III Monalisa Quidproquo Midsummer Festival

PROGRAM AND BOOK OF ABSTRACTS

8th - 11th July 2014

FINAL PROGRAMME

CTCs Tuesday July 8, 2014 @CRO Aviano

14.00 ALFONSO COLOMBATTI: **"CTCs @CRO"**

14.30 WILHELM HUCK (Nijmegen): **"Microfluidics for the study of single cells"**

15:10 FABIO DEL BEN/ MATTEO TURETTA: "**A metabolism-based approach to detect circulating tumor cells"**

15.40 LEONARDO VENTURELLI: **"Using magnetic nano-particles for mesenchymal cells recognition and isolation"**

16.00 coffee break

16.30 ELISABETTA ROSSI: **"Measuring CTCs using the Veridex CellSearch apparatus"**

STEM CELLS Wednesday July 9, 2014 - Aula Interdipartimentale, Piazzale Kolbe 4

12:10 WILHELM HUCK: **"Cells and gels: mechanical forces influence cell behavior"**

13.00 lunch break

14:00 DANIELA CESSELLI: **"My take on personalized medicine"**

14:30 GIUSEPPE GIANFRANCESCHI: "**Human Cardiac Stem Cells obtained from failing hearts are characterized by a pharmacologically reversible dysfunction in the Autophagy-Lysosome Pathway"**

14:50 DAMIANO MANGONI: **"Stem cells isolated from the human glioblastoma microenvironment enhance the aggressiveness of glioma-initiating stem cells through the release of exosomes"**

15:10 ELISA MAZZEGA: **"Mechanosensing and human cardiac stem cells"**

15:30 ROSSANA DOMENIS: **"Different outcome on T cell effector function between glioma- associated stem cells- and glioma-stem cells-derived exosomes"**

16:00 CLAUDIO BRANCOLINI: **"I, Claudius, and cancer"**

16:40 CAMILLA LUNI: **"One-step high-throughput reprogramming and differentiation on a chip"**

BIOSENSORS Thursday July 10, 2014 - Aula Interdipartimentale, Piazzale Kolbe 4

14.00 MATTEO CASTRONOVO: **"Functional properties of DNA-RNA nanostructures on surfaces"**

14.40 ALEX STOPAR: **"Functional properties of DNA nanostructures in solution"**

15.00 LUCIA CORAL: **"Implementing a high throughput sensor with DNA origami"**

15.20 LUIGI XODO: **"Role of guanine repeats in transcription regulation"**

15:50-16:10 coffee break

16.20 MARYSE NKOUA **"Novel approach for detection of miRNAs target based in AFM Nanografting arrays"**

17:00 DANIELE BORIN: **"Superhydrophobic micromechanical pillars as a tool for highly parallel detection and direct measurements in liquid"**

17.30 MARTINA TARDIVO: **"Microelectromechanical Sensor for Circulating Biomarkers detection: Pillars functionalization with Ab anti-PSMA"**

THEORY Friday July 11, 2014 – Aula Interdipartimentale, Piazzale Kolbe 4

14:00 ALEX RODRIGUEZ: **"Clustering by fast search-and-find of density peaks"**

14:40 PILAR COSSIO: **"Atomic Force Spectroscopy from brittle to ductile"**

15:20 FEDERICO FOGOLARI: **"Distance-based conformational and configurational entropy of proteins from molecular dynamics simulations"**

16:00-16:30 coffee break

16:30 ALESSANDRA CORAZZA: **"Nuclear Magnetic Resonance as a tool for the assessment of protein-ligand interaction."**

17:00 SARA FORTUNA: **"Coupled binders for the development of novel nanodevices for protein recognition"**

17:30 MIGUEL SOLER: "**Computational design of peptides as novel binders for beta-2-microglobulin recognition"**

18:00 CEDRIX DONGMO: **"Comparison of Structural Stability and Identification of possible Binding Sites of Tranthyretin-based biomarker systems"**

19:30 DINNER AT RAMANDOLO

CTCs

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CTCs @ CRO

Alfonso Colombatti, Division of Experimental Oncology 2 – CRO-IRCCS, Aviano

The majority of tumor-related deaths occur as a result of our current lack of effective treatments for metastatic disease. One factor is that metastatic lesions are highly heterogeneous compared to primary tumors. Nevertheless, the majority of treatments are currently based upon the characteristics of the primary tumor. Thus, while disease outcome is ultimately determined by metastatic spread, repetitive metastatic biopsies upon disease recurrence or progression are often difficult to perform and can be a significant source of morbidity for patients, even if this could provide important informations. This suggests that many patients receive sub-optimal treatments. Metastasis correlates with the presence of tumor cells in the peripheral blood, referred as circulating tumor cells (CTCs).

More recently, the detection of CTCs in the early localized tumor stage are becoming a potential surrogate marker of early dissemination useful for refining prognoses, monitoring response to treatment, and providing molecular characterization of residual disease after systemic therapy. However, thus far, the clinical uses of CTCs have focused mainly on enumeration.

In addition, still many questions remain unanswered regarding the biology of CTCs, how best to enumerate and characterize them and the path to general clinical acceptance for technology platforms, for patient diagnosis, prognosis, and response to therapy, as well as for accelerating oncologic drug development.

