RegioneLombardia

DALLA SCIENZA DEI MATERIALI ALLA BIOMEDICINA MOLECOLARE

Percorsi scientifico-formativi per giovani ricercatori

Università degli Studi di Pavia
Aula Foscolo
Martedì 17 novembre 2009

Programma

10.00 **Saluti/interventi del Rettore e del Presidente della Regione (o suo delegato)**

10.30 **Il progetto (C. Balduini, P. Mustarelli)**

10.40 **Comunicazioni di giovani ricercatori (20’) (moderatore: V. Bellotti)**

G. Dacarro
Dalle superfici funzionali nanostrutturate ai biosensori ottici innovativi

F. Bragheri
Pinze ottiche e stretcher ottico: dispositivi biofotonici in fibra per analisi di singole cellule

M. Santagostino
Nuovi ruoli dei telomeri e della telomerasi

S. Giorgetti
Nuovi interattori della β2-microglobulina con capacità antifibrillogenica

La valutazione dei revisori (E. Rizzarelli, G. Tettamanti, R. Tubino)

12.20 **Considerazioni conclusive (A. De Maio)**

12.30 **Buffet e visione poster**

FROM FUNCTIONAL NANOSTRUCTURED SURFACES TO INNOVATIVE OPTICAL BIOSENSORS

Giacomo Dacarro
CILSOMAF, c/o Dipartimento di Fisica "A. Volta", via Bassi 6, Pavia – giacomo.dacarro@gmail.com

Molecular Self-Assembled Monolayers (SAMs) can be formed on SiO_2 surfaces (*i.e.* glass, quartz, SiO_2/Si) by wet reaction of $(RO)_3Si$---X (trialkoxysilanes) molecules. Several functional molecules can be covalently bound to the surface simply reacting –X group on the monolayer with a chemically complementary –Y group [1](fig. 1a). Exploiting the affinity of some organic moieties (thiols and amines) towards transition metals we can attach both metal ion complexes and metal nanoparticles (fig. 1b). Different synthetic protocols may be adopted both in the SAM formation and in the NP deposition steps, leading to the possibility of conrolling the stability and the morphology of the NP-covered surfaces. Some transition metals (copper and silver) have a well known antibacterial activity. Functionalized surfaces loaded with metal ions and nanoparticles showed a good microbicidal effect minimizing the quantity of metal ions released in solution.

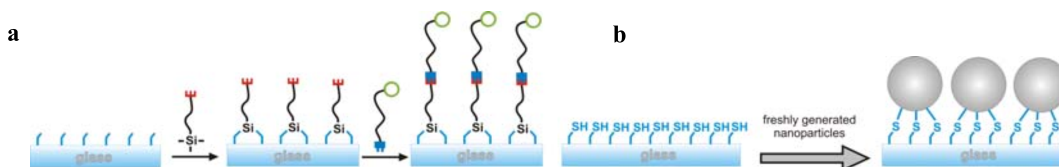

Figura 1

Exploiting the same SAM deposition and growth approach a fluorophore (tetramethylrhodamine) monolayer was bound to the surface of a a-$Si_{1-x}N_x$:H multilayer. A directional enhancement of the emission by more than one order of magnitude was observed when the rhodamine monolayer was coupled to a surface resonant mode called Bloch Surface Wave (BSW) in a one-dimensional photonic crystal [2]. The use of BSW and properly functionalized surfaces could be useful for the realization of fluorescence based high sensitivity optical biosensors.

Finally a hybrid photonic-plasmonic device has been realized in order to achieve physico-chemical diagnostics of surfaces and analytes with nanometric resolution. The device was designed for simultaneous measurement of morphology (using AFM microscopy) and chemical composition (by Raman scattering) of a sample. The device integrates an adiabatic metal waveguide with nanometric curvature radius on a commercial AFM cantilever. This acts simultaneously as an atomic force microscope tip and as a plasmonic resonant nanoantenna for Raman signal, leading to a physico-chemical mapping of analytes with nanometric size and resolution [3].

[1] Pallavicini P.; Dacarro G.; Galli M.; Patrini M. Spectroscopic evaluation of surface functionalization efficiency in the preparation of mercaptopropyltrimethoxysilane self-assembled monolayers on glass. *Journal of Colloid and Interface Science* 2009, *332*, 432–438.
[1] Liscidini M.; Galli M.; Shi M.; Dacarro G.; Patrini M.; Bajoni D.; Sipe J. E. Strong modification of light emission from a dye monolayer via Bloch surface waves. *Optics Letters* 2009, *34(15)*, 2318-2320.
[1] De Angelis F.; Das G.; Candeloro P.; Patrini M.; Galli M.; Di Fabrizio E. et al. Nanoscale chemical mapping using three-dimensional adiabatic compression of surface plasmon polaritons. *Nature Nanotechnology*, in press (Nov 2009).

FIBER OPTIC TWEEZERS AND OPTICAL STRETCHER: BIOPHOTONIC TOOLS FOR SINGLE CELL STUDIES

F. Bragheri, L. Ferrara, P. Minzioni, I. Cristiani
Dept. of Electronics, University of Pavia, Italy

In molecular and cellular biology an impelling demand has arisen for the development of tools able to select, isolate and monitor single cells or cell clusters. Studies performed at a single cell level have revealed extremely heterogeneous behaviors and they provided unpredictable information, opening new scenarios for the comprehension of the basic biological mechanisms and for the early detection of several diseases [1].
The exploitation of optical forces represents an accurate, non-invasive and gentle manipulation technique for individual cell studies [2]. Of great interest is the manipulation of individual cells, which, combined with fluorescence analysis, represents a flexible tool for cell monitoring and sorting. In addition new insights can derive from the investigation of the viscoelastic properties of trapped cells through the application of intense optical forces, able to cause a significant deformation of the cytoskeleton. The degree of cytoskeleton deformability is characteristically altered by many diseases, including cancer, and provides a unique and reliable marker of the cell status [3].
In this work we will present our recent results on the development of two integrated biophotonic tools that exploit optical forces to provide complementary information on cell properties: a fiber optical tweezers (OT) and an optical stretcher (OS). The attention will be mainly focused on the experiments performed through OS, whose set-up is based on an optical trap obtained through two counterpropagating beams emitted by two optical fibers. The fibers are mounted in correspondence of a microfluidic channel in which a controlled flow of the cell suspension under test is present. Once trapped, the cells are progressively stretched by increasing the optical power.
Preliminary results obtained by probing the viscoelastic properties of human erythrocytes have already provided important indications for the study of circulatory complications due to diabetes.

1. D.G. Grier, Nature 424, (2003) 810.
2. C. Liberale, P. Minzioni, F. Bragheri, F. De Angelis, E. Di Fabrizio, I. Cristiani, Nature Phot. 1, (2007) 723
3. J. Guck, R. Ananthakrishnan, H. Mahmood, T. J. Moon, C.C. Casey Cunningham, J. Kas, Biophys. J. 81 (2001) 767.

