Development of Triggerable NanoCluster biosensor for Prostate Cancer diagnosis

My research project is focused on the development of nanostructured biosensors for early detection of Prostate Cancer. In this I have assembled two different triggerable nanosensors composed by interacting nanoparticles that respond to the presence of a specific protein biomarker. One of these sensors is designed for *ex vivo* application to detect exosomal Prostate Specific Membrane Antigen (PSMA) in urine samples. The second one is designed to detect Prostate Specific Antigen (PSA) in blood and its proprieties would make it suitable for *in vivo* application.

Introduction

Pathologies such as cancer can worsen for years before been detected. Unfortunately, complete eradication of cancer is strictly related to the possibility to perform diagnosis at early stage of the disease (or of the relapse of the pathology after treatment). Considering Prostate Cancer, which is the second most diagnosed cancer in men, survival rate drops from nearly 100% if Prostate Cancer is diagnosed early to 30% when it is diagnosed in advanced state[1]. Importantly, diagnostic capabilities in clinics have not improved in the last decades, since currently achievable sensitivity level of diagnostic assays used in clinics is hampered by several factors. Firstly, nowadays there is no ideal unique biomarker for early cancer detection and, secondly, there is an intrinsic limit of detection proper of any analytical technique. Considering ELISA, the main bioanalytical technique used in clinics for protein detection, recognition of the biomarker relies on the interaction between an antigen and an antibody ad the limit of detection is dictated mainly by the attainment of chemical equilibrium between the two molecules[2,3]. The sensitivity of ELISA permits the detection of an antibody with a dissociation constant of 10^{-9} M in standardised clinical tests, and this value can decrease down to 10^{-11} M only when reaction is performed at its best conditions. As a consequence, the limit of detection of ELISA strictly depends on the concentration of the biomarker and does not depend on volume of the sample. One way to overcome this limitation relies on the identification and quantification of a biomarker not with a chemical equilibrium based reaction (such as ELISA), but with an irreversible recognition step of the analyte by the sensor. These kind of reactions have the potential to ameliorate current diagnostic capabilities because sensitivity level depends on the absolute number of molecules (instead of concentration) and, as a consequence, would increase with the screening of larger sample volumes. With this in mind, we developed triggerable metal-based nanoarchitectures able to respond to the presence of a protein biomarker. These sensors are composed by differently decorated Gold Nanoparticles (NPs) that interact together because they present on their surface complementary DNA sequences. These DNA sequences have been designed appropriately to anneal stably in absence of the analyte and the interaction between NPs that expose these complementary sequences results into a cluster of nanoparticles. In addition, the recognition of the target protein by the sensor occurs because these DNA sequences include an aptamer specific to the biomarker. The presence of the analyte destabilizes the interaction between DNA sequences and triggers the release of single Gold NPs from the cluster in an irreversible way. The choice of Gold NPs permits to monitor the release of single NPs from the cluster and variation in its size exploiting Gold NPs optical proprieties. In addition, isolated fractions of single reacted NPs from unreacted clusters can be quantified with higher sensitivity by ICP-MS measurements.

In order to achieve early Prostate Cancer detection, we selected two protein biomarkers for our sensors: Prostate Specific Membrane Antigen (PSMA) for Prostate Cancer early detection and Prostate Specific Antigen (PSA) for early detection of Prostate Cancer biochemical recurrence. Despite Prostate Cancer starts to be regularly screened in men older than 50 years old performing digital rectal examination and Prostate Specific Antigen (PSA) testing, PSA has a low specificity as biomarker in Prostate Cancer diagnosis[4,5] and causes important cases of over diagnosis. Prostate Specific Membrane Antigen (PSMA)[6] is a promising alternative biomarker and can discriminate Prostate Cancer more specifically than PSA[7]. In fact, PSMA expression importantly increases in Prostate Cancer and, in particular, in

aggressive and metastatic Prostate Cancer [8,9]. Importantly, PSMA is able to discriminate between Prostate Cancer and healthy patients in urine samples and in urinary exosomes[10–12]. On the other side, despite PSA is poorly specific for Prostate Cancer early detection, determination of PSA serum level is the main tool to monitor biochemical recurrence[14] in patients that underwent Radical Prostatectomy. However, depending on the limit of detection of the PSA assay, recurrence can remain unnoticed if PSA falls in the undetectable range[15] and increasing our sensing ability would result into an earlier detection, and thus treatment, of recurrence of the disease. For these reasons, we developed two different Nanostructured biosensors able to detect either PSA or PSMA to perform respectively early detection of biochemical recurrence or of cancer onset. In particular, PSMA responsive sensor is designed for *ex vivo* analysis or urinary exosomes, whereas PSA responsive sensor is composed by ultrasmall renal clearable NPs that would make it suitable for an *in vivo* application, permitting the screening of large volume samples.

