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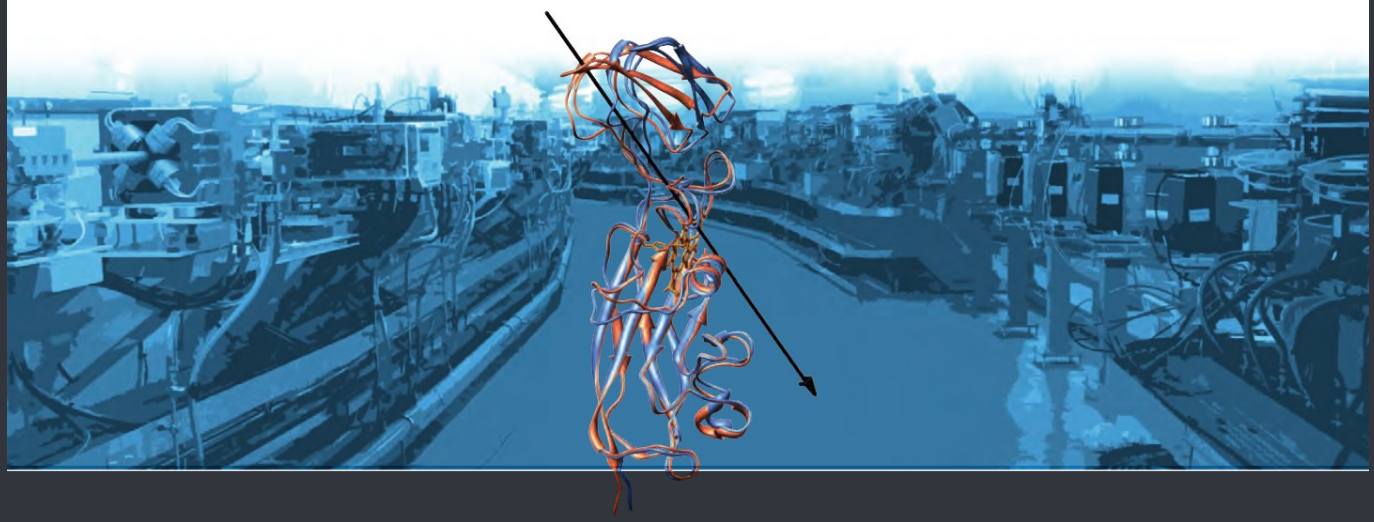
Abstracts of the 16th CONGRESS

SPANISH BIOPHYSICAL SOCIETY

6 - 8 June 2017 | Sevilla (Spain)



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Tuesday, 6th June

SPECIAL SESSION
Satellite Meeting - Workshop on New and Notable Biophysics — Auditorium
Chairs: María García-Parajo / Rafael Fdez-Chacón

Experimental measurement of binding energy, selectivity and allostery using fluctuation theorems ALEMANY, Anna	**11:00h - 11:30h**
Cargo selection by the retromer coat complex: A mechanism for recycling transmembrane proteins from endosomes HIERRO, Aitor	**11:30h - 12:00h**
Live-cell structural biology: towards the mechanics of exocytosis GALLEGO, Oriol	**12:00h - 12:30h**
Functional connectivity between neuronal partners according to cortical brain states GARCÍA-JUNCO, Pablo	**12:30h - 13:00h**

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SYMPOSIUM - Concurrent Session
S1 - Protein Structure, Dynamics and Function — Auditorium
Chairs: Marta Bruix / Antonio Díaz-Quintana

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SYMPOSIUM - Concurrent Session
S2 - Membrane Structure and Function
Supported by CPL/Elsevier
Chairs: Félix Goñi / Jesús Salgado
Lecture Room

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The proppin atg18 shows oligomerization upon membrane binding PÉREZ LARA, Ángel	**18:50h - 19:00h**

SPONSORED SEMINAR
Wyatt Technologies
Lecture Room

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S1 - Protein Structure, Dynamics and Function
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S2- Membrane Structure and Function
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Chairs: Félix Goñi / Jesús Salgado
Lecture Room

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Wednesday, 7th June

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SYMPOSIUM - Concurrent Session
S4 - Receptors, Channels and Transporters
Organized by the Spanish Channel Network
Chairs: Rafael Fdez. Chacón / Vicente Aguilella
Lecture Room

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Chair: Jesús Pérez Gil
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LAFeBS Lecture
Chair: Juan Carmelo Gómez
Auditorium

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SYMPOSIUM - Concurrent Session **S5 - Supramolecular Complexes** Chairs: José Carrascosa / José M. Mancheño	Auditorium
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NETWORKING	1st Floor Hall
Exhibitors, Posters and Refreshments	**17:00h - 17:30h**

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Structural basis of RNA polymerase I activation FERNÁNDEZ-TORNERO, Carlos	**18:40h - 18:50h**

SYMPOSIUM - Concurrent Session **S6 - Cellular Biophysics** Jointly organised by the Spanish and Portuguese Biophysical Society **Chairs**: Francisco Barros / Nuno Santos	Lecture Room
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SPECIAL SESSION	Auditorium
SBE Assembly	**20:00h - 20:30h**

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SYMPOSIUM - Concurrent Session S7 - Biophysics of Carbohydrates and Nucleic Acids Chairs: Fernando Moreno / Pedro Nieto	Auditorium
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SYMPOSIUM - Concurrent Session **S8 - Biointerfaces, Biofilms and Nanobiophysics** Chairs: Miquel Pons / Ana Azuaga	Lecture Room
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NETWORKING	1st Floor Hall
Exhibitors, Posters and Refreshments	**10:30h - 11:00h**

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SYMPOSIUM - Concurrent Session **S7 - Biophysics of Carbohydrates and Nucleic Acids** Chairs: Fernando Moreno / Pedro Nieto	Auditorium
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Analysis of the different structures of pulmonary surfactant collectin SP-D by atomic force microscopy ARROYO, Raquel	**11:50h - 12:00h**
Virtual high throughput screening (VHTS) of small mechanoactive molecules for controlling the mechanical stability of HIV-1 receptor RODRÍGUEZ, Bárbara	**12:00h - 12:10h**
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NETWORKING	Italian Pavilion
Lunch Break	**13:15h - 14:30h**

NETWORKING	1st Floor Hall
Exhibitors and Posters	**14:30h - 15:00h**

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PLENARY LECTURE **Manuel Rico - Bruker Prize** Chair: Antonio Ferrer	Auditorium
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PLENARY LECTURE **Enrique Pérez-Paya Prize** Chair: Antonio Ferrer	Auditorium
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Elucidating structures of sugars by high resolution spectroscopies COCINERO, Emilio	**15:50h - 16:10h**
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PLENARY LECTURE **SBE-33 Prize** Chair: Antonio Ferrer	Auditorium
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Biophysical approaches to assess transport properties of β-barrel channels QUERALT-MARTÍN, María	**16:55h - 17:15h**

NETWORKING	1st Floor Hall
Exhibitors, Posters and Refreshments	**17:15h - 17:45h**

PLENARY LECTURE **Closing Lecture** **Chair:** Antonio Ferrer	Auditorium
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SPECIAL SESSION	1st Floor
Prizes and Closing	**18:30h - 19:00h**

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Abstracts of Talks

1 Workshop on New and Notable Biophysics

Chairs: María García-Parajo / Rafael Fernández-Chacón

Experimental measurement of binding energy, selectivity and allostery using fluctuation theorems

A. Alemany,[a,b] J. Camunas,[c] F. Ritort[d]

[a] *Hubrecht Institute, Utrecht, Netherlands.* [b] *University of Barcelona, Barcelona, Spain.* [c] *Standford University, Palo Alto, United States of America.* [d] *Universitat de Barcelona, Barcelona, Spain.*

Thermodynamic bulk measurements of binding reactions rely on the validity of the law of mass action and the assumption of a dilute solution. Yet important biological systems such as allosteric ligand-receptor binding, macromolecular crowding, or misfolded molecules may not follow these assumptions and require a particular reaction model. In this talk we introduce a fluctuation theorem for ligand binding and an experimental approach using single-molecule force-spectroscopy to determine binding energies, selectivity and allostery of nucleic acids and peptides in a model-independent fashion. A similar approach could be used for proteins. This work extends the use of fluctuation theorems beyond unimolecular folding reactions, bridging the thermodynamics of small systems and the basic laws of chemical equilibrium.

Cargo selection by the retromer coat complex: A mechanism for recycling transmembrane proteins from endosomes

A. Hierro

CIC bioGUNE, Derio, Spain

Recycling prevents waste, reduces consumption and maintains balance. The ability to return receptors to their original location relies on signals and mechanisms that orchestrate their selective packing. Living cells constantly recycle receptors, proteins and lipids with a direct impact on nutrient uptake, re-sensitisation to environmental signals, immune surveillance and waste management. Endosomes are key recycling compartments where the biosynthetic and endocytic pathways intersect. Here, the fate of sorting receptors is directly linked to their selective recruitment into tubulo-vesicular carriers. Retromer is a multi-protein complex that recycles transmembrane cargo from endosomes to the trans-Golgi network and the plasma membrane. Our results will focus on how retromer complex couples membrane recruitment with cargo selection.

Live-cell structural biology: towards the mechanics of exocytosis

O. Gallego

IRB Barcelona, Barcelona, Spain

Structure-function analyses are fundamental to understand the mode of action of the cellular machinery. However, cellular functions result from the concerted action of complicated systems of protein assemblies and other cellular components, which complexity cannot be reconstituted in a tube. For instance, during exocytosis the secretory vesicles are first tethered to the plasma membrane and subsequently fused with it to release the cargo. Each of these steps is executed by different protein complexes that are somehow coordinated in time and space. Additionally, the activity of each complex requires the interaction with specific biological membranes. Thus, despite in vitro techniques can reconstruct isolated protein complexes up to the atomic scale, models where cellular functions are executed by individual protein assemblies are intrinsically limited.

PICT technique ("Protein interactions from Imaging of Complexes after Translocation") provides a unique combination of advantages that allow the detection and the quantitative description of macromolecular interactions in vivo. We have combined PICT with fluorescence localization and computational integration of structural data to determine the 3D structure of protein complexes directly in living cells. Using this approach, we have reconstructed the exocyst, a conserved multisubunit assembly that is responsible to tether secretory vesicles during exocytosis (Picco et al., 2017). The 3D architecture of the exocyst bound to a vesicle allowed us to start building a mechanistic model for exocytosis.

Functional connectivity between neuronal partners according to cortical brain states

P. García-Junco, Clemente,[a,b] T. Ikrar,[c] E. Tring,[a] X. Xu,[c] D. Ringach,[a] J. Trachtenberg[a]

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Synaptic inhibition orchestrates both spontaneous and sensory-driven activity in the cerebral cortex, and it's generated by interneurons reciprocally connected to other cortical neurons. Neurons expressing parvalbumin (PV), somatostatin (SOM) and vasoactive intestinal peptide (VIP) are the three largest and non-overlapping classes of interneurons in the mouse cortex. Several studies have shown cortical interactions between these groups of interneurons and their excitatory partners, but the functional meaning of the connections are poorly understood. An important question in systems neuroscience is how behavioral state modulates the processing of sensory signals. To gain further insights into the impact of behavioral state on local cortical circuitry, we employ a novel approach based on resonant scanning 2-photon imaging of large populations of identified cortical neurons in frontal cortex of behaving mice. GCAMP6 calcium sensors were used to image activity of excitatory and inhibitory neurons, using cell type specific CRE-driver lines that also expressed a red fluorescent protein. Our data identify a novel dual role of VIP interneurons to modulate the gain of excitatory neurons. During arousal, pyramidal neurons receive both indirect VIP→SOM cell-mediated disinhibition and direct VIP cell-mediated inhibition. An expected outcome from this circuitry is that variability in the net balance of inhibition and disinhibition generates a heterogeneous response of excitatory neurons, some of which are enhanced during arousal as others are suppressed. The net effect on individual cells is expected to shift their operating point, modulating the gain of pyramidal neurons during arousal.

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2 Plenary Lectures

Opening Session

Chairs: Irene Díaz-Moreno / Miguel A. De la Rosa

Acute oxygen sensing: molecular mechanisms and medical impact

J. López-Barneo

Institute of Biomedicine of Seville (IBiS), University Hospital "Virgen del Rocío"/CSIC/University of Seville, Sevilla, Spain

Oxygen (O_2) is necessary for oxidative phosphorylation, the major source of energy for the cells. Adaptive responses, which can be acute or chronic, have evolved to minimize the detrimental effects of O_2-deficiency (hypoxia). Unraveling the mechanisms underlying O_2 sensing by cells is among the major advances in modern biomedical research. During sustained (chronic) hypoxia, transcription factors are activated to induce (in hours or days) the expression of "O_2-sensitive" genes, which decrease the cellular needs of O_2 and increase O_2 supply to the cells. In mammals, hypoxia also triggers fast (in seconds) life-saving cardiorespiratory reflexes (hyperventilation and sympathetic activation) to increase gas exchange in the lungs and delivery of O2 to critical organs, such as the brain and heart. These acute responses to hypoxia are mediated by cells of the "homeostatic acute O_2 sensing system", which contain O_2-regulated ion channels. The main arterial chemoreceptors are glomus cells in the carotid body, which express K^+ channels that are inhibited by hypoxia. This leads to depolarization, Ca^{2+} influx and the release of transmitters that activate nerve fibers impinging upon the respiratory center. The mechanism whereby glomus cells "sense" changes in O_2 tension to signal membrane K^+ channels has remained elusive. We have shown that genetic disruption of the quinone binding site in mitochondria complex I selective abolishes acute O_2 sensing, and proposed a model in which accumulation of reduced quinone during hypoxia increases mitochondrial NADH and reactive O_2 species to signal membrane K^+ channels. Gene expression analyses suggest that O_2-sensitive cells have a signature profile, composed of specific metabolic enzymes and transporters, ion channels, and subunits of mitochondrial electron transport. Knowledge of the molecular mechanisms

of acute O_2 sensing helps design more efficient therapies for severe and highly prevalent diseases in the human population.

Tumour suppressor p53: structure, aggregation and rescue

A. Fersht

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The tumour suppressor p53 is an archetypical intrinsically disordered protein, having two stably folded domains, with the remaining 37% of its sequence disordered. If p53 and its apoptopic pathways are functional, cancer cells are doomed. Consequently, it has the most frequently mutated gene in cancer. Reactivation of inactivated mutant p53 is an attractive target for the development of anti-cancer drugs. To provide a rational basis for designing such drugs, we performed a rigorous biophysical analysis of the inactivation of p53 before designing candidate molecules and testing with cancer cell lines. Some 30% of inactivating mutations just lower the stability of p53. Further, p53 is kinetically unstable and those oncogenic mutants even more so, rapidly aggregating. We solved the structure of the most common oncogenic mutants. One highly destabilized mutant, Y220C, has a mutational surface cavity. We developed small compounds that raise its melting temperature and restore its activity. They rescue the activity of Y220C in cancer cell lines and induce apoptosis. To probe the feasibility of preventing aggregation by other routes, we analysed the aggregation mechanism. p53 aggregates to give an amorphous structure that gives the characteristic diffraction pattern and bind the dyes diagnostic of amyloid fibrils. A detailed phi-value analysis shows that the first step in aggregation is the extensive unfolding of the p53 core domain followed by the binding binding and extensive unfolding of a second core domain, with many beta strands participating. Several aggregation prone sequences in the protein self- and cross-aggregate. Mutation of any one of those sequences does not prevent formation of the amorphous aggregate. Our most successful molecule for the general rescue of p53 mutants reacts covalently with two surface-exposed thiol groups, stabilising p53 thermodynamically and also eliciting cytotoxic effects specifically in cancer cells.

ISMAR Lecture

Chair: Jesús Pérez-Gil

Multiple spectroscopies reveal dynamic oligomerization of functionally active GPCRs

A. Watts

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G protein-coupled receptors (GPCRs) play a pivotal role in cellular signalling, highlighted by the fact that they form the target for ~40% of marketed pharmaceuticals. While evidence has been accumulating for the existence and functional significance of GPCR oligomers, the matter is still under debate, in part due to lack of consensus on morphological aspects, such as the receptor interfaces involved in oligomerisation, and their possible dynamic nature [1]. Neurotensin receptor 1 (NTS1) is one of few GPCRs that can be produced in E. coli in an active state, and has been implicated in a variety of conditions including schizophrenia and various cancers. NTS1 has been demonstrated by us to show lipid dependent functionality [2-5] and dimerise in lipid bilayers [6], however, there is still no structural or dynamics information on the receptor dimer. Here, we combine single-molecule [5], and ensemble FRET, DEER, and in silico approaches to probe NTS1 dimerisation. The results support the presence of a concentration-dependent dynamic equilibrium between monomers and dimers, which could provide a means of regulation of receptor signalling and biased coupling in vivo.

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LAFeBS Lecture

Chair: Juan Carmelo Gómez-Fernández

Aquaporins: exploring gating and function in the plant kingdom

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Universidad de Buenos Aires, Buenos Aires, Argentina

Aquaporins are small transmembrane proteins ubiquitously expressed in biological membranes that facilitates water exchange. Assembled as homo/hetero tetramers -and in contrast to ion channels- each monomer works as a functional transport unit. In the plant kingdom seven aquaporin subfamilies are described and, in particular, the plasma membrane intrinsic proteins (PIPs) seems crucial in controlling osmotic permeability (Pf) at the plasma membrane. These PIP aquaporins also represent a highly abundant and conserved subfamily that has been historically divided into two subgroups due to their differences: PIP1 and PIP2. In terms of their function, all PIPs show capacity to rapidly adjust the Pf by means of a gating response. A close state seems to prevail under certain stimuli as cytosolic pH decrease, intracellular Ca^{2+} concentration increase and dephosphorylation of specific Serines. Many PIP1s also show another feature that clearly distinguishes them from any PIP2. These PIP1 fail to reach the PM when expressed alone, but they can succeed if they are coexpressed with a PIP2. Therefore, in terms of activity, PIP aquaporins can rapidly adjust membrane water permeability by means of two mechanisms: channel gating and channel translocation of PIP subunits (PIP1 and PIP2, organized -or not- in mixed tetramers). Evidences indicate that these mechanisms are not only highly conserved among species but their juxtaposition enhances the dynamics of the response. In particular, we propose that heterotetramerization, serine phosphorylation status and pH sensitivity affects aquaporin gating and thus would rule the Pf of a membrane that express PIPs when fast responses are mandatory. The functional properties of this interaction and physiological consequences are addressed in order to understand the relevance of the cell to cell pathway in the hydraulics dynamics not only as a physiological challenge but also as a response to adverse plant environmental conditions.

RSEF-SBE Lecture

Chair: José Adolfo Azcarraga

Dynamics and physics of cancer: Tumor and immune cell interactions

J. Seoane

Nonlinear Dynamics, Chaos and Complex Systems Group Departamento de Física Universidad Rey Juan Carlos, Madrid, Spain

In this plenary talk we present our work in Dynamics and Physics of Cancer [1,2,3,4]. In particular, our study uses in silico experiments and mathematical analyses to characterize the transient and asymptotic dynamics of the cell-mediated immune response to tumor growth. An hybrid probabilistic cellular automaton model describing the spatio-temporal evolution of tumor growth and its interaction with the cell-mediated immune response is developed. The model parameters have been adjusted to an ordinary differential equation model, which has been previously validated [2] with in vivo experiments and chromium release assays. We utilize the cellular automaton model to investigate and discuss the capacity of the cytotoxic cells to sustain long periods of tumor mass dormancy, as commonly observed in recurrent metastatic disease. This is joint work with Alvaro G. López and Miguel A. F. Sanjuán (Spain).

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Closing Lecture

Chair: Antonio Ferrer

Single particle cryo-EM of membrane proteins in lipid nanodisc

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In the last few years, major technological breakthroughs enabled single particle cryo-EM to become the technique of choice for structure determination of many challenging biological macromolecules. Atomic structures of many membrane proteins that are refractory to crystallization have now determined by this method, including our previous work of determining the atomic structures of TRPV1 and TRPA1. In most of these studies, membrane proteins were solubilized in detergent, or detergent-like amphipathic polymers ("amphipols"). However, maintaining purified proteins in a near-native lipid bilayer environment is crucial for visualizing specific and functionally important lipid–protein interactions, and more importantly, for maintaining protein functionality. The next technical challenge for single particle cryo-EM of membrane proteins is to enable atomic structure determination of integral membrane proteins in a native or native-like lipid bilayer environment.
Lipid nanodisc uses membrane-scaffolding proteins (MSP) to reconstitute integral membrane proteins into lipid nanoparticles. This highly native-like system is the first choice for a general platform for single particle cryo-EM of membrane proteins. We tested the feasibility of using lipid nanodisc in atomic structure determination of relatively small integral membrane proteins, such as TRPV1. We reconstituted TRPV1 ion channel in lipid nanodiscs, and determined atomic structures of nanodisc-embedded TRPV1 in three different conformations. These structures revealed locations of some annular and regulatory lipids that form specific interactions with the channel. Such specific phospholipid interactions enhance binding of a spider toxin to TRPV1 through formation of a tripartite complex. Our structures also reveal that, in the absence of vanilloid agonist, a phosphatidylinositol lipid occupies the capsaicin-binding site of TRPV1, providing important clues about physiological mechanisms of channel regulation.

3 SBE Prizes

Chair: Antonio Ferrer

Manuel Rico - Bruker Prize

The long and winding road towards autophagy

A. Alonso

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Our interest in the changes induced by several agents on the architecture of lipid bilayers started when, as a PhD student, I observed the 'fusion', i.e. lysis and reassembly, of small unilamellar vesicles in the presence of detergents. Then followed our model of (true) membrane fusion promoted by phospholipase C, and the involvement of diacylglycerol-driven non-lamellar phases in the fusion mechanism. In the last decade our interest has focused on ceramide, a molecule deceptively similar in structure to diacylglycerol, although endowed with vastly different properties. More recently we have applied our experience to the study of the role of lipids in the growth of the autophagosome, a cellular structure occurring at the early stages of autophagy. We have been able to describe how lipid geometry and bilayer curvature modulate autophagosomal elongation mediated by proteins of the Atg8 family. We have also quantified the binding of cardiolipin to LC3 and other Atg8 homologues, and described the negative effect of cardiolipin oxidation in these initial steps of autophagy. Ceramides are also important modulators of autophagy. In collaboration with G. Velasco (UCM, Madrid) we have demonstrated that the dihydroceramide/ceramide ratio mediates the cytotoxic autophagy of cancer cells via autolysosome destabilization. Our current studies include the interaction with Atg3 proteins with lipid membranes, and the lipidation reaction occurring between Atg8 and phospholipids.

