, it is crucial to perform both electrochemical and optical studies for obtaining the most favorable sensing conditions for the detection of the target protein, i.e. Vascular Endothelial Growth Factor (VEGF_{165}) an important cancer marker that plays a key role in angiogenesis.
Purpose of the STSM

The objective of this STSM was to test the binding activity of different DNA sequences of aptamers by means of surface plasmon resonance imaging (SPRi) in order to assemble an aptamer-based biosensor sensitive to ochratoxin a (OTA) and vascular endothelial growth factor (VEGF₁₆₅).

Sequences of aptamers used were: OTA-SH: 5’-SH-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3’, V7t1-SH: 5’-SH-TTT TTT TTT TTT TTT TTT AGA TGG GCC GGG CAG GTG GGG GTG T-3’, VEGFapt_SH: 5’-SH-TTC CCG TCT TCC AGA CAA GAG TGC AGG G-3’. The first aptamer OTA-SH was only sensitive to OTA. Part of these studies already began last year, thus a completion of the experiments was essential. VEGFapt and V7t1 aptamers both sensitive to VEGF₁₆₅, were selected in order to analyze its individual response. In all cases, the immobilization technique employed was thiol-chemisorption. Among other tasks to be completed, was to find out the optimal conditions for the regeneration of the biosensor after detecting VEGF₁₆₅, since parallel experiments based on multiwalled carbon nanotubes (MWCNTs)-dendrimers-aptamers platform were tested in our home institution for sensing the same analyte. We were also interest in comparing theses two platforms and select the most satisfactory for VEGF₁₆₅ detection.

Description of the work carried out during the visit

The first week of the STSM was dedicated to the preparation of gold surfaces for immobilization of thiolated sequences of aptamers: OTA-SH, V7t1-SH and VEGFapt-SH. Figure 1 illustrates the biosensor assembling. This first chip was prepared containing the three probes mentioned above plus one thiolated sample of unspecific DNA sequence (5’-SH-GTG GTGTCACAGGAAGATT-3’). After aptamers attachment to the gold surface, a layer of co-thiols composed of 11-Mercaptoundecanol (MUD) and 6-Mercaptohexanol (MCH) ensured the proper blocking of the surface in those places where no probes were immobilized. This sensor was exposed to 1 µM OTA and the changes in the signal were monitored by SPRi.
Gold surface was regenerated in 100 mM HCl. 50 mM NaOH were also tested for regeneration, showing that the acidic treatment is the best when working with OTA.

![Gold chip surface after immobilization of different sequences of aptamers, surface blocking with co-thiols layer and subsequently washing by deionized water (left). Gold chip surface schema containing three aptamers immobilized on the chip: OTA-SH, V7t1-SH, VEGFapt-SH. Unspecific DNA sequence, gold and buffer blank spots are also represented (right).]

**Figure 1.**

During the second week another chip was prepared, this time containing aptamers sensitive only to VEGF_{165}. This biosensor was also covered by the co-thiols layer used in the first chip. As analyte, different concentrations of VEGF_{165} were injected on SPRi device and binding signal followed. Intermediate regeneration steps in 50 mM NaOH for VEGF were utilized before addition of each VEGF_{165} concentration: 30 nM, 50 nM, 100 nM, 200 nM. This regeneration procedure turned out the best results for cleaning the surface of VEGF_{165}. After last VEGF_{165} injection (200 nM), we performed an additional sandwich assay by using V7t1 and VEGFapt aptamers to investigate the affinity of each aptamer to human recombinant VEGF_{165}. 50 mM NaOH and 100 mM HCl were injected this time for regenerating the surface. NaOH was used for cleaning the protein and HCl for removing the aptamers. After the surface was again free of analyte, BSA (bovine serum albumin) in 200 nM concentration was added as a control sample.

In the third week, we reproduced a similar chip as in the first week but in this case the analyte tested was VEGF_{165} in concentrations: 10 nM, 30 nM, 100 nM, 200 nM. Immobilized probes were V7t1-SH, VEGFapt-SH and we also added OTA-SH aptamer to the sensor, this probe was later selected as blank control for this chip. Sensor regeneration was also performed in 50 mM NaOH.
Description of the main results obtained

Regarding to the first chip prepared for monitoring the affinity of aptamers to OTA, we have observed that since molecular weight of OTA is relatively small (403.8 Da) and it was dissolved in an organic solvent- acetonitrile, the interaction of the solvent with the surface was strong and interfered with the response signal of the aptamers producing also a non-specific binding. After regeneration, we have confirmed that even when the surface was clean, in such a big concentration, the OTA solvent could possibly interact with the aptamers immobilized on the surface.

It should be taken into account that for OTA detection by SPRi, due to the solvent combination used, big variation of the refractive index from the running buffer with regard to the testing solution occurred. For this reason, operatively, for the detection of OTA we have subtracted the whole binding signal from the acetonitrile signal, taken as reference.

Further, a sandwich assay for the VEGF was studied. In particular, the second chip was dedicated to analyze the VEGF<sub>165</sub> binding affinity to its respective aptamers V7t1 and VEGFapt, alternatively used as capturing and secondary binding aptamers. In Figure 2 are shown the results of this assay. In both cases, BSA signal was used as reference control, since non-specific interaction of VEGF protein with the surface was detected in the first round of experiments when taking working buffer spot as blank reference control. On left-side graph (a) is represented the sandwich assay taking in account just the V7t1 aptamer as platform of immobilization.