The recent merging of the research laboratories with the clinical laboratory department will allow the full implementation in an adequate clinical setting where it will be possible to robustly control both pre- and analytical variables and set up standard operating procedures for the comparative clinical evaluation of CTC assays in order to validate the new CellFind against CellSearch instruments.

"Microfluidics for the study of single cells"

Wilhelm T. S. Huck, Radboud University Nijmegen, Institute for Molecules and Materials

In recent years there has been an enormous interest in exploiting droplet-based microfluidic devices for performing on-chip biochemical reactions including enzyme kinetics, protein crystallization, PCR and in vitro translation and transcription. Picoliter droplets of water in oil emulsions can be created inside microfluidic devices and can be merged, split and sorted using electric fields, while the contents can be analyzed with sensitive optical techniques. The very high throughput of these devices, up to 10,000 per second, drives research in this area to include directed evolution on-chip and the discovery of 'rare' cells.

Protein, metabolite, as well as mRNA levels, show a large cell-to-cell variation both in a resting state and after exposure to stimuli, even in cells bearing the same genotype. Moreover, in many fields, minority subpopulations of cells are often subject of interest to the scientific community. This statement applies especially to stem cell science and tumor immunology, where cells of interest often comprise only a small fraction of cells in a sample. One example are circulating tumor cells that are found in frequencies as low as 1-10 cells per mL of whole blood (which roughly contains 5 billion cells) in patients with metastatic disease. In this talk, I will discuss recent progress in probing the heterogeneity in cytokine secretion by individual T-cells, differentiating tumor cells from healthy cells in the blood, a quantifying mRNA levels in individual tumor cells.

A metabolism-based approach to detect circulating tumor cells

Fabio del Ben

We report recent advances in setting up a device to count CTCs, based on an innovative metabolism-based assay. We encapsulate single cells in picoliter droplets with a microfluidic technique, and exploit this peculiar environment to detect Warburg effect measuring pH of the extracellular medium at a single cell level using a ratiometric fluorescent dye. We will report the established experimental setting together with recent results of measurements of peripheral blood mononuclear cells and cancer cell lines of metastatic breast cancer, lung adenocarcinoma and glioblastoma.

Using magnetic nano-particles for mesenchymal cells recognition and isolation

Venturelli Leonardo[1], Nappini Silvia [1], Dal Zilio Simone[1], Turetta Matteo [2], Del Ben Fabio [2], Cesselli Daniela [2], Cojoc Dan [1], Scoles Giacinto[1,2].

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Introduction

The techniques used nowadays for CTCs enrichment involve Antibodies (Ab)[1] for specific target recognition on membrane. Recently, to overcome Ab cost, an innovative system based on the anaerobic metabolism[2] of cancer cells that leads to a higher uptake of glucose and glutamine with respect to healthy cells, has become a new powerful tool. Thus we have developed a Magnetic Nanoparticles (MNPs) based method for mesenchymal tumoral cells recognition. Mesenchymal cells were able to internalize MNPs functionalized by glucose with a higher and valuable amount respect to epithelial tumoral cells.

Materials and Methods

$CoFe_2O_4$ Magnetic Nanoparticles were synthesized as described by Massart[3-5] and subsequently functionalized by covalent bonding with 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (**2-nbdg**). MDA-MB-231, MCF-7 and JHH-6, HepG2 tumoral cell lines have been studied for breast cancer and hepatoblastoma respectively. In both cases mesenchymal cells (MDA-MB-231 and JHH-6) internalize a higher and valuable amount of MNPs respect to epithelial ones. The internalization was proved by fluorescence optical microscope and by inner evaluation of tumoral cells by cutting them with Focused Ion Beam technique, previously fixed (PFA 4%, Et-OH 99%) and Chromium deposition (20nm).

White Blood Cells were collected from healthy patient at Ospedale della Misericordia, Udine.

A microfluidic device, consisting of a channel of 100 μm in width, 30 μm in height and 50 mm in length was developed in polydimethylsiloxane (PDMS) by soft optical lithography technique for future application of sorting under magnetic gradient.

Conclusions

Glucose shell allows us to specifically recognize tumoral cells due to their anaerobic metabolism. No White Blood Cells internalized MNPs in our studies. This system will provide in the next future a unique and innovative method for identification and isolation of mesenchymal Circulating Tumoral Cells from whole blood.

References

1) Nagrath S., Toner M., Isolation of rare circulating tumor cells in cancer patients by microchip technology; *Nature*, 2007, 450, 1235-9.

2)Butler EB, Tan M., Stalling the engine of resistance: targeting cancer metabolism to overcome therapeutic resistance ; *Cancer Res*., 2013 May1; 73(9):2709-17.

3)C.R. Massart, *Seances Acad. Sci*., 1980, Ser. C, 291, 1–3.

4)C.R. Massart, *IEEE Trans. Magn*., 1981,17, 1247–1248.

5)C.R. Massart, *US Pat*. 4329241, 1982.

Measuring CTCs using the Veridex CellSearch apparatus

Elisabetta Rossi, Università di Padova

One of the most promising developments in translational cancer medicine has been the emergence of circulating tumor cells (CTC) as a minimally invasive multifunctional biomarker. CTCs in peripheral blood originate from solid tumors and are involved in the process of haematogenous metastatic spread to distant sites for the establishment of secondary foci of disease. The emergence of modern CTC technologies has enabled serial assessments to be undertaken at multiple time points along a patient's cancer journey for pharmacodynamic (PD), prognostic, predictive, and intermediate endpoint biomarker studies. CTCs is a multifunctional biomarker and we focuses on the potential of CTCs as PD endpoints through CTC enumeration and phenotype characterization with CellSearch system using specific markers or with the molecular characterization.