Clustered NPs based biosensor assembly and working principle

Nanoparticle based biosensors were assembled as follow: particles were decorated with selected and complementary nucleotide sequences that lead to the formation of the clusters and trigger the release of single NPs upon the recognition of the target protein. Two main structures of nucleotide sequences were designed, named dimeric and trimeric, and a control sequence (Scrambled) that does not respond to the analyte (*fig.1a*). In the dimeric and trimeric structure two or three NPs are differently functionalised in order to assemble the cluster. Both in dimeric and trimeric clusters, the interaction among particles is controlled by three nucleotide sequences: A, B and a sensing strand that includes an aptamer specific for the target protein. The sensing strand is composed by two parts with different function: a portion that anneals to A and confers structural and thermal stability to the cluster and a second unit that consist in the aptameric sequence. The aptamer is partially annealed to B strand, whereas the rest of the sequence is not annealed and this condition is necessary to ignite the recognition of the target protein. In fact, in absence of the analyte (PSMA or PSA) the annealing between the aptamer and B strand is thermally stable. However, the presence of the biomarker will favour the interaction between the aptamer and the target protein instead of the one between the aptamer portion complementary to B and B strand itself. As a consequence, the aptamer loses some of its interaction with B sequence and this translates into a reduction of the melting temperature between these two strands, producing the release of B, thus of the Gold NPs decorated with it (*fig. 1b*). If performed *in* vitro, this kind of reaction is an equilibrium based reaction with a certain dissociation constant. However, the outcome of the reaction when the cluster is incubated with the analyte indicate that the equilibrium of this reacion is strongly moved towards the dissamble of the cluster and the release of single nanoparticle. On the other side, if this reaction takes place in bloodstream *in vivo*, we can really obtain an irreversible recogntion step because of the biological conditions: released single NPs in circulation and presence of nucleases will defintely impede the back anneal of the nanoparticle to the cluster.

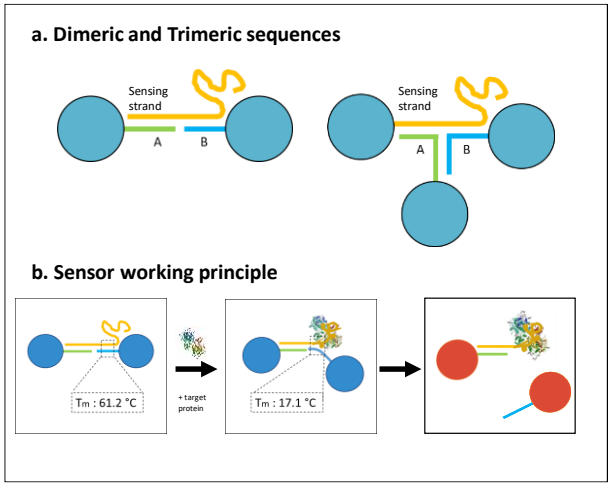

Figure 1 : (a) The two structures of NPs Clusters Dimer (upper left) and Trimer (upper right) (lower left) and DNA sequences involved. (b) working principle of the sensor highlighting the reduction of the melting temperature between the sensing strand and B after the interaction of the system with the target protein.

1. PSMA biosensor

The first sensor developed is designed to respond to the PSMA protein for *ex vivo* application. This sensor is composed of 13 nm diameter Gold NPs, which have good optical proprieties and show plasmon resonance proprieties that depends on the size of the cluster. The DNA sequences for PSMA responsive cluster include the aptameric sequence specific for PSMA[13] and were firstly designed *in silico* with DINAMelt online software. Melting temperatures were then confirmed with UV-Vis absorbance at 260 nm (*Table 1*) and are comparable with those calculated *in silico*. Then, NPs clusters were obtained incubating nanoparticles that expose complementary sequences on their surface together with the aptamer. Cluster size was measured with Dynamic Light Scattering (DLS) and UV-Vis spectrum: solution of single nanoparticles shows a plasmonic peak at 520 nm, whereas clustered particles have a plasmonic peak at 540 nm and increased hydrodynamic radius at DLS. To confirm that clusters assembled because of complementary sequences we measured melting curves of the clusters following variation in size change at DLS incubating the clusters at different temperatures. We observed that clusters disassemble upon reaching DNA sequences melting temperature (*Table 1*), consistently with the dependence of assembly stability on the hybridization of the nucleotide sequences.