SMALL MOLECULES AND MACROMOLECULES FOR THE INHIBITION OF B2 MICROGLOBULIN FIBRILLOGENESIS

Sofia Giorgetti
Department of Biochemistry, University of Pavia, Italy

Discovery of new molecules able to inhibit protein fibrillogenesis “in vitro” and “in vivo” represents a challenging demand for treatment of amyloid disease. Recent advances in the elucidation of the mechanism of amyloidogenesis of β2 microglobulin (β2-m) are offering the unique opportunity to discover new anti-amyloidogenic compounds. Small polycyclic molecules like tetracyclines and high affinity antibodies raised against the amyloidogenic protein precursor represent two prototypic classes of interactors for potential pharmaceutical exploitation. Two methods of fibrillogenesis and one cell toxicity test have been used for the identification of active compounds. From a series of ten analogues of tetracyclines we have singled out the two best anti-amyloidogenic compounds, able to inhibit the fibrillogenesis of β2-m with an IC_{50} of approximately 50 μM and capable solubilising preformed fibrils at an IC_{50} of 250 μM. Both compounds abrogate the cytotoxicity of oligomeric β2-m. Whereas the tetracyclines exert their activity through a low affinity interaction with the amyloidogenic conformers, the second class of compounds we have tested (monovalent nanobodies) displays an anti-amyloidogenic capacity through a nanomolar affinity against β2-m and its highly amyloidogenic species. For this class of compounds we have been able to identify how the contact of the nanobody with specific regions of β2-m can switch off its amyloidogenic propensity. Comparative analysis of the effectiveness of these molecules in the inhibition or disorganization of amyloid fibrils are essential preliminary steps toward the identification of therapeutic strategies of potential pharmacological applications in patients affected by dialysis related amyloidosis.

NEW ROLES OF TELOMERES AND TELOMERASE

Marco Santagostino, Solomon Nergadze, Lela Khoriauli, Valerio Vitelli, Alexandra Smirnova, Pamela Vidale, Manuel Lupotto and Elena Giulotto
Dipartimento di Genetica e Microbiologia, Università di Pavia

Telomeres are the extremities of linear chromosomes and, in vertebrates, are composed by tandem repeats of TTAGGG hexameres tightly associated to proteins. Telomeres are essential for the maintenance of chromosome integrity. Telomerase, the enzyme responsible for the synthesis of telomeric repeats, is inactive in normal somatic cells and this is the reason why telomeres shorten at each cell division causing cellular senescence. On the contrary, telomerase is reactivated in the majority of human tumours. Telomeres and telomerase are a particularly hot topic; in fact, the 2009 Nobel price for Medicine was awarded to Elisabeth Blackburn, Carol Greider and Jack Szostak who made essential contributions to this field.

Since their discovery, telomeres have been considered transcriptionally silent. In collaboration with the group of Joachim Lingner (ISREC, Lausanne) we demonstrated that telomeres are transcribed into Telomeric Repeat-containing RNA molecules (TERRA) that are associated to telomeric chromatin [1]. In collaboration with Claus Azzalin (ETHZ, Zurich), we recently demonstrated that the transcription of TERRA is driven by specific promoters localized in subtelomeric regions and proceeds towards the extremities of the chromosomes [2], supporting the idea that TERRA exerts a fundamental role in telomere biology and genome stability.

Telomeric repeats are also found at internal sites of chromosomes where they give rise to the so-called Interstitial Telomeric Sequences (ITSs). We demonstrated that ITSs were inserted in the genome by telomerase during the repair of DNA double strand breaks that occurred in the germ line in the course of evolution [3], indicating that this enzyme, besides its role in telomere maintenance, is also involved in DNA repair. In order to investigate whether ITSs are simply marks of ancient DNA breaks, or play a functional role, we carried out an extensive *in silico* analysis of the ITSs in the human genome. Our results suggest that the presence of ITSs can influence gene expression and, in particular, alternative splicing. In addition, we identified several telomeric repeat-containing transcripts derived from ITS loci; therefore, ITSs may also have a role in the maintenance of genome stability by contributing to the production of TERRA molecules.

1) Azzalin, C.M., Reichenbach, P., Khoriauli, L., Giulotto, E. and Lingner, J. [2007] “Telomeric repeat-containing RNA and RNA surveillance factors at mammalian chromosome ends”, Science, Vol. 318, pp. 798-801
2) Nergadze, S.G., Farnung, B.O., Wischnewski, H., Khoriauli, L., Vitelli, V., Chawla, R., Giulotto, E., Azzalin., CM. [2009] “CpG-island promoters drive transcription of human telomeric repeat-containing RNA”, RNA, Vol. 15, Oct 22. [Epub ahead of print]
3) Nergadze, S.G., Santagostino, M.A., Salzano, A., Mondello, C. Giulotto, E. [2007] “Contribution of telomerase RNA retrotranscription to DNA double-strand break repair during mammalian genome evolution”, Genome Biol., Vol. 8, pp. R260

MONOAMINE OXIDASES AND LSD1: SIMILAR CHEMISTRY FOR NEUROTRANSMITTER AND CHROMATIN MODIFICATION

Andrea Mattevi, Federico Forneris, Dale E. Edmondson, Claudia Binda
Department of Genetics and Microbiology, University of Pavia, via Ferrata 1, 27100 Pavia, Italy and Departments of Biochemistry and Chemistry, Emory University, Atlanta, Georgia 30322 USA

Monoamine oxidases (MAOs) and the histone demthylase LSD1 are evolutionarily related enzymes that catalyze the oxidative deamination of their substrates. They represent a spectacular example of how similarities in the chemistry of the catalyzed reaction can constrain evolution, despite different biological functions and cellular localizations. MAOs bind the outer mitochondrial membrane and play a central role in the metabolism of neurotransmitters such as dopamine and serotonin. MAO's rise to prominence in the biomedical community originated in the early fifties from Zeller's finding that MAO was the target for hydrazine inhibition which could function in treating depression. Since then, a huge number of MAO inhibitors have been developed and several of them have been used for the treatment of Parkinson's disease and depression. Our structural studies have shown that most of the known MAO inhibitors function through a mechanism-based mode that generates a covalent adduct with the FAD cofactor.
LSD1 is a more recently discovered enzyme. It is responsible for the demethylation of Lys4 of histone H3. LSD1 is implicated in tumorigenesis and there are increasing efforts to identify LSD1 inhibitors. The crystal structure of LSD1 reveals a different substrate-binding site but similar catalytic machinery compared to those exhibited by MAO structures. This similarity is proving to be particularly insightful, prompting researchers to exploit the knowledge gained from MAO inhibition studies to develop effective LSD1 inhibitors. We shall present a comparative analysis of LSD1 and MAOs with a focus on the relevance of the structural investigations for understanding the mechanisms drug action and for the design of new inhibitor molecules targeting these amine oxidase enzymes.

NUMERICAL AND EXPERIMENTAL DEMONSTRATION OF A SINGLE-FIBER PROBE FOR OPTICAL TRAPPING AND ANALYSIS

Francesca Bragheri, Paolo Minzioni, Ilaria Cristiani
CNISM and Department of Electronics, University of Pavia, Italy
Carlo Liberale, Francesco De Angelis, Enzo Di Fabrizio
Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi Magna Graecia di Catanzaro, Italy

After the first demonstrations of particle manipulation by means of the radiation force exerted on a particle by a tightly focussed laser beam, optical tweezers have found a variety of applications both in biology and fundamental physics [1]. At the state of the art, the classical optical tweezer (OT) configuration is based on the use of a laser beam that is tightly focussed, by means of a high numerical aperture (NA) microscope objective, in the medium containing the particles to be trapped. Although powerful, the range of application of this technique is actually limited by the necessity to use a large and complex apparatus. We have recently proposed [2] a new technique to obtain a fiber-optic-tweezer that was designed by successfully combining two concepts: the use of non-standard fibers, and the achievement of light focusing with high NA by using total-internal-reflection (instead of refraction) at the interface between fibers and surrounding-medium. In the following we will indicate such a tweezer as TOFT (total-internal-reflection-based optical fiber tweezer).
In this work we present a numerical study and experimental demonstration of the TOFT performances. The optical force distribution has been carefully analyzed in the Mie regime through numerical simulations of the optical forces [3, 4] in order to identify the most critical aspects of the tweezers configuration. The trapping efficiency of the TOFT has been evaluated by using a new parameter, representing the escape energy per unit power, which allows obtaining important information on the trap.
By using TOFT structure, simultaneous 3D trapping and fluorescence measurements are demonstrated [2].