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IDEAS

"Cells and gels: mechanical forces influence cell behavior"

Wilhelm T. S. Huck, Radboud University Nijmegen, Institute for Molecules and Materials

The engineering and modelling of cell micro-environment has proven highly valuable for the elucidation of key cellular processes that govern both tissue development and self-renewal. Progress was enabled in part by the emergence of novel surface patterning tools based on photo- and soft-lithography. Recent studies have shown that the spatial distribution of extra-cellular matrix (ECM) anchoring as well as cell-cell bridging sites is critical to cellular behaviour both in vitro and in vivo, as it provides a local asymmetric environment to the cell.

One specific aspect that has received relatively little attention is the influence of cellular shape and matrix topology on keratinocytes responsiveness. Furthermore, mechanical triggers such as cell shape and the stiffness and topology of the underlying matrix have been proposed to be as important as soluble inducers. Much work has focussed on the differentiation process of mesenchymal stem cells into differ¬ent lineages guided by matrix elasticity, however, little is yet known about other cell types. Human epidermal skin stem cells (keratinocytes) are attached to a basement membrane in vivo and are therefore ideal for studying anchorage-dependent proc¬esses within the cell.

To study the effect of matrix stiffness on keratinocyte differentiation, both polyacrylamide hydrogels as well as PDMS substrates of stiffnesses spanning three orders of magnitude have been prepared. Varying the monomer to crosslinker ratios produces substrates with iden¬tical surface chemistry, but different mechanical properties. All substrates have been characterised using microindentation and it is the aim of this work to correlate the differentiation behaviour of keratinocytes on surfaces of varied stiffnesses with the mechanical environment the cells sense in vivo

In this talk, I will show results of epidermal stem cell differentiation on a range of soft substrates as well as on controlled cellular patterns that limit collective behaviour, and dissociate the different chemical, mechanical and topological parameters involved in dictating cell phenotype.

Recent papers:

Role of the extracellular matrix in regulating stem cell fate, F.M. Watt, W.T.S. Huck, Nature Rev. Mol. Cell. Biol. 2013, 14, 467-473

Extracellular-matrix tethering regulates stem-cell fate, B. Trappmann, J.E. Gautrot, J.T. Connelly, D.G. Strange, Y. Li, M.L. Oyen, M.A. Cohen Stuart, H. Boehm, B. Li, V. Vogel, J.P. Spatz, F.M. Watt and W.T.S. Huck, Nat. Mater. 2012, 11, 642-649

Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions, J. T. Connelly, J. E. Gautrot, B. Trappmann, D. W. M. Tan, G. Donati, W. T. S. Huck, F. M. Watt., Nature Cell Biology

Human Cardiac Stem Cells obtained from failing hearts are characterized by a pharmacologically reversible dysfunction in the Autophagy-Lysosome Pathway.

G. Gianfranceschi1, E. Athanasakis1, E. Mazzega1, A. Caragnano1, D. Cesselli1, N. Finato1, G. Scoles1, CAB. Beltrami1 , AP. Beltrami1.

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Background: Human Cardiac Stem Cells (hCSCs) obtained, in vitro, from failing explanted hearts (E-hCSCs) distinguish themselves from those isolated from healthy donors (D-hCSCs) by senescence markers and compromised functionality, that reduce their in vivo cardiac reparative ability.

Since the autophagy lysosome pathway (ALP) plays a pivotal role in cellular homeostasis by controlling both cellular clearance and response to nutrients, defects on ALP may be associated to ageing and heart failure progression.

Aims: to monitor the efficiency of ALP in hCSCs isolated from patients with heart failure and to develop a drug-based strategy able to boost ALP activity.

Methods and Results: 14 D-hCSCs and 20 E-hCSCs, obtained from healthy and failing human hearts, respectively, were compared in terms of cell surface immunophenotype and senescence marker presence. Although the two groups of cells shared a similar immunophenotype, E-hCSCs showed a significant enrichment in the fraction of senescent cells (p16+, γH2A.X+Ki67-).

Real Time PCR was performed to investigate 3 D- and 3 E-hCSCs for transcriptional profile identifying 452 genes downregulated in E-hCSCs ($p<0.05$), 16 of which are involved in ALP.

Consistently, the lysosomal compartment of 7 D- and 5 E-hCSCs was monitored by FACS analysis after staining with lysotracker and acridine orange and E-hCSCs displayed lysosomes less functionals than the D-HCSCs ones.

Since in the course of autophagy lysosomal function is activated via mTORC1 suppression, we evaluated mTORC1 activity by western blot analysis. In particular we focused our attention on pS6K, in parallel with the autophagic markers Atg3, Atg7, LC3II, p62. Altogether results showed that E-hCSCs were characterized by an enhanced activity of mTORC1 and an arrest in autophagic degradation.

Moving from these elements we develop a three days drug treatment of E-hCSCs with 10nM Rapamycin (TORC1 inhibitor). This pharmacologic strategy was able to reduce mTORC1 activity, to potentiate the lysosomal functionality, to improve the autophagic flux and to reduce the fraction of senescent cells.