Manuel Rico - Bruker Prize

Spatiotemporal organization of biological membranes using nanophotonic tools

M.F. García-Parajo

ICFO, Castelldefels, Spain

The quest for optical imaging of biological processes at the nanoscale has driven in recent years a swift development of a large number of nanoscopy techniques. These, so called, super-resolution methods are providing new capabilities for probing biology at the nanoscale by fluorescence. While these techniques conveniently use lens-based microscopy, the attainable resolution severely depends on the sample fluorescence properties. True nanoscale optical resolution free from these constrains can alternatively be obtained by interacting with fluorophores in the near-field. Indeed, near-field scanning optical microscopy (NSOM) using subwavelength aperture probes is one of the earliest approaches sought to achieve nanometric optical resolution. More recently, photonic antennas have emerged as excellent alternative candidates to further improve the resolution in the near-field by enhancing electromagnetic fields into regions of space much smaller than the wavelength of light. Here, I will describe our efforts towards the fabrication of different 2D antenna arrays for applications in nano-imaging and spectroscopy of living cells with unprecedented resolution and sensitivity. In particular, I will show that in-plane dimer antennas provide giant fluorescence enhancement factors up to 104—105 times, together with nanoscale detection volumes in the 20 zL range. We have taken advantage of the superior optical performance of these in-plane antennas arrays together with their extreme planarity to enquire on the nanoscale dynamics of multicomponent lipid bilayers. Our results reveal for the first time the coexistence of fluctuating nanoscopic domains on both Liquid order and Liquid disorder phases of mimetic membranes, in the microsecond scale and with characteristic sizes below 10nm. These nanoscale assemblies might be reminiscent to those naturally occurring in living cells that in the absence of proteins and/or other stabilizing factors, are poised to be highly transient.

Enrique Pérez-Paya Prize

Elucidating structures of sugars by high resolution spectroscopies

E. Cocinero

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Sugars are one of the major building blocks in biology, playing numerous key roles in living organisms. We present several studies on carbohydrates exploiting an experimental strategy which combines microwave, laser spectroscopies in high-resolution, computation and synthesis. Laser spectroscopy offers high sensitivity coupled to mass and conformer selectivity, making it ideal for polysaccharides and glycopeptides studies. On the other hand, microwave spectroscopy coupled with ultrafast laser vaporization provides much higher resolution and direct access to molecular structure of monosaccharides. This combined approach provides not only accurate chemical insight on conformation, structure and molecular properties, but also benchmarking standards guiding the development of theoretical calculations. In order to illustrate the possibilities of a combined

microwave-laser approach we present results on the conformational landscape and structural properties of several monosaccharides,[i],[ii] polysaccharides[iii] and glycopeptides including microsolvation and molecular recognition processes.[iv]

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Enrique Pérez-Paya Prize

Benefits of diversity: from molecular organization to cell signaling

C. Manzo

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Cellular signaling is regulated by biochemical interactions that are ultimately controlled by molecular diffusion. Recent advances in fluorescence microscopy have allowed the visualization of single molecules in living cells at unprecedented spatiotemporal resolution, revealing that the heterogeneity of the cellular environment produces exotic molecular motions that deviate from Brownian behavior [1]. These findings have stimulated new questions about the mechanisms generating these phenomena, as well as regarding their implications for cell biology. In this context, we have studied a transmembrane receptor involved in the capture of pathogens, which motion exhibits anomalous diffusion with signatures of weak ergodicity breaking [2]. Through the study of receptor mutants, we have been able to correlate the receptors motion to its molecular structures, lateral organization and interactions, thus establishing a link between nonergodicity and biological function. In addition, we have quantitatively interpreted the receptor dynamics through a stochastic model of random motion with random diffusivity on scale-free media [3,4], and we are attempting to gain further insight into the molecular causes of this complex diffusion. Our work highlights the role of heterogeneity in cell membranes and proposes a connection with function regulation. In addition, our models offer a theoretical framework to interpret anomalous transport in complex media, such as those found, e.g., in soft condensed matter, geology, and ecology.

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SBE-33 Prize

Optogenetic activation of receptor tyrosine kinases

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Receptor tyrosine kinases (RTKs) are a large family of membrane receptors that sense growth factors and regulate a variety of cell behaviors in health and disease. We engineered RTKs that can be selectively activated by low-intensity blue light. We selected light-oxygen-voltage (LOV)-sensing domains for their ability to activate RTKs by light-activated dimerization. Incorporation of LOV domains resulted in robust activation of relevant RTKs and the induction of cellular signaling in human cells with high spatio-temporal precision. Furthermore, light faithfully mimicked complex mitogenic and morphogenic cell behavior induced by growth factors. Next, we used light-activated RTKs to create an optogenetics-assisted drug screening platform. Our all optical approach obviates the addition of chemical activators or reporters, and reduces the number of operational steps. Using this platform, we screened a small library of kinase inhibitors, and we found that tivozanib specifically blocks the ROS1 orphan receptor, which is critically involved in lung cancer. Finally, we applied our light-activated RTKs to optically manipulate cell signaling in vivo. We generated a light-based fly model to trigger proliferative behavior during development, and to rescue cellular degeneration in a Parkinson's disease fly model. These results suggest that engineered light-activated receptors promise a fast and precise approach to control signaling in cells and living animals.

SBE-33 Prize

Biophysical approaches to assess transport properties of -barrel channels

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-barrel channels are wide pores that allow the passage of different types of ions, water and small hydrophilic molecules. Electrophysiology has been proved as the perfect tool to evaluate the functional properties of these systems, characterized by their multiionic transport. In particular, in this presentation I will discuss how planar bilayer electrophysiology at the single-channel level in combination with different theoretical and experimental approaches has a great potential to unveil the transport mechanisms of protein channels, from current and selectivity to thermodynamics.

4 S1 - Protein Structure, Dynamics and Function

Chairs: Marta Bruix / Antonio Díaz-Quintana

Nitrogen Signaling: New Structure-Centered Discoveries

V. Rubio

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The nitrogen flow affects all species in the biosphere and is a key, highly regulated process. Bacteria, cyanobacteria and plants play particularly important roles in the assimilation branch of this process. They have a dedicated system for nitrogen regulation, centered around the very widespread regulatory protein PII, which is under allosteric regulation by 2-oxoglutarate and nucleotides and by postranslational modification of its flexible T-loop. My laboratory was central in clarifying how PII controls arginine accumulation in cyanobacteria (and plants) and how it carries out in cyanobacteria its gene regulatory roles via an adaptor protein, PipX, and a transcriptional regulator, NtcA. More recently, we have contributed to advance our understanding of this regulatory system. We will review here these advances, including: 1) DNA targeting of this regulatory system and ways of deactivation of the cyanobacterial transcriptional regulator NtcA; 2) additional regulatory functions of PipX when in complex with PII; 3) the structure of PipX in solution and the role of its C-terminal helix in PipX extra signaling; 4) extension of PII signaling to exteme salt environments, highlighting adaptation of signaling to high salinity; 5) structure of the transcriptional regulator AmtR of the industrial microoganism Corynebacterium glutamicum, as first step towards understanding PII regulation in this organism; 6) structure of postranslationally modified PII from E. coli and the postranslational modification cascade; and 7) expanding the PII regulatory universe via PipY, a pyridoxal-dependent protein. Our efforts have included collaborations with other laboratories (Drs. Bonete, Contreras, Neira and Pineda, in Alicante, Elche and Valencia) and have have brought novel understanding of nitrogen regulation centered on protein PII across different phyla.

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Expanding the Mitochondrial Links to the DNA Damage Response

M.A. De la Rosa, K. González-Arzola, A. Guerra-Castellano, S.M. García-Mauriño, C. Elena-Real, F. Rivero-Rodríguez, A. Velázquez-Cruz, S. Curran-French, A. Díaz-Quintana, I. Díaz-Moreno

IIQ - cicCartuja, Universidad de Sevilla & CSIC, Sevilla, Spain

Genome integrity is constantly battered by genotoxic agents. These can induce DNA damage that leads to cell death if not properly repaired. Most studies on the DNA repair process have focused on yeast and mammals, in which histone chaperones have been revealed as key regulators for DNA to be accessible to repair machinery. However, knowledge of their exact role in DNA damage response is far from complete, in particular in plants. Our recent studies reveal that the closely related histone chaperones human SET/TAF-I and plant NRP1 are similarly involved in nucleosome assembly following DNA break in humans and plants, respectively [1,2], and that both histone chaperones interact with cytochrome *c* in the cell nucleus upon DNA damage. We have used Nuclear Magnetic Resonance (NMR), Isothermal Titration Calorimetry (ITC), Surface Plasmon Resonance (SPR) and Molecular Docking (MD) to provide a structural insight into the complex formed by cytochrome c with each histone chaperone. Cytochrome *c* competitively hinders the binding of SET/TAF-I and NRP1 to core histones, thus locking their histone binding domains and inhibiting their nucleosome assembly activities [1,2]. These findings also indicate that the underlying molecular mechanism of nucleosome disassembly/reassembly needed for DNA repair is highly conserved throughout evolution.

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Rational development of bicyclic peptides targeting the Grb7 cancer target

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The design of potent and specific peptide inhibitors to therapeutic targets is of enormous utility for both proof-of-concept studies and for the development of potential new therapeutics. Here we describe the development of a specific inhibitor of the Grb7-SH2 domain involved in cancer progression. Grb7 is an adapter protein, aberrantly co-overexpressed with erbB-2 and identified as an independent prognostic marker in breast cancer. Grb7 signals the activation of erbB-2 which plays a key role in disregulated cell growth in cancer. Grb7 also mediates signalling from focal adhesion kinase (FAK) exacerbating cell migration and the metastatic potential of cells. It is thus a prime target for the development of novel anti-cancer therapies. We have structurally characterised a cyclic peptide (G7-18NATE) that is a specific inhibitor of Grb7 and inhibits cellular growth and migration in cancer cell lines1. Based on this we have developed a series of second generation bicyclic peptides that show enhanced affinity and maintained specificity for the Grb7-SH2 domain as analysed using SPR2. Interestingly, X-ray crystallographic structural studies revealed an unexpected binding mode resulting in inhibitor redesign3. We have also developed cyclic peptides that incorporate carboxymethylphenylalanine and carboxyphenylalanine as phosphotyrosine mimetics, and shown using X-ray crystallography the way in which this also contributes to improved binding4.

Finally, we have shown that by combining these two strategies we are able to achieve peptides with affinities in the nM range that still maintain target specificity.

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Mitochondrial alterations in apoptosis at the single molecule level

A.J. García-Sáez

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The permeabilization of the mitochondrial outer membrane (MOM) is a key step in the regulation of apoptosis, a form of programmed cell death. Bax is a proapoptotic member of the Bcl-2 family that, during apoptosis, accumulates at discrete sites in the MOM, called apoptotic foci, to mediate its permeabilization. We showed that this process requires a conformational change in Bax, which partially opens its pore-forming hairpin, and is accompanied by self-assembly into multiple oligomeric species based on dimer units. This results in the opening of membrane pores, which can be fully or partially delineated by Bax molecules. Based on this, we proposed a new models for the molecular mechanism of Bax in MOM permeabilization. We also provide new insight into the interplay between Bax and other components of the apoptotic foci, which form complex macromolecular assemblies to orchestrate this key apoptotic event.

Molecular basis of the interaction of the human Apoptosis Inducing Factor with its nuclear partners

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The Apoptosis Inducing Factor (AIF) was first discovered as a caspase-independent cell death promoter that also plays a vital role in mitochondria, where it is normally confined. In healthy mitochondria, AIF contributes to the maintenance and stability of several respiratory chain complexes and is present in a monomer-dimer equilibrium regulated by NADH/NAD+ levels. AIF dimerization is undergoing upon NADH oxidation, which is accompanied by conformational rearrangements of the reductase and apoptotic domains. These observations suggest an interconnection between the mitochondrial and apoptotic activities of AIF which increases the interest of the intriguing relation between redox states and cellular activities. After cell death induction, AIF is liberated into cytosol, and then translocated to the nucleus where induces DNA degradation. The interaction between human AIF and the DNA occurs in an independent manner based on electrostatic interactions. The lethal activity of AIF requires its interaction with nuclease proteins as cyclophilin A. Here, we use different biophysical techniques to in vitro characterize the hAIF interaction with its nuclear partners. In addition, we also analyze the influence of the hAIF redox state in the modulation of such interactions.

Post-Translational Tyrosine Phosphorylation Bursts Cytochrome c Dynamics

A. Díaz-Quintana,[a] A. Guerra-Castellano,[a] R. Del Conte,[b] S.M. García-Mauriño,[a] K. González-Arzola,[c] P. Turano,[d] I. Díaz-Moreno,[a] M.A. De la Rosa[a]

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Cytochrome c is a key modulator of life-death signaling in mammalian and plant cells1-3. Accordingly, its post-translational modifications relate to diverse pathological situations. Indeed, phosphorylation of tyrosine 48 (Tyr48) occurs upon ischemia-reperfusion injury4. Hence, the structural and functional features affected by this modification were analyzed through the replacement of Tyr48 with the non-canonical amino-acid p-carboxymethyl-phenylalanine (pCMF). Notably, analysis of thermal unfolding suggests a destabilization of the weakest folding unit of the protein, which houses the mutation5.

2D- and 3D-NMR spectra were recorded to assign signals of reduced Y48pCMF cytochrome c species. NOE integration, distance geometry analysis and restrained molecular dynamics allowed for the modelling of the protein's 3D structure6, which is similar to that reported for the wild-type species7. Nevertheless, the two structures differ at the loop containing the amino-acid substitution and its surroundings. Analysis of relaxation rates and amide heteronuclear NOE values indicate enhanced dynamics around the mutation site. Appropriately, hydrogen exchange experiments indicate an increase of solvent accessibility in this region upon mutation. Strikingly, these changes affect well-known functional sites of cytochrome *c*. Thus, the data offer a hint about how Tyr48 phosphorylation affects diverse physiological processes.

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Structural basis for broad neutralization of HIV-1 through the molecular recognition of 10E8 helical epitope at the membrane interface

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The mechanism by which the HIV-1 MPER epitope is recognized by the potent neutralizing antibody 10E8 at membrane interfaces remains poorly understood. To solve this problem, we have optimized a 10E8 peptide epitope and analyzed the structure and binding activities of the antibody in membrane and membrane-like environments. The X-ray crystal structure of the Fab-peptide complex in detergents revealed for the first time that the epitope of 10E8 is a continuous helix spanning the gp41 MPER/transmembrane domain junction (MPER-N-TMD; Env residues 671-690). The MPER-N-TMD helix projects beyond the tip of the heavy-chain complementarity determining region 3 loop, indicating that the antibody sits parallel to the plane of the membrane in binding the native epitope. Biophysical, biochemical and mutational analyses demonstrated that strengthening the affinity of 10E8 for the TMD helix in a membrane environment, correlated with its neutralizing potency. Our research clarifies the molecular mechanisms underlying broad neutralization of HIV-1 by 10E8, and the structure of its natural epitope. The conclusions of our research will guide future vaccine-design strategies targeting MPER.

5 S2 - Membrane Structure and Function

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Chairs: Félix Goñi / Jesús Salgado

Membrane Fusion/Fission Yin-Yang in the Pulmonary Surfactant Complexes

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Formation and maintenance by the pulmonary surfactant system of surface active films at the respiratory surface is crucial to stabilize the lung against physical forces acting along the demanding breathing mechanics. For that purpose, surfactant requires the essential participation of small very hydrophobic proteins, SP-B and SP-C. These proteins are assembled by pneumocytes into tightly packed lipid-protein complexes that, once secreted, experiment remarkable structural transformations required for the homeostasis of the alveolar spaces. Lack or dysfunction of SP-B is incompatible with life. This protein assembles into supramolecular complexes able to promote the rapid flow of surface active species towards the interface, and at the same time, the formation of a highly cohesive multilayered structure providing maximal mechanical stability. These actions are associated with the ability of SP-B to promote membrane-membrane interactions and membrane fusion. Surfactant protein SP-C, on the other hand, considered the most hydrophobic protein in proteome, is an small palmitoylated transmembrane peptide producing deep perturbations into surfactant membranes, which end in their fragmentation to form small vesicles of 25-30 nm. These small vesicles are likely uptaken and recycled by pneumocytes and macrophages. The opposed actions of SP-B and SP-C towards membranes is mutually modulated, suggesting that their concerted action is a key feature to sustain pulmonary surfactant performance at the respiratory airspaces.

Pores and membrane remodelling by amphipathic peptides in single vesicles

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Pore formation, domain remodelling, fusion and fission of membranes are separated functions executed by a variety of proteins and protein complexes. However, similar activities can also be performed by a large number of amphipathic peptides. Throughout decades many studies have been conducted to elucidate the mechanisms behind these processes, but the success has been very limited because of the lack of direct structural information. Here we study the leakage, membrane remodelling and fission induced by fragments of apoptotic proteins of the Bcl2 family, using single vesicle fluorescence microscopy. The leakage occurs stochastically within the vesicle population and the kinetics can be analysed for individual vesicles using exponential models. We find quantal per vesicle leakage rates, which allows obtaining the distribution of pore size and the density of pores in the membrane. In parallel, some peptide versions are able to induce lateral domains in the membranes, accompanied by fusion and fission of vesicle, and the different activities are closely related the presence of cardiolipin in the lipid composition. I will discuss the connections between these apparently distinct functions based on the background intrinsic curvature of the lipids and the stretching and line tension effects exerted by the peptides.

The proppin atg18 shows oligomerization upon membrane binding

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PROPPINs (-propellers that bind polyphosphoinositides) are PtdIns3P and PtdIns(3,5)P2 binding autophagy related proteins that contain two phosphatidylinositolphosphate (PIP) binding sites in a conserved FRRG motif. Here we present the membrane binding characterization of the PROPPIN Atg18 from Pichia angusta. Rapid kinetic experiments suggest that the initial Atg18-membrane binding is driven by non-specific PIP interactions and the FRRG motif retains the protein in the membrane by binding two PIP molecules. Additionally, we studied the residues involved on the membrane binding using FRET and cross-linking experiments. Surprisingly, cross-linking experiments with liposome bound Atg18 yielded several intermolecular cross-linked peptides, which indicated Atg18 oligomerization. Later, we confirmed the Atg18 oligomerization by FRET-based stopped-flow measurements. All together, we demonstrated that Atg18 rapidly oligomerizes upon membrane binding while it is mainly monomeric in solution.

Phospholipid-Membrane Protein Selectivity: AFM-FS and Fret Studies

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Since transmembrane proteins (TMPs) crystals are more frequently obtained with enough purity for x-ray diffraction studies than decades ago, their action mechanisms may become elucidated. One of the pending issues is the actual interplay between transmembrane proteins and membrane lipids. There are strong evidences on the involvement of specific lipids on membrane proteins function, as the potassium channel KcsA or the secondary transporter LacY, which activities are related with the presence of anionic phospholipids as phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), respectively. We have approached this issue by implementation of atomic force microscopy (AFM), AFM in force spectroscopy (FS) mode and Förster resonance energy transfer (FRET). We will present in this communication the observations performed with the AFM on the distribution of LacY in binary supported lipid bilayers (SLBs) of PE and PG. The preference of LacY for fluid phases in SLBs as observed by AFM will be discussed under the basis of FS measurements. Single molecule force spectroscopy (SMFS) will be used to characterize the unfolding force (Fu) required to extract LacY from different lipid environments. FRET measurements between pyrene labeled phospholipids and the single tryptophan mutant (LacY-C154G) will evidence the selectivity between specific PE species and the protein. The body of results will be discussed within the framework of the flexible surface model (FSM) of the membrane.

Systematic lipidomics to uncover new membrane lipid functions

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The control of membrane lipid homeostasis is an essential process that allows cells to maintain both their energetic balance and the structural integrity of their different membrane systems. However, despite the importance of this process, and although most of the enzymes involved in lipid metabolism have been already identified, little is known about the regulatory mechanisms. In order to have a broader overview about how membrane lipid metabolism is orchestrated in cells, we present in this work a strategy that allows the monitoring of lipid changes in cells using a large-scale RNAi screening of human kinases combined with targeted lipidomic analysis by mass spectrometry. Statistical analysis of the screening highlights some genes whose knockdown induces changes in sphingolipid levels. Among them, cells lacking bromodomain-containing protein 3 (BDR3) have been found to have an increase in ceramide levels in different human cell lines, together with a decrease in glucosylceramide. Also, other changes in glycerophospholipids (i.e. ether-lipids, saturated lipids) can be detected, which shows that a membrane lipid remodelling takes place upon BRD3 knockdown by siRNA or by CRISPRi technology. These changes are accompanied by an increase in oxidative stress levels and affect plasma membrane properties, as detected in vivo using mechanosensitive membrane probes. We propose a metabolic and/or functional crosstalk between ether-lipids and sphingolipids

that may be important for the adaptation of cells against oxidative stress, showing the importance of maintaining membrane lipid homeostasis to preserve cell viability under stress conditions.

Photoacoustic effect applied on cell membranes: Direct observation by multi-photon laser confocal microscopy

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The photoacoustic effect consists of the formation of sound waves following light absorption by a material upon exposure to a short and intense light pulse. With highly-absorbing materials, the pressure wave can be strong enough to cause mechanical distress in soft matter e.g. cell membranes. Carbon nanoparticles can be used to exploit this effect, as they absorb light very intensively in the infrared region and emit powerful pressure waves. By using a multi-photon confocal laser microscope, in which we can irradiate the sample with different laser wavelengths, we have directly observed and recorded this effect in human red blood cells and Chinese hamster ovarian cells. At low energy, these mechanical shocks cause disruption of cell membrane integrity, opening transient pores through which compounds of interest may be internalized before the gaps in the membrane are self-repaired. At higher energies nevertheless the number and/or extension of the pores seem to be excessive for the cell to survive, and an irreversible process of death is started. This technique opens an interesting field of study on the photoacoustic effect in micro- and nano-systems, and its possible technological applications.

High-resolution studies of protein-lipid interactions using fluorinated lipids and biomolecular 19F NMR

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High resolution NMR studies on the structure, dynamics and molecular interactions of lipids are notoriously hampered by poor spectral ^{1}H dispersion. Uniform isotopic labeling with ^{13}C would allow to enhance resolution by 1H,^{13}C correlation spectroscopy, but their 13C spectra still show incomplete dispersion and signal splitting from 1JCC coupling. Also, ^{13}C would be no unique marker for lipids in interaction studies with ^{13}C labeled proteins. Thus we propose sparse chemical labeling with fluorine (100% ^{19}F) in the lipid chain to greatly enhance spectral resolution indirectly, via fluorine induced deshielding of nearby ^{1}H, and directly, via editing in a ^{19}F dimension with surpassing spectral dispersion, simplicity, and intensity. Both deshielding and JH,F coupling reach up to 4 bonds, suggesting an optimal sparse fluorination scheme to minimise the biophysical impact of this chemical modification, suppress signal splitting from 1JFF coupling, and preserve a high 1H density for intermolecular 1H(lipid),1H(protein) NOE contacts. As a first example for this novel class of membrane mimics we obtained di-(4-fluoro)heptanoylphosphocholine, 4F-DHPC7, that forms stable

micelles of similar size as DHPC7. Both 4F-DHPC7 and DHPC7 micelles solubilize and stabilise two representative membrane proteins: (i) photosensory rhodopsin II with 7 transmembrane helices, and (ii) outer membrane protein X with a -barrel fold. Differences in the ^{1}H,^{15}N NMR fingerprint spectra recorded in 4F-DHPC7 and DHPC7 are small, confirming similar protein structures, and correlate with residues near the fluorine position in modelled micelles, suggesting a new method to gauge protein immersion depth. A first ^{19}F filtered NOESY experiment indeed revealed unambiguous NOE contacts between 4-F-DHPC7 and a bihelical integrin fragment. Tests on more fluorinated lipids and membrane proteins with optimised biomolecular ^{19}F NMR experiments are now required to further develop the promising approach.