[1] A. Ashkin, "Acceleration and trapping of particles by radiation pressure," Phys. Rev. Lett., 24, 156-158, (1970).
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[3] P. Minzioni, F. Bragheri, C. Liberale, E. Di Fabrizio, I. Cristiani, "A novel approach to fiber-optic tweezers: Numerical analysis of the trapping efficiency", IEEE J. Sel. Top. Quantum Electron. 14, 151 (2008).
[4] F. Bragheri, P. Minzioni, C. Liberale, E. Di Fabrizio, I Cristiani, "Design and optimization of a reflection-based fiber-optic tweezers", Opt. Express, 16, 17647 (2008)

DEHYDROEPIANDROSTERONE AND CORTISOL: EFFECTS ON GNB2L1 HUMAN PROMOTER REGION CODING FOR RACK-1 SCAFFOLD PROTEIN

Buoso Erica[1], Molteni Elisabetta[1], Lanni Cristina[1], Govoni Stefano[1], Corsini Emanuela[2] e Racchi Marco[1].
[1]*Department of Experimental and Applied Pharmacology, Center of Excellence in Applied Biology, University of Pavia, Italy.*
[2]*Department of Pharmacological Sciences, University of Milan, Italy.*

Background: The immune system follows, with aging, a gradual decline which involves also a dysfunction of kinases such as protein kinase C (PKC). This event is related to decreased level of RACK-1 which is the PKC scaffold protein. Different evidence indicate that in the elderly a correlation between RACK-1 down-regulation and low plasmatic dehydroepiandrosterone (DHEA) levels occurs. It has also been determined that DHEA administration to leucocytes of elderly subjects restored levels of RACK-1 expression. Despite these biochemical studies, very little is known about RACK-1 gene expression and regulation. To study direct binding sites for DHEA on GNB2L1 promoter region we developed a luciferase-reporter vector (GW luc basic) for the *in vitro* study of deletion mutants (Δ) and we recently mapped a consensus sequence for Glucocorticoid Response Element (GRE) [1].
Aim: Employing a dual-luciferase reporter assay system we investigated, in THP-1 cells, whether physiological cortisol concentrations, 0,1μM and 0,5 μM, which correspond respectively to minimum and maximum plasmatic peak, were able to influence GNB2L1 promoter activity.
Results: After cortisol treatment, luciferase activity of Δ1 and Δ9 deletion mutant was strongly decreased by both concentrations. On the contrary, cortisol did not influence luciferase activity of Δ6 deletion mutant, since this mutant is lacking in GRE sequence. Furthermore, cortisol concentrations were able to influence significantly, although with different capacity, RACK-1 expression at mRNA and protein level. As DHEA and glucocorticoid imbalance is important during aging, we investigated their possible interaction in GNB2L1 promoter activity control. After incubation of transfected THP-1 cells with 10nM and 100nM DHEA for different times (16h, 24h, 48h, 72h) we added 0,1 μM and 0,5 μM cortisol for 6h. We observed that DHEA contrasted the potent inhibitory cortisol activity at both concentrations only after 72h. The same experiments were conducted in real-time PCR and in Western Blotting showing that DHEA was able to contrast cortisol effect also before 72h.
Conclusions: These results highlight that DHEA may have a possible double effect on RACK-1 expression, since they suggest that DHEA may prevent glucocorticoid receptor binding to GRE sequence and influence RACK-1 promoter expression acting at different and yet to be explored sites outside our cloned sequence.

[1] Del Vecchio I, Zuccotti A., Pisano F., Canneva F., Lenzken S., Rousset F., Corsini E., Govoni S., Racchi M. Functional mapping of the promoter region of the GNB2L1 human gene coding for RACK1 scaffold protein. Gene 2009; 430:17-29.

AMYLOID PRECURSOS PROTEIN PROTEOLYSIS IN HUMAN PLATELETS IS REGULATED BY CALMODULIN

I. Canobbio, S. Catricalà, L. Cipolla, C. Balduini, M. Torti
Department of Biochemistry, University of Pavia, Italy, Pavia, Italy

Abnormal metabolism of the amyloid precursor protein, APP, results in the accumulation of amyloid peptide Aβ in the brain, causing the onset of Alzheimer disease. Aβ and proteolytic fragments of APP are also present in human plasma, and mainly derive from circulating platelets. Platelets express APP, and metabolise APP to produce the soluble fragments sAPPα, sAPPβ and Aβ. Very little is known about the exact function of APP in the cardiovascular system. In this study, we have investigated the metabolism of APP in human platelets.
Two different forms of APP are expressed in human platelets. A soluble fragment of 120 kDa, possibly including both sAPP and sAPP , is stored into -granules and is released upon platelet stimulation. A 140 kDa full length protein, named APP_{FL}, is expressed on platelet surface, and accounts for about ten percent of total platelet APP. APP_{FL} undergoes proteolysis upon stimulation of platelet with physiological agonists. Agonist-induced proteolysis of APP_{FL} occurs independently of platelet aggregation, but is inhibited in the presence of EDTA. Proteolysis of APP_{FL}, but not secretion of soluble APP fragments, is observed also upon incubation of platelets with the cell permeable calmodulin (CaM) antagonist W7. W7-induced APP_{FL} proteolysis is time and dose-dependent and is prevented by preincubation with the metalloproteinase inhibitor GM6001 or with EDTA. We have found that APP co-immunoprecipitates with CaM in resting platelets, and we are currently characterizing the interaction in vitro between the recombinant intracellular domain of APP and CaM. These results indicate that, in platelets, proteolysis of the extracellular domain of APP resembles the shedding of other known surface receptors, including GPIbα, GPV and GPVI, and demonstrate that the balance between amyloidogenic and non amyloidogenic metabolism of APP can be regulated by calmodulin.

AMYLOIDOGENIC PROTEINS AND CAPILLARY ELECTROPHORESIS: FROM FOLDING TO SMALL MOLECULE BINDING

Colombo, R.[1], Carotti, A.[2], Catto, M.[2], Racchi, M.[3], Lanni, C.[3], Verga, L.[4], De Lorenzi, E.[1]

[1]University of Pavia, Department of Pharmaceutical Chemistry, Viale Taramelli 12, Pavia, Italy. [2]University of Bari, Department of Pharmaceutical Chemistry, Bari, Italy. [3]University of Pavia, Department of Experimental and Applied Pharmacology, Pavia, Italy [4]Policlinico S.Matteo, Department of Infectious Diseases Foundation IRCCS, Pavia, Italy.

Alzheimer's disease is a form of amyloidosis, a disorder where the conformational changes of a protein ultimately lead to the deposition of insoluble fibrils. The formation of amyloid fibrils is a dynamic and complex process, as it implies the formation of different intermediate species at equilibrium, including non-covalently associated soluble oligomers, protofilaments and protofibrils, according to a nucleation-elongation mechanism. The characterisation of such a process can be partially elucidated by a variety of spectroscopic techniques, that nevertheless yield population-average data. In this respect capillary electrophoresis is an excellent complementary tool that enables the separation and the detection of these species during their formation and just before fibril deposition. We exploited this potential of CE with Abeta42 peptide, which is found in the brain of Alzheimer's disease patients. The availability of CE methods capable of monitoring these processes (1) is extremely valuable, as they can be used as a starting point for co-incubation studies with small molecules, to investigate the effect exerted on the equilibrium of these intermediate species, and to verify, by electron microscopy, whether there is a delay or an inhibition of fibril formation. Here we show the effect that two drugs with similar structure have on a toxic oligomeric (>100kDa) intermediate of the fibrillogenesis of Abeta42 (2, 3). The promising results obtained may have therapeutic implications.