Sequence	***Tmelt In silico (DINAMelt)***	***Tmelt In vitro (260 nm abs)***	***Tmelt (DLS)Nanoscluster***
antiPSMA dimer	56 °C	53 °C	52.5°C
antiPSMA trimer	46.5°C	41 °C	35°C
Scrambled	56°C	58°C	50°C

Table 1: antiPSMA and Scrambled sequences melting temperatures and relative clusters melting temperatures.

Then, we tested biosensing response of clusters to PSMA in conditions of growing complexity. We first tested the sensor with human recombinant PSMA and then with PSMA positive exosomes, which are the real biological target of the sensor. When we incubated the cluster with human recombinant PSMA, we observed that the dimeric cluster starts to show sensing ability at PSMA concentration of 50 pM, as shown by DLS measurements (*fig.2*), and does not show a definite trend when incubated with BSA as negative control (up to physiological level). Scrambled cluster shows no response when incubated either with PSMA or BSA, confirming the specificity of our sensor. The trimeric cluster responsive to PSMA did not show any relevant response to human recombinant PSMA and was not further studied. Cluster response to PSMA was also followed measuring variations in the plasmonic shift of the sensor. In this case we observed a reduction of the plasmonic peak wavelength from 544 nm (with no PSMA) to 538 nm with a PSMA concentration of 50 nM (*fig.3a*). DLS and spectra measurements indicate that the cluster reduces its size when incubated with higher PSMA concentration, releasing single nanoparticles.

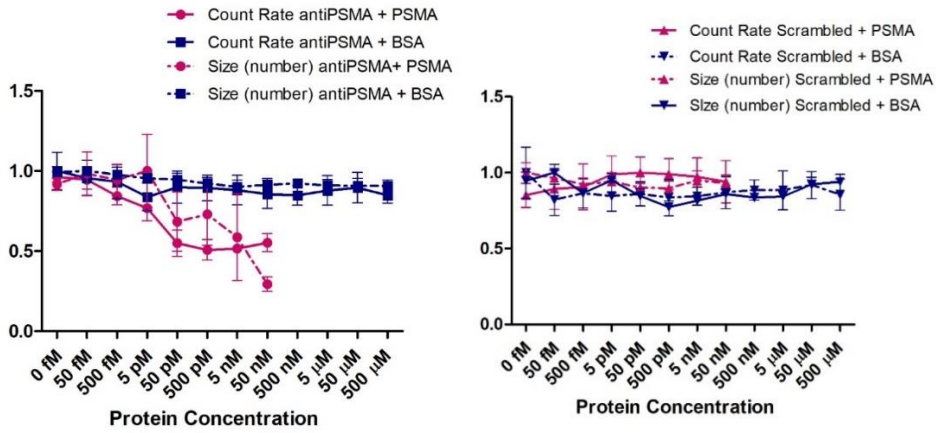

Figure 2 DLS measurement of Dimeric Cluster (antiPSMA and Scrambled) response to PSMA and BSA. Count Rate and Number Mean are normalized

Then, since the presence of PSMA positive exosomes in urine is discriminant to perform Prostate Cancer diagnosis, PSMA responsive clusters were incubated with different concentration of exosomes. Exosomes were isolated and characterised from Prostate Cancer cell lines PC-3 (PSMA negative) and LNCaP (PSMA positive). Cluster response to exosomes was monitored at UV-VIS and DLS. In this case we observed an increase in size at DLS and plasmonic shift towards longer wavelength when the cluster was incubated with LNCaP exosomes at increasing concentration (*Fig.3b*). Curiously, this trend is opposite to the one observed when clusters were incubated with purified PSMA protein. Considering this data, it is tempting to assume that in presence of exosomes clusters not only release single NPs but also adhere and accumulates to the surface of exosomes because of NPs able to interact with PSMA. In order to characterize the final product of this reaction we are currently setting a method based on size exclusion to separate single NPs from NPs on exosomes surface and unreacted clusters.

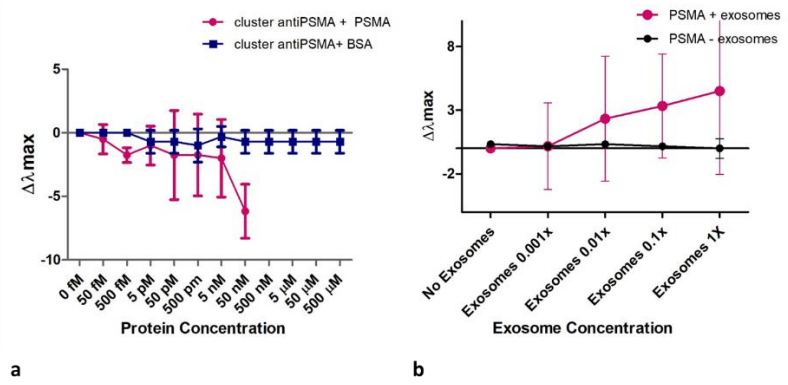

Figure 3: Plasmon peak shift of the cluster when incubated with hrPSMA (a) or when incubated with PSMA positive exosomes (b).