Conclusions: we demonstrated that E-HCSCSs are characterized by a blunted ALP. The pharmacologic inhibition of TORC1 on one hand reactivated the pathway and on the other hand contrasted senescence offering promising perspectives to improve E-HCSCS cardiac regenerative efficiency.

TITLE: Stem cells isolated from the human glioblastoma microenvironment enhance the aggressiveness of glioma-initiating stem cells through the release of exosomes.

Damiano Mangoni

INTRODUCTION: Several recent studies highlight the importance of tumor-supporting cells in cancer progression and the role played by exosomes in tumor-stroma interactions. Recently, we optimized a method to isolate and grow in culture a population of human Glioma-Associated Stem Cells (GASC) that, although devoid of genetic alterations, are characterized by stem cell properties, aberrant growth features, a gene expression profile typical of tumor supporting cells and are able to modify the biological properties of the commercially available glioblastoma cell lines A172 and U87. We wanted to verify whether the pro-tumorigenic effect of GASC is due to the exosomal fraction.

METHODS: Exosomes were isolated from GASC culture supernatants by ExoQuick-TC and their presence verified by FACS, Atomic Force Microscopy and NanoSight. We compared the capacity of the unfractioned-GASC supernatant, exosome-depleted GASC supernatant and GASC-derived exosome to modify growth kinetic, migration ability and anchorage-independent growth of both A172 cells (n=4) and glioblastoma-initiating stem cells (GSC) isolated from the same surgical biopsies (n=3). Supernatants and exosomes obtained from the human fibroblasts Wi38 were used as controls.

RESULTS AND CONCLUSION: GASC-derived exosomes have a diameter ranging from 40 to 134 nm, express the exosome-specific markers CD9 and CD63 and can be actively internalized by A172 cells. When grown in the presence of unfractioned- GASC supernatant and GASC-derived exosomes, both A172 and GSC showed a significant decrease in the population doubling time and a significant increase both in migration speed and anchorage-independent growth. These changes were not present when A172 and GSC were grown in the presence of exosome-depleted GASC supernatants or Wi38-derived products.

In conclusion, GASC enhance the aggressiveness of glioma cells through an exosome-mediated mechanism.

Studying the molecular cargo of exosomes could be important to understand the tumor biology, opening the way to new therapeutic approaches aimed at interfering with the tumor-stroma interplay.

Title: Mechanosensing and human cardiac stem cells.

Elisa Mazzega

Abstract

Cells can sense and adapt both to chemical and mechanical features of the surrounding environment. While the effect of chemical stimulations such as cytokines is widely known, most of the molecular implications of the mechanical stimulation are still poorly understood. In particular, we addressed the effect of specific alterations of the mechanical properties of the pathologic heart tissue on adult progenitors stem cells (CSC). We would like to study the biological effect in terms of proliferation (cell density and Ki67), senescence (γH2AX), stemness (OCT_4, c-Kit) and differentiation potential (MHC, SMA, CD31).

We first consider two features of the pathological condition: tissue stiffness, using polyacrylamide gels as a substrate with tunable properties, and increased mechanical overload, employing a specifically designed microfluidic chip.

From preliminary results, cells derived from healthy donors (D-CSC) showed an increased proliferation on stiffer substrates compared to R-CSC. Interestingly, both D-CSC and R-CSC have a strong response in terms of proliferation after prolonged cyclical stretch.

DIFFERENT OUTCOME ON T CELL EFFECTOR FUNCTION BETWEEN GLIOMA-ASSOCIATED STEM CELLS- AND GLIOMA-STEM CELLS-DERIVED EXOSOMES.

Domenis R1, Bourkoula E1, Mangoni D1, Beltrami CA1, Cesselli D 1 and Gri G1

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Introduction: Immunosuppression is considered to be a pivotal factor in tumor growth and progression, as well as in tumor response to immunotherapeutic agents. Although malignant gliomas expressed tumor-associated and tumor specific antigens, that should make these tumors detectable by the immune system, many factors work in concert to inhibit anti-glioma immunity. There is increasing evidence that exosomes may contribute to cancer progression by influencing different immune cell types, namely effector T cell, regulatory T cell and NK cell. We have demonstrated that glioma associated stem cells (GASC)-derived exosomes support the malignant properties and aggressiveness of human glioma-stem cells (GSC) thus, we analyse the immunomodulatory properties of GASC-derived exosomes and compare to GCS-derived ones.

Methods: Exosomes have been isolated from GASC and GSC supernatants by ExoQuick. Their immunomodulatory proprieties have been evaluated on peripheral blood lymphocytes from healthy donors unstimulated or activated with anti-CD3 and anti-CD28. Expression of activation markers, proliferation and apoptosis/necrosis have been measured by flow cytometry. Cytokine production was determined using Bio-Plex cytokine assay.

Results: We found that both GSC- and GASC-derived exosomes are able to inhibit the T-cell activation markers,CD69 and CD25, and to downregulate IL-2 production upon stimulation, while not affecting cell viability. A specific inhibitory effect was observed on IL-1 and IL-6 production after treatment with GASC-derived exosomes and on IL-5 and INF-γ production with GSC-derived exosomes. Only GSC-derived exosomes inhibited activated T cell proliferation. Interestingly, GSC-derived exosomes, but not GASC-derived ones, were able to induce high levels of IL-1β, IL-6 and IL-10 production by unstimulated T cells.