To gain complementary information about oligomer size distribution the aggregation process of Abeta42 was also monitored by Flow Field Flow Fractionation. This technique can separate aggregating samples and particles with dimensions up to micrometers and so we could explore a time window that is prohibited to CE and to any other separative technique, that is, when insoluble material is already formed. Our results confirmed the presence of a first peak with a hydrodynamic radius of 5 nm, possibly corresponding to the globular aggregates of 60 kDa species migrating in CE and of a second peak corresponding to the most toxic oligomers. When sample began to precipitate, MALS detection revealed the presence of very large species and provided indication of the gyration radius. The gyration radius obtained should approximately correspond to a length of 1.5 μm, consistent with fibril dimensions also monitored by TEM (4).

(1) S. Sabella, M. Quaglia, C. Lanni, M. Racchi, S. Govoni, G. Caccialanza, A. Calligaro, V. Bellotti, E. De Lorenzi, *Electrophoresis 2004*, 25, 3186-3194.
(2) Colombo, R., Carotti, A., Catto, M., Racchi, M., Lanni, C., Verga, L., Caccialanza, G., De Lorenzi, E., *Electrophoresis 2009,* 30, 1418-1429.
(3) Colombo R. et al. Patent n. MI2008A000366.
(4) Rambaldi, D.C., Zattoni, A., Reschiglian, P., Colombo, R., De Lorenzi, E., *Anal. Bioanal. Chem. 2009*, 394, 2145-2149.

MONOAMINE OXIDASES AND LSD1: SIMILAR CHEMISTRY FOR NEUROTRANSMITTER AND CHROMATIN MODIFICATION

Andrea Mattevi, Federico Forneris, Dale E. Edmondson, Claudia Binda

Department of Genetics and Microbiology, University of Pavia, via Ferrata 1, 27100 Pavia, Italy and Departments of Biochemistry and Chemistry, Emory University, Atlanta, Georgia 30322 USA

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AMYLOIDOGENIC PROTEINS AND CAPILLARY ELECTROPHORESIS: FROM FOLDING TO SMALL MOLECULE BINDING

Colombo, R.[1], Bertoletti, L.[1], Regazzoni, L.[2], Carini, M.[2], Maffei Facino, R.,[2], Aldini, G.,[2] De Lorenzi, E.,[1]
[1]*University of Pavia, Department of Pharmaceutical Chemistry, Viale Taramelli 12, Pavia, Italy*
[2]*University of Milan, Department of Pharmaceutical Sciences "Pietro Pratesi", Via Mangiagalli 25, Milano, Italy*

Affinity Capillary Electrophoresis (ACE) can be usefully exploited to describe protein-ligand non covalent complexations, either with the aim of rapidly screening a library of chemical entities or to obtain a more detailed insight into affinity constants and binding stoichiometry, once a hit has been identified. ACE is a versatile analytical approach that encompasses many different methods and depending on the mode chosen, it is possible to describe a large variety of interactions, from low-to-intermediate up to high affinity complexations. The principal advantages of ACE, compared to other separative or spectroscopic techniques, are the small volume consumption, the speed of analysis, and the fact that the complexation takes place in free solution, thereby preserving biological interactions. To show how this technique works, a specific example will be presented, where the protein target is β_2-microglobulin (β_2-m) and the putative ligands are a series of sulfonated compounds.

β_2-m is a small amyloidogenic protein normally present on the surface of most nucleated cells and responsible for Dialysis-Related Amyloidosis (DRA), which represents a severe complication of long term hemodialysis. A therapeutic approach for this amyloidosis could be based on the stabilization of β_2-m through the binding to a small molecule, and consequent inhibition of protein misfolding and amyloid fibril formation. The lack of a binding site for non-polypeptidic ligands on the β_2-m structure makes it difficult both the identification of functional groups responsible for the binding and the search of hits to be optimized. A few compounds have been previously described to weakly bind β_2-m, including the drug suramin, a bis-hexasulfonated naphtyl urea (1).

Taking suramin as an initial reference, it will be shown how a screening of 200 sulfonated/suramin-like molecules by the ACE mode known as DCCE (dynamic complexation capillary electrophoresis) successfully leads to the identification of one ligand, named 573, that further shows interesting anti-fibrillogenic properties (2, 3). The low sensitivity of ACE in terms of identification of very low affinities, prompted us to use the same protein-target system to set up a novel screening by high resolution LTQ-Orbitrap Hybrid Mass Spectrometry. The unique sensitivity and accuracy of this state-of-the-art mass spectrometer and the high throughput of analysis enabled us to rapidly cross-check the screening potential of both techniques, to have a fast feedback on the ACE results, to fish out hits previously discarded as very weak binders.

(1) De Lorenzi, E., Grossi, S., Massolini, G., Giorgetti, S., Mangione, P., Andreola, A., Chiti, F., Bellotti, V., Caccialanza, G. Capillary electrophoresis investigation of a partially unfolded conformation of β_2-microglobulin. *Electrophoresis,* 23 (2002) 918-925.
(2) Quaglia, M., Carazzone, C., Sabella, S., Colombo, R., Giorgetti, S., Bellotti, V., De Lorenzi, E. Search of ligands for the amyloidogenic protein beta$_2$-microglobulin by capillary electrophoresis and other techniques *Electrophoresis* 26 (2005) 4055-4063.
(3) Carazzone,C, Colombo R., Quaglia M., Mangione M, Raimondi S., Giorgetti S., Caccialanza G, Bellotti V., De Lorenzi E. Sulphonated molecules that bind a partially structured species of β_2-microglobulin also influence refolding and fibrillogenesis *Electrophoresis* 29 (2008) 1502-1510.

COMPOSITE PROTON MEMBRANES FOR POLYMER ELECTROLYTE FUEL CELLS

A. Magistris, P. Mustarelli, A. Carollo, E. Quartarone, S. Grandi
Dept. of Physical Chemistry, University of Pavia, Italy

Acid-doped polybenzimidazoles-based films are particularly appealing as high temperature membranes because of their relatively high proton conductivity without humidification. PBI contains basic functional groups that can interact with strong acids, such as phosphoric acid, allowing proton migration along the anionic chains via a Grotthuss mechanism. Anyway, a technological limit of these systems for fuel cells applications is related to the loss of the free acid in presence of water, which may cause a drop of proton conductivity during operation, and limits the use of PBI-based membranes at temperatures higher than 150°C.

In this work we propose a possible way to reduce the leaching of the free acid in the polybenzimidazole membranes. We report the development of new H_3PO_4-doped membranes, based on composite PBI, containing different amounts of silica functionalised with imidazole groups.

The actual effect of filler on the polymer protonation degree and acid retention capability is discussed. The influence of the concentration of filler on the electric properties is studied by the impedance spectroscopy technique. The thermal and microstructural properties are investigated by thermal analysis and scanning electron microscopy, respectively. Preliminary electrochemical tests of MEAs are shown and compared with those perfomed on PBI membranes without filler.

NONLINEAR COEFFICIENTS OF HAFNIUM DOPED LITHIUM NIOBATE CRYSTALS

I. Cristiani[1], P. Minzioni[1], J Parravicini[1], Jin Yu[1], L. Tartara[1], V. Degiorgio[1], E.P. Kokanyan[2]

1. Electronics dept., University of Pavia, Via Ferrata 1, 27100 Pavia Italy

2. Institute for Physical Research, National Academy of Sciences of Armenia, 378410, Ashtarak-2, Armenia

Lithium niobate is a very interesting material for the realization of nonlinear optical devices. A strong limitation to the utilization of wavelength converters based on ferroelectric crystals comes from the photorefractive effect that causes beam distortion and a reduced efficiency of nonlinear interactions. Recent measurements [1] performed by our group have shown that Hafnium-doped LN crystals (Hf:LN) exhibit a significant reduction of photorefractivity. An important step toward the possible utilization of Hf:LN in wavelength converters is the measurement of the effect of Hf doping on the nonlinear properties of the crystals. In this work we present measurements of the second-order nonlinear coefficient d_{31} as a function of the Hf concentration for a set of Hf:LN crystals. We recall that the nonlinear coefficient important for application is d_{33} , and not d_{31}. However, several investigations on doped LN crystals have shown that the behavior of d_{31} is the same as that of d_{33}.