2. PSA biosensor

The second NPs based biosensor is designed to respond to the presence of PSA for *in vivo* applications. In fact, prompt recognition of PSA in serum of patients that underwent radical prostatectomy is crucial to achieve early detection of biochemical recurrence. The application of triggerable nanoclustered sensor *in vivo* would permit to screen the total blood volume in a real irreversible way, also because of biological conditions (i.e. presence of nucleases) in bloodstream. In this case the biosensor is composed by single 2nm of diameter AuNPs, whose size makes them renal clearable thus suitable for *in vivo* application. In fact, ultrasmall NPs are rapidly (in few hours)

excreted in urine. PSA sensor is designed to quantify serological PSA by measuring the level of released reacted single NPs in urine with ICP-MS measurements. As for the previous sensor, particles were decorated with selected and complementary ssDNA sequences that lead to the formation of the nanoparticle clusters. We designed two dimeric clusters: one that includes the aptamer sequence responsive to PSA[16] (antiPSA) and one that contain a control Scrambled sequence. AntiPSA sequences and control Scrambled sequences were firstly designed *in silico* with the DINAMelt online software. Melting temperature of sequences were then confirmed with absorbance measurements at 260 nm (*Table 2*). Then, antiPSA and Scrambled cluster were assembled incubating NPs that expose complementary sequences and the aptamer. Increase in size of the clusters from single NPs was measured either by DLS measurement and by UV-plasmon resonance shift. Chemico-physical characterisation of sensors was concluded measuring the melting temperature of clusters at DLS (*Table 2*). From melting temperatures measurements, we conclude that this set of sensors are stable at body temperature (37°C) and that the interaction of NPs is due to the DNA sequences.

Sequence	***Tmelt In silico (DINAMelt)***	***Tmelt In vitro (260 nm abs)***	***Tmelt (DLS) Nanocluster***
antiPSA	55.6	58 °C	55°C
Scrambled	55.6°C	58°C	57.5°C

Table 2: antiPSA and Scrambled sequences melting temperatures and relative clusters melting temperatures.

Conclusion

In conclusion, two different nanostructures for Prostate Cancer early detection have been assembled. PSMA responsive cluster has good thermal stability and a sensing response either with human recombinant PSMA and with PSMA positive exosomes. PSMA responsive cluster shows a decrease in size when incubated with human recombinant PSMA, indicating the release of single NPs from the cluster upon the interaction with the target protein. On the other side, when PSMA responsive cluster was incubated with PSMA positive exosomes, we observed an increase in size of the interacting systems. This is probably due to the interaction of the NPs decorated with the aptameric sequence on PSMA present on the surface of the exosome. This process, however, indicates the presence of effective interaction between the sensor and PSMA-positive exosomes even at low (10^{10} exosomes/ml) exosome concentration. The second sensor is responsive to PSA and has been fully characterised for what concern thermal stability of involved DNA sequences and for chemico-physical proprieties of the clusters, which resulted thermally stable. Melting curves of this set of clusters indicate also that their assembly is due to the annealing of the involved DNA sequences.

References:

(1) Etzioni, R.; Urban, N.; Ramsey, S.; McIntosh, M.; Schwartz, S.; Reid, B.; Radich, J.; Anderson, G.; Hartwell, L. The Case for Early Detection. *Nat. Rev. Cancer* **2003**, *3* (4), 243–252. https://doi.org/10.1038/nrc1041.

(2) Zhang, S.; Garcia-D'Angeli, A.; Brennan, J. P.; Huo, Q. Predicting Detection Limits of Enzyme-Linked Immunosorbent Assay (ELISA) and Bioanalytical Techniques in General. *Analyst* **2013**, *139* (2), 439–445. https://doi.org/10.1039/c3an01835k.

(3) Giljohann, D. A.; Mirkin, C. A. Drivers of Biodiagnostic Development. *Nature* **2009**, *462* (7272), 461–464. https://doi.org/10.1038/nature08605.

(4) Filella, X.; Foj, L. Prostate Cancer Detection and Prognosis: From Prostate Specific Antigen (PSA) to Exosomal Biomarkers. *International Journal of Molecular Sciences*. 2016. https://doi.org/10.3390/ijms17111784.