6 S3 - Protein Folding, Misfolding and Stability

Chairs: Javier Sancho / Óscar Millet

Rational thermostabilization of a three-state protein and testing of the role of a native basin intermediate

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Rational stabilization of proteins against thermal denaturation may facilitate their use in biotechnological and medical applications and provides an opportunity to test our understanding of protein energetics. Specially challenging are proteins not showing two-state unfolding equilibria. Long-chain flavodoxins (non covalent complexes between apoflavodoxin and FMN) are electron transfer proteins involved in essential bacterial reactions. Apoflavodoxin thermal unfolding is characterized by accumulation of an evolutionary conserved partly unfolded intermediate whose structure has been determined for the Anabaena protein. The intermediate belongs to the native basin as it is significantly populated at room temperature. Relevant thermostabilization of three-state proteins, such as this one, can only be achieved by specific intervention in the unstable subdomain. Following this simple rule we have designed and combined stabilizing mutations to produce a thermostable apoflavodoxin variant (ΔTm = 32 °C) with cooperative two-state equilibrium behaviour, thus lacking the equilibrium intermediate of the WT protein. General reasoning has attributed important roles in protein binding and catalysis to native basin intermediates, but proof is sometimes elusive. Comparison of WT and thermostable apoflavodoxin variant allows to assess the functional importance of this particular intermediate. Our analyses indicate that it only exerts minor influences in folding, FMN binding, protein-protein interaction, electron transfer rates and overall tridimensional structure, which argues for a lack of biological relevance of this evolutionary conserved intermediate and raises some concern for unsubstantiated attributions of adaptative value to similar features in other proteins.

Molecular Mechanisms for Cellular Protein Quality Control

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Cells have immensely crowded interiors and, to function successfully, they require quality control mechanisms to reorganise misplaced contents. The fate of hydrophobic proteins that have become exposed to the cytoplasm is decided by a collaboration between cochaperone SGTA (small, glutamine-rich, tetratricopeptide repeat protein alpha) and the BAG6 complex, whose operation relies on multiple transient and subtly discriminated interactions with diverse binding partners. These conspire to determine whether an exposed hydrophobic patch will be refolded by chaperones to safety within a protein's core, or delivered to an appropriate membrane or recycled via the ubiquitin/proteasome pathway. It seems these fates are under constant evaluation and can be changed or reversed in response to changing circumstances. I will present our latest structural and functional results on SGTA and its interactions (with an E3 ligase and a selection of hydrophobic substrates) using NMR spectroscopy and a range of complementary biophysical techniques such as Small Angle X-ray Scattering (SAXS), electron paramagnetic resonance (EPR) and native mass spectrometry.

Reliable structural and energetic model of the "unfolded state" of proteins

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The traditional structural and functional representation of proteins, limited to their native folded state has been challenged by a new description where partially or completely unfolded conformations are increasingly important. The poor knowledge about the non-native conformations, such as the partially unfolded intermediate states, the intrinsically unfolded domains, and the ensemble of conformations known as the "unfolded state", constitutes a pressing problem. In this work we aimed at developing a realistic structural and energetic model for the "unfolded state" of proteins that allows to calculate one thermodynamic property, the unfolding enthalpy (ΔHu), as a first step towards predicting stability (ΔGu). From one model protein we performed MD simulations both of the solved wild type structure (the folded state) and of a randomly, sufficient selected subset from 2200 unfolded structures released from the ProtSa server (an 'a priori' representation of the "unfolded state") for each of these two proteins. We take into account the solvent effect on the calculation of the theoretical ΔHu by differentiating between the contributions of the first water shells ("biological waters") and that the bulk. Despite huge absolute enthalpies are obtained from these simulated states, i.e. folded and unfolded, the estimation of ΔHu (ΔHu = Hu – Hf – ΔHsolv-eff) is accurate and reproducible. A second related estimation, the calculation of the unfolding specific heat variation (ΔCpu) is also carried out. Quantum corrections and rmsd/system-size effects are assessed. ΔCpu estimation also match well with experimental results. Statistical analyses to warranty convergence of results and to establish the minimal number of unfolded structures required to reliably represent the "unfolded state" are performed. We propose the present

methodology to reliably establish the "unfolded state" (a "minimum unfolded state") in proteins with similar characteristics to that here modelled.

Putative Role of Hypercooperative Hydrogen Bonds in Stabilizing an Amyloid-Like Pathological Conformation of TDP-43, a Protein Linked to Amyotrophic Lateral Sclerosis

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As the world's population ages, a large increase in dementia is predicted for future decades. TDP-43, an essential RNA-binding protein, forms aggregates in >95% of sporadic ALS cases and is also tied to Alzheimer's Disease. TDP-43 is composed of a folded N-terminal domain, two central RRM RNA-binding domains, and a disordered, 150-residue long C-terminal region. The latter is essential for forming functional assemblies that regulate RNA translation but is also key for pathological aggregation. Previous studies showed that TDP-43 aggregates resist harsh treatment and seed new aggregates which spread and "infect" new cells [1]. The Gln/Asn rich motif (residues 341-367) of the C-terminal region is necessary for efficient aggregation of TDP-43 [2]. The objective of our work is to determine the structure of this aggregate and the basis of its stability. Utilizing extensive spectroscopic characterization and computational experiments, we propose that this motif forms a beta hairpin that oligomerizes to adopt an amyloid-like conformer. We also show that Gln and Asn side chain hydrogen bonds in amyloids may possess an extraordinary hypercooperativity, which could decisively stabilize TDP-43 aggregates. A study of TDP-43 aggregates from ALS patient brains showed that the Gln/Asn-rich motif is protected in vivo from Gln/Asn deamidation, Ser phosphorylation and Met oxidation [3], which is in line with our conformational model. The high conformational stability afforded by Gln/Asn H-bond hypercooperativity could explain the stability of these TDP-43 aggregates, and perhaps also those of other pathological amyloids, like polyglutamine aggregates, as well as functional amyloids like Sup35 (GNNQQNY) and CPEB3 (QQQQRQQQQ) implicated in memory consolidation.

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Remodeling of RepE conformation by the molecular chaperones DnaK and DnaJ

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Hsp70 chaperones, together with their Hsp40 cochaperones and nucleotide exchange factors, are essential components of the cellular protein homeostasis network. The Hsp70 chaperone system is involved in multiple essential functions as protein folding, transport across membranes, assembly of macromolecular structures, prevention of protein aggregation and reactivation of aggregates in cooperation with Hsp100 chaperones in bacteria and fungi, and Hsp110 in metazoans. Especially interesting is the ability to modify the function of naturally occurring proteins by conformational

remodeling. A good model to study this process is the bacterial protein RepE, the initiation factor of mini F plasmid replication. RepE function depends on its oligomeric state: while the dimer acts as repressor of plasmid replication, monomers are activators. Notably, monomerization of RepE is facilitated by DnaK and DnaJ, the main representatives of the Hsp70 and Hsp40 families in bacteria. Here we present bulk biochemical and biophysical data, as well as nanopore single molecule studies of the interaction of RepE with the chaperones and specific DNA sequences.

Pharmacological chaperones as a novel therapeutic intervention line for congenital erythropoietic porphyria

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The group of pathologies produced by a lack of activity in some of the enzymes of the heme group biosynthesis is generically known as porphyria. Normally the loss of activity is produced by mutations in the amino acid sequence of said proteins and the type of porphyria depends on the specific enzyme causing the mutation. Specifically, congenital erythropoietic porphyria (CEP), also known as Günther's disease named after the author who described it in 1911, is a hereditary disease and the least frequent of the porphyrias (affecting > 1 in 1000000 people). This disease is a consequence of a malfunction in the uroporphyrinogen III synthase (UROIIIS), which is an enzyme of 260 amino acid residues (in the human isoform) catalyzing the cyclization of the linear tetrapyrrole hydroxymethylbilane to produce macrocycle uroporphyrinogen III (or urogen III), the precursor of the heme groups, siroheme, F340, vitamin B12 and chlorophyll. Here, we describe a novel putative treatment for congenital erythropoietic porphyria (CEP). In particular, we have demonstrated that a repurposed pharmacological chaperone enhances the catalytic activity of uroporphyrinogen III synthase (UROIIIS) by increasing its stability and intracellular concentration levels. Further, the compound reduces the levels of uroporphyrinogen (UROI) and its derivatives in relevant models of the disease.

Increased vulnerability of human NQO1 towards cancer-associated inactivation through divergent evolution

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Human NAD(P)H:quinone oxidoreductase 1 (NQO1) is a FAD-dependent enzyme involved in the antioxidant defense, activation of cancer pro-drugs and stabilization of oncosuppressors such as p53 and p73 [1]. A single nucleotide and cancer-associated polymorphism in NQO1 (P187S) causes its loss-of-function due to inactivation and destabilization of the enzyme by altering the dynamics of the FAD binding site and the C-terminal domain [2, 3, 4]. NQO1 is also a paradigm of the relationship between human flavoproteome stability and the bioavailability of flavin precursors [5].

Our experimental and structural analyses support that cancer-associated vulnerability of

NQO1 towards inactivation is linked to divergence of several stabilizing amino acids along primate evolution. Accordingly, we identify and characterize reverse mutations that protect the P187S polymorphism towards inactivation in vitro and inside cells. The protective role of these mutations is futher discussed in the context of a structural and thermodynamic mechanism of rescue involving a population-shift in the conformational ensemble of apo-P187S.

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PolyQ Tracts as Efficient C-capping Elements for Coiled-coils

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Poly-glutamine (polyQ) tract expansions have been linked to a variety of neurodegenerative diseases. The conservation of such sequences points to a relevant role, which is suggested to involve their organization into secondary structure elements. For the particular case of the androgen receptor (AR) we recently reported that the Leu-rich segment N-terminal to the polyQ tract acts as a helical N-capping sequence that propagates helicity into the tract itself [1]. Based on that, we have acquired in vitro CD and NMR as well as in silico MD data on a battery of peptides showing that the helicity of the sequence positively correlates with the number of glutamines in the tract up to the values found in the average human population (16-25 residues, depending on ethnicity), and that helix stabilization depends on glutamine sidechain-mediated hydrogen bonds. This supports a C-capping role for the polyQ tract, as a minimum number of glutamine residues is required to stabilize the helicity while further growth of the tract is detrimental because of increased aggregation rates. Proteome analysis shows that regions predicted to fold into coiled-coils are highly enriched in adjacent sequences N-terminal to polyQ tracts, thus providing the grounds for a general role of such tracts as C-caps for these helical elements.

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The role of hydrophobic matching on transmembrane helix packing in biological membranes

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The concept of Hydrophobic matching refers to the pairing between hydrophobic core of the membrane (MB) and the transmembrane (TM) domain of the protein, in order to avoid unfavorable exposure of hydrophobic surfaces to a hydrophilic environment. Hydrophobic matching was been widely studied experimentally and computationally. Glycophorin A (GpA), which represent one of the best suited and most studied models for helical transmembrane (TM) segment packing and MB protein folding, has been used as in vitro experiment for understand Hydrophobic matching, showing that the surrounding hydrophobic environment length can modify the GpA TM segment dimer/monomer equilibrium. However, there is not, up to date, a direct confirmation of this effect and its implication on MB protein folding in vivo. In this work we explore the concept of hydrophobic matching in vivo using chimeric GpA proteins with different length TM domain. Results show, equally in Escherichia coli cells (Tox RED assay) and human derived cells (BiFC assay), in contrast to previous in vitro studies, that all tested chimeric proteins can homo-dimerize through the TM domain in an in vivo context, concluding that biological membranes can accommodate transmembrane homo-dimers with a wide range of hydrophobic lenghts. Furthermore, hetero-dimers with a large length disparity between their monomers were also tolerated. Nonetheless, length differences between transmembrane helices hindered the dimer/monomer equilibrium confirming the impact of the hydrophobic matching on helix packing in vivo.

New NMR experimental techniques: Protein structural compactness and transient conformational exchange dynamics

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Atomic resolution and dynamic information of biological macromolecules are mandatory to understand their biological function at molecular level. However macromolecules are dynamic ensembles so alternative high-energy conformations can play important function roles. Therefore, there is a real demand of new experimental techniques that provide access to study dynamic properties of macromolecules by NMR. Intrinsically disordered proteins (IDPs) are characterized by high local mobility and lack of stable three-dimensional structure allowing them to interact with a large variety of binding partners. We propose taking advantage on the different relaxation dynamics of IDPs and globular proteins to understand the structural compactness and solvent accessibility by new NMR experiments based on a set of selective measurements of T1 relaxation delays (I).

NMR spectroscopy is a unique technique to study conformational dynamics over a wide-range of time scales, from picoseconds to hours. Real-Time NMR provides information of transient populated states based on the acquisition of NMR experiments with fast-pulsing methods. On the other hand relaxation-dispersion (RD) methods enable detection of conformational exchange dynamics of excited protein states that take place on the micro- to millisecond time scale but. Then we propose to combine both techniques as a powerful tool to study site-resolved conformational dynamics occurring in timely unstable or transient populated protein states (II).

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7 S4 - Receptors, Channels and Transporters

Organized by the Spanish Channel Network

Chairs: Rafael Fernández-Chacón / Vicente Aguilella

New insights into molecular function of large- conductance voltage- and calcium- activated potassium channels (BK) and calcium nanodomains

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In neurons, sites of Ca^{2+} influx and Ca^{2+} sensors are located within 20-50 nm, in subcellular "Ca^{2+} nanodomains". Such tight coupling is key for the functional properties of synapses and neuronal excitability. Two main players act together in nanodomains, coupling Ca^{2+} signal to membrane potential: the voltage-dependent Ca^{2+} channels (Cav) and the large conductance Ca^{2+} and voltage-gated K^+ channels (BK). BK channels are characterized by synergistic activation by Ca^{2+} and membrane depolarization, but the molecular mechanism underlying channel function is not completely understood. Information about isolated moieties of the channel has been obtained using different approaches. Nevertheless, the specialized behavior of this channel must be studied in the whole protein complex at the membrane to determine the complete range of structures and movements critical to its in vivo function.

In our laboratory we combine genetics, biochemistry, electrophysiology and spectroscopy, which we correlate with protein structural analysis, to investigate the real time structural dynamics underlying the molecular coupling of Ca^{2+}, voltage and activation of BK channels at the membrane as well as their structural organization and clustering with Cav complexes in reconstituted nanodomains taking advantage of imaging techniques. In addition, BK subcellular localization and role in Ca^{2+} neuronal nanodomains make these channels perfect candidates as reporters of local changes in

[Ca2+] restricted to specific subcellular regions close to the membrane. We have created fluorescent variants of the channel that report BK activity induced by Ca2+ binding, or Ca^{2+} binding and voltage. We aim to optimize and deploy these novel optoelectrical reporters to study relevant Ca^{2+}-induced processes both in cellular and animal models. Overall, optically-active BK channels with spectrally-separate photoactivation and FRET modules offer many possibilities for the study of activation in mammalian cells.

Organization of receptors, ion channels and transporters along the neuronal surface

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Biological membranes are composed of two sheets of phospholipids with embedded integral and peripheral proteins, acting not only as a boundary of living cells but also as an interface among cells and their organella. Among integral proteins, molecular cloning has revealed over 200 genes encoding neurotransmitter receptors, ion channels and transporters in mammals, making them the most diverse subset of plasma membrane proteins. The precise location of neurotransmitter receptors, ion channels and transporters along the dendro-somato-axonic surface of the neurons, as well as at intracellular sites, is an important factor in determining its functional impact. However, they are not evenly distributed on the neuronal surface and depending on the protein subtype, are instead concentrated at different compartments. One factor necessary to understand their role in neuronal function is to unravel their specialized distribution and subcellular localization within a cell, and this can only be achieved by electron microscopy. The SDS-digested freeze-fracture replica labelling (SDS-FRL) technique is a powerful and cutting-edge approach for quantitative investigation of localization and the two-dimensional distribution of membrane molecules at a nano-scale spatial resolution. SDS-FRL was developed by combining a conventional freeze-fracture replica technique with an immunogold labelling approach for the molecules of interest. I will introduce a rationale, advantage and disadvantage of the SDS-FRL technique, in comparison with the conventional immunoelectron microscopy techniques, showing clear evidence that this highly sensitive approach provides comprehensive information on the organization of neurotransmitter receptors, ion channels and transporters in any given neuron and demonstrating their unique localization in a protein type-, subunit-, brain region-, cell type- and compartment-dependent-manner.

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Differential modulation of Kv1.3/Kv1.5 complexes by Kcne4

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The voltage-dependent potassium channel Kv1.3 is widely expressed in the immune system. Kv1.3 is present in T and B-lymphocytes as well as macrophages and dendritic cells, controlling their activation and proliferation. This channel is one of the responsible actors of the chronic activation during autoimmune diseases, such as multiple sclerosis. In fact, a pharmacological control of the channel alleviates symptoms of disease.

Kv1.3 may interact with a large collection of proteins. Thus, other Kv1 subunits, regulatory -subunits (i.e. Kcne4) and other proteins like caveolin may form a heteroligomeric complex named the Kv1.3 channelosome. These interactions modify either Kv1.3 traffic or tune the functional activity resulting in potential alterations in cell physiology. Here we analysed the effect of both Kv1.5 and Kcne4 in the functional activity of a Kv1.3 functional complex.

As Kv1.3 and Kv1.5 can form functional heterotetramers with a variable stoichiometry, we generated a Kv1.3-Kv1.5 protein tandem chimera of a 1:1 fixed ratio. We describe the behaviour of the heterotetramer in this ratio regarding traffic and function. As Kcne4 is able to interact and modulate Kv1.3, but not Kv1.5, homotetrameric channel, we performed experiments in the presence or absence of KCNE4. We describe a differential effect of Kcne4 over the Kv1.3 channelosome depending on the presence of Kv1.5. Also, we demonstrate that our model recapitulates what it is found in dendritic cells.

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Characterization of the dual-membrane specifity of rabphilin 3A

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Rabphilin 3A is a membrane traffic protein that contains a tandem C2AB-domain located in its C-terminal that is responsible for the Ca2+-dependent phospholipid binding and mediates interactions with regulatory proteins like SNAP25, CASK, Anexin A4 and Miosin V. We report here functional analyses to characterize the molecular determinants of the Rabphilin3A interaction with membranes. By using Isothermal Titration Calorimetry we have determined the affinities and thermodynamic properties of these interactions and the results indicate that the C2AB domain binds preferentially to membranes containing PIP2 and phosphatidylserine in the presence of Ca2+. This is an exothermic reaction driven mainly by enthalpy changes. Dynamic Light Scattering assays have demonstrated that the main aggregation capacity resides in the C2B domain. Site-directed mutagenesis of key residues located at the different interacting surfaces of the C2AB domain shows that each one plays differential roles in the tandem. These is due to a collection of conserved key functional residues, but at the same time each one possess differential amino acids that confer them special abilities to interact with the membrane and with other proteins. These findings provide functional explanation about how these domains are regulated by a dual-target mechanism and reveal how this

family of proteins can employ subtle structural changes to modulate their sensitivity and specificity to various cellular signals.

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Transmembrane interactions of Bcl-2 proteins

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Interactions among pro- and anti-apoptotic members of the Bcl-2 (B-cell lymphoma 2) protein family modulate the permeabilization of the outer mitochondrial membrane (MOMP) and thus control commitment of cells to apoptosis. Deregulation of this interaction network generates an imbalance between cell death and survival and contributes to the pathophysiology of several diseases such as neurodegenerative disorders and cancer. Almost all the members of the Bcl-2 protein family have a C-terminal transmembrane domain that has been traditionally considered a mere membrane anchor. The interactions of transmembrane domains and their contribution to protein function are poorly understood. Here, we demonstrate, in biological membranes, that interactions between the transmembrane domains of Bax and anti-apoptotic Bcl-2 proteins represent a previously unappreciated level of apoptosis regulation.

Computational approaches to the study of the TRPV1 channel activation and modulation

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Transient receptor potential (TRP) ion channels constitute a notable family of cation channels involved in the ability of organisms to detect noxious mechanical, thermal and chemical stimuli that gives rise to the perception of pain. One of the most experimentally studied agonist of TRP channels is capsaicin, which is responsible for the burning sensation produced when chili pepper is in contact with organic tissues. Understanding how TRP channels are regulated by capsaicin and other natural products is essential to high impact pharmacological applications, particularly those related to pain treatment. By selected examples from the work we have carried out, I will provide an overview of the current knowledge we have about activation, permeation and selectivity of TRPV1, one of the so-called 'human molecular thermometers'.

A modular model of presynaptic function

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Synapses of almost every type exhibit large dynamic changes in connection strength during ordinary use; the phenomenon is termed short-term plasticity. The resulting input/output function computed in the time domain varies greatly between synapses. Multiple presynaptic mechanisms are involved, but the identity of the mechanisms, how they interact, and the implications for biological computation are not understood. Our quantitative analysis of rate-limiting mechanisms in synaptic vesicle trafficking questioned the assumption that mass action of freely diffusing vesicles plays a role, and generated a mathematically simpler modular model where presynaptic terminals could be thought of as assemblies of individually-tuned frequency filters, operating in parallel. The modular model provides a starting point for developing a general theory for the role of synaptic dynamics in biological computation. I will discuss how and why we developed the new model and a series of cell biology experiments that test its validity.

Analysing TRP channels using state-of-the-art artificial bilayer methodology

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Thermal Transient Receptor Potential (TRP) channels belong to an important class of receptors for drug screening as they are involved in sensations like pain and taste. TRP channels are found widely distributed throughout the mammalian central and peripheral nervous systems and are sensitive to temperature, ligands and mechanic stimulation. However, using electrophysiological assays, TRP channels are difficult targets to analyze due to their multiple and diverse pathways of activation and the requirement of a precise temperature control. Here we demonstrate that TRP channels can be analyzed in a reliable manner with artificial bilayer recordings. In particular, we studied different reconstituted TRP channels (purified human TRP-A1, TRP-V1, TRP-V3 and TRP-M8) using Nanion's recently introduced Orbit mini setup. Planar lipid bilayers were formed by painting lipids in organic solvents over Micro Electrode Cavity Arrays (MECA) in a highly inert polymer. The reconstitution of TRP channels were achieved by adding the purified proteins directly to the bilayers. The TRP channels were activated either by specific ligand or by temperature protocols using the integrated temperature control system of the Obit mini setup with a precision of $\pm$ 1°C. The data obtained from experiments involving artificial bilayers were compared to data obtained from experiments on stably-transfected HEK cell lines (Millipore, Charles River) using an automated patch clamp platform (Patchliner, Nanion) with temperature control.

Structure of the homodimeric androgen receptor ligand-binding domain

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The androgen receptor (AR) plays a crucial role in normal physiology, development and metabolism as well as in the aetiology and treatment of diverse pathologies such as androgen insensitivity syndromes (AIS), male infertility, neurodegeneration and prostate cancer (PCa). We have shown that dimerization of AR ligand-binding domain (LBD) is induced by receptor agonists but not by antagonists. The 2.15-Å crystal structure of homodimeric, agonist- and coactivator peptide-bound AR-LBD unveils a 1,000-Å2 large dimerization surface, which harbours over 40 previously unexplained AIS- and PCa-associated point mutations. An AIS mutation in the self-association interface (P767A) disrupts dimer formation in vivo, and has a detrimental effect on the transactivating properties of full-length AR, despite retained hormone-binding capacity. The conservation of essential residues suggests that the unveiled dimerization mechanism might be shared by other human nuclear receptors. Our work defines AR-LBD homodimerization as an essential step in the proper functioning of this important transcription factor and opens novel and unexplored therapeutic avenues to design personalized drugs against castration resistant prostate cancer.