We considered a series of congruent Hf:LN crystals grown by the Czochralski technique, as described in Ref.1. Hf^{4+} impurity ions were introduced into the melt in the form of HfO_2 in increasing amounts going from 0 to 5 mol%. We performed second-harmonic-generation (SHG) measurements by using, as the optical source the picosecond pulses of an optical parametric oscillator the picosecond pulses of an optical parametric oscillator (OPO) pumped by a mode-locked Q-switched Nd:YAG laser. Each Q-switching event gives rise to a train of 24 pulses having the duration of 23 ps and 4MW peak power. We set the OPO wavelength at 1120 nm and we varied the temperature of every crystal under test, in such a way to satisfy type I phase-matching for SHG. The measurements were performed in a non-depletion regime for the pump intensity. In Fig. 1 we present the nonlinear coefficients obtained for different dopant concentrations. The values have been normalised to that of the congruent crystal and have a precision of about 5%. Our results indicate that the nonlinear coefficient d_{31} slightly decreases by increasing Hf concentration. Up to 3% doping, the coefficient reduction is lower then 10%, while it decreases more significantly for higher dopant concentrations. In particular, in the 4 mol % and 5 mol% Hf:LN crystal, d_{31} reduces by about 22% and 35% respectively in comparison with congruent LN. It is interesting to notice that a similar behaviour has been predicted and experimentally demonstrated also in Mg doped LN[2]. Considering the data reported in Fig. 1, one can reasonably predict that crystals with a doping level of about 2 mol% should present a nonlinear coefficient almost equal to that of the undoped ones. In Fig. 2 we report the normalised SHG efficiency for all the considered crystals as a function of temperature. As expected the amount of Hf incorporation affects significantly the wavelength dependence of refractive indices, leading to a strong spreading of the phase matching temperatures for the various crystals. In addition the temperature acceptance for SHG increases by increasing the Hf concentration (the sample thickness is 2.9mm for the undoped crystal, and 3.5÷3.6mm for the others). This fact could indicate the presence of disorder or a non perfect homogeneity of the more heavily doped crystals, in agreement with the reduction of the nonlinear coefficients. It is therefore possible that the observed decrease of d_{31} is not an intrinsic effect, but comes from a reduced optical homogeneity of the grown crystals. We are presently investigating this point.

In conclusion this work demonstrates that Hf:LN with dopant concentration below 3% exhibits a nonlinear efficiency comparable to that of the congruent crystals. Hence, considering that 3% Hf:LN presents enhanced resistance to photorefractive damage, Hf:LN represents a very attractive candidate for the development of nonlinear devices.

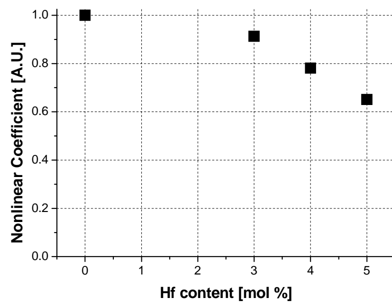

Fig. 1 Nonlinear coefficient normalised to the value measured in the congruent crystal

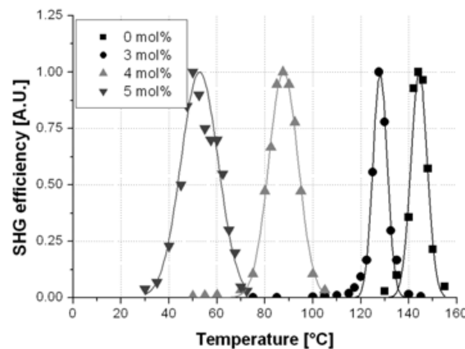

Fig. 2 Normalised efficiency of the the crystals under test as a function of the phase matching temperature

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ABNORMAL DIFFERENTIATION PROPERTIES OF MESENCHYMAL STEM CELLS IN THE MURINE MODEL OF OSTEOGENESIS IMPERFECTA BRTLIV

R. Gioia[1], C. Panaroni[1,2], R. Besio[1], A. Lupi[1], I.Villa[3], G. Merlini[4], G. Palladini[4], J.C. Marini[5], G. Cetta[1], R. Tenni[1], A. Rossi[1], A. Forlino[1]

[1]*Department of Biochemistry, University of Pavia, Italy;*[2]*Istituto Clinico Humanitas, Rozzano, Italy*
[3]*Bone Metabolic Unit San Raffaele Scientific Institute,Milan, Italy*[4]Amyloidosis Research and Treatment Center, Biotechnology Research Laboratories, Fondazione IRCCS Policlinico San Matteo, Department of Biochemistry, University of Pavia, Italy.
[5]*Section on Connective Tissue Disorders, BEMB, NICHD,NIH, Bethesda, USA*

The brittle bone disease Osteogenesis Imperfecta (OI) is a genetic disorder characterized mainly by bone fragility, fractures, skeletal deformities and short stature. The classical autosomal dominant forms of OI (type I-IV) are caused by mutations in the genes COL1A1 and COL1A2, coding for the α1 and α2 chains of type I collagen, respectively. Recently, forms of OI with recessive transmission (type VII-VIII), caused by mutations in the genes CRTAP and P3HI involved in collagen hydrossilation, have been described, whereas the molecular basis of OI type V (dominant transmission) and VI (recessive transmission) are still unknown.

The BrtlIV mouse is the first knock-in murine model for the moderate severe form of classical OI, carrying a glycine substitution in the α1 chain of type I collagen (G349C) and reproducing the clinical features of type IV OI patients [1].

In our study we used this murine model, which is available in our laboratory, to investigate the mesenchymal stem cell (MSC) differentiation properties in OI. Bone marrow MSCs are able to differentiate in several cell types, such as chondrocytes, myocytes, adipocytes and osteoblasts and it is known that equilibrium exists between adipocytes and osteoblasts differentiation. In particular we analyzed the ability of MSCs to differentiate toward adipocytes and osteoblasts respectively. Spines (2-3 cm) from wild-type (WT) and BrtlIV 2 month old female mice were dissected and cleaned from soft tissues, and total bone marrow was collected by bone crushing. Mesenchymal stem cells were isolated by plastic adhesion. Colony Forming Unit-Fibroblasts (CFU-F) number was evaluated by Giemsa staining following 1 week of culture. No difference was observed in the CFU-F number between WT and BrtlIV mice. Differentiation into adipocytes was induced by 10^{-8}M dexamethasone, 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine and 125 μM indomethacin, while differentiation into osteoblasts was induced by 10^{-8}M dexamethasone, 10 mM β-glycerol phosphate and 0.2 mM ascorbic acid 2-phosphate. Adipocytes colonies were identified by Oil Red O staining, while mineralization was detected by Von Kossa staining and measured using the LEICA Application Suite v3.0 software. Mesenchymal stem cells from BrtlIV mice differentiated into adypocites more than cells from WT mice (p=0.05); on the contrary osteogenesis was favoured in WT cells compared to the BrtlIV ones. Real Time PCR data confirmed a higher expression of the osteogenic markers type I collagen, Runx2, osteopontin, osterix and bone sialoprotein 2 in WT cells.