Conclusion: This study suggests that only GSC-derived exosomes per se are able to affect cytokine production from resting T cell while both cell type-derived exosomes, although differently, downregulate T cell immune response.

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ONE-STEP HIGH-THROUGHPUT REPROGRAMMING AND DIFFERENTIATION ON A CHIP

Camilla Luni

An emerging need in biological and clinical studies is to increase the number of patients or patient-derived samples, in view of the profound impact of human population heterogeneity, in terms of genetic profiles and ethnicity, on biological and therapeutical responses. Conventional strategies of drug discovery for development of ad hoc patient-specific therapies need to be substantially improved to offer cost-effective and feasible new solutions for both common and rare diseases. Human induced pluripotent stem cells (hiPSC) opened a breakthrough new perspective on research and clinical applications, but have not been included in translational procedures applicable to study inherited pathologies and human heterogeneity yet. The down-scale of the reprogramming process could provide a unique opportunity to derive cost-effective hiPSCs and valuable human in vitro tissues. The full process of tissue-specific cell derivation involves multiple steps, from cell reprogramming and expansion for several passages to differentiation towards a final phenotype. A recent method for the expression of reprogramming factors is based on the use of modified mRNA (mmRNA). This delivery system is non-integrating, transient, and after multiple transfections of the reprogramming factors is able to produce vector-free hiPSCs in short time with high efficiency, to the point that the production of clinical-grade hiPSC is now feasible, although new technological advancements are required. We developed a comprehensive micro-scale platform with a translational potential for a population-based cell reprogramming and programming without any intermediate stage of expansion. mmRNA technology and chemically defined long-term culture environments were adopted to safely and efficiently derive clinical-grade hiPSCs with unmatched minimum requirements of reagents (few microliters per day). Microfluidic-derived hiPSC emerged as soon as 7 days from the initial transfection and all clones tested exhibited a pluripotent phenotype. The reprogramming of ~100 cells

per sample in feeder-free conditions and the direct and stable expansion of hiPSC with chemically-defined media were effective. Due to the transient nature of mmRNAs, we verified that the expansion passages after colony formation are not required to fully exploit hiPSC differentiation potential in tissue engineering applications. Microarray analyses revealed no significant differences between fresh and expanded hiPSC obtained without the use of integrating or long-lasting viral vectors. In the perspective of obtaining population-based tissue libraries, we further optimized the micro-scaled reprogramming system obtaining an efficiency of up to 16% with the exclusive use of mmRNAs. A fully automated microfluidic chip was developed to simultaneously derive hiPSC from 192 independent samples without need of human intervention for the entire process. Pluripotent cells were then directly programmed into functional cell types valuable for in vitro human assays. Without any expansion procedure, we obtained advanced cardiac and hepatic phenotypes in less than one month, starting from human fibroblasts. Our technology paves the way to high-throughput screening studies on hiPSC-derived functional tissues, after robust and systematic tissue production. This work represents an important step towards the effective use of hiPSC-derived tissue libraries for studies of large cohorts of patients for personalized medicine.

BIOSENSORS

Functional properties of DNA-RNA nanostructures on surfaces

Shiv K. Redhu (1), **Matteo Castronovo** (2,3,4), and Allen W. Nicholson (1,2)

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The important need in discovery research and diagnostics to detect biomarkers at currently inaccessible amounts has spurred the development of detection technologies at the nanoscale. Self-assembled nanodevices are conceptually promising, as they could enable sample detection and analysis in a largely autonomous fashion. This behavior would minimize sample handling and processing, thus reducing sample loss, and also minimize experimentally-imposed bias. Noncoding RNA molecules, including micro(mi) RNAs, are relevant biomarkers of specific disease states.

Atomic force microscopy (AFM) is used here to detect RNA-DNA hybrids that are formed through RNA capture by a laterally-confined, self-assembled, single-strand(ss) DNA matrix, several microns in dimension. Addition of a short (21 nt) RNA to the nanomatrix causes an increase in matrix height, while the subsequent addition of RNase H in the presence of Mg^{2+} produces a permanent height decrease, reflecting the processing of the RNA-DNA hybrids. An exceptionally large, stable increase in height is observed when the RNase H reaction proceeds in the absence of the catalytic Mg^{2+} cofactor. The RNase H-dependent height increase is only observed with RNA-DNA hybrids exhibiting a hybrid density above a specific threshold. These data indicate formation of a stable RNase H-hybrid assemblage, Thus, RNA binding by a dense ssDNA nanomatrix can be detected by the noncatalytic behavior of RNase H, thus preserving the integrity of the bound RNA. The implications of these results in RNA biomarker detection are discussed.