Calcium Signal Transduction in the Calmodulin/Kv7.1 Channel Complex

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The cardiac IKs channel (KCNQ1/KCNE1) is a major repolarization current in the heart adequating diastolic filling time in the face of accompanying accelerated heart rate, while the M-current (KCNQ2/KCNQ3) is a key controller of neuronal excitability. Both are under dynamic control by the phospholipase C cascade, which causes reduction on PIP2 levels and release of Ca^{2+} from IP3 sensitive intracellular stores. While the action of PIP2 in gating is thought to be direct on the channel, Ca^{2+} regulation is thought to be mediated by calmodulin (CaM), which binds to an intracellular site of the channel known as helix A + helix B. The current hypothesis regarding Ca^{2+} gating posits that CaM wraps around helix B under resting conditions, and, when CaM becomes loaded with Ca^{2+}, it embraces both A+B helices simultaneously, causing a large structural rearrangement. We have examined this issue by monitoring conformational changes triggered by Ca^{2+} using FRET. KCNQ1 is well positioned to integrate changes in intracellular Ca2+ into an alteration in action potential duration, consistent with our results, showing that Ca^{2+}-responsive of considerable FRET change. In contrast, Ca^{2+} cause minimal FRET changes in KCNQ2 channels when are loaded with Ca^{2+}, according with our NMR results suggest that the AB module behaves as a rigid body around which CaM accommodates, both loaded with and without Ca^{2+}. Our investigation is focused on determining the regions responsible of Ca^{2+} signal transduction in KCNQ1 and KCNQ2 channels. For this, we have constructed different chimeras between KCNQ1 and KCNQ2 and we are analyzing

FRET changes in response to Ca^{2+}. In combination with NMR studies, we expect to obtain a full description trajectory changes at atomistic level which led to gating of potassium channels by Ca^{2+}.

8 S5 - Supramolecular Complexes

Chairs: José L. Carrascosa / José M. Mancheño

Structural Insights on Regulation of Lytic Machineries in the Pneumococcal Divisome

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Separation of daughter cells during bacterial cell division requires that the septal cross wall be split by peptidoglycan hydrolases. In Streptococcus pneumoniae D,D-carboxypeptidase DacA and L,D-carboxypeptidase DacB function in a sequential manner while essential endopeptidase PcsB is regulated by the transmembrane FtsEX complex. The crystal structure of DacB, radically different to that of DacA, contains a mononuclear Zn^{2+} catalytic center located in the middle of a large and fully exposed groove. Two different conformations were found presenting a different arrangement of the active site topology. The critical residues for catalysis and substrate specificity were identified. The crystal structure of full-length PcsB shows an unprecedented dimeric structure in which the unique V-shaped coiled-coil domain of each monomer acts as a molecular tweezers locking down the catalytic domain of its dimeric partner in an inactive configuration. This finding strongly suggests that the release of the catalytic domains requires an ATP-driven conformational change in the FtsEX complex, which is most likely conveyed towards the catalytic domains through a set of coordinated movements of the -helices forming the coiled-coil domain of PcsB. Recent findings on regulation of the different lytic machineries in this system will be discussed.

Architecture of heteromeric AMPA-type glutamate receptors

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AMPA-type glutamate receptors (AMPARs) are neuronal ion channels that mediate fast excitatory neurotransmission and drive synaptic plasticity, the molecular mechanism underlying memory formation. In the brain AMPARs exist predominantly as heterotetramers of various combinations of four subunit subtypes (GluA1-4) and can associate with more than 30 proteins. Subunit composition and auxiliary proteins modulate gating kinetics, ion permeation, pharmacology, and trafficking. Here we combined X-ray crystallography, cryo-EM, normal mode analysis, crosslinking and electrophysiology to get insights into the architecture of heterotetrameric AMPARs. We initially focused on the N-terminal domains (NTDs), which comprise 50% of the receptor and are sequence-diverse between subunits, playing a role in receptor heteromeric assembly. Crystal structures of GluA2/3 and GluA2/4 NTD heterotetramers revealed a novel compact arrangement that we could trap in full-length receptors by cysteine crosslinking. We used the crosslinked protein to determine a cryo-EM structure of a full-length GluA2/3 heterotetramer, which showed a compact arrangement resembling the NTD crystal structures and deviating from GluA2 homomers determined previously. Cryo-EM models also showed two different conformations in the ligand binding domain, which binds glutamate and undergoes structural rearrangements that trigger channel opening. We finally used cysteine crosslinking experiments to investigate the conformation of other homo- and heterotetramers, which allowed us to dissect the rules of subunit positioning and confirmed that AMPARs are highly dynamic. Our data highlight the structural diversity of the NTD layer of AMPARs, a potential platform for synaptic protein binding, and illustrate the potential of cryo-EM to isolate conformational states of dynamic proteins.

Homologous Histone Chaperones Human SET/TAF-I and Plant NRP1: Similarly Inhibited by Cytochrome c in Cell Nucleus

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Genome integrity is constantly under attack by genotoxic agents. These can induce DNA damage leading to cell death if not properly repaired. While most studies on the DNA repair process have focused on yeast and mammals, in which histone chaperones have been revealed as key regulators making DNA accessible to repair machinery, knowledge of the exact role of histone chaperones in DNA damage response is far from complete, particularly in plants. Our recent studies reveal that the closely-related histone chaperones, human SET/TAF-I and plant NRP1, are similarly involved

in nucleosome assembly following DNA breaks in humans and plants, respectively [1,2]. Furthermore, both histone chaperones were found to interact with cytochrome c in the cell nucleus upon DNA damage. To provide structural insight into the complex formed by cytochrome c with each histone chaperone, nuclear magnetic resonance, isothermal titration calorimetry, surface plasmon resonance and molecular docking were used. Cytochrome c competitively hinders the binding of SET/TAF-I and NRP1 to core histones, thus locking their histone-binding domains and inhibiting their nucleosome assembly activities [1,2]. The findings also indicate that the underlying molecular mechanism of nucleosome disassembly/reassembly needed for DNA repair is highly conserved throughout evolution.

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Mechano-chemical characterization of dynamin-mediated membrane fission

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The dynamin family of proteins are unique GTPases involved in membrane fission and fusion events throughout the cell. Dynamin is necessary for internalizing essential nutrients, is tightly coupled to cell signalling events, and has been linked to neuropathies and myopathies. As a vesicle invaginates, dynamin oligomerizes as a spiral around the neck of the vesicle generating dynamin-lipid tubes that constrict upon GTP hydrolysis, causing the fission of the neck and release of the vesicle . The ability of dynamin to constrict mechanically the underlying lipid bilayer makes it unique among GTPases as a mechano-chemical enzyme. However, the mechano-chemical processes governing the operation of dynamins at the molecular level are still under debate. Using optical tweezers, we have developed an experimental assay to measure the real-time activities of Dynamin 1 and Dynamin 2 proteins as they constrict individual membrane nanotubes and the effect of GTP analogues on their reactions. Our results shed light into the mechanical operation of these enzymes and reveal significant differences between their mechano-chemical cycles.

Structure-function studies with Δ1-pyrrolin-5-carboxylate synthetase (P5CS), a key bifunctional player in amino acid biosynthesis, inborn disease and stress resistance

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Δ1-Pyrrolin-5-carboxylate synthetase (P5CS), a bifunctional enzyme, catalyzes in multicellular eukaryotes the first two steps of proline synthesis. These steps are also the first of ornithine synthesis

in animals. In plants, P5CS is very important for stress resistance and is feed-back inhibited by proline. In mammals one isoform is inhibited by ornithine. In humans, P5CS deficiency (P5CSD) is a rare inborn error associated with either a cutis laxa/developmental delay/metabolic phenotype or with a presentation of complicated spastic paraplegia, and exhibits both recessive or dominant inheritance depending on the mutation. We determined the crystal structure of the enzyme that corresponds in bacteria to the glutamate-5-kinase domain and the structural mechanism of its inhibition by proline. We identified the first dominant human mutation in P5CS deficiency and were centrally involved in reporting the spastic paraplegia phenotype. We now try to understand the double phenotype and dual type of inheritance of the human deficiency on the basis of a hypothesis of negative dominance. We have produced recombinantly the human and the Arabidopsis thaliana enzymes, developed assays to determine their enzyme activities and substrate and inhibition kinetics, characterized the oligomeric structure of the human enzyme by showing that it is a basic dimer that associates at least into tetramers in equilibrium with higher oligomers in a concentration-dependent way, and made site directed mutagenesis studies to test the effects of the clinical mutations. Attempts at structure determination have failed thus far from the crystallographic side, but they are yielding clear-cut results by single particle electron microscopy. Our findings (still in progress at the time of writing) will be used to discuss the negative dominance hypotyhesis for explaining the dual inheritance of the deficiency.

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Herpesvirus DNA packaging machinery

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Herpesviruses are infectious agents causing a number of human diseases, including two types of cancer. There are 8 human herpesviruses, all of them being pathogens. No vaccines are available against herpesviruses and current antiviral drugs are, in some cases, problematic because of their serious side effects. Herpesviruses are complex and highly evolved dsDNA viruses, some of them encoding more than 200 proteins. Despite belonging to 4 different families, all herpesviruses share a common morphology. Virions consist of large icosahedral capsids into which the genome is packaged. The packaging machinery includes a large portal particle, at one vertex of the capsid, and a terminase complex that cleaves the replicated DNA concatemer into unit genomes, while pushing them through the portal into preformed capsids. Thus, herpesviruses are evolutionary related to tail bacteriophages and share with them a similar packaging system. Advances in our structural studies on both herpesvirus and bacteriophage packaging machines, using a combination of X-ray diffraction and cryo-EM techniques, will be presented. In addition, a structure-driven development of new antiviral compounds targeting the herpesvirus terminase will be presented.

Uncovering the Flexible Architecture of a Complex Macromolecular Machine in DNA Repair at 4-5 Å Using Cryo-EM

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Our group is dedicated to improve the mechanistic understanding of large macromolecular complexes important in DNA repair and DNA damage signalling. Many of these processes are regulated in the cell by multi-subunit macromolecular complexes. Elucidating the structure of these large complexes provides relevant information about how they function. Recent advances in cryo-electron microscopy (cryo-EM) methods allow approaching these complex machineries, but dealing with structural flexibility and heterogeneity is still a major issue in order to reach high resolution. In my talk, I will introduce recent advances in the cryo-EM methodology, and where this field is moving. I will describe examples from our current research in the laboratory to introduce the potential of this methodology, but also major areas of difficulty. I will describe several multi-subunit complexes that work in the DNA damage response, and which combine rigid and flexible regions. The structure of these flexible complexes is been analysed using a specific strategy for each region in the complex. Rigid interactions can be resolved at high resolution, whereas flexible regions could only be resolved at medium resolution after extensive classification of the data into sub-populations grouping molecules in a similar conformation. The work that I present is a joint effort of most members in our group, together with collaborators in the UK. This collaborative approach has been important to address the difficulties found when analysing these large and flexible macromolecular complexes.

Structural studies of the CCT-gelsolin complex

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The eukaryotic cytosolic chaperonin CCT (Chaperoning Containing T-CP1) is a molecular machine involved in assisting the folding of proteins that regulate in important cellular processes. This chaperonin consists of a large cylindrical oligomer formed by two rings each one built by eight different subunits (~60kDa). It was originally thought that the function of CCT was the folding of the cytoskeletal proteins actin and tubulin but subsequent studies have shown that CCT interacts with a wide range of proteins. Some of these proteins bind to CCT but do not requiere interactions with CCT in order to get its proper folding and to be functional. Gelsolin is an actin filament severing protein that increases actin dynamics by generating filament ends for further actin polymerization, and previous studies have shown its interaction with CCT. This binding is slow and gelsolin is accumulated over time on CCT suggesting that this protein is not a real folding substrate of the chaperonin. In fact, although bacteria lack CCT, gelsolin can successfully be produced as a native soluble protein in bacteria. Therefore, CCT could have a regulatory effect on gelsolin, acting indirectly in the actin filament dynamics. The main aim of this project is the structural characterization of the CCT-Gelsolin complex using electron-microscopy in order to elucidate the binding mechanism that mediates such interaction and whether CCT has an actual role on actin dynamics regulation. To face this goal, our first step has been to carry out binding assays between CCT and Gelsolin adding DTSSP (3,3´-dithiobis[sulfosuccinimidylpropionate]) to crosslink the complex, which was

later purified by gel filtration and this sample is being used for further structural characterization which is currently in progress.

Architecture of the yeast Elongator complex

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The highly conserved eukaryotic Elongator complex performs specific chemical modifications on wobble base uridines of tRNAs, which are essential for proteome stability and homeostasis. The complex is formed by six individual subunits (Elp1-6) that are all equally important for its tRNA modification activity. However, its overall architecture and the detailed reaction mechanism remain elusive. Here we show the structures of the yeast Elp123 sub-complex and fully assembled Elongator solved by negative stain electron microscopy, showing that two copies of the Elp1, Elp2 and Elp3 subunits form a symmetric two-lobed scaffold, which binds Elp456 asymmetrically. Furthermore, we use crosslinking mass spectrometry to identify the global interaction network of Elongator and employ an integrative modeling approach to localize all individual subunits and generate a topological model of the full complex. We validate our model, which is consistent with previous studies on the individual subunits, by complementary biochemical analyses. Our study provides a structural framework on how the tRNA modification activity is carried out by Elongator.

Structural basis of RNA polymerase I activation

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Biosynthesis of the eukaryotic ribosome starts with ribosomal RNA production by RNA polymerase I (Pol I), a process that is critical to regulate cell growth and proliferation. Binding of initiation factor Rrn3 activates Pol I, fostering recruitment to ribosomal DNA promoters. In the past, we determined the crystal structure of yeast Pol I, a 14-subunit complex composed of more than 80,000 atoms with a total mass of 590,000 Da, at 3.0 Å resolution [1]. The structure represents the latent state of the enzyme, which is dimeric and exhibits an open DNA-binding cleft with an extended loop blocking the active site. We now obtained the electron cryomicroscopy structures of monomeric Pol I and of the Pol I-Rrn3 complex at 4.9 and 7.7 Å resolution, respectively [2]. As expected, monomeric Pol I presents a partially-closed DNA-binding cleft and an accessible active site. Rrn3 binds on the enzyme stalk and restrains its conformation, thus generating a surface for the interaction with promoter-bound initiation factors. Our structural studies shed light on how the Pol I enzyme is activated to transcribe ribosomal DNA.

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9 S6 - Cellular Biophysics

Jointly organised by the Spanish and Portuguese Biophysical Societies

Chairs: Francisco Barros / Nuno Santos

Reciprocal coupling between cell cycle and primary cilia through Kv10.1 potassium channels

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The primary cilium is critical for morphogenic and growth factor signaling. Ciliogenesis and cell cycle progression are tightly linked, and only quiescent cells produce primary cilia, through yet unclear mechanisms. Kv10.1 is a voltage-gated potassium channel frequently overexpressed in tumors while virtually undetectable in tissues outside the brain. When present in tumor cells, it promotes cell proliferation and resistance to hypoxia, and confers worse prognosis. Conversely, its inhibition or knockdown reduces tumor progression in vivo. We observed that Kv10.1 is expressed in tumor cells upon E2F1 activation –often aberrantly induced in cancer–. E2F1 binds to the promoter of Kv10.1 in a time frame compatible with the G2/M transition of the cell cycle both in tumor cells and normal tissues. Since at any given time point a very restricted fraction of cells is at the G2 phase of the cycle, this explains why Kv10.1 expression is undetectable in healthy peripheral tissuesExpression extends over the whole span of G2 and M phase. In normal cells, the expression would thereafter be shut off, until the next G2 phase is reached. Kv10.1 is targeted to the ciliary membrane, where it is found in the proximity of the basal body. Kv10.1 participates in primary cilium disassembly in G2. Interference with ciliary localization results in the inability to induce ciliary disassembly and also abolishes the tumorigenic properties of Kv10.1 Consistently, knockdown of Kv10.1 results in a longer duration of G2/M, induces the presence of primary cilia in proliferating cells, and disrupts the regulation of the Sonic hedgehog pathway. In summary, Kv10.1 potassium channel participates in triggering ciliary disassembly before entry into mitosis, and it

does so in tumor as well as normal cells. Therefore, modulation of ciliogenesis by the Kv10.1 ion channel is likely to be a major mechanism underlying its tumorigenic effects.

Lipid Domains in Biological Membranes

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Sphingolipids (SLs) have emerged as an important class of lipids due to their bioactive role in a number of cellular events and in disease. The evidence that several SL species participate in the formation of lipid domains and that this might underlie their biological mechanism of action has fostered research in the biophysical aspects of bioactive SLs. However, studies addressing the biophysical implications of SLs under physiological conditions are still missing. The present study shows that increase in ceramide levels upon activation of TNF receptor drives profound changes in membrane properties through the formation of highly-ordered Ceramide-enriched domains. We further show that these domains exist in intracellular vesicles that colocalize with endocytic markers. Our results link the biophysical changes induced by Ceramides to important cell processes and emphasize the existence of Ceramide-enriched vesicles with distinctive biophysical properties that might function as intracellular signaling platforms.

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Characterization of the Rabphilin3A and SNAP25 interaction in PC12 cells

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Endless eukaryotic cell biological processes rely on membrane fusion, including the synaptic transmission. The liberation of neurotransmitters to the synaptic space is a deeply complex process that needs an accurate control of the recruitment of the many proteins involved. Numerous of these proteins share a common structural motif: the C2 domains, which are regulated by their ability to bind Ca^{2+}, phospholipids and other proteins, providing them with the capability to fine-tune the broad range of vesicle release modes. Rabphilin3A (Rph3A) is a membrane trafficking protein involved in the Ca^{2+}-dependent regulation of secretory vesicle exocytosis in neurons and neuroendocrine cells. In this work, we have used in situ protein ligation assay (PLA) to characterize the molecular determinants driving the Rph3A-SNAP25 interaction in PC12 cells. We observed that these interactions occur both in the cytosol and at the plasma membrane. These signals correspond to populations of transport and synaptic vesicles that might contain Rph3A-SNAP25 and vesicles docking respectively. Furthermore, staining HA-Rph3A and myc-SNAP25 by immunofluorescence demonstrated that both proteins localize as expected. Site-directed mutagenesis of important aminoacidic residues located at the C2B bottom -helix as well as two regions of the SNAP25-N helix showed that these two motifs are important for the Rabphilin3A-SNAP25 interaction at the plasma membrane and

contribute to explain some of the numerous steps and interaction events which take place at the presynaptic neuron.

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PROSAS-CNA: A proton accelerator for cancer therapy and research

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The andalusian health service (SAS) and the spanish National Accelerator Centre (CNA) promote the installation of a proton accelerator, with an energy of about 250 Me,[e] to treat different types of Cancer, using the technique known as Proton Therapy. Proton Therapy is based on the physical properties of protons. When they go through biological media, they deposit most of their energy at a given depth, which is determined by the incident beam energy. This is known as the Bragg peak. The status of the project, the medical and technological applications, as well as the opportunities that it opens in radiobiology, will be presented.

Lactate sensing by carotid body glomus cells

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The carotid body (CB) is a multimodal chemoreceptor organ in which glomus cells sense changes in blood O_2, hypoglycemia, CO_2, and pH. Recently, it has been suggested that CB glomus cells can also detect an increase in blood lactate, although the underlying mechanisms are unknown. We have investigated the effects of lactate on dispersed single glomus cells and in CB slices. Extracellular L-lactate (2-10 mM, sodium salt) increased glomus cell secretion rate, evaluated by amperometry, without occluding the secretory response to hypoxia and both stimuli (hypoxia and lactate) had additive effects. In accord with the amperometric data, isolated glomus cells responded to both, lactate and hypoxia, with an external Ca^{2+}-dependent increase in cytosolic [Ca^{2+}], as well as accumulation of NADH and reactive oxygen species (ROS). However, the kinetics of NADH increase induced by lactate were faster than those elicited by hypoxia. These data suggest that hypoxia and lactate increase NADH levels and activate glomus cells through separate signal transduction pathways. Increase in extracellular lactate seems to produce accumulation of lactate in the cytosol, which gives rise to NADH generated during the conversion of lactate to pyruvate. In contrast, hypoxia accumulates NADH primarily in the mitochondria, due to slow down of NADH/quinone oxidoreductase activity, which indirectly changes NAD(P)H levels in the cytosol (Fernández-Agüera et al., Cell Metab 2015). Activation of glomus cells by lactate may play an important role in CB-mediated regulation of respiration during exercise and in pathological conditions presenting lactacidemia.

High fibrinogen levels promote erythrocyte-erythrocyte adhesion: a cardiovascular risk factor in heart failure and arterial hypertension patients

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Erythrocyte aggregation is an indicator of cardiovascular risk, which is influenced by plasma fibrinogen concentration. Fibrinogen levels are elevated during cardiovascular diseases. Our main goals were to understand how fibrinogen-erythrocyte binding influences erythrocyte aggregation and how it constitutes a cardiovascular risk factor in essential arterial hypertension (EAH) and chronic heart failure (CHF). Fibrinogen-erythrocyte and erythrocyte-erythrocyte adhesion measurements were conducted by atomic force microscopy (AFM)-based force spectroscopy. Upon increasing fibrinogen concentration, there was an increase in the work and force necessary for cell-cell detachment, both for healthy donors and EAH patients. Nevertheless, higher values were obtained for the EAH patients at each fibrinogen concentration. Fibrinogen-erythrocyte (un)binding forces were higher in EAH and CHF patients, when compared with the control group, despite a lower binding frequency. Ischemic CHF patients showed increased binding forces compared to non-ischemic patients. Erythrocyte deformability (assessed as elongation index) results show that heart failure patients presented higher erythrocyte deformability than the control group at lower shear stresses, and lower deformability at higher shear stresses. This indicates that patients' erythrocytes are more deformable than those from healthy donors in blood vessels with larger internal diameters; however, in smaller-diameter vessels the opposite trend exists. Finally, a 12-month clinical follow-up shows that CHF patients with higher fibrinogen-erythrocyte binding forces, probed by AFM at the beginning of the assessment, had a significantly higher probability of being hospitalized due to cardiovascular complications on the subsequent year. Our results show that AFM can be a promising tool for clinical prognosis, pinpointing those patients with increased risk for cardiovascular diseases.