Bortezomib, a drug commonly used for the treatment of multiple myeloma, acts by inhibiting proteasome activity and thus by improving, at the MSCs level, the intracellular amount of Runx2, one of the first molecules involved in osteoblasts differentiation. We tested *in vivo* its use to improve the ability of OI MSCs to differentiate toward osteoblastic lineage. WT and BrtlIV 5 weeks old mice were treated with intraperitoneal injections of Bortezomib (0.3 mg/kg) for three weeks, and then sacrificed at two months of age. Mesenchymal stem cells were isolated and differentiated in culture as described above. Long bones (femurs and tibiae) were used for pQCT analysis, to evaluate bone geometrical properties. After Bortezomib treatment, the difference between WT and mutated cells in mineralization ability disappears, as well as the difference observed in adipogenesis. pQCT analysis revealed that the differences existing, before the treatment, between WT and BrtlIV mice bones, the last being smaller, thinner and with a reduced density, stayed after the treatment. Following Bortezomib administration a significant improvement was anyway found in BrtlIV mice in the cortical density, cortical thickness and endosteal circumference of femur and in the cortical density of tibia.

In conclusion, we demonstrated that a difference existed in mesenchymal stem cells differentiation ability between WT and BrtlIV mice and that it could be eliminated, although not reversed, by the use of a protreosomal inhibitor. The higher tendency toward adipocytes differentiation of mutant MSCs could contribute to determine the differences observed by pQCT in the geometrical properties in long bones and the identification of drug specifically targeted to eliminate such preferential differentiation could be a possible cure for OI.

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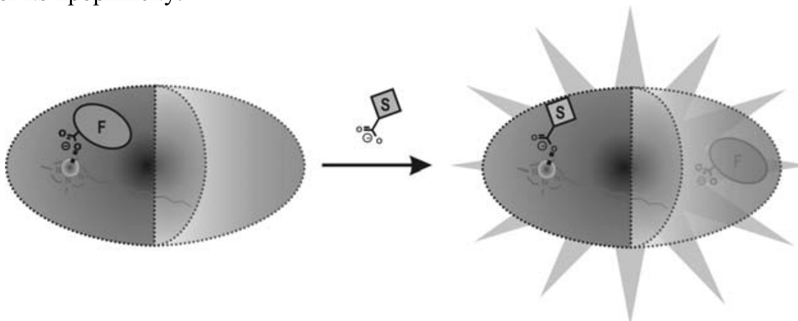
A MICELLAR SELF-ASSEMBLED DEVICE THAT MEASURES THE LIPOPHILICITY OF NON STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs) WITH AN OFF-ON FLUORESCENT RESPONSE

F. Denat,[b] Y. A. Diaz Fernandez,[a] P. Pallavicini,[a] L.Pasotti[a] and N.Sok[b]

a: Dipartimento di Chimica Generale, Università di Pavia, v.le Taramelli, 12 – 27100 Pavia, Italy; b: Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR CNRS 5260, 9 avenue Alain Savary - BP 47870 - 21078 DIJON cedex – France

The penetration inside hydrophobic microenvironments (i.e. cellular membranes) of pharmaceutical molecules influences dramatically the biological activity of drugs. In fact, lipophilicity is an important parameter to consider in drug design and pharmacokinetics studies. Moreover, in the recent area of drug delivery by means of micellar carriers, the same property plays an obvious role in the drug load capacity of a chosen carrier. Generally lipophilicity is quantified by means of the octanol-water partition coeficient (logP). We have recently described a micellar self-assembled ON-OFF sensor for the liphophilicity of fatty acids based on a multicomponent approach, using a Zn(II) cation inside a macrocylic lipophilized ligand as the receptor, forming a ternary complex with a fluorophore when solubilised in Triton X-100 micelles.[1] This original system have now been improved, applying it to measure the lipophilicity of the widespread NSAIDs and changing the response style from ON-OFF to the more valuable OFF-ON. We changed the metal into the lipophilic macrocyclic receptor from Zn^{2+} to Cu^{2+} and the fluorophore from Pyrene carboxylate to Coumarine343. When Coumarin is in its carboxylate form and bound to the copper(II) macrocyclic complex inside the micelles, the fluorescence is OFF. When lipophilic carboxylate anions are able to penetrate inside the micelles and displace the coumarine from Cu(II), the fluorescence turns ON. We checked the system with the series of fatty acids, $CH_3(CH_2)_nCOOH$, with $0<n<14$, at pH 7.4 where they are fully deprotonated. The intensity of fluorescence revival depends on the chain length, giving a direct experimental signal of its penetration inside the micelle, and hence of its lipophilicity.



We applied the device to evaluate the lipophilicity of a series of NSAIDs. The fluorescence revival intensities were found to sharply correlate with the log p values reported in literature. We have also determined the pKa of these NSAIDs in the micellar solutions of Triton X-100. The change of pKa respect to the values in water also correlate with the observed revived fluorescence intensities. Being the variation of the observed pKa a function of the partition between bulk water and the micellar core, that is in turn dependent on the molecular lipophilicity, also this data demonstrate that our system is capable of evaluating NSAIDs lipophilicity by means of a fluorescence change.

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NEW PBI-BASED MEMBRANES AS ELECTROLYTES FOR PEMFCs

A. Carollo, V. Kurdakova, A. Magistris, P. Mustarelli, E. Quartarone

[a.] *Dept. of Physical Chemistry, University of Pavia, Via Taramelli 16, 27100 Pavia Italy*

The current research on polymer electrolytes for fuel cells is focused on the optimization of a membrane working at about120°C and low humidity levels (<30%), which are the real operative conditions in case of automotive applications. Among a wide variety of tested polymer systems, PBI-based membranes, doped with phosphoric acid, are considered the best alternative to Nafion, due to their high conductivity even with no or low humidification and promising electrochemical performances. At present, these membranes suffer of a relevant drawback: the leaching of the free acid during fuel cell operation, which impede their use below at least 150°C. In this paper we present an experimental strategy which will consist in the improvement of the acid retention capability of the membrane. Therefore, new polymeric architectures, based on polybenzimidazole, are synthesized with an increased number of basic sites, differently interspaced along the polymer backbone. Subsequently, composite membranes are prepared by dispersing in the previously prepared matrices micro- and nanosized silica, which differ for morphology, microstructure and chemical nature.

Both in situ-electrochemical tests and impedance spectroscopy are performed to evaluate the the MEA performances and durability.

RELATIONSHIPS BETWEEN OOCYTE LEGACY AND EMBRYONIC DEVELOPMENTAL COMPETENCE IN THE MOUSE.

Michele Bellone, Chiara Vasco, Maurizio Zuccotti and Silvia Garagna
Laboratorio di Biologia dello Sviluppo. Università degli studi di Pavia.

The early stages of mammalian development are sustained by the presence of transcripts and proteins that have been produced and stored in the oocyte during folliculogenesis. This supply is utilised by the 2-cell stage in the mouse, at which time zygotic gene activation (ZGA) occurs and novel transcripts and proteins are expressed by the embryonic genome. An altered maternal contribution or a negative influence of the culture conditions to a correct expression of zygotic genes may leed to a developmental block at the time of ZGA. Using two model studies of mouse oocytes that cease development at the 2-cell stage, we tried to further our understanding of the oocyte's developmental legacy required to develop beyond the 2-cell stage. First, performing reciprocal MII-chromosome plates transfer between MII oocytes of a non-blocking inbred strain and MII oocytes of a blocking outbred strain, we have demonstrated that development beyond the 2-cell stage does not depend on the MII genome, but instead it relies on a cytoplasmic factor(s) already present in ovulated non-blocking oocytes, but absent, inactive or quantitatively insufficient in blocking oocytes. Second, we have compared the pattern of transcription of two types of mouse MII oocytes, one which is developmentally competent (MII^{SN} oocyte), the other that ceases development at the 2-cell stage (MII^{NSN} oocyte). Gene expression profiles of MII^{SN} and MII^{NSN} oocytes were analysed by both microarray and single cell RT-PCR. We have identified a number of gene expression networks and pathways whose misregulated expression would contribute to a developmental block. In particular, we found that the Oct-4 transcription factor is important for the regulation of genes (i.e., *Stella* and *Foxj2*) necessary for a correct pre-implantation embryonic development; when Oct-4 was down-regulated in MII^{NSN} oocytes, *Stella* and *Foxj2* were down- or up-regulated, respectively. Also, we found that Oct-4 down-regulation correlates with the up-regulation of eighteen genes involved in apoptosis and mitochondrial dysfunction. Parallel to a molecular, we have undertaken a morphological analysis of mouse antral oocytes with the aim of defining morphological parameter useful to predict their developmental competence. For this purpose, the position of the nucleus within the ooplasm has been determined prior to fertilisation. We found that those oocytes with a central nucleus are more capable to complete pre-implantation development than those with an eccentric one but, since this parameter can be influenced by culture conditions, it should be used in combination with the observation of the chromatin organisation (SN or NSN), in order to have a more accurate prediction of the developmental potential.