After addition of VEGF<sub>165</sub> the SPR binding signal was recorded and VEGFapt aptamer was subsequently injected. An increase in the signal was observed after injection of the second aptamer indicating that this aptamer also bind to VEGF<sub>165</sub> as expected. On the right graph (b) is illustrated the same sandwich assay but taking in consideration only the signal given by VEGFapt aptamer.
Figure 2. Sandwich assay for V7t1 and VEGFapt aptamer both immobilized on gold surface in concentrations 1 µM, 5µM and 10 µM respectively. After immobilization of both aptamers, 200 nM of VEGF protein was added followed by injection of 1 µM of VEGFapt aptamer. Subsequently 1 µM of V7t1 was aggregated and the signal recorded after every single step of sandwich formation a) Sandwich assay taking in account signal changes over V7t1 aptamer, b) Sandwich assay taking in account signal changes over VEGFapt aptamer. In both cases, interaction of BSA with V7t1 and VEGFapt was taken as reference control.

In this case, binding signal to VEGF165 was also detected but with less intensity showing that VEGFapt aptamer has less affinity to VEGF165 in the sandwich assay probably due to the fact that VEGFapt aptamer has binding affinity to the heparin-binding domain (HBD) of VEGF165 while V7t1 aptamer binds to the two binding sites present in VEGF165: heparin-binding domain (HBD) and receptor binding domain (RBD). Figure 3 shows a schematic representation of the VEGF binding to both aptamers.

Figure 3. V7t1 aptamer binds to heparin binding domain (HBD) and receptor binding domain (RBD) present in VEGF, while VEGFapt aptamer binds only to HBD of the protein.
The use of BSA signal subtraction was justified by the unspecific binding observed of VEGF protein on blank reference control spots, probably due to the high number of disulfide bonds present in the VEGF\textsubscript{165} structure that could interact with the co-thiol groups used for blocking the surface as well as the high salts content of the working buffer. We observed this effect in the SPRi flow cell image as slightly colored spots resting on the co-thiols surface. The findings obtained by SPRi are of crucial importance for the assembling of an aptamer-based biosensor, indicating that chemistry techniques for immobilization such as those including biotinylated aptamers could considerable reduce this effect. Sensor regeneration in NaOH and HCl ran satisfactory.

Finally, (during the third week), affinity of the VEGF\textsubscript{165} to V7t1-SH and VEGFapt-SH aptamers was studied taking OTA aptamer (OTA-SH) probe as reference. The optimal concentration of the aptamers to be used in the sandwich-like assay was eventually optimized after the binding of 200 nM VEGF\textsubscript{165} to V7t1-SH and VEGFapt-SH immobilized directly on the gold surface.

Results are presented in Figure 4. Binding signal corresponds to immobilized aptamers on the chip surface (in the configuration adopted in Figure 1). It is worth to noting that V7t1 has a tail of 17 thymine bases that clearly influences the mobility of the aptamer anchored on the gold, this fact could be clearly observed in aptamers having a concentration lower than 10 µM where V7t1 aptamer present a higher binding affinity than VEGFapt. At 10 µM concentration, both aptamer present a similar affinity to VEGF\textsubscript{165}.

This is an important result that serves us to confirm the optimal amount of aptamers sensitive to VEGF\textsubscript{165} for biosensor assembling to be immobilized on gold surface when working with direct thiol attachment.
Figure 4. SPR signal after binding of V7t1 and VEGFapt aptamers to 200 nM VEGF$_{165}$. V7t1 and VEGFapt were immobilized on the gold surface in concentrations: 1 µM, 5 µM and 10 µM respectively. OTA-SH aptamer signal was taken as blank control.

- **Projected publications/articles resulting or to result from the STSM (if applicable)**

The results obtained during this STSM will serve as complementary information for a biosensor project that we are currently developing in our home institution concerning to the elaboration of a VEGF$_{165}$ biosensor using aptamers as elements of recognition but using a more complex platform of immobilization. This mission gave us also significant knowledge about the aptasensor surface behavior with regard to VEGF$_{165}$. Additionally, it elucidated the interaction of the protein itself with thiol groups that has to be reduced in order to avoid non specific interactions on the surface. STSM also provided useful tips about the influence of structural configuration of aptamers and its effect in the binding activity to the ligand of interest, in this case VEGF$_{165}$. Moreover, the visit has also brought me new perspectives on aptamers-based sensor development and its applications on fluidic systems and on-lab chip systems. Information acquired from this STSM will be used for comparing two biosensors assays devoted to VEGF$_{165}$ detection: one based on direct immobilization of thiolated aptamers on gold surface and the other one, using MWCNTs-dendrimers and carboxylated aptamers as transduction platform on the gold. SPRi surface information, as well electrochemical methods will be also confronted for these two assays. Achieved results from this analysis will be presented at the conference Bioelectrochemistry 2013 to be held in Bochum, Germany on 17th March 2013 under the name of "Development of an Aptamer-based Biosensor for Detection of Vascular Endothelial Growth Factor" which has been selected as a poster contribution.

- **Confirmation by the host institute of the successful execution of the mission**

A separately report from the host institution will be send confirming the completion of the mission.
I would like to express my thanks to the COST action TD1003 that supported this work, to my home institution Comenius University in Bratislava, in particular to my supervisor Prof. Dr. Tibor Hianik, as well as the host institution Universita degli Studi di Firenze, that made it possible the completion of this STSM. Especially thanks go to Prof. Dr. Maria Minunni, Dr. Simona Scarano and Maria Laura Ermini for their helpful dedication and constructive guidelines during my whole stay in Florence.