Synaptic and extra-synaptic functions of a vesicle associated co-chaperone

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Nerve terminals are able to maintain the continuous release of neurotransmitters during extended periods of time at locations far away from the cell soma. For example, presynaptic terminals from tonic motorneurons receive from 300.000 to 500.000 action potentials per day (Hennig and Lomo, Nature 1985) imposing on SNARE complexes a heavy-duty cycling of protein folding and unfolding reactions. Cysteine String Protein-alpha (CSP-alpha) is a synaptic vesicle protein that, together with Hsc-70 and SGT (small glutamine-rich protein), forms a chaperone complex essential to maintain a functional pool of SNAP25 and to promote SNARE complex assembly (Chandra et al., Cell 2005; Sharma et al. Nat. Cell Biol. 2011). In humans, mutations in the DNAJC5 gene, that codes CSP-alpha, cause autosomal-dominant adult onset neuronal ceroid lipofuscinosis, that leads to seizures and early death in young adults (Noskovà et al. Am J Hum Genet 2011; Benitez at al. PLoS One 2011). Interestingly knock-out mice lacking CSP-alpha suffer from early lethality

due to presynaptic degeneration (Fernández-Chacón et.al., Neuron 2004). We have found that motorneurons require CSP-alpha to maintain the readily releasable vesicular pool and synaptic vesicle recycling (Rozas., et al., Neuron 2012). Interestingly, in central neurons, we have shown that CSP-alpha prevents activity-dependent degeneration of GABAergic synapses in high firing rate parvalbumin-positive neurons, indicating that high-neural activity increases synapse vulnerability and CSP-alpha is essential to maintain presynaptic function under a physiologically high-activity regime (García-Junco-Clemente et al., JNeurosci. 2010). In my talk I will discuss recent unexpected findings that uncover unanticipated functions of CSP-alpha beyond the maintenance of synaptic vesicle trafficking at the nerve terminals.

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Effective reconstitution of HIV-1 gp41 transmembrane-domain derived peptides displaying the neutralizing MPER epitope on the surface of lipid bilayers

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The envelope glycoprotein (Env) gp41 subunit plays a pivotal role in HIV-1 entry by promoting the merger of the host cell and virus membranes. Although recently reported structural data help to elucidate the mechanistic basis of gp41 ectodomain functioning during the process, much less is known on the organization and functional roles of the following membrane-proximal external region (MPER) and the transmembrane domain (TMD). However, it is known that the MPER-TMD connecting segment harbors a conserved sequence targeted by the broadly neutralizing antibodies 4E10 and 10E8 pointing out to an active role of these region in the fusion process. Here, based on previous examination of a series of C-terminal-truncation mutants (Yue et al. (2009) J. Virol. 83, 11588), we report attempts to reconstitute the MPER-TMD segment in lipid bilayers with a phospholipid to cholesterol molar ratio similar to that of the viral membrane. We have used two different peptides to represent the MPER-TMD segment, namely, MPER-TMD1 and MPER-TMD2, spanning Env gp41 residues 671-700 and 671-709, respectively, which would act as platforms to expose the 4E10/10E8 MPER epitopes at membrane surfaces. Infrared spectroscopy (IR) measurements demonstrated the efficient reconstitution of the MPER-TMD1 sequence as a predominant a-helix in membranes while MPER-TMD2 retained a significant fraction of unfolded-aggregated structures. Moreover, as evidenced from antigenicity and immunogenicity assays, and supported by Molecular Dynamics Simulations, MPER-TMD1 bearing vesicles, but not MPER-TMD2 bearing ones, effectively exposed the MPER C-terminal stretch, harboring the 4E10/10E8 MPER epitopes, on the surface of cholesterol-enriched membranes. These findings provide new clues for the design and development of peptide-liposome vaccines targeting the MPER vulnerability site on Env.

Measuring lipid membrane properties using a mechanosensitive fluorescence probe

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To measure the chemical-mechanic states of lipid membranes, once needs various tools, many of which being incompatible with cell biology protocols. Applying lessons from nature, we developed a mechanosensitive fluorescent probe, the twisted dithienothiophene. This push-pull probe, change planarization state in function of his environment, and we have taken full advantage of this mechano-probe potential and we calibrated based on membrane tension, fluidity and different lipid composition by measuring the push-pull fluorescence lifetime. Likewise, we are able to use this fluorescent probe on life cells, for visualize differences between organelles, membrane tension and membrane composition.

Real time measurements of Exo and Endocytosis in SMA mouse model expressing SypHy

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In our laboratory we generated a new line of transgenic mice expressing synaptophysin and pHluorin (SypHy) as a fusion protein, in a SMA mouse model. This tool allowed us to study synaptic vesicle recycling in motor nerve terminals in real time by monitoring the fluorescence changes taking place during exocytosis and endocytosis. In the Smnko/ko-SypHy transgenic mouse, electrical stimulation of the axons that innervate the TVA muscle produced a fluorescence increase in nerve terminals due to the fusion of SVs with the plasma membrane and the exposure of pHluorin to the extracellular pH (7.4). Following stimulation, fluorescence returned to resting levels as vesicles were endocytosed and reacidified. We studied the time course of fluorescence decay in the SMA-SypHy transgenic mouse line. We calculated both t1/2 and t1/e values of the mean fluorescence responses in WT and SMA mutant mice expressing SypHy and found that there was no significance difference between these phenotype. This result suggest that endocytosis is not impaired in SMA mutant mice as the contrary to recently found.

10 S7 - Biophysics of Carbohydrates and Nucleic Acids

Chairs: Fernando Moreno-Herrero / Pedro Nieto

A new consensus GC-DNA motif for the ancient Smad4 family

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All activated Smad transcription factors (R-Smads) require Smad4 as a partner for gene regulation, development, and regeneration. The GTCT motif has been considered the main Smad binding site for all R-Smads and Smad4 proteins. Remarkably, Smad proteins also occupy the human genome at GC-rich cis-regulatory elements (CREs), lacking GTCT sites. Indeed, several GC motifs have been identified as Smad1/5 binders in the BMP pathway; however, specific GC-sites have not been characterized for Smad4 and for TGF- activated Smad2/3 proteins. Here, we elucidate the structural basis for the specific and high affinity binding of human Smad4 to new GC-motifs from the Goosecoid (Gsc) promoter, a mesoendoderm differentiation gene. These sites were narrowed down using EMSA and CRISPR/Cas9 deletion experiments and high-resolution NMR and X-ray crystallography. Binding to the GTCT sites was shown in the past, to interact specifically with 3-bp. Remarkably, using the same DNA-binding region, Smad4 binds to the new sites reading 4-bp. The complex of Trichoplax adhaerens Smad4 MH1 domain with the same DNAs suggests that binding to these motifs is conserved along Smad4 evolution. We also discovered that the plasticity of the MH1 domain -hairpin to recognize several DNA sequences is favored by its conformational flexibility, which provides a basis for understanding the functional adaptability of Smad proteins, and of Smad4 in particular, as a shared mediator in TGF- and BMP signaling pathways.

Combined Magnetic Tweezers and TIRF microscopy to visualize DNA-protein interactions

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The combination of Magnetic Tweezers (MT) with TIRF microscopy allows the simultaneous correlation of mechanical measurements of biomolecules and direct visualization of DNA-protein interactions. The strength of combining these two techniques relies on the advantages they have separately. MT permit the tracking of several DNA molecules in parallel, while a force is applied in a controlled manner. TIRF microscopy exhibits a superior signal to noise ratio over other fluorescence-based techniques, with an evanescent field of a few hundreds of nm from the surface. The drawback is that long DNA molecules need to be stretched across the surface of the flow cell. Here, we present the implementation of a laterally pulling device and its subsequent calibration in flow cells and capillaries. TIRF microscopy was also implemented in this lateral Magnetic Tweezers setup and characterized using fluorescently labelled beads and quantum dots. DNA binding by the B. subtilis protein Spo0J/ParB was studied using the combined setup. Fluorescently labelled ParB proteins were able to bind non-specifically along DNA molecules producing a constant emission signal, consistent with the dynamic exchange of protein. Condensation of DNA by ParB was prevented using lateral pulling.

The intervening domain from MeCP2 enhances the DNA affinity of the methyl binding domain and provides an independent DNA interaction site

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Methyl-CpG binding protein 2 (MeCP2) preferentially interacts with methylated DNA and it is involved in epigenetic regulation and chromatin remodelling. Mutations in MeCP2 are linked to Rett Syndrome, the leading cause of intellectual retardation in girls and causing mental, motor and growth impairment. Unstructured regions in MeCP2 provide the plasticity for establishing interactions with multiple binding partners. We present a biophysical characterization of the methyl binding domain (MBD) from MeCP2 reporting the contribution of flanking domains to its structural stability and dsDNA interaction. The flanking disordered intervening domain (ID) increased the structural stability of MBD, modified its dsDNA binding profile from an entropically-driven moderate-affinity

binding to an overwhelmingly enthalpically-driven high-affinity binding. Additionally, ID provided an additional site for simultaneously and autonomously binding an independent dsDNA molecule, which is a key feature linked to the chromatin remodelling and looping activity of MeCP2, as well as its ability to interact with nucleosomes replacing histone H1. The dsDNA interaction is characterized by an unusually large heat capacity linked to a cluster of water molecules trapped within the binding interface. The dynamics of disordered regions together with extrinsic factors are key determinants of MeCP2 global structural properties and functional capabilities.

Atomic force microscopy shows that TubR bends the DNA forming a loop at tubC

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Most partition systems responsible for plasmid segregation and maintenance are composed by three components: a DNA centromere sequence, a centromere-binding protein (CBP), and a motor protein. According to the nature of the motor protein these systems can be classified into type I (Walker-A ATPase), type II (actin-like ATPase) and type III (tubulin-like GTPase). Clostridium botulinum phage c-st encode a type III partition system that likely function during the phage lysogenic phase and makes this organism a prototype model for the study of DNA segregation. Here, the motor protein (TubZ) moves the phage DNA through its interaction with the partitioning complex (centromere sequence -tubC- and the CBP –TubR-). We use Atomic Force Microscopy (AFM) to image the interaction of TubR with tubC that expands along 500 bp. We found that TubR binding induces the deformation of DNA, bending it into one or two loops at saturated protein concentrations. This interaction is specific, as control experiments on random DNA did not show any deformation. Additionally, at TubR saturating conditions we did not observe any DNA aggregation or further DNA spreading. AFM volumetric analysis suggests the protein is a monomer. The typical size of closed loops is 15-20 nm in diameter, providing an estimation of 20 monomers per loop. The size of the loop is compatible with published models of TubZ filaments suggesting that this may be the structure recognized by TubZ. Preliminary AFM experiments with TubZ and TubR-DNA showed clustering of TubZ around the loop structures. All together, our data suggest a model where TubR binds and spreads along tubC while inducing a bend in the DNA.

DNA synthesis determines the binding mode of the human mitochondrial single-stranded DNA-binding protein

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Single-stranded DNA-binding proteins (SSBs) play a key role in genome maintenance, binding and organizing single-stranded DNA (ssDNA) intermediates. Multimeric SSBs, such as the human mitochondrial SSB (HmtSSB), present multiple sites to interact with ssDNA, which has been shown in vitro to enable them to bind a variable number of single-stranded nucleotides depending on the

salt and protein concentration. It has long been suggested that different binding modes might be used selectively for different functions. To study this possibility, we used optical tweezers to determine and compare the structure and energetics of long, individual HmtSSB-DNA complexes assembled on preformed ssDNA, and on ssDNA generated gradually during 'in situ' DNA synthesis. We show that HmtSSB binds to preformed ssDNA in two major modes, depending on salt and protein concentration. However, when protein binding was coupled to strand- displacement DNA synthesis, only one of the two binding modes was observed under all experimental conditions. Our results reveal a key role for the gradual generation of ssDNA in modulating the binding mode of a multimeric SSB protein and consequently, in generating the appropriate nucleoprotein structure for DNA synthetic reactions required for genome maintenance.

Bacterial Surface Glycans. Novel Bacteria-Based Microarray and QCM Approaches for In-Situ Assessment of Glycan-Lectin Interactions

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Bacterial surfaces display a diversity of carbohydrate structures that can be recognized by host receptors, as anti-carbohydrate antibodies and lectins of the innate immune system, for triggering defense responses. Analysis of such glycan-protein interactions using purified bacterial components may not reflect the real bacteria-receptor interplay, since the carbohydrate structures are not tested in their natural environment. We have developed two novel methods for exploring interactions directly on the bacterial surface, based on the generation of bacteria microarrays (1,2) and quartz crystal microbalance (QCM) sensor chips (2). Bacteria microarrays have proved to be useful for exploring the presence of accessible carbohydrate epitopes and for detecting strain-selective binding of antibodies and lectins, also giving information on binding avidity. In addition, QCM bacteria chips enable the analysis of the affinity and kinetic parameters of lectin binding, providing further insights into the interactions occurring at the bacterial surface. Illustrative examples of the development, validation and application of these microarray and QCM strategies will be presented.

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Design of novel glycopeptide-based cancer vaccines

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Mucin MUC1 is an O-glycoprotein overexpressed in various tumors. While in healthy tissues, the peptide sequence of this protein carries complex oligosaccharides, in cancer cells, it shows simple and truncated carbohydrates, such as the Tn antigen (-O-GalNAc-Ser/Thr). These antigens are exposed to the immune system and can interact with it. Due to this unique characteristic, partially glycosylated MUC1 derivatives are attractive antigens for the development of therapeutic vaccines for the treatment of cancer.[1] Currently, considerable effort is dedicated to synthesizing MUC1 derivatives that can elicit a strong immune response. However, the identification of the significant structural elements involved in the presentation of the antigen, as well as the recognition process of these antigens by anti-MUC1 antibodies remains partly unclear. We are developing a multi-disciplinary approach that combines synthesis, X-ray diffraction, nuclear magnetic resonance and molecular modeling to identify these structural features (Figure).[2,3] Our results provide valuable hints for the design of efficacious cancer vaccines.

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Deciphering the conformational code behind the indirect readout of DNA sequences

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The correct recognition of DNA sequences by proteins is crucial for important biological events as DNA replication, transcription, and regulation of gene function. Several cases of binding can be explained by a direct readout mechanism, where the proteins recognize a specific DNA sequence, interacting at the molecular level with specific nucleobases in specific positions. A second model has been suggested to explain protein-DNA recognition for those cases where the binding was confirmed but it was impossible to find a consensus sequence: The indirect readout mechanism. In this cases specific conformational features of the DNA, which are local in nature and sequence-dependent, give to DNA a particular shape recognized by the protein. The shape adopted reflects DNA's ability to exist in diverse conformational sub-states at the backbone and base level giving rise to structural polymorphisms.

Combining the new parmBSC1 force field and the latest knowledge in the area of DNA conformations in the helical space, we present the most complete and unified description, at the tetranucleotide level, of the different existing polymorphisms and their interconnections. For this purpose we ran microsecond-long Molecular Dynamics (MD) simulations on a newly designed an optimized sequence library containing all the unique 136 tetranucleotides. Using this dataset, and the previous μABC data (from the Ascona B-DNA Consortium), we carried out our analysis on more than 75 μs of accumulated simulation time, involving a total of 52 different sequences, and at least 6 replicas of each tetranucleotide. Our results reconcile with unprecedented accuracy the major theoretical and experimental sources of knowledge on DNA structural polymorphisms (X-ray crystallography, $^{1}H/^{31}P$-NMR, data mining of databases, and MD simulations), and reveal the

possible sub-state combinations that DNA could adopt and which ultimately conform the basic code needed for understanding the indirect readout mechanism.

TIA-1 RRM23 binding and recognition of target oligonucleotides

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TIA-1 is an RNA-binding protein involved in splicing and translational repression. It mainly interacts with RNA via its second and third RNA recognition motifs (RRM2 and RRM3, respectively), with specificity for U-rich sequences directed by RRM2. It has recently been shown that RRM3 also contributes to binding, with preferential binding to C-rich sequences. In this work, we designed UC-rich and CU-rich 10-nt sequences for engagement of both RRM2 and RRM3 and demonstrated that the TIA-1 RRM23 construct preferentially binds the UC-rich RNA ligand (5'-UUUUUACUCC-3'). Interestingly, this binding depends on the presence of Lys274, located at the C-terminus of RRM3, and binding to equivalent DNA sequences occurs with similar affinity. Small-angle X-ray scattering was used to demonstrate that, upon complex formation with target RNA or DNA, TIA-1 RRM23 adopts a compact structure, showing that both RRMs engage with the target 10-nt sequences to form the complex. We also report the crystal structure of TIA-1 RRM2 in complex with DNA to 2.3 Å resolution, providing the first atomic resolution structure of any TIA protein RRM in complex with oligonucleotide. Altogether, our data support a specific mode of TIA-1 RRM23 interaction with target oligonucleotides consistent with the role of TIA-1 in binding RNA to regulate gene expression.

Deciphering the long distance-glycosylation preferences of GalNAc-Ts

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GalNAc-Ts are a large family of glycosyltransferases that uniquely feature both a catalytic and lectin domain and that are responsible of the mucin-like glycosylation found in higher eukaryotes. To date, the underlying molecular basis of how GalNAc-Ts present distinct long distance-glycosylation preferences remained elusive. Here, through the first crystal structures of complexes of GalNAc-T2 with glycopeptides, we show how the activity profile of GalNAc-T2 is dictated by conformational heterogeneity and relies on a flexible linker located between the catalytic and the lectin domains. Our results also shed light on how GalNAc-Ts generate dense decoration of proteins with O-glycans, and suggest that the flexible linker dictates the lectin domain rotation, which in turn is responsible for the long distance-glycosylation preferences.

11 S8 - Biointerfaces, Biofilms and Nanobiophysics

Chairs: Miquel Pons / Ana I. Azuaga

Engineered proteins as scaffolds for functional nanostructures and materials

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Self-assembly of biological molecules into defined functional structures has a tremendous potential in nanopatterning, and the design of novel biomaterials and functional devices. Molecular self-assembly is a process by which complex three-dimensional structures with specified functions are constructed from simple molecular building blocks. We present the supramolecular assembly of modular repeat proteins, in particular designed consensus tetratricopeptide repeats (CTPRs), and their application as building blocks for the generation of functional nanostructures and biomaterials. CTPR proteins can be assembled into self-standing thin films,[1] monolayers,[2] and thin nanometer fibers.[3] In this work, we show the use of CTPRs as scaffolds to template: (1) Photoactive organic molecules.[4] In particular, CTPR proteins are used to organize organic chromophores, while preserving their structure. The unique self assembly properties of CTPRs have been exploited to generate ordered photoconductive films of the protein-porphyrin conjugates. (2) Gold Nanoparticles.[2] CTPR are used to template gold nanoparticles into ordered monolayers, and into structured thin films providing conductivity properties to the materials. (3) Fluorescent nanoclusters.[5] We show the ability of CTPR proteins to stabilize fluorescent gold nanoclusters. Since the structural and functional integrity of the protein template is critical for applications, protocols that retain the protein structure and function have been developed. A CTPR module with specific binding capabilities has been successfully used to stabilize nanoclusters and tested as a sensor.

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Seeking fresh air in biofilms through an oxygen-sensitive toxin-antitoxin system

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Classical toxin-antitoxin regulatory systems in bacteria are based on the formation of stable complexes in which the toxin activity is masked. Degradation of the labile antitoxin under appropriate conditions releases the toxin. We shall describe a new toxin-antitoxin system formed by Hha (acting as a toxin) and TomB (the antitoxin). The antitoxin catalyses the oxidation by molecular oxygen of the single cysteine residue in the toxin to charged sulphinic/sulphonic acid forms. This causes an imbalance of a tight electrostatic network in Hha and results in the loss of structure, and therefore, inactivation of the toxin. The interaction between toxin and antitoxin, which could only be mapped by paramagnetic relaxation enhancement NMR, is transient. The structure of the antitoxin was solved by NMR but required special acquisition conditions as this proteins is also slowly oxidized by air. The Hha-TomB system is the first example of a new class of toxin-antitoxin systems. The Hha-TomB pair contributes to regulate the spread of biofilms, probably through the presence of oxygen gradients inside the biofilm, allowing selective dead of only the cells buried in the biofilm interior and creating channels through which nutrients may enter and bacterial cells released to colonize new sites.

DNA Amplification in Double Emulsion Templated Vesicles

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The emerging field of synthetic biology applies a vision inherited from engineering to create gene circuits that mimic the genetic pathways of living cells. The encapsulation and proper functioning of these gene circuits within aqueous compartments or vesicles constitute a first step towards the development of artificial cells [1]. Unfortunately, the production of these artificial cells remains as a proof-of-concept due to the poor encapsulation efficiency of conventional methods for vesicle production. Here, we propose to use microfluidic technologies to fabricate thousands of identical vesicles, efficiently encapsulating a gene amplification system within their cores [2]. Using this approach, we recreate a DNA amplification process that utilizes the phi 29 DNA polymerase [3]. We quantify this DNA amplification from the fluorescence emission of Evagreen®, a fluorophore that only emits if bound to double-stranded DNA. This engineered system may help understand gene evolution since it enables the study of the role of stochasticity in DNA amplification.

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Label-free, multiplexed, single-molecule analysis of protein-DNA complexes with nanopores

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Protein interactions with specific DNA sequences are crucial in the control of gene expression and the regulation of replication. Single-molecule methods offer unique capabilities to unravel the mechanism and kinetics of these interactions. Here we develop a nanopore approach where a target DNA sequence is contained in a hairpin followed by a ssDNA. This system allows DNA-protein complexes to be distinguished from bare DNA molecules as they are pulled through a single nanopore detector, providing both the equilibrium and kinetic constants of the interactions. We show that this approach can be used to test the inhibitory effect of small molecules on complex formation, and their mechanisms of action. In a proof of concept, we use DNAs with different sequence patterns to probe the ability of the nanopore to distinguish the effects of an inhibitor in a complex mixture of target DNAs and proteins. We anticipate that the use of this technology with arrays of thousands of nanopores will drive the discovery of new inhibitors of transcription factor binding.

Lipid nanotubes from freestanding lipid membranes

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Structures such as lipid nanotubes play an important structural role in different cellular organelles such as the Golgi apparatus, endoplasmic reticulum and mitochondria, but also in inter and intra-cellular exchanges and cellular migration. The study of their properties and biophysics is often carried on vesicles, supported lipid bilayers or living cells. In these approaches, it is challenging to achieve asymmetric lipid distribution, dynamic buffer control and zero curvature. In contrast, the use of a freestanding lipid bilayer on a microfluidic device present additional advantages such as easy access to both sides of the membrane, possibility to create several membranes in a same device, possibility to circulate different solutions, and full compatibility with optical techniques. We show a novel and versatile method to study lipid nanotubes by combining optical tweezers with freestanding lipid membranes formed inside a microfluidic chip. The bilayers were assembled over a hole inside a microfluidic device and lipid nanotubes were formed via two methods: 1) by pulling a lipid patch anchored to bead by streptavidin-biotin interactions or 2) by pushing the bead across the membrane. The tension, bending rigidity and the force required to pull the nanotubes will be discussed. Our method of forming tubes from freestanding bilayers provides a robust platform, not only for nanotubes studies, but also for further study of protein-membrane interactions under controlled conditions on each side of the membrane, and modulated membrane complexity.