IN UTERO TRANSPLANTATION RESCUES THE BONE PHENOTYPE IN THE OSTEOGENESIS IMPERFECTA MURINE MODEL BRTLIV.

Cristina Panaroni 1,2,3, Roberta Gioia 1, Anna Lupi 1, Roberta Besio 1, Egon Perilli 4, Aurora Farina 5, Marco Casasco 5, Isabella Villa 6, SA. Goldstein 7, Giuseppe Cetta 1, Joan C. Marini 8, Antonio Rossi 1, Annalisa Frattini 2, Paolo Vezzoni 2,3, Antonella Forlino 1.
1 Department of Biochemistry, University of Pavia, Italy; 2 ITB-CNR, Milan, Italy; 3 Istituto Clinico Humanitas, Rozzano, Italy; 4 Istituti Ortopedici Rizzoli, Bologna, Italy; 5 Department of Sperimental Medicine, Section of Histology and Embryology, University of Pavia, Italy; 6 Bone Metabolic Unit San Raffaele Scientific Institute, Milan, Italy; 7 Orthopaedic Research Laboratories, Department of Orthopaedic Surgery, University of Michigan, Ann Arbor, Michigan, USA; 8 BEMB, NICHD, NIH, Bethesda, USA

Osteogenesis Imperfcta (OI) is a brittle bone disease caused mainly by mutations in the genes coding for Type I collagen. OI is characterized by bone fragility and deformity. BrtlIV is a knock in murine model for Classical OI, carrying a Gly349Cys substitution in the alpha1(I) chain. We evaluated an in utero cell therapy treatment for OI using the murine model BrtlIV. The bone marrow cells isolated from eGFP mice were injected into E14.5 embryos and mice were analyzed at 2 m. The engraftment, detected in various organs by microscopy, was quantified in bone by confocal microscopy and in different tissues by FACS and by Real Time PCR. Analysis of type I collagen extracted from bone revealed a reduction of the mutant collagen in treated mice, indicating that donor cells, differentiated to osteoblast/osteocytes, synthesized up to 20% of all type I collagen in bone. Raman spectroscopy revealed a significant reduction of heterogeneity in mineralization around donor cells in bone. PQCT showed a statistical increase in trabecular and cortical density in long bones epiphysis of Brtl treated animals and micro CT analysis of mid-shaft femur detected improvement in both geometric parameters and mechanical properties.

CONFORMATIONALLY ALTERED p53: A POTENTIAL PREDICTIVE MARKER FROM MCI TO ALZHEIMER'S DISEASE?

Serena Stanga[1], Marco Racchi[1], Daniela Uberti[2], Giuliano Mazzini[3], Elena Sinforiani[4], Maurizio Memo[2], Stefano Govoni[1] and Cristina Lanni[1]

[1] Dept. of Experimental and Applied Pharmacology, Centre of Excellence in Applied Biology, University of Pavia; [2] Dept. of Biomedical Sciences and Biotechnologies, University of Brescia; [3] IGM-CNR, Histochemistry and Cytometry, University of Pavia; [4] Lab. of Neuropsychology, IRCCS Fondazione "Casimiro Mondino", Pavia

Background: According to the current clinical criteria, definite Alzheimer's disease (AD) can only be diagnosed following neuropathological examination of brain samples, obtained by biopsy or autopsy. Furthermore, when evaluating the intermediate state between normal aging and established AD, known as mild cognitive impairment (MCI), not all MCI patients progress to AD and hence there is a need of a reliable prediction tool able to identify which patients with MCI will progress to AD. Candidate biochemical markers for AD should be molecules representing some of the cerebral pathogenetic processes typical of AD or representing altered metabolic or cellular processes possibly identified either in brain or peripheral tissues from affected patients. The current inability of clinical criteria to accurately identify this at-risk group underscores the importance of developing biomarkers able to potentially supplement the clinical approaches. Recently a role for conformationally altered p53 as a novel candidate biomarker for early onset AD has been described. The aim of our work is to investigate the usefulness of this method especially for younger patients, thus supporting its putative application for subjects with MCI and earlier in the clinical course of AD.
Methods: We used a rapid and quantitative flow-cytometric approach to investigate the different expression of conformationally altered p53 among MCI, AD and non-AD subjects. Parallely we perform the molecular genetic analysis for the apolipoprotein E (APOE) genotype.
Results: We found that peripheral blood cells from MCI specifically expressed increased levels of unfolded p53 compared to age-matched controls. We found that the expression of conformationally altered p53 is age-dependent. For our preliminary data analysis we have arbitrarily worked out the related cut-points by linear regression, taking as reference linear fit of controls, thus dividing the subjects in specific age interval segments. Young ($\leq$ 70 years) MCI patients showed levels of conformationally altered p53 comparable to those measured in AD patients, but significantly different from subjects of control group. Furthermore after having subjected MCI patients to a geriatric visit after two years from the enrollement, we found that 14 patients on 28 were converted to AD diagnosis. In this MCI converted group, the changes in the amount of conformationally altered p53 were independent from distribution of ε4 allele of apolipoprotein E (APOE). MCI patients without the ε4 allele showed a higher amount of conformationally altered p53 compared to respective controls, thus suggesting that the presence of the ε4 allele of APOE does not influence the level of conformationally altered p53 expression.
Conclusions: We found that high values of unfolded blood p53, which has been linked to AD pathology, may be considered as high risk factor for the conversion to AD and when p53 and APOEε4 genotype are combined as indipendent predictors of conversion to AD they yield to sensitivity and specificity values comparable to those obtained by a combination of CSF biomarkers. Therefore, we suggest that measurement of conformational p53 state can be useful as an easy accessible adjunctive diagnostic tool in identifying those at-risk group of MCI patients who progress to AD.

A GC-RICH MINISATELLITE DRIVES TRANSCRIPTION OF HUMAN TELOMERES

V. Vitelli[1], S.G. Nergadze[1], L. Khoriauli[1], M. Lupotto[1], C.M. Azzalin[2], E. Giulotto[1]

1 Dip. di Genetica e Microbiologia "A. Buzzati-Traverso", Univ. degli Studi di Pavia, Pavia, Italy
2 Institute of Biochemistry (IBC), ETHZ-Eidgenössische Technische Hochschule Zurich, Zurich, Switzerland

The longstanding dogma that telomeric ends are transcriptionally inactive have been overturned by the discovery, in several eukaryotes, of a heterogeneous population of telomeric repeats-containing RNAs (TERRA) (Azzalin et al, Science 2007). These RNA molecules, ranging from 100 nt to 10.000 nt, are transcribed by RNA Polymerase II starting from different subtelomeric loci. TERRA is a component of shelterin, the nucleoproteic complex of the telomeric tract, and it may function as a regulator of the stability of the chromosome ends and as a suppressor of telomerase activity. Here we show that in human cells, a CpG dinucleotide-rich island, cloned from chromosome X subtelomere, sustains the expression of a reporter gene (eGFP). Further molecular dissection of the region showed that a 29 bp minisatellite alone, comprised within this region, is endowed with promoter activity. In addition, a 61 bp repeat element acts as insulator between this promoter and subtelomeric genes. An in-silico analysis showed that sequences >93% identical to this promoter are present at several subtelomeres, and possibly contribute to the transcription of TERRA molecules.