Functional properties of DNA nanostructures in solution

Alex Stopar (1,2), Lucia Coral (1,2), Stefano Di Giacomo (3), and Matteo Castronovo (1,2,4)

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The application of water-soluble, self-assembled DNA nanostructures towards developing innovative approaches to biomolecular sensing or carrying and target-specific delivering drugs, greatly depends on the capacity of such DNA nanostructures to interact with other functional biomolecules in complex biological systems such as the cellular environment. Recent studies suggest that in cell lysate (Qian, Yan, & Meldrum, 2011), or in living cells (Amir et al., 2014), or in animal model (Lee et al., 2012) DNA nanostructures are more stable than the single- and double-stranded molecular DNA, while previous work of our group demonstrated that the activity of restriction enzymes within surface-supported self-assembled monolayers (SAMs) of dsDNA molecules depends on steric hindrance, and is inhibited when the DNA density is higher than a certain critical threshold. Collectively, these results suggest that the enhanced stability of self-assembled DNA nanostructures compared to other naturally available DNA forms, is due to the inherent high level of packing. There is, however, a lack of information on the structural factors that determine enzymatic reactions in such highly dense DNA nanostructures.

We studied the behavior of 12 different restriction enzymes towards two well-known, 2D DNA nanostructures, a triangle and a rectangle, both originating from the same single stranded DNA scaffold (M13mp18), by denaturing gel electrophoresis analysis of the reactions products. Whereas the DNA scaffold sequence naturally includes a number of restriction sites (regardless the 2D shape), our results suggest that most of the restriction enzymes tested differently access to their sites as a function of the 2D shape. The restriction site accessibility towards the enzyme must depend on the pattern of staples inherent the DNA nanostructure, which is varies with DNA nanostructure's shape. In addition, our data analysis suggests that restriction sites within such a highly-dense DNA nanostructures are mostly accessible if surface-exposed,, and in contrast, are nearly inaccessible where facing to an adjacent double helical segment.

Implementing a high throughput sensor with DNA origami

Lucia Coral (1,2), Alex Stopar (1,2), Emmanouil Athanasakis (3), and Matteo Castronovo (1,2,4)

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In the attempt of establishing a quantitative approach to control the interactions between self-assembled DNA nanoparticles and nucleases, we experimentally studied the effect of 14 different enzymes on two, different DNA Origami originated from the same ssDNA scaffold (M13mp18) and obtained by folding the scaffold with two significantly different sets of staple, ssDNA sequences (comprising ~200 staples each). The M13mp18 scaffold naturally contains from 1 to 26 sites per each of the selected enzymes. We found that some of these enzymes produce shape-dependent fragmentation patterns. Our hypothesis is the enzymatic activity is steric regulated by the spatial arrangement of restriction sites within the DNA nanostructure and by their orientation along the DNA helices. We have been developing a novel protocol for detecting and quantifying the efficiency of nucleases action in a restriction site-specific fashion, by means of RT-PCR amplification.

We plan to apply our preliminary results to the development of a water-soluble nanodevice, consisting in a logic gate coupled to a memory unit, able to detect the presence of a micro (mi) RNA in solution. Specifically, the device will be comprised of a three-dimensional (3D) DNA Origami and a steric-regulated restriction enzyme in solution (see previous results on surface-bound nucleic acids monolayers, Redhu S.K. *et al*., Sci. Rep. 2013): 3D DNA Origami will serve as a matrix for imprinting the presence of a miRNA molecule by the site-specific and sterically modulated action of the ancillary restriction enzyme. Capturing a miRNA, by hybridization on a complementary probe that protrudes from the Origami surface, and is located near one restriction site, will enable (or inhibit) permanent site-modification by the restriction enzyme. In our detection scheme, we plan to detect miRNA-specific fragments of ssDNA staples by means of standard methods for DNA analysis, routinely available in biomedical laboratory. We expect our detection approach will permit the efficient "storage" of miRNA-related biological information that is otherwise subjected to alteration due to the rapid degradation of RNA content in biological samples.

Novel approach for detection of miRNAs target based in AFM Nanografting arrays

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Techniques to detect and quantify DNA and microRNA (miRNA) molecules have a crucial role for genomic research. Significant quantitative reverse-transcriptase polymerase chain reaction (qRT - PCR) studies reported that miRNAs are expressed differently in patients having cancer and cardiovascular disease, therefore miRNAs could be used as a biomarkers for the diagnosis and prognosis of human diseases[1]. However, miRNAs detection is still a quite challenging task, due to their easy degradation and non-compatibility with conventional amplification schemes. Novel routes based on nanotechnology to improve the sensitivity of miRNA detection are therefore desirable. We propose here to use Atomic Force Microscopy (AFM) based nanografting to produce DNA nanoarrays with variable molecular density capable of rapid and accurate detection of DNA/miRNA targets, through the measurement, via AFM, of the different nanomechanical response of ssDNA and DNA/miRNA hybridized nanopatches. With our device, we are able to avoid any labelling and/or amplification scheme and to detect more than one sequence of miRNAs on a same assay with so far 100pM resolution.

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Superhydrophobic micromechanical pillars as a tool for highly parallel detection and direct measurements in liquid

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Abstract

Micro and nano resonators have experienced a wide diffusion in the last decades for molecular detection, due to their extreme sensitivity, ease of fabrication and potential high throughput. In very demanding conditions (ultra high vacuum and cryogenic temperature), the ultimate limit of a single proton mass was demonstrated [1]. Such kind of resonators are gaining attention also as biomolecular sensors, to be used at room temperature and ambient pressure. In these conditions, the limit of detection is reduced to attogram (10^{-18}g) in air [2] and to nanogram (10^{-9}g) in liquid [3], due to the damping of the viscous medium. This effect can the application of these devices to real time biological assays. To overcome this limitation, several approaches have been proposed, from the actuation of resonators at higher modes [4], to the fabrication of cantilevers in contact with the liquid only on one side [5] or embedding a microfluidic channel [6].