Liquid Microjets in XFELs: Last Train to Molecular Heaven

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High intensity, coherent X-rays (X-ray free electron lasers, XFELs) have become the current paradigmatic tool in high resolution molecular analysis of biological samples. X-ray scattering of a molecule is possible at the Angstom resolution with a sufficiently strong radiation input (Doniach 1996, J. Synchrotron Rad. 3, 260-267). However, the first word storming one's mind when hearing about the interaction of a normally fragile biological molecule and an ultra-high intensity energy input is destruction. A pioneering but hardly recalled work (Solem 1986, J. Opt. Soc. Am. B 3, 1551-1565) anticipated for the first time "the remarkable fact that, at sufficiently high intensity, an image of diffraction-limited resolution can be captured before the specimen is obliterated". In the year 2000, Hajdu and co-workers (Neutze et al. 2000, Nature 406, 752-757) made the first precise prediction on the energy strength and short pulse time needed to image a protein (lysozyme) before destruction. A time lapse of about 10 years was necessary to build an XFEL with a sufficiently short pulse rate (10-20 fs.) and make experiments with biological samples (protein crystals) (Chapman et. al. 2011, Nature 470, 73-78). The enormous success of this achievement, reflected by the explosive interest raised in the scientific literature, was brought in a humble but crucial engineering vehicle (see figure 1 in Chapman et al. 2000): a Flow-Focusing® microjet (Ganan-Calvo 1998, Phys. Rev. Lett. 80, 285-288; DePonte et al. 2008, J. Phys. D: Appl. Phys. 41, 195505) acting like a ultra-smooth, high-speed micro-train carrying the samples towards their glorious last station destiny: an ultra-high intensity, ultra-short X-Ray spot getting an image of their molecular structure. Here, we provide an overview of current sample introduction strategies in XFELs, their stringent demands and challenges, and how liquid microjets produced by strongly focused hydrodynamic or electrohydrodynamic means deliver.

Orb2/CPEB amyloid: similarities and differences with pathological amyloids

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Ordered proteinaceous aggregates called "amyloids" are typically associated with neurodegenerative diseases and cognitive impairment. Contrasting to this, the amyloid-like state of the neuronal RNA binding protein Orb2/CPEB in Drosophila was recently implicated in memory consolidation, but it remains unclear what makes this functional amyloid-like protein to behave in such a diametrically opposed way. By means of an array of biophysical, cell biological and behavioural assays we have characterized the structural features of Orb2 from the monomer to the amyloid state. Remarkably, we found that Orb2 shares many structural traits with pathological amyloids, including the intermediate toxic oligomeric species, which can be sequestered in vivo in hetero-oligomers by pathological amyloids. Nevertheless, unlike pathological amyloids, Orb2 rapidly forms amyloids and its toxic intermediates are extremely transient, which indicates that kinetic parameters differentiate this functional amyloid from pathological amyloids. Furthermore, we also observed that QBP1, a well-known anti-amyloidogenic peptide, interferes with long-term memory in Drosophila. Our results provide structural insights into how the amyloid-like state of the Orb2 protein can stabilize

memory and be nontoxic. Furthermore, they provide a mechanism for how memory processes are often affected in amyloid-based diseases.

Analysis of the Different Structures of Pulmonary Surfactant Collectin SP-D by Atomic Force Microscopy

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We have performed a structural and quantitative characterization of recombinant human pulmonary surfactant protein D (rhSP-D), a C-type (Ca2+-dependent) lectin belonging to the collectin family. It is found mainly in alveolar spaces, participating in the innate immune defense of the lungs. SP-D monomer contains four structural domains: an N-terminus domain, a collagen region, a -helical coiled-coil neck and a C-terminus carbohydrate recognition domain (CRD). Monomers form trimers through folding of the collagenous region into triple helices and the assembly of a coiled-coil bundle of -helices in the neck region. These trimers are stabilized by two disulfide bonds in the cysteine-rich N-terminal domain. Trimers associate into higher order oligomers whose size and conformation is sensitive to environmental factors and the conditions during purification and storage. Despite extensive studies carried out to characterize the oligomerization process of SP-D, the pathway and type of interactions involved in the formation of large oligomers, are not clearly understood.

In the current study, the protein produced in mammalian CHO cells, was analyzed by Atomic Force Microscopy (AFM) and electrophoresis. The goal has been the determination of the distribution of oligomeric forms, the exploration of the possible oligomerization pathway and the description of the conformational diversity of rhSP-D. AFM experiments revealed that rhSP-D is a mixture of trimers, hexamers, dodecamers, and larger oligomers ("fuzzy balls"), with the most abundant structure being the dodecamer under the conditions of these experiments. Same kind of structures were found in human SP-D, used as a control. Moreover, we have developed a cross-linking protocol to detect the presence of SP-D dodecameric forms by PAGE-SDS, in which dodecamer is only visualized after chemical crosslinking and in the presence of denaturing agents, indicating the importance of hydrophobic interactions in dodecamer formation.

Virtual High Throughput Screening (VHTS) of small mechanoactive molecules for controlling the mechanical stability of HIV-1 receptor

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Viral infections are one of the major causes of death worldwide. The HIV-1 virus, responsible of AIDS disease, has become an epidemic which continues to spread at an alarming rate of approximately 2 million new cases per year. Traditional cell and molecular biology have tried to fight this pathology but have proven insufficient. Molecular mechanomedicine is an emerging field in nanobiology focused on the study of the effect of newtonian mechanical forces in the structure and properties of proteins related with human health, key to which are membrane proteins such as CD4. It is believed that these proteins are subjected to mechanical forces that modify not only their tertiary structure but also their secondary structure (mechanical changes), which enable them to regulate different cell functions. In this sense, HIV-1 makes use of CD4 membrane protein as a mechanical anchor in order to infect T-lymphocites. There is experimental evidence that viral infections have a mechanical component indispensable for them to be carried out. In other words, for the infection to take place, membrane proteins need to undergo mechanical changes. The latter, can be measured by means "single molecule techniques", such as atomic force spectroscopy (AFS). It has been proven that Ibalizumab, a humanized anti-CD4 monoclonal antibody which inhibits HIV-1 infection, binds to CD4 making it mechanically more stable. We therefore believe that a relationship between the mechanical stability of CD4 and HIV-1 infection may exist. In particular, an increase in the mechanical stability of CD4 may inhibit HIV-1 infection. With this goal in mind, we made use of VHTS to identify commercially available small molecules with the ability to control the mechanical properties of CD4. AFS experiments confirmed that our molecule candidates identified by VHTS bind efficiently to D1D2CD4 and are able to mechanically stabilize the mechanical properties of CD4, as much as or even to a higher extent Ibalizumab does.

Unfolding pathway of the cancer-associated NQO1 enzyme studied at the single molecule level

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NQO1 is a FAD-dependent NAD(P)H quinone oxidoreductase that activates cancer pro-drugs and stabilizes oncosuppressors such as p53 and p73. Disease associated mutations usually result in intracellular enzyme inactivation, dysregulation and instability. It has been previously shown that the mutant P187S strongly destabilizes the NQO1 dimer in vitro, resulting in a loss-of-function and cancer-associated polymorphism. However, a combination of FAD and the inhibitor Dicoumarol seems to revert these alterations. It has been suggested that FAD and Dicoumarol stabilize NQO1 against degradation. Here we apply nanopore force spectroscopy to analyze the stability of the protein against vectorial unfolding. We found that addition of FAD and Dicoumarol changes the unfolding pathway resulting in slower unfolding, which may explain the lower degradation rate observed in vivo. Our results suggest that protein unfolding through nanopores capture the basics of protein unfolding by the proteasome in the absence of ubiquitination.

12 Seminars

The light scattering toolkit for characterization of proteins and other bio-macromolecules

L. Rouzic

Wyatt Technology, Toulouse, France

Biophysical characterization and protein quality assessments are central capabilities in any laboratory that seeks qualified, reproducible results in biomolecular or biochemical research. This seminar describes a comprehensive suite of tools based on static and dynamic light scattering that provides biophysical characterization and quality screening from first-principles. Common uses of the light scattering toolkit include determination of molecular weight and size, native oligomeric or aggregation state, protein-protein binding parameters, and the composition of glycoproteins or other conjugated macromolecules, net charges. Beyond molecules, light scattering tools characterize vesicles, virus-like particles and other macromolecular assemblies for size, conformation and structure. All measurements are performed entirely in solution and without any form of labeling, offering valuable alternatives to less general methods. Separation by Fied Flow Fractionation will be also presented.

Easy and Rapid Analysis of Protein Interactions and Stability in Solution

A. Justies

NanoTemper Technologies GmbH, Munich, Germany

Binding affinities, protein stabilities, and protein aggregation are crucial properties, which must be determined in basic and pre-clinical research up to formulation processes. The search for an optimal combination of biophysical techniques can be challenging in various points. The goal is to achieve the maximal information content, sensitivity and robustness in an easy, fast and precise way.

Here we describe two technologies, nanoDSF and MicroScale Thermophoresis, which can facilitate and accelerate your experiments and giving you additional insight in your system. nanoDSF is a label free approach to determine protein stability in solution. By the combination of a dual-UV technology it is possible to obtain high resolution unfolding curves with an unmatched reproducibility to determine thermal and chemical stability. In combination with a back-reflection optic, which determines aggregation in parallel to the melting, this is a tool for determining long term stability or storage conditions. Additional applications are for example buffer and detergent screenings, ranking mutant stabilities, batch comparisons, and quality control.

MicroScale Thermophoresis (MST) quantifies biomolecular interactions in immobilization-free assay. It measures the motion of molecules along microscopic temperature gradients and detects changes in their hydration shell, charge or size. Almost all interactions between molecules and virtually any biochemical process are linked to a change in size, charge and/or conformation of molecules which alter this hydration shell and therefore can be detected and quantified by MST. The presentation will cover the basic principle of both technologies and show you some practical applications.

Introducing the new Malvern Microcal PEAQ DSC and Viscosizer

T.D.R. Pacheco-Gómez

Malvern Instruments, Malvern, United Kingdom

Malvern-Panalytical, is a market leading provider of instrumentation for biophysical analysis. This presentation introduces the latest technology developments, and explores their application in protein and small molecule analysis. Two new-to-market technologies will be introduced the Viscosizer TD and the new PEAQ DSC. The Viscosizer - Taylor Dispersion platform offers a rapid way of very accurately measuring small changes < 0.1 mpas in sample viscosity, and offers size analysis for proteins and small molecules samples, that would not be analysable by conventional light scattering or multi-angle light scattering technologies. In 2017, the new MicroCal PEAQ automated differential scanning calorimeter (DSC) was launched, the key features and developments will be explained and how this "gold standard" instrument offers information rich data for use in both the University and Pharmaceuticals sectors.

Characterization of biomolecules: structure, conformation, thermodynamics and kinetics

A. García

Paralab S.L., Barcelona, Spain

We will present the instrumental novelties that will help us to control the biophysical parameters in our daily work with biomolecules, whether we are investigating in thermodynamics, kinetics, affinity or structure solving. We will explore through Applied Photophysics Capabilities and Evolution of CD and The Future of CD Spectroscopy: The Power of Quantitative Circular Dichroism (qCD). Obtaining thermodynamic parameters such as Tm and Hvan't Hoff more quickly and accurately. Novelties in Real-Time Label Free kinetics with Bio-Layer Interferometry (BLI) and Surface Plasmon Resonance (SPR) by Pall-ForteBio. Obtaining kinetic constants and affinity immediately without microfluidics. We will introduce the new Rigaku X-ray Diffractometers for protein crystallography. Learn about the new 2D Hybrid Photon Counter (HPC) detectors manufactured by Rigaku and how the combination with rotating anodes will help you solve the most complicated structures in unmatched times.

Abstracts of Posters

13 P1 - Protein Structure, Dynamics and Function

Kinetics and thermodynamics in the protein-ligand interactions during activity in the bifunctional FAD synthetase from Corynebacterium ammoniagenes

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Prokaryotic bifunctional FAD synthetases (FADSs) are bimodular enzymes exhibiting ATP:riboflavin kinase (RFK) activity in its C-terminal module and FMN:ATP adenylyltransferase (FMNAT) activity in its C-terminal. These activities provide the organism with the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) cofactors. The RFK activity of FADS from *Corynebacterium ammoniagenes* (*Ca*FADS) has a strong inhibition at mild concentrations of the RF substrate. Selective inhibition of key enzymes is a common tool to regulate metabolic pathways. Since FMN and FAD act as cofactors in a plethora of flavoproteins and flavoenzymes in all living organisms, inhibition of the RFK activity in some family members might contribute to the flavin cellular homeostasis, and, therefore, it is a topic worthy of study. Here we use a truncated *Ca*FADS variant that only contains the C-terminal RFK module, being it similarly functional in the RFK activity as the full length enzyme. The steady-state characterization of this variant indicates that besides inhibition by the RF substrate, both of the reaction products, ADP and FMN, also inhibit the RFK activity. The use of pre-steady-state kinetics collectively with isothermal titration calorimetry allows us to present a kinetic and thermodynamic explanation of such inhibitory behavior related to ligand binding that is coherent with the structural conformational changes occurring during the RFK catalysis in *Ca*FADS. Furthermore, these methods can be also used to evaluate the behavior of the full-length enzyme.

Mitochondrial dysfunction in response to cytochrome c phosphorylation at position 48

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The regulation of mitochondrial activity allows cells to adapt to changing conditions and respond to oxidative stress. Mitochondrial dysfunction can lead to hypoxia-related pathologies. The phosphorylation of tyrosine-48 (Tyr-48) in cytochrome *c* is related to a wide range of human diseases due to the pleiotropic role of the latter in cell life and death. However, its analysis is difficult due to the low yield of purified phosphorylated cytochrome *c* obtained from cell extracts, as well as the lack of knowledge about the specific kinases involved. Therefore, the course taken involved the analysis of Tyr-48 in a phosphomimetic mutant, Y48*p*CMF C*c*, bearing a close resemblance to cytochrome *c*, and developed through the optimization of non-canonical amino acid *p*-carboxymethyl-L-phenylalanine (*p*CMF) synthesis.[1]

It is noteworthy that the Y48*p*CMF mutation significantly destabilizes the Fe-Met bond in the ferric form of cytochrome *c*, thereby lowering the pK_a value for the alkaline transition of the heme-protein to physiological pH. The NMR structure of the resulting mutant reveals significant conformational shifts and enhanced dynamics around *p*CMF that could explain changes observed in its functionality. The phosphomimetic mutation impairs cytochrome *c* diffusion between respiratory complexes, enhances hemeprotein peroxidase activity and hinders caspase-dependent apoptosis. Our findings provide a framework to further investigate the modulation of mitochondrial activity by phosphorylated cytochrome *c* and to develop novel therapeutic approaches based on its pro-survival effects.[2]

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Effect of protein aggregation and protein structure on magnetite formation

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Magnetotactic bacteria (MTB) are a diverse group of microorganisms that have in common the ability to passively align and swim along the Earth's magnetic field. This is because MTB biomineralize magnetite or greigite crystals through a controlled biomineralization process. Magnetosome nanocrystals are surrounded by a lipid bilayer forming the magnetosome organelle. Being also biocompatible, magnetosomes could be used in many biomedical applications as in cell separation, etc. MamC from Magnetococcus marinus MC-1 has been shown to control the size of magnetite crystals in in vitro experiments, thereby demonstrating its potential as a candidate protein for the production of magnetite nanoparticles possibly useful in medical and other applications. However, the importance of the structure and aggregation state of the protein on the resulting biomimetic nanoparticles has not been yet assessed. One method normally used to prevent the aggregation of integral membrane proteins is the introduction of detergents during protein purification. In this study, results from protein aggregation following the addition of three detergents are presented. Magnetite particles formed in the presence of MamC purified using these three detergents were compared. Our results show that detergents alter the structure of the folded recombinant protein, thus preventing the ability of MamC to control the size of magnetite crystals formed chemically in vitro. Furthermore, we show that the introduction of detergents only at the dialysis process during the protein purification prevents its aggregation and allows for correct, functional folding of MamC. These results also indicate that the population of the active protein particles present at a certain oligomeric state needs to be considered, rather than only the oligomeric state, in order to interpret the ability of magnetosome recombinant proteins to control the size and/or morphology of magnetite crystals formed chemically in vitro.

Understanding the mechanism of bacteriophage T7 DNA delivery system inside the bacterial cytoplasm

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The majority of bacteriophages protect their genetic material by packaging the nucleic acid in concentric layers to an almost crystalline concentration inside protein shells (capsid). This highly condensed genome also has to be efficiently injected into the host bacterium in a process named ejection. The assembly and maturation of bacteriophages take place through a series of concerted steps where macromolecular complexes play specific functions with high efficiency. Tailed phages represent an extreme case of optimization of the infective process. Bacteriophage T7 belongs to the *Podoviridae* family and has a short, non-contractile tail formed by a tubular structure surrounded by fibers. Besides the tail, T7 uses a second protein complex to puncture the *E. coli* bacterial membrane required for DNA ejection. This complex, named the "core", is found inside of the viral head in the mature virus that it is postulated to be translocated through the tail during infection building a protein channel across the bacterial membrane. Correlation of the structural analysis of

these complexes by cryo-EM with their biochemical and biophysical properties would allow to build a complete model for understanding the molecular mechanism of T7 DNA ejection process.

Peripheral Membrane Interactions Boost the Engagement by an Anti HIV-1 Broadly Neutralizing Antibody

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The 4E10 antibody displays an extreme breadth of HIV-1 neutralization and therefore constitutes a model system for structure-guided vaccine design and immunotherapeutics. In this regard, the relevance of auto-reactivity with membrane lipids for the biological function and development of this antibody is still subject of controversy. To address this issue, here we have compared membrane-partitioning capacities of the 4E10 antibody and several of its variants, which were mutated at the paratope surface in contact with the membrane-interface. We have first used a physical separation approach (vesicle flotation), and subsequently carried out quantitative fluorescence measurements in an intact system (spectroscopic titration), using 4E10 Fab labeled with the polarity-sensitive 4-Chloro-7-Nitrobenz-2-Oxa-1,3-Diazole (NBD) probe. Moreover, recognition of epitope peptide in membrane has been probed by photo-cross linking using a Fab that incorporated the genetically encoded unnatural amino acid p-benzoylphenylalanine (pBPA). The experimental data rule out stereospecific recognition of viral lipids as a requirement for function, but support nonspecific electrostatic interactions between 4E10 basic residues and acidic phospholipids in membranes. Membrane-partitioning energetics indicates that 4E10 behaves as a peripheral membrane protein, using in concert interactions mediated by solvent-exposed hydrophobic and basic residues for enhancing its ability to binding viral membrane-associated ligand epitope. The implications of these findings for the natural production and biological function of this antibody are discussed.

Cooperation between the C-terminal domains of nucleoplasmin is key to stabilize and transfer the histone octamer to DNA

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Histone chaperones are key components of the machinery that regulates chromatin dynamics. We have previously shown that the distal face of nucleoplasmin (NP), the first histone chaperone described, is the protein region involved in substrate binding, and that the complexes formed between NP and the distinct core histones differ in stoichiometry and overall architecture. One NP pentamer binds five H2A-H2B dimers, with each C-terminal domain interacting with one dimer. In contrast, a more complex structure is obtained in the case of H3-H4 and the histone octamer, in which two NP pentamers face each other through their distal face, encaging the basic ligand. In this work, we characterize the NP/octamer complex and present its cryoEM structure. We also demonstrate that post-translational NP modifications contribute to stabilize the NP/histone

octamer complexes and that several C-terminal domains must cooperate to form stable complexes with H3-H4 and histone octamers, in contrast with what is observed with H2A-H2B dimers. Finally we show that, in vitro, NP is capable of transferring the histone octamer to DNA, assembling nucleosomes. This activity could have biological relevance in processes in which the histone octamer must be rapidly removed from or deposited into the DNA, such as DNA replication in the first steps of embryo development and transcription in highly active genes.

Membrane insertion of a Dengue virus NS2A segment. A computational study

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Non-structural NS2A protein of Dengue virus is essential for viral replication but poorly characterized because of its high hydrophobicity. We have previously shown experimentally that NS2A possess a segment, peptide dens25, known to insert into membranes and interact specifically with negatively-charged phospholipids. In order to characterize its membrane interaction we have used two types of molecular dynamics membrane model systems, a highly mobile membrane mimetic and an endoplasmic reticulum membrane-like model. Using the membrane mimetic system, we have been able of demonstrating the spontaneous binding of dens25 to the negatively-charged phospholipid DVPA containing membrane whereas no binding was observed for the membrane containing the zwitterionic phospholipid DVPC. Using the ER-like membrane model system, we demonstrate the spontaneous insertion of dens25 into the middle of the membrane; it maintaining its three-dimensional structure and presenting a nearly parallel orientation with respect to the membrane surface. Both charged and hydrophobic amino acids, presenting an interfacial/hydrophobic pattern characteristic of a membrane-proximal segment, are responsible for membrane binding and insertion. Dens25 might control protein/membrane interaction and be involved in membrane rearrangements critical for the viral cycle. These data should help us in the development of inhibitor molecules that target NS2A segments involved in membrane reorganization.

Hijacking the retrograde trafficking by the L. pneumophila effector RidL

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Our group is involved in the study of protein effectors from *Legionella pneumophila* that allow the formation of the intracellular replicative niche. More precisely, the protein of interest in this project is RidL, one of the few effectors from *L. pneumophila* required for infectivity. Previous studies have demonstrated that RidI interacts with retromer, an heteropentameric complex involved in recycling transmembrane proteins from endosomes. Yet, the hijacking mechanism of RidL remains unknown. The goal of this project is to elucidate such mechanism and propose new therapeutic targets to fight legionella infection. In this regard, I present an integrated set of biophysical and biochemical results, ongoing models and future directions.

EPR spectroscopy as an electronic and structural tool to study photosynthetic cytochrome c550

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Cytochrome C_{550} (Cc_{550}) is associated with photosystem II (PSII) in some photosynthetic organisms such as cyanobacteria, diatoms and red algae. The heme group in this protein has the characteristic covalent link to the protein chain and is coordinated to two histidine side chains. Although its function is not determined, a redox role has been proposed based on the redox potential measured when the protein is bound to the PSII. In addition, the redox potential of Cc_{550} decreases substantially when it is measured in solution in the soluble Cc_{550} form. The parameters governing this redox potential variation are not known. A change in the electronic levels of iron due to a rearrangement of the axial ligands has been hypothesized. Also, a change in the hydrophobicity of heme environment has been postulated.

Here we use Electron Paramagnetic Resonance (EPR) techniques to study two variants of Cc_{550} from the cyanobacteria *Synechocystis sp.*and the diatom *Phaeodactylum tricornutum* to determine the energy distribution of iron electronic levels. Our findings show that variations in relative energy between bound and unbound states and among the different variants are discrete and of comparable magnitude. The comparison of crystal structures, EPR spectra and the sequence rules out the hypothesis of a major rearrangement of axial ligands and rather indicates a different distribution of charges in the heme. The study of nuclear energy levels of iron using 2D pulse EPR techniques of both variants reveals more details about the geometry of the heme site, such as the effect of tilting of one of the axial imidazole rings or its rotation with respect to each other or to the heme molecular frame.