(5) Lomas, D. J.; Ahmed, H. U. All Change in the Prostate Cancer Diagnostic Pathway. *Nat. Rev. Clin. Oncol.* **2020**. https://doi.org/10.1038/s41571-020-0332-z.

(6) Chang, S. S. Overview of Prostate-Specific Membrane Antigen. *Rev. Urol.* **2004**, *6 Suppl 10* (Figure 1), S13-8.

(7) Gorges, T. M.; Riethdorf, S.; von Ahsen, O.; Nastaly, P.; Röck, K.; Boede, M.; Peine, S.; Kuske, A.; Schmid, E.; Kneip, C.; et al. Heterogeneous PSMA Expression on Circulating Tumor Cells - a Potential Basis for Stratification and Monitoring of PSMA-Directed Therapies in Prostate Cancer. *Oncotarget* **2016**, *7* (23), 34930–34941. https://doi.org/10.18632/oncotarget.9004.

(8) Hupe, M. C.; Philippi, C.; Roth, D.; Kümpers, C.; Ribbat-Idel, J.; Becker, F.; Joerg, V.; Duensing, S.; Lubczyk, V. H.; Kirfel, J.; et al. Expression of Prostate-Specific Membrane Antigen (PSMA) on Biopsies Is an Independent Risk Stratifier of Prostate Cancer Patients at Time of Initial Diagnosis. *Front. Oncol.* **2018**, *8* (DEC), 1–7. https://doi.org/10.3389/fonc.2018.00623.

(9) Ross, J. S.; Sheehan, C. E.; Fisher, H. A. G.; Kaufman, R. P.; Kaur, P.; Gray, K.; Webb, I.; Gray, G. S.; Mosher, R.; Kallakury, B. V. S. Correlation of Primary Tumor Prostate-Specific Membrane Antigen Expression with Disease Recurrence in Prostate Cancer. *Clin. Cancer Res.* **2003**, *9* (17), 6357–6362.

(10) Mitchell, P. J.; Welton, J.; Staffurth, J.; Court, J.; Mason, M. D.; Tabi, Z.; Clayton, A. Can Urinary Exosomes Act as Treatment Response Markers in Prostate Cancer? *J. Transl. Med.* **2009**, *7* (of 8), 1–13. https://doi.org/10.1186/1479-5876-7-4.

(11) Øverbye, A.; Skotland, T.; Koehler, C. J.; Thiede, B.; Seierstad, T.; Berge, V.; Sandvig, K.; Llorente, A. Identification of Prostate Cancer Biomarkers in Urinary Exosomes. *Oncotarget* **2015**, *6* (30), 30357–30376. https://doi.org/10.18632/oncotarget.4851.

(12) Li, P.; Yu, X.; Han, W.; Kong, Y.; Bao, W.; Zhang, J.; Zhang, W.; Gu, Y. Ultrasensitive and Reversible Nanoplatform of Urinary Exosomes for Prostate Cancer Diagnosis. *ACS Sensors* **2019**, *4* (5), 1433–1441. https://doi.org/10.1021/acssensors.9b00621.

(13) Boyacioglu, O.; Stuart, C. H.; Kulik, G.; Gmeiner, W. H. Dimeric DNA Aptamer Complexes for High-Capacity-Targeted Drug Delivery Using Ph-Sensitive Covalent Linkages. *Mol. Ther. - Nucleic Acids* **2013**, *2* (May), e107. https://doi.org/10.1038/mtna.2013.37.

(14) Tourinho-Barbosa, R. R.; Srougi, V.; Nunes-Silva, I.; Baghdadi, M.; Rembeyo, G.; Eiffel, S. S.; Barret, E.; Rozet, F.; Galiano, M.; Cathelineau, X.; et al. Biochemical Recurrence after Radical Prostatectomy: What Does It Mean? *Int. Braz J Urol* **2018**, *44* (1), 14–21. https://doi.org/10.1590/S1677-5538.IBJU.2016.0656.

(15) Freedland, S. J.; Sutter, M. E.; Dorey, F.; Aronson, W. J. Defining the Ideal Cutpoint for Determining PSA Recurrence after Radical Prostatectomy. *Urology* **2003**, *61* (2), 365–369. https://doi.org/10.1016/S0090-4295(02)02268-9.

(16) Srivastava, M.; Nirala, N. R.; Srivastava, S. K.; Prakash, R. A Comparative Study of Aptasensor Vs Immunosensor for Label-Free PSA Cancer Detection on GQDs-AuNRs Modified Screen-Printed Electrodes. *Sci. Rep.* **2018**, *8* (1), 1–11. https://doi.org/10.1038/s41598-018-19733-z.