In our group we are working with micropillars, that are vertically oriented, tapered oscillators used as mass microbalances [7]. Due to their geometry, pillars can be fabricated in very dense arrays and, through a proper hydrophobic treatment, a superhydrophobic Cassie-Baxter state can be obtained [8]. In this configuration, liquid pins the top surface of pillars without wetting the lateral walls or the ground of the matrix. In this way, during its lateral oscillation, a pillar is moving in air, maintaining higher mass resolution than the one of an horizontal cantilever fully immersed in liquid.

In this talk the technique for highly parallel detection of pillars in vacuum/air is presented, and several approaches for direct measurement of resonance frequency in liquid are discussed, showing the potentiality, the limits and the solution that have been considered.

Finally, the future plans about parallel functionalization of pillars with a photocleavable linker will be illustrated.

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Microelectromechanical Sensor for Circulating Biomarkers detection: Pillars functionalization with Ab anti-PSMA

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The availability of antibodies with high affinity, together with the unmatched sensitivity at extremely low production cost offered by micro- and nano- electromechanical system (MEMS, NEMS), allows us to develop a promising new class of biosensors for the detection of circulating biomarkers at low concentration, Point of Care oriented and extremely competitive in terms of analysis time and costs, compared with current diagnostic systems (i.e. ELISA test) [1].

A promising alternative to usual MEMS sensors is offered by vertical (pillars) and horizontal cantilevers. Pillars, in particular, have a small sensitive area, in the range of few μm^2, spotted on larger volumes, so that the response in the diffusion limited regime is three order of magnitude faster. This enables us to reach detector saturation still in short time at reduced concentration of incubation, comparing with usual protocol in which incubations occur in hours at μM concentration. Moreover, because of their small mass, pillars have a high sensitivity, and a detection limits of 10 pM can be reached in a range of minutes [2].

This new technology has been applied to the detection of prostate specific membrane antigen (PSMA), a tumor marker for prostate cancer that, in spite of the observed strong correlation between its expression and tumor occurrence, has not yet found application in tumor early diagnosis, because of the lack of a reliable detection method[3].

In order to gain, for Pillars technology, a relevance in biomedical applications and in particular in PSMA detection, a suitable interface between biological biomarkers and silicon based MEMS should be engineered.

Here, an anti PSMA antibody characterized by high affinity (D2B, $Kd<10^{-9}M$)[4], was chemically modified introducing an –SH functional group, and was used to form a monolayer on the surface of both pillars and horizontal cantilevers, previously coated with a layer of gold.

The functionalization occurs in solution, the presence of Abs on the sensors surface has been first confirmed by an enzymatic immunodetection (anti-mouse Ab-HRP plus TMB) and then tested against incubation time; we found that, on pillar, at μM concentration, the saturation can be reached after only 10 minutes. Moreover, using a nM concentration also the data dispersion can been reduced from 30% to 10%, so an homogenous monolayer, corresponding to 20% surface coverage ($2*10^{12}$ molecules/cm^2) was achieved.

We tested the activity of our D2B functionalized surfaces acquiring the mass detected by the sensors, after the incubation with a solution containing different concentration of antigen PSMA, from 100nM to 100pM. As result, the curve mass VS PSMA concentration indicates that the minimum concentration at which PSMA can be detected is around 3nM.

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THEORY

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"Atomic Force Spectroscopy from brittle to ductile"

Pilar Cossio

The quantitative analysis of single-molecule force spectroscopy experiments is greatly facilitated by the availability of analytic expressions for the force dependence of macromolecular rupture rates. However, limited forms of the potential energy surfaces that describe the rupture behavior are currently available. Here, we introduce a wide class of continuous one-dimensional differentiable potential surfaces that cover a broad range of rupture scenarios. At the brittle extreme, the distance between the metastable minimum corresponding to the intact state and the transition state to rupture is independent of an applied force; in the ductile limit, the distance decreases exponentially with increasing force. The analytical dependence of the mean rupture force on the logarithm of the force loading rate is found to vary from the linear behavior of the Bell-Evans model to a more rapid increase at higher pulling speeds. The potential surfaces introduced here allow us to reconcile bulk protein unfolding experiments, high-speed force spectroscopy experiments, and molecular dynamics simulations covering more than 10 orders of magnitude in force loading rates.

"Distance-based conformational and configurational entropy of proteins from molecular dynamics simulations"

Federico Fogolari

The talk will present the use of nearest neighbor entropy estimator in the context of most common applications in protein thermodynamics and will develop to

some extent the subject by addressing the entropy of folding and rototranslational entropy loss.

The talk is organized as follows:

i) methods are first reviewed and linked to the formalism used;

Sample applications are discussed:

ii) reference entropies for amino acids in unfolded proteins are obtained from a

database of residues not participating in secondary structure elements;

iii) the conformational entropy of folding of beta2-microglobulin is computed from molecular dynamics snapshots;

iv) backbone conformational entropy is computed for four molecular dynamics simulations of the EPAC protein and compared with order parameters (often used as a

measure of entropy) and overall conformational entropy;

v) the conformational and rototranslational entropy of binding is computed from

simulations of 20 tripeptides bound to the peptide binding protein OppA.