Molecular Recognition between Human Mitochondrial Cytochromes: The Cytochrome c1/Cytochrome c Interaction

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The cytochrome bc_1 (Cbc_1) complex is a membrane-bound component of the mitochondrial electron transport chain. The complex, which catalyzes electron transport from ubiquinol to cytochrome *c* (C*c*), comprises three redox centers: cytochrome *b*, the Rieske protein and cytochrome c_1 (Cc_1). Cc_1 is responsible for the reduction of C*c*, which, in turn, results in the transfer of electrons to cytochrome *c* oxidase (C*c*O).The reaction is essential for cellular bioenergetics, insofar asit is coupled with proton translocation leading to ATP formation. Recently, our group has reportedthat plant C*c* shows two binding sites on Cc_1.[1] The first, or so-called *proximal* site, is suitable for electron transfer, whereas the second, or distal site, located near the Rieskeprotein,is involved in

the channeling of C*c* molecules towards C*c*O.[1-3] Given this, this work aims to determine whether the C*c*-C$c_1$two-binding site model is conserved through out evolution and, specifically, can be found in the interaction between the two corresponding human hemeproteins. First, in close collaboration with the *European Integrating Structure Platform* (*Instruct*, PID1163), several constructions of the soluble N-terminal domain of Cc_1 were designed. Among these, a triple Cc_1 mutant lacking non-heme-coordinated cysteines and containing a bacterial periplasmic signal allowed for the expression of human Cc_1 as a recombinant protein. Physicochemical properties, including redox potential, were then analyzed. Preliminary measurements byisothermal titration calorimetry suggest that the C*c*:Cc_1 stoichiometry of 2:1 is presentin both plants and humans.

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Role of HIGD1A and Cytochrome c Proteins in Mitochondrial Respiratory Chain Supercomplexes

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Recently, cryo-electron microscopy was used to elucidate the structure of the respirasome, a mitochondrial supercomplex containing complexes I, III and IV (CI, CIII and CIV, respectively).[1,2] Despite having been developed at high resolution, the model lacks membrane proteins HIG1 domain family 1A and 2A (HIGD1A and HIGD2A), which have been identified as supercomplex adaptors. Indeed, HIGD2A is necessary for the assembly of the respirasome, whereas HIGD1A has been recognised as a positive regulator of cytochrome *c* oxidase (from CIV), with an impact on cell survival and tumour growth.[3] In addition, new studies suggest a putative stabilization of the CI/CIII and CIII/CIV supercomplexes by mobile carriers coenzyme Q and cytochrome *c* (C*c*), respectively.[4,5]

The pro-survival activity of HIGD1A is related to the inhibition of C*c* release and the decrease in caspase activation.[6] This effect is dependent on the soluble N-terminal, 26-amino-acid-long domain of HIGD1A, which is oriented towards the mitochondrial intermembrane space.[6] The C*c*/N-term HIGD1A interaction was characterized by combining measurements obtained through isothermal titration calorimetry and nuclear magnetic resonance, revealing transient C*c*/N-term HIGD1A contacts in the supercomplex context. Further experiments will be carried out using the full-length HIGD1A bound to GFP to improve solubility.

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Membrane activity of Adenylate Cyclase Toxin involves formation of tunable size pores with toroidal features

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Adenylate Cyclase Toxin (ACT or CyaA) secreted by the whooping cough bacterium Bordetella pertussis, belongs to the RTX (Repeats in ToXin) family of exoproteins with pore-forming activity. Pore formation by RTX-toxins leads to cell death by dissipation of ionic gradients and membrane potential across the cytoplasmic membrane of target cells. Beyond this, very little is known on the molecular structure or even the nature of the membrane-integrated pores these toxins form. Here we provide the first visualization of ACT molecules in membrane pore structures. We reveal that ACT clusters into heterogeneous oligomeric assemblies presenting a broad distribution of different architectures, including monomers, lines, arcs, and full rings that perforate the membranes. Remarkably we find that ACT pore size is not constant as typically observed in purely proteinaceous channels, but evolves with time, and depends on protein concentration, suggesting a toroidal mechanism. Our data support a new model for the nature of ACT-mediated lesions, in which the toxin delineates pores of different sizes to permeabilize cell membranes. A pore tunable in size adds a new regulatory element in the ACT-mediated cytotoxicity, which may lead to different pore sizes in different physiological scenarios or different cell types.

Human pathogen *Fusarium oxysporum* α-mating pheromone: ten amino acids code for structure and function

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The ascomycete fungui *Fusarium oxysporum*is a highly destructive plant pathogen and an emerging human pathogen. During sexual development, ascomycete fungi produce two types of peptide pheromones: a and α. The function of a-pheromones seem to be highly conserved among ascomycetes; however, until very recently (1) no structural information have been described at atomic detail. To perform their function as sexual chemoattractants, α-pheromones should be sensed by membrane receptors Ste2 and Ste3. In order to understand the mechanisms of interaction and activation of its receptors, structural information regarding these pheromones is essential. Solution NMR was employed to characterize the structures of the α-pheromone peptide in different environments. In all conditions, α-pheromone adopts a defined secondary structure. The central residues are crucial to define the structure in solution, the β-turn centered in Gly 6 and Gln 7 is a hallmark of the α-pheromone structure. Atomic interactions with membrane mimetics have ALSO been identified by NOE data. DPC and Gemini surfactants were employed as suitable membrane mimetic, as they form appropriate micelles for NMR studies. The large proportion of Trp (3/10 residues) play an important role in the interaction with micelles, thus their position in sequence is critical for their specificity. By NMR we have quantified the intermolecular interaction at atomic level. Based on the NMR results, a 3D model of the interaction between pheromones and micelles is proposed. Finally, the presence of two Cys residues adds the possibility of being reduced or oxidized.

We studied changes in the interaction with membranes associated to this redox process that can be important for the recognition of its receptors.

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Non-enzymatic modification of hen egg white lysozyme by glycolaldehyde proves that intermolecular crosslinks are involved in the aggregation of glycated proteins

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Reactive carbonyl compound can modify proteins through a process known as protein glycation (PG). Diabetes-derived hyperglycemia triggers this process, which involves the development of diabetes-related diseases.[1] Moreover, PG boosts protein aggregation (PA) increasing the development of aggregation diseases in diabetic people.[2] Regardless its biological relevance, the mechanism that links PG with PA remains unclear. To delve into this process, we have used a broad range of biophysical techniques (e.g. AFM, NMR, fluorescence, etc.) to study the glycation of hen egg white lysozyme (HEWL) with glycolaldehyde (GLA), a reactive α-hydroxyaldehyde that increases its physiological concentration under diabetes mellitus.[3] Our data prove that HEWL glycation occurs through two different concentration-dependent mechanisms. At low HEWL concentrations ([HEWL]$\leq$2mM), its glycation results into the formation of non-crosslinking fluorescent advanced glycation end-products (AGEs). This process does not alter the HEWL native structure but depletes its enzymatic activity without inducing PA. However, upon increasing HEWL concentration, the mechanism gradually shifts towards the formation of intermolecular crosslinking AGEs, which trigger the formation of covalently linked insoluble spherical-like aggregates.[4] These results differ from the aggregation-modulation mechanism that we earlier described for HEWL glycated with ribose.[5] Altogether, our data provide new insights into the mechanism that links PG with PA, and demonstrates the strongly dependence of the glycated-protein aggregation mechanism on the chemical nature of the glycating agent.

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Coordinated activity of the human mitochondrial DNA polymerase and SSB proteins at the replication fork

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Mitochondrial DNA polymerase gamma (Polγ) is the sole polymerase responsible for replication of the mitochondrial genome. It is well established that defect in mtDNA replication lead to mitochondrial dysfunction and disease. To date, approximately 150 disease mutations in Pol γ have been identified, which places Pol γ as a major locus for mitochondrial disease. To understand the molecular basis of these diseases, it is important to define the molecular mechanisms that govern the enzymatic activity of Polγ. To this end, we are using optical tweezers to: 1) study the real-time kinetics of individual Pol γ molecules during primer extension and strand displacement DNA synthesis and, 2) to determine the effect of human mitochondrial single stranded binding proteins (HmtSSB) on these reactions. Our results show that HmtSSB strongly modulates the real time kinetics of Pol γ. During primer extension HmtSSB plays a crucial role establishing the template-primer structure at the polymerase active site, increasing in this way the polymerase processivity and nucleotide incorporation rate. During strand displacement, DNA binding of HmtSSB to the displaced strand destabilizes the replication fork, favoring the polymerase advance. We will discuss the implication of these findings on the mtDNA replication context.

Crystal structures of PipY from Synechococcus elongatus, a paradigm for the intriguing COG0325 family of PLP-binding proteins

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Cyanobacteria, key players in the nitrogen/carbon flows of the biosphere, are the focus of our joint efforts to decipher regulatory networks in these organisms. A key player, protein PipX, shuttles between protein PII and the transcriptional regulator NtcA, depending on the nitrogen abundance, effecting on transcriptional regulator PlmA when PII-bound, or on NtcA. Since the gene for PipX immediately precedes the *pipY* gene, making an operon with it in *Synechococcus elongatus*, we tried to clarify PipX-PipY functional interactions. As a first step, we produced and purified PipY, finding that it is monomeric (gel filtration) and that, judged from its absorption spectrum, hosts pyridoxal phosphate (PLP), which slowly dissociates from it. Incubation of aged PipY with PLP revealed a K_D PLP ~30 μM. Reaction with the PLP-targeting antibiotic D-cycloserine revealed very low affinity of PipY for it. PipY crystal structures (~1.9 Å resolutions) in PLP-bound and apo forms showed the modified TIM barrel that characterizes the fold type III PLP enzymes. Unlike these enzymes, PipY was single-domain and monomeric, and lacked residues that are crucial for catalysis. Thus PipY is highly unlikely to be an enzyme. The high exposure of the PLP, its modest affinity for the protein, and the conformational changes in PipY associated with PLP dissociation, support a role of PipY in vitamin B6 homeostasis, possibly as a provider of PLP to PLP-dependent enzymes. Our detailed structural data, and the comparison with the structure of the yeast orthologue reported previously and with the PDB-deposited structures of three bacterial *COG0325*products, make of

PipY a structural paradigm for the universal *COG0325* gene family, traced back to LUCA. Our findings help understand the roles of the mutations found in the human orthologue as the cause of vitamin B6-dependent epilepsy.

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Fluorescence studies reveal a high-affinity interaction between the neurotoxic amyloid β-peptide and calmodulin

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Many works have been focused on Amyloid β-peptides (Aβ), a major hallmark of Alzheimers disease (AD), and their interactions with different proteins. Although Aβ neurotoxicity develops with cytosolic calcium dysregulation, our knowledge about Aβ and calcium regulation and signaling is still limited. One of the proteins that plays a major multifunctional role in calcium signaling is calmodulin (CaM). In this work, using fluorescent derivatives of CaM (Badan-CaM), we show that Aβ binds with high affinity to CaM. Furthermore, we have been able to fine tune that the 25-35 domain of Aβ form a novel binding motif for CaM which is responsible for this high affinity interaction. The low values obtained for the dissociation constants of Aβ from CaM point out that CaM is one of the cellular targets with highest affinity for neurotoxic Aβ peptides. Therefore, this novel high affinity Aβ-CaM interaction opens a new gateway to further understand the mechanism involved in the neurotoxic effect of Aβ and also to consider the potential of calmodulin and calmodulin-derived peptides as therapeutic agents in AD.

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Discovery of inhibitors of the ferredoxin-NADP+ reductase from the Xanthomonas citri subsp. citri phytopathogen

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Plant type Ferredoxin-NADP(H) reductases (FNRs, EC 1.18.1.2) constitute a family of FAD containing enzymes that deliver NADPH or low potential one-electron donors to redox-based metabolisms in plastids and bacteria. Based on phylogenetic analysis, FNRs present in most prokaryotes (collectively known as FPRs) have been classified into two subclasses represented by the *Azotobacter vinelandii* (subclass I) and the *Escherichia coli* (subclass II) [1]. *Xanthomonas axonopodis citri*subsp. *citri* (*Xcc*) is a Gram-negative bacterium responsible for citrus canker, a disease that affects most commercial citrus crops and has economic impact worldwide [2]. Its*fpr* gene encodes a subclass I FPR (*Xcc*FPR). The role of *Xcc*FPR nor its potential substrate have been elucidated, but its involvement in the oxidative stress response of *X. citri*via interaction with ferredoxin XAC1762 has been proposed [3]. Therefore, *Xcc*FPR is relevant for the pathogen survival and the inhibition of its activity might represent an effective treatment against citrus canker. We started performing a high-throughput screening (HTS) of a library of 11120 druglike compounds to find inhibitors of *Xcc*FPR based on its diaphorase activity. We selected 43 HTS hits and narrowed them down to 5 primary hits that showed IC_{50} values in the low micromolar range and successfully abolished the activity of *Xcc*FPR. The four best inhibitors were assayed *in vivo* on plate cultures, and two of them showed bacterial growth inhibition. Based on the best primary hit, secondary hits were selected and one of them improved the characteristics of the primary one. Type of inhibition of this hit was determined and its effect on plastidic type FNR. This work is in progress but represents a promising pathway for the development of phytosanitary compounds against citrus canker propagation.

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Proton release and uptake in a membrane protein traced with microsecond resolution by a pH-sensitive vibrational probe

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Infrared (IR) spectroscopy has been successfully used in the past to probe the dynamics of internal proton transfer reactions during the functional mechanism of many membrane proteins, but has remained mostly silent to protonation changes in the aqueous medium. In the present work we have overcome such limitation and resolved proton release and uptake events in the light-driven proton-pump bacteriorhodopsin (BR) by selectively monitoring vibrational changes of buffer molecules with 6 μs resolution. As a pH-sensitive vibrational probe we used 2-(*N*-morpholino)ethanesulfonic acid, MES, and its perdeuterated form. Thus, internal and external proton transfer reactions of BR and potentially in other proteins can be now simultaneously probed in a single time-resolved FT-IR experiment, allowing for the comparison of proton release and uptake events with other molecular processes within the protein at unprecedented detail. We exploited this technical achievement to test our current understanding of the proton pumping mechanism of BR, in particular regarding the proton release and uptake steps. We demonstrate for the first time that the so-called *continuum* band, extending from 2300 to well below 1700 cm^{-1}, consists of two independent spectral contributions. The first contribution corresponds to deprotonation of the proton release complex (PRC), a complex in the EC domain where an excess proton is shared by internal water molecules. From kinetic and spectral considerations we tentatively assign the second component of the *continuum* band to the proton uptake complex (PUC), a complex with an excess proton reminiscent to the PRC but located in the cytoplasmic (CP) domain. Integrating additional results, we propose a revised version of the proton transfer reactions responsible for the light-driven vectorial proton translocation by BR that challenges the current standard model.

Towards the mechanochemical characterization of the human mitochondrial replisome

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Mitochondria are eukaryotic organelles, responsible for cellular energy generation. Besides their role in energy production, mitochondria are essential for cellular activity regulation, e.g. cell signaling and cell death. Mitochondria have their own DNA (mtDNA) and, in general, human cells contain thousands of mtDNA copies. A reduction in the number of mitochondrial DNA molecules or accumulation of mtDNA mutations may cause so-called 'mitochondrial diseases' that, in humans, affect tissues highly dependent on mitochondrial metabolism, such as brain, heart , liver, skeletal muscles and kidney tissues [1]. Therefore, mitochondrial dysfunction and, partly, mitochondrial diseases occurrence, in some measure, depend on effectiveness and accuracy of mtDNA replication. The mitochondrial DNA replication machinery is much simpler than its nuclear DNA equivalents. The 'minimal replisome', that is capable of processive DNA synthesis, can be reconstituted in vitro with just three proteins: the TWINKLE DNA helicase, the single-stranded DNA binding protein (mtSSB) and the mitochondrial DNA polymerase γ [2]. The mechanism of human mtDNA replication has not

yet been fully characterized. It is unclear how the proteins, involved in mtDNA replication, act at the replication fork. In present work, we aimed to detect and characterize the human mitochondrial DNA helicase activity at a single molecule level.

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Functional and structural differences between Sorting Nexin 3 and Sorting Nexin 12

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Protein recycling is essential for cell homeostasis. Several systems have been developed to perform this process. Among them, Retromer, a heteropentameric protein, is involved in recycling of proteins (known as cargos) from late endosomes to Golgi apparatus or plasma membrane [1,2]. Retromer is composed of the Cargo Selection Complex (CSC), formed by Vps35, Vps26 and Vps29; and accessory proteins called Sorting Nexins (SNX). CSC was thought to be the unique responsible of cargo recognition, and mutations on its sequence causes severe diseases like Parkinson or Alzheimer [3]. Intense research has been done on Retromer and its interaction with cargos and SNX, and recent results prove the importance of both CSC and SNX for cargo recognition [1]. SNX are a family of proteins that contain a PX domain, able to recognize phosphoinsitides (PI), specially PI(3)P [4]. They are involved in protein-lipid interaction, specially in the endocytic network. A heterodimer of SNX1/2 and SNX5/6 was thought to be the putative partners of CSC, but recent results have demonstrated the interaction with others SNX, like SNX3, in cargo recognition and recycling [1]. SNX12 is a PX containing SNX highly similar to SNX3. Although it was thought to be expressed at low levels, recent results show high expression in mice brain [5]. Furthermore, based on the structure of SNX3 [1] and the not yet published SNX12 structure solved in our lab, we try to demonstrate differences in PIP recognition between SNX3 and SNX12, what would be a starting point to differentiate this two similar proteins, thought to be redundant in function until now. Furthermore, this result would open a new insight in SNX–CSC biology, implicating high relevance of SNX in cargo recognition and sorting.

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pyDockDNA: A pyDock upgrade for protein-DNA docking

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Structural prediction of protein-DNA interactions can contribute to the understanding of essential cell processes at molecular level, such as those related to gene transcription and regulation. Very often, protein-DNA are structurally modelled by protein-protein docking tools, but the association between proteins and DNA has specific characteristics regarding the energetics and conformational flexibility of the nucleic acids that are not fully captured with standard protein docking methods.

A few computational methods for protein-DNA docking have been reported such ParaDock [1], but the field is still in development.

Here we have explored the use of different energy-based functions for the scoring of rigid-body protein-DNA docking poses generated by FTDock. We have implemented the different terms of the scoring function used in pyDock [2], that is, van der Waals, desolvation and electrostatics terms. Charges for the DNA molecule have been defined from Amber 94 force-field. Different parameters and combinations of these functions have been tested on an available DNA-protein docking benchmark [3]. For the scoring of the initial rigid-body protein-DNA docking decoys, best results are obtained with a combination of electrostatics and van der Waals terms. The protocol is implemented in a new module called pyDockDNA. Success rates and limitations according to protein and DNA flexibility, DNA type, etc. will be discussed.

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Exploring computational tools for protein modeling in cryo-electron microscopy density maps: I-tasser and Amber

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The number of near-atomic resolution maps of proteins obtained using cryo-electron microscopy (cryo-EM) is increasing rapidly, due to availability of new microscopes and especially new direct electron detectors. Despite the advantage of cryo-EM to directly image proteins frozen in solution, modeling atomic structures *de novo* on those maps is still a difficult problem. In contrast to X-ray diffraction, in cryo-EM the resolution is typically not sufficient to identify all the side chains of the protein.

Here we explore how several computational tools can help modeling proteins on cryo-EM maps at near-atomic resolution, using homology modeling and molecular dynamics (MD). Structural motifs in proteins are widely conserved in all living organisms, and usually a sequence identity of 30% is enough for tools like I-tasser [1] to identify the secondary structure and provide a rough model of a given domain. After building a model with I-tasser, we used a cryo-EM map at 4 Å to perform rigid fitting of this model into the density, from the beginning to the end of the map following the mainchain, and backwards to avoid biasing. Using Amber to run MD simulation of the model [2], it is possible to set a soft restrain force constant towards the map. Amber force field is keeping the right geometries for the protein while is fitting better into the density along the simulation. Once the model reaches a stable conformation inside the map, we can identify some well-placed side chains to validate our model.

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The formation of carboxymethyl lysine and carboxyethyl lysine modify the protective capacity of alpha-synuclein on the reactive oxygen species and free radical formation

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α-Synuclein (α-syn) is a nuclear and synaptic intrinsically disordered protein.[1] In Parkinson's disease, it tends to aggregate by forming intracellular Lewy bodies,[2] which finally induce the neuronal death.[3] Several hypotheses have been proposed to explain the toxicity of α-syn aggregates. Among them, it can be highlighted their capacity to induce the formation of reactive oxygen species (ROS) and free radicals under the presence of free metal ions.[4] However, this does not result from a direct ROS-active form of the α-syn since *in vitro* studies have proven that α-syn is able to chelate Cu^{2+} and decrease the formation of ROS by radical scavenging and redox silencing.[5]

These results raise now the question whether chemical modifications (such as those associated to the non-ezymatic glycation) would affect the α-syn redox silencing capacity. Therefore, we have modified the fifteen Lys side chains in α-syn to finally have carboxymethyl lysine (CML) or carboxyethyl lysine (CEL). These modified α-syn have been used to study how the modifications influence the α-syn capacity to bind Cu^{2+}, to scavenger $OH^{\cdot}$ and $O_2^{\cdot -}$ radicals, or to inhibit their formation. Overall, the obtained results reveal that CEL and CML reduce the redox silencing capacity of α-syn, and support the suggestions about the contribution of these post-translational modifications on the high oxidative stress observed inside of neurons in Parkinson's disease.

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Insights into the Nop7 and Erb1 interaction, essential for the assembly of PeBoW complex in ribosome biogenesis

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Ribosome biogenesis is one of the most essential pathways in eukaryotes in which numerous groups of proteins participate. One of these complexes is the PeBoW complex (in mammals) or Nop7-subcomplex (in yeast). The mammalian PeBoW complex, composed of Pes1, Bop1 and WDR12 (Nop7, Erb1 and Ytm1 in yeast) is critical for the processing of the 32S pre-ribosomal RNA. Several studies were carried out to characterize the interactions between these proteins. Erb1 is considered to be the core of the complex due to the fact that it physically interacts with both, Nop7 and Ytm1. We have previously described the interaction between Erb1 and Ytm1 through their β-propellers domains (Wegrecki *et al*, 2015). However, the association between Nop7 and Erb1 is not clear yet. In this work, the heterodimer formed by Nop7 and Erb1 both from *Chaetomiumjjj thermophilum* and *Saccharomyces cerevisiae* were expressed at large-scale in *E. coli* (DE3) BL21 CodonPlus (RIPL) and purified using affinity and size exclusion chromatography (gel filtration). Bio-layer Interferometry experiments were used to calculate the binding affinity (K_D values) for the interaction between Nop7 and Erb1. Moreover, it has been possible to purify the complex by gel

filtration. Our data suggest that the N-terminal domains both from Nop7 and Erb1 are required for the binding and heterodimer complex formation.

Cytochrome c and its Interaction with the Histone Chaperone ANP32B: Tackling the Chaperone Disordered Regions

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Extra-mitochondrial cytochrome *c* (C*c*) has recently emerged as an inhibitor of histone chaperones, namely SET/TAF-1β in mammals and NRP1 in plants, upon DNA damage. Likewise, C*c* can target other histone chaperones, such as mammalian Acidic leucine-rich Nuclear Phosphoprotein 32 family member B (ANP32B). ANP32B modulates mRNA nucleocytoplasmic trafficking upon Thr244 phosphorylation, ANP32B comprises four Leucine-Rich Repeats (LRR) at its N-terminal end and an unstructured Low-Complexity Acidic Region (LCAR) at the C-terminal end.

Here, we used co-immunoprecipitation assays to confirm the cytoplasmic interaction between ANP32B and C*c* upon DNA damage. Then, we analyzed such interaction by Isothermal Titration Calorimetry (ITC) and Nuclear Magnetic Resonance (NMR) spectroscopy. Notably, wild-type (WT) full-length ANP32B and its phosphomimetic T244E mutant interacted with C*c*, whereas a deletion mutant lacking the LCAR region failed to bind the hemeprotein, confirming that the LCAR region is responsible for the ANP32B-C*c* interaction.