BOTH SUBUNITS OF HUMAN TELOMERASE ARE REQUIRED TO IMMORTALIZE PRIMARY FIBROBLASTS FROM *EQUUS* SPECIES

Elisa Magnani[1], Pamela Vidale[1], Solomon G. Nergadze[1], Gael Cristofari[2], Chiara Mondello[3] and Elena Giulotto[1]
1. Dipartimento di Genetica e Microbiologia, Università di Pavia, Pavia, Italy
2. Ecole Normale Superieure de Lyon, Lyon, France
3. Istituto di Genetica Molecolare, CNR, Pavia, Italy

It was shown that primary cells from several mammalian species (human, bovine, mouse, rat, sheep) can acquire unlimited proliferative potential upon ectopic expression of human TERT. However, when we transfected fibroblasts from donkey, Burchelli's zebra and Grevyi's zebra (all belonging to the genus *Equus*) with human TERT, we were unable to prolong their proliferative capacity. This was probably due to the incompatibility of the human catalytic subunit with the equine RNA component, as suggested by an *in vitro* assay in which telomerase activity was measured in a rabbit reticulocyte lysate containing human TERT and horse TERC expression vectors. We then introduced both the subunits of human telomerase in the same cell lines and were able to restore its activity, allowing *Equus* fibroblasts to proliferate beyond their senescence limit. In the immortalized cells, telomeres were greatly elongated and the karyotype was aneuploid or near tetraploid. Interestingly, in Burchelli's zebra cells a small supernumerary telomere-less, probably circular, chromosome was gradually selected. Ectopic expression of the two subunits of human telomerase can then be viewed as a general method to immortalize primary cells from endangered species. These cellular systems may also help in elucidating the role of telomerase in cellular immortalization.

INSERTION OF TELOMERASE RNA RETROTRANSCRIPTS AT DNA DOUBLE-STRAND BREAK SITES DURING MAMMALIAN GENOME EVOLUTION

Solomon G. Nergadze[1], Marco Santagostino[1], Chiara Mondello[2] and Elena Giulotto[1]
[1]*Dip. Genetica e Microbiologia "A. Buzzati-Traverso", Università degli Studi di Pavia, Via Ferrata 1, 27100 Pavia, Italy;* [2]*Ist. Genetica Molecolare, CNR, Via Abbiategrasso 207, 27100 Pavia, Italy*

Introduction
In vertebrates, tandem arrays of TTAGGG hexameres are present at both telomeres and at intrachromosomal sites, giving rise to interstitial telomeric sequences (ITSs). We previously showed that, in primates, ITSs were inserted during the repair of DNA double-strand breaks and proposed that they could arise either from the capture of telomeric fragments or from the action of telomerase (Nergadze et al, Genome Res. 14: 1704, 2004). A direct involvement of telomerase in ITS insertion is conceivable in view of the mounting evidence for the sharing of factors between the machineries for DNA double strand break repair and telomere maintenance.
Results
In this work a comparative analysis of four mammalian genomes (human, chimpanzee, mouse and rat) provided two novel observations strongly supporting the hypothesis of telomerase involvement in ITS formation: 1) ITSs were preferentially introduced at DNA double-strand break sites where a few nucleotides homologous to the telomeric hexameres were exposed; 2) at several rodent ITS loci, a fragment retrotranscribed from the 3' end of telomerase RNA (TERC), far away from the canonical template, is adjacent to the telomeric repeats (TERC-ITS loci). At these loci, the telomeric repeats and the TERC 3' retrotranscripts are in opposite orientation. We then cloned the loci orthologous to 9 mouse TERC-ITS from 13 species of the genus *Mus* and demonstrated that the 3' and the 5' portions of the RNA were inserted at the same time by a complex mechanism. Finally, mutational analysis of the TTAGGG arrays in different species, suggests that they were introduced in the ancestral genome as telomeric hexameres. In conclusion, our results suggest that telomerase, the main enzyme working at telomeres, can also contribute to the repair of DNA double-strand breaks. These data do not allow us to estimate the probability of ITS insertion in mammalian genomes, however, considering that hundreds of interstitial telomeric sequences were created in the species under study through this pathway and that many others should have occurred without being fixed during evolution, we can conclude that the frequency of these peculiar repair events is not negligible. These telomerase driven repair processes occurring during evolution constitute a previously undescribed mechanism of genome plasticity and support the hypothesis that telomerase derives from an ancestral retrotransposition.

BIOCHEMICAL AND HISTOLOGICAL ANALYSIS IN THE GROWTH PLATE OF A MURINE MODEL OF DIASTROPHIC DYSPLASIA

M. Facchini, B. Gualeni, F. De Leonardis, A. Forlino, F. Pecora, G. Cetta, R. Tenni, and A. Rossi
Department of Biochemistry "A. Castellani", University of Pavia, Pavia.

The *diastrophic dysplasia sulfate transporter (DTDST* or *SLC26A2)* gene encodes for a sulfate/chloride antiporter expressed on the surface of many cell types, including chondrocytes.

Mutations in this gene cause a diminished inorganic sulfate uptake into the cell, leading mainly to synthesis and secretion of undersulfated proteoglycans (PGs) in the cartilage extracellular matrix. These defects cause a family of chondrodysplasias with different clinical severity; among them diastrophic dysplasia (DTD) is the moderate/severe form, characterized by dwarfism, progressive joint disease and malformed skeleton.

The dtd mouse is a *knock-in* mouse, characterized by a phenotype that recapitulates the essential aspects of human DTD. Thus, this animal model is a powerful tool to better elucidate the role of PG sulfation in skeletal development [1].

In particular we observed that long bones of dtd mice are shorter when compared to those of wild-type animals, suggesting an altered endochondral ossification process.

We analysed PG sulfation and, to clarify how it affects bone growth, we performed histomorphometric and chondrocyte proliferation studies on the growth plate of 3 weeks old mice.

For proliferation analysis mice were injected intraperitoneally with BrdU and sacrificed after 2 hours. Chondrocytes in the proliferative zone of the growth plate that incorporated BrdU during the labelling time were detected by immunohistochemistry. Our results demonstrated a significant decrease of chondrocyte proliferation in the growth plate of mutant mice compared to wild-type animals.

Histomorphometric measurements were performed on tibial sections stained with toluidine blue. In mutant animals the relative height of the hypertrophic zone and the average number of cells per column in the proliferative and hypertrophic zone were significantly reduced compared to wild-types.

In order to quantify PG undersulfation, we analysed the sulfation of chondroitin sulfate disaccharides both in the growth plate and in the zones of the growth plate (resting, proliferative and hypertrophic). The whole growth plate or the different zones were isolated from tibial sections with a microdissector (Eppendorf) and, after enzymatic digestion of the tissue with chondroitinase ABC, disaccharides released from chondroitin sulfate PGs were labeled with a fluorescent dye and analysed by reverse phase HPLC. Our data showed a significant undersulfation in the growth plate in toto and in all the zones of dtd mice compared to wild-type animals. In particular, the percentage of non-sulfated disaccharide in mutant mice was about 2 fold higher than in wild-types.

This relevant undersulfation of chondroitin sulfate PGs leads to alterations in the mechanical properties of the extracellular matrix and in signaling pathways, as demonstrated by histomorphometric and proliferation studies. Indeed we observed a reduced chondrocyte proliferation in the proliferative zone and a subsequent delayed differentiation in hypertrophic cells, suggesting the involvement of the Ihh-PTHrP signaling.

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