Implications and development will be discussed.

Coupled binders for the development of novel nanodevices for protein recognition

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An ideal binder should be capable of capturing with high affinity, sensitivity, and specificity a target molecule, such as an organic molecule or a biomolecule. Examples include antibodies and minibodies typically optimised in vivo, as well as DNAs and RNAs based aptamers evolved in vitro, and peptides optimised in silico.

While the attention is usually towards the development and optimization of new and existing binders, a complementary approach consists in the design of polydentate binders. It is in fact well known in coordination chemistry that polydentate binders have enhanced affinity compared to the affinity of a collection of monodentate binders, the simplest example being that of dicarboxilic acids binding a metal with stronger affinity with respect to that of the corresponding uncoupled acids (the so-called "chelating effect"). This characteristic can be exploited to design generic binders with enhanced affinity towards larger molecules such as proteins. Nature itself exploits the synergy amongst coupled multiple binding sites in biological systems. For instance antibodies are capable of binding their target thanks to a number of coupled loops. The structure is rigid and the loops sequence can be varied to enhance both binding affinity and selectivity towards a certain target. Similarly, rigid synthetic scaffolds such as calixarenes and porphirins have been used to enhance the affinity of peptides loops by coupling multiple loops together [Y. Hamuro, et al. Angew Chem Int Ed 1997; H. S. Park, et al. J Am Chem Soc 1999]. However, these these scaffolds are little versatile and are usually designed to pinpoint a single binding site.

Here we explore the use of flexible linkers. We will theoretically show with the use of a minimal model and Monte Carlo simulations that flexible linkers are a viable option to enable two binding moieties to reach two different sites on a target as the free energy cost of dissociating two coupled binders is higher than that of dissociating two monodentate binders (chelating-like effect). Further work will involve the application of this setup to a real system by designing coupled binders for the lysozyme, a 14.3 kDa model system well suited for the NMR characterisation of its interaction sites. By pinpointing the design principles for effective coupled functionalities and by focusing on their flexible linker we will provide new general design strategies for novel nanodevices.

Computational design of peptides as probes for the recognition of Beta-2-microglobulin.

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In the last two decades, biomarkers have appeared among the most promising tools in cancer management. Typical receptors for proteins are antibodies optimized in vivo. However, while monoclonal antibodies are widespread accepted binders for gold standard tests such as ELISA (Enzyme-Linked ImmunoSorbent Assay), they have a number of important drawbacks: their production is costly, time consuming, is carried out in vivo, and does not allow for a control over the specificity of their binding site, in turn not allowing fine-tuning the bio-responsiveness of a device.

Short peptides represent one of the most promising alternative to antibodies [1] allowing for a rational design of a generic binder because of the number of different molecules that can be obtained by combining the natural amino-acids, the possibility of automated synthesis [2], and the possibility to introduce further modifications to enhance binding capability. A peptide design approach proposed by Laio et al. has already been successfully used

for drug recognition [3], and protein recognition [4]. Now they have proposed a new algorithm for drug recognition. The code, based on a combination of Molecular Dynamics, cluster analysis, Binding Energy analysis, and replica exchange Monte Carlo, is able to optimize simultaneously the sequence and the conformation of small peptides in order to reach a high binding affinity to a target organic molecule.

Here we adapt the new algorithm to generate peptides capable of binding a biomolecule with high affinity. We choose beta-2-microglobulin (B2M), biomarker part of the OVA1 panel for the prediction of ovarian cancer, as a test case since its suitability for the liquid NMR study allows the direct comparison between the computer generated structures and the experimental system. Based on the system composed by the binding of B2M and the human histocompatibility antigen HLA-A2 [5], we choose the binding site of B2M taking into account the experimental accesibility of optimized peptides. We obtained 40 different sequences of soluble peptides binding to B2M with a Binding Energy lower than -23 kcal/mol. At present, the generated peptides are being screened both computationally and experimentally.

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Comparison of Structural Stability and Identification of possible Binding Sites of Tranthyretin-based biomarker systems

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The possibility to design new ligands and receptors for protein recognition in silico is a key step towards the development of new diagnostic tools. The design approach strongly relies on protein structure which should be pretty stable with few unfolding events. This work focuses on Tranthyretin biomaker protein (TTR), which has been shown to be implicated in amyloidogenic pathologies such as familial amyloid polyneuropathy (FAP) and familial amyloid cardiomiopathy (FAC). Amyloid formation from natively folded proteins involves partial and/or global unfolding of the native state. This suggests that the regions of TTR that form the subunit interfaces in the tetramer are likely destabilized in the monomer, facilitating conformational fluctuations to a potentially amyloidogenic intermediate state. Getting structural information about this system and localizing its possible binding sites is of paramount importance for the development of a binder which could link to the protein both to recognize it or to limit its misfolding event. Aim of this work has been to test the stability over time and determine the appropriate binding sites of TTR biomarker protein by accessing and comparing the structures of both wild type (WT-TTR), PDB code 1F41 and double point mutant F87M/L110M (M-TTR), PDB code 1GKO for the design of new ligands for the recognition of this biomarker protein using explicit solvent molecular dynamics simulations with Gromacs softwar

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