The LCAR contains the topogenic KRKR motif near T244. Hence, we tested two synthetic peptides containing LCAR residues from 231 to 251. T244 was replaced by glutamate in the second one. Both peptides were able to bind C*c*; albeit the T244E mutant exhibited a higher affinity.

Simulations by Replica Exchange Molecular Dynamics (REMD) using distance restraints derived from 2D ^{1}H-^{1}H TOCSY, NOESY and ROESY NMR spectra showed that both peptides adopted a compact structure, despite secondary structure predictions suggesting that the LCAR is fully unstructured. Brownian Dynamics calculations using C*c* and the peptide structures derived from REMD showed that both peptides explored the heme-surrounding cleft and part of the opposite-side of C*c*, in agreement with the chemical-shift perturbations maps inferred from NMR titration assays. Further functional assays will be performed to shed light onto the functional relevance of the ANP32B-C*c*interaction.

Activation of the NGF receptor TrkA is regulated through homo- and hetero-dimer transmembrane domain interactions with p75 neurotrophin receptor

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An unsolved question in neurotrophin signaling is the molecular mechanism of how p75 modulates TrkA activation and the structural basis of their interaction. p75 sensitizes TrkA to lower concentrations of NGF but the mechanism is unknown. Here we found that TrkA activation is modulated by homo and hetero transmembrane domain (TMD) interactions with itself and with p75. NMR spectroscopy, cysteine-scanning mutagenesis and functional studies revealed the structural basis of TrkA-TMD dimerization and the active dimer interface. NGF induce a rotation of the transmembrane domain of TrkA. NMR titration experiments reveal the formation of a heterocomplex between the transmembrane domains of TrkA and p75 through an interaction the N-terminus of the TMD a-helix. In the absence of NGF p75 keeps TrkA activity in check by reducing TrkA autoactivation. These findings support a conformational mechanism of TrkA activation by p75 and provide a new structural framework for understanding the nature of the NGF high-affinity complex.

c-Src protein self-association induced by lipid anchoring

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The non-receptor protein kinase c-Src participates in numerous cell signalling pathways that play crucial roles in maintaining the cell physiology. Identified as the first proto-oncogene, its deregulation has been related to several human cancers [1]. Its structure consists of five distinct domains, from N-terminus to the C-terminus: myristoylated SH4, Unique Domain, SH3, SH2 and the Kinase Domain or SH1. c-Src binds to the lipid membrane through the insertion of its myristoyl chain and the electrostatic interaction of the positively charged SH4 domain residues with the negatively charged lipids. Recent studies [2], demonstrated the formation of stable c-Src dimers on supported lipid bilayers. Self-association of the isolated SH4 domain has also been observed and leads to the formation of large clusters, when the domain is directly fused to reporter proteins [3]. The neighbour Src domains, seem to play a role in restricting the oligomerization to discrete dimers. In this study, we use Surface Plasmon Resonance and site-directed mutagenesis to investigate the structural determinants of Src self-association on lipid membranes.

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Structural insights into the cargo binding specificity of the retromer complex

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Retromer is a protein coat that mediates endosomal protein sorting and trafficking. It assembles on endosomes and forms tubular vesicles that return specific transmembrane proteins to the plasma membrane or to the *trans*-Golgi network. Therefore, the rescued cargo proteins avoid the lysosomal degradation pathway. Retromer's cargo includes cellular transmembrane proteins such as, signaling receptors, ion channels, transporters or enzymes. Defects in retromer impair various cellular processes and underlie some forms of Alzheimer's disease and Parkinson's disease. The retromer complex comprises a VPS26-VPS29-VPS35 heterotrimer and various combinations of sorting nexin (SNX) proteins that contribute to membrane recruitment and formation of recycling tubules. We published recently an X-ray crystallographic analysis of a four-component complex comprising the VPS26 and VPS35 subunits of retromer, the sorting nexin SNX3, and a recycling signal from the divalent cation transporter DMT1-II. This analysis identifies a binding site for canonical recycling signals at the interface between VPS26 and SNX3. To further analyse the cargo binding we have solved the structure of retromer with other cargo proteins. These interactions have been validated by isothermal titration calorimetry and mutational analysis. The results presented here reveal the molecular details of the cargo binding specificty of the retromer complex for the canonical recycling signal.

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Structural and Functional Characterization of a Novel Bacterial Glycosyltransferase Family Involved in Pathogenesis

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Bacterial pathogens have evolved distinct ways of colonizing host cells and promote infection. Many human intestinal bacterial pathogens such as *Salmonella, Shigella* and enteropathogenic/ enterohemorrhagic *Escherichia coli* utilize type III secretion systems to deliver virulence effector proteins into the host to promote colonization and interfere with antimicrobial host response. Among the type III effectors, the NleB protein has been shown to be essential for virulence of enteric pathogens. NleB is a glycosyltransferase that has been shown to interact with host cell death-domain-containing proteins, GlcNAcylate a specific arginine on these and thereby inhibiting death receptor signalling and preventing host cell apoptosis. Preliminary data suggest a phage origin for NleB protein with up to two isoforms present in *E. coli* cells, *nleb* wt that codifies for NleB1 and *nleb* V2 that codifies for NleB2 whose target is unknown. According with the activity tests, the transfer of glucose by recombinant NleB1 seems to be restricted to the death domain of the FAD protein. This finding is supported with the observation of a soluble stable complex of NleB1 with FADD-DD domain that produce promising crystals under crystallization assays.

However, this data is very preliminary thus more effort is required to structurally characterize NleB1 catalytic mechanism as a model and extrapolate this knowledge to the whole new family of NleB orthologues. Furthermore, the proposed research over arginine GlcNAcylation constitutes a complete new research field in cell signalling and bacterial virulence.

Molecular mimicry to exploit the host-cell ubiquitination pathway

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Ubiquitination of target proteins is mediated by the sequential action of three types of enzyme: Ub–activating enzymes (E1s), Ub–conjugating enzymes (E2s), and Ub ligases (E3s). *Legionella pneumophila*, the causative agent of Legionnaire's disease, exploits the host ubiquitination machinery by producing its own E3 ligase mimics, which hijack the E2–Ub complex to target different host proteins for ubiquitination and subsequent degradation. Legionella pneumophila encodes its own molecular mimics of E3 ligases, including the effector protein RavN, thereby subverting the ubiquitin pathway for its own benefit during infection. Using protein crystallography, we have revealed that the fold of RavN shows only residual resemblance to conventional eukaryotic E3s. The N-terminal region of RavN displays a U-box-like motif that lacks the central alpha helix commonly found in other U-box domains, indicating that RavN is an E3 ligase relic that has undergone significant evolutionary alteration. Yet its mode of interaction with E2 enzymes, host proteins that are important for the ubiquitin transfer reaction, has been preserved throughout evolution, and substitution of amino acid residues within the predicted E2 binding interface render RavN inactive.

Molecular basis for the interaction between cytochrome c and its novel apoptotic target 14-3-3ε

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Cytochrome *c*(C*c*) is a mitochondrial protein which results essential to triggering apoptosis, when it is released into the cytosol. Traditionally, the activation of the apoptosome, and thereby the activation of the caspases, was considered the only cytosolic role of C*c*, through its binding with Apaf-1. However, our group evidenced that C*c* interacts with several nuclear and cytosolic targets upon cell death stimuli. Among these novel apoptotic targets, 14-3-3ε stands out due to its importance in several processes, such as cellular cycle and apoptosis. Thus, the study of the interaction between C*c* and 14-3-3ε rises special relevance for the understanding of the apoptosis signalling.

In this work, we tackle the interaction of C*c* with 14-3-3ε using Isothermal Titration Calorimetry (ITC) and Nuclear Magnetic Resonance (NMR). Calorimetry assays show C*c* binds 14-3-3ε at two different binding sites, with binding affinities in the μM range. The combined use of site directed mutagenesis on 14-3-3ε and ITC assays allows the identification of the concave groove and the convex face of 14-3-3ε as C*c*-binding sites. Furthermore, both ITC and ^{19}F NMR titrations of C*c* with the C-terminal tails of 14-3-3ε expose these tails interact directly with C*c*. In addition, the

C*c* NMR perturbation map reveals how the metallo-protein recognizes 14-3-3ε using the rim of the heme group.

Finally, a structural docking for the C*c* / 14-3-3ε complex is presented based on molecular dynamics along with restrain-driven HADDOCK computations. Then, our data provide molecular basis for a novel cytosolic interaction involving C*c*, enhancing its apoptotic function.

NMR tools for the structural studies of IDPs

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Intrinsically disordered proteins, IDPs can be associated with a range of functions, and they play a main role in protein interaction networks. This has brought an increased interest in characterizing it. X-ray crystallography can not be used to characterize IDPs as their mobility prevents the formation of suitable crystals. NMR spectroscopy emerges as the unique tool to obtain structural and dynamic information at atomic resolution.

Chemical shifts and J-coupling constants are a valuable source of structural information. Sequential assignment of NMR spectra of proteins is a prerequisite to obtain theses parameters. NMR assignment of IDPs by conventional HN-detected methods is hampered by the small dispersion of the amide protons chemical shifts and exchange broadening of amide proton signals. Therefore alternative assignment strategies are needed. We have proposed an approach base on the acquisition of two ^{13}C-detected experiments to directly correlate the chemical shifts of two consecutive ^{13}C-^{15}N groups in proteins.[1]

On the other hand, although chemical shifts are highly useful for identifying transiently populated secondary structure elements in intrinsically disordered proteins (IDPs), further evidence can be obtained from the analysis of J couplings. A coupling that may be useful in this sense is $^{2}J_{N(i)Ca(i-1)}$. The $^{2}J_{N(i)Ca(i-1)}$ coupling has been proposed as a valuable indicator to identify secondary structure elements in folded proteins[2] and has proven to be useful for characterizing structures of IDPs. Here we present an approach base on the acquisition ^{13}C-detected experiments and Non Uniform Sampling methodology to measure it.

This approach has been tested with different IDPs, Nupr1 (93 residues), IF7 (86 residues), hCPEB-Nt (128 residues).

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DNA replication in Mycobacterium tuberculosis: structural and biochemical characterization of the replicative DNA polymerase DnaE1 and its unique PHP-exonuclease

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High fidelity DNA synthesis is essential for all organisms and depends on a proofreading 3'-5' exonuclease that is associated with the replicative DNA polymerase. Contrary to humans and the model organism *Escherichia coli*, the proofreading activity in the major pathogenic bacteria *Mycobacterium tuberculosis* (Mtb) is performed by an intrinsic domain of the replicative DNA polymerase DnaE1, the PHP domain. The mechanism of action of the PHP-exonuclease is unknown. Resistance to antibiotic in Mtb is caused by point mutations that occur during DNA replication. Hence, understanding the mechanisms that regulate replication fidelity in Mtb is crucial in the search for new therapies to treat one of the major global health problems. We have solved the crystal structure of the Mtb DnaE1 polymerase and biochemically characterized its PHP-exonuclease. We have identified a tri-nuclear Zn center in the PHP domain coordinated by nine conserved residues. Interestingly, the PHP active site in DnaE1 shows remarkable similarities to the active site of *E. coli* endonuclease IV (Endo IV), an enzyme that also cleaves DNA. All these observations allow us to propose a mechanism for DNA hydrolysis by the PHP-exonuclease based on the mechanism of action of Endo IV. Finally, the unique PHP-exonuclease active site of Mtb appears an attractive target for specific inhibition as is not found in eukaryotes, making the likelihood of cross reactivity to human exonucleases small. Therefore, this work provides new insights in understanding replication fidelity in Mtb and will be a valuable tool in the development of novel treatments that target DNA replication in *Mycobacterium tuberculosis*.

HuR Revealed as a Novel Target for Cytochrome c under DNA Damage

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Human antigen R (HuR) is a ubiquitously expressed RNA Binding Protein (RBP), whose coding region comprises three independent RNA Recognition Motifs (RRMs) and a hinge region between RRM 2 and 3; where the nucleo-cytoplasmic shuttling sequence is located. This RBP regulates the splicing, stability and translation of a diverse range of mRNAs, including that of Cytochrome *c* (C*c*). C*c*, in turn, is a multi-functional heme protein which plays a moonlighting role in cells, participating in their energetics, DNA damage response and apoptosis. We recently detected the interaction between HuR and C*c* in cells submitted to DNA damage by co-immunoprecipitation assays. Moreover, the signaling networks of HuR and C*c* converge by targeting ANP32B and SET/TAF-1β proteins which act as PP2A inhibitors. These findings suggest a novel cell death pathway regulated by the C*c*-HuR axis.

To explore the structural features of the C*c*-HuR complex, we performed Nuclear Magnetic Resonance (NMR) titrations using the following HuR constructs: RRM12, RRM23 and RRM3. We

first tried HuR RRM12, but did not observe any interaction with C*c*. Next, we examined HuR RRM23 and RRM3 constructs, which were purified using the anionic detergent N-Laurylsarcosine (sarkosyl) in order to overcome the poor solubility of the third domain of HuR. NMR titrations using ^{15}N-labeled C*c* show no chemical-shift perturbations upon adding either ^{14}N-HuR RRM23 or ^{14}N-HuR RRM3. However, the 1D-NMR Met80-εCH_3 resonance of reduced C*c* broadens upon addition of the ^{14}N-HuR RRM23 construct. Thus, we speculate that sarkosyl encapsulates HuR inside detergent micelles.

Accordingly, we dispensed with sarkosyl and fused HuR RRM23 and RRM3 constructs with a GST tag to enhance HuR solubility. Indeed, HuR RRM3 has successfully been purified, allowing us to perform further structural analyses.

CXCR4 transmembrane region VI governs its actin-dependent dynamic clustering, signaling and cell response

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A current challenge in cell motility studies is to understand how the dynamics and spatiotemporal organization of chemokine receptors at the cell membrane influences their function. Using single-particle tracking and super-resolution microscopy, we found that CXCR4 forms monomers, dimers and small nanoclusters on the T cell membrane, which became larger after binding of its ligand, CXCL12. The actin cytoskeleton and the co-receptor CD4 acting in an orchestrated fashion regulate the lateral mobility of CXCR4 and signaling strength. In CXCR4 transmembrane region VI, we identified the structural residues crucial for receptor clustering, and generated an oligomerization-defective CXCR4 mutant that did not cluster in response to CXCL12 and showed severely impaired signaling. We demonstrate that structural motifs of CXCR4 and local organizers of the cell membrane regulate the distribution, cluster size and function of this receptor and define new targets to intervene in the in vivo functions associated to these inflammatory mediators.

The Instruct Image Processing center (I2PC): support to structural biologists

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The Instruct Image Processing Center (I2PC) at the CNB-CSIC is the European Reference Center for infrastructure provision in Image Processing in Transmission Electron Microscopy and X-ray Microscopy. We provide support to structural biologists, helping them to maximize the extraction of biological knowledge from their electron microscopy images in three different platforms: Instruct, iNEXT and Corbel. In iNEXT we give support at sample level, with analysis of EM grids and acquisition of EM images using a FEI Talos Arctica with a Falcon III direct detector. In Instruct we give support for full EM image processing using SCIPION framework, including movie alignment, particle picking, classification, volume reconstruction and atomic structure determination. In Corbel we link the structural data with genomics and proteomics databases, with annotations of 3D protein structures at residue level using proteomic and genomic sources including UniProt and ENSEMBL databases, diseases and genomic variants, protein domain families, disordered regions, short linear motifs and immunological epitopes.

14 P2 - Membrane Function Structure and Function

LC3/GABARAP interaction with cardiolipin: Implications for mitophagy

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Mitochondria are essential organelles for the regulation of cellular energy homeostasis and cell death. The elimination of damaged mitochondria through selective autophagy (mitophagy) is therefore critical for maintaining proper cellular functions. How the cell recognizes dysfunctional cellular components which should be degraded is still unclear. The phospholipid cardiolipin (CL) has been proposed to play a role in selective mitochondrial degradation. CL externalization to the outer mitochondrial membrane would act as a signal for the autophagic machinery to start the process, in which the LC3B protein would be involved. This protein belongs to LC3/GABARAP family, composed of the six orthologs of yeast protein Atg8 identified as yet in mammals. The existence of this variety of closely related proteins raises the question of whether each of them has a different role in autophagy, either in the selection of cargos or in the various steps of autophagosome biogenesis. In order to explore which of the orthologs could be involved in mitophagy, we have performed a quantitative analysis of CL interaction with the LC3/GABARAP family members LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2, using model membranes. Furthermore an *in vivo* approach has been carried out to determine the co-localization of these proteins with mitocondria in cells, upon induction of mitophagy by rotenone, a mitochondrial complex I inhibitor that induces CL externalization to the outer mitochondrial membrane. We have observed that LC3A, LC3B and LC3C but not GABARAP or GABARAPL2 interact with CL-containing vesicles. Moreover, in human glioblastoma cells rotenone-induced autophagy leads to LC3B translocation to mitochondria and subsequent delivery of mitochondria to lysosomes. Our results support the notion that each human Atg8 ortholog might play a specific role in different autophagic processes.

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Graphene oxide interaction with model and cell membranes

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The evaluation of toxicity for the proper use of graphene oxide (GO) in biomedical applications involving intravenous injections is crucial but the GO circulation time and blood interactions are largely unknown. It is thought that GO may cause physical disruption of red blood cells (RBC), haemolysis. The aim of this work is to characterize the interaction of GO with model membranes and use this knowledge to improve the haemocompatibility of GO with human RBC. We have shown that GO interacts with both, neutral and negatively-charged lipid membranes; binding is decreased beyond a certain concentration of negatively-charged lipids, and favored in high-salt buffers. After this binding occurs, some vesicles remain intact, while others are disrupted and spread over the GO surface. Neutral membrane vesicles tend to break down and extend over the GO, while vesicles with negatively-charged membranes are mainly bound to the GO without disruption. GO also interacts with RBC, and causes haemolysis in human RBC; haemolysis is decreased when GO is coated with lipid membranes, particularly with pure PC vesicles.

Understanding the molecular structure and biophysical properties of the pulmonary surfactant system: from the pneumocyte to the air-liquid interface

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Pulmonary surfactant is thought to form a multi-layered interfacial film into the air-liquid interface, where it is located to carry out its biophysical function: reduce surface tension to estabilize the respiratory surface along breathing dynamics. Nevertheless, not much is known about the structure of newly synthesized lung surfactant as it is assembled into lamellar bodies. Lamellar bodies (LBs) are the specialized organelles in which these complexes are stored before being secreted and adsorbed into the air-liquid interface. Understanding how lipids and proteins are organized has been quite difficult because of the absence of a native model of freshly synthesized pulmonary surfactant. Therefore, synthesis, packing, secretion, and reorganization of LBs to form the multi-layered interfacial film, are still poorly understood processes in terms of lipid-protein interactions and biophysical properties. Here, we describe a new approach to obtain a surfactant with similar properties to pristine pulmonary surfactant: human amniotic fluid lung surfactant (AFS). AFS is a highly structured, dehydrated and organised lipid-protein complex suggesting LB-like structural features. Biophysical behaviour analysed using a captive bubble surfactometer shows an efficient interfacial adsorption and spreading. Moreover, AFS is a dynamic-sensitive surface active material, which is able to reduce surface tension during fast compression-expansion cycles even though it does not produce the same low surface tensions throughout slow compression-expansion cycles. Furthermore, AFS is more resistant to serum or meconium inhibition than surfactant obtained from

bronchoalveolar lavages of porcine lungs used commonly as reference. This research opens new opportunities to investigate lipid-protein interactions and the supramolecular structure of membranes in the lung surfactant system, as well as the development of new surfactant preparations of potential therapeutic application.

Surfactant protein SP-C and cholesterol modulate phase segregation in lung surfactant membranes

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Breathing is sustained by the presence of lung surfactant (LS), a surface active agent lining the alveolar air-liquid interface. This lipid-protein complex develops a complex meshwork of lipid assemblies that reduce surface tension, and so allows lungs to compress and expand normally with minimal energy requirements. Cholesterol is the major neutral lipid in surfactant, but its precise role is a matter of debate. In some instances, cholesterol presence can impair surfactant function, an effect that can be reversed by the presence of the lipopeptide SP-C, the smallest and most hydrophobic protein present in LS complexes. In this work, the combined effect of SP-C and cholesterol on LS-mimicking membranes has been assessed by deuterium Nuclear Magnetic Resonance (2H-NMR) and Electron Spin Resonance (ESR). Our results show that SP-C induced phase segregation at physiological temperatures leading to the generation of a highly ordered, possibly interdigitated, phase and a liquid crystalline phase. All the lipids forming part of the LS model system, DPPC, POPC and POPG, were affected by SP-C-induced phase segregation to different extents, revealing SP-C specificity for certain lipid environments. The combination of SP-C and cholesterol also resulted in an ordering effect, but evidence of phase segregation was only observed at 30°C. The use of deuterated cholesterol did not show signs of any specific lipid-protein interactions, whereas SP-C palmitoylation appeared as an important factor maximizing the differences among lipids forming part of every phase. These results illustrate how the complex surfactant structure can be finely tuned by a balanced action of cholesterol and SP-C, which display combined effects regulating surfactant structure and dynamics.

The Effects of Pterostilbene and Resveratrol on The Biomechanic, Biochemical and Histological Parameters in Streptozotocin-Induced Diabetic Rats Gastrocnemius Muscle

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INTRODUCTION: Diabetes Mellitus (DM) is believed to have negative effects such as skeletal muscle atrophy, lower muscle mass, weakness and reduced physical capacity. A great number of studies have reported that antioxidant resveratrol and pterostilbene treatments could enhance the various metabolic disorders associated with diabetes. The aim of this study is to investigate the comparative effects of pterostilbene (PTS) (trans-3,5-dimethoxy-4l-hydroxystilbene) and resveratrol (RSV) (trans-3,5, 4l-trihydroxystilbene) applied at different doses in the treatment of streptozotocin (STZ)-induced diabetic myopathy. MATERIALS AND METHODS: Eighty rats of Wistar albino species were used. The animals were divided into eight groups (n = 10): control (non-diabetic); diabetic (DM); DM+10 mg/kg PTS; DM+20 mg/kg PTS; DM+40 mg/kg PTS; DM+10 mg/kg RSV; DM+20 mg/kg RSV and DM+(10+10) mg/kg PTS/RSV combination. At the end of the 5-week experimental period, the right gastrocnemius muscles of the rats were examined biomechanically, while the left ones were examined histologically. In addition, blood glucose, serum insulin and malondialdehyde levels were analyzed in blood samples taken from rats. FINDINGS AND RESULT: The skeletal muscle isometric contraction forces shown a decrease with diabetes were observed to have increased more with PTS antioxidant applications as compared to RSV applications. Blood glucose, serum insulin and malondialdehyde levels in diabetic rats were found to be closer to normal level with PTS applications. When the skeletal muscle electron microscopic images of diabetic rats treated with antioxidants were examined, Those in the mix treatment group were observed to showing better healing in Type-1 DM compared to the other diabetic and treatment groups. We suggest that PTS antioxidant treatments could be a better therapeutic nutraceutical alternative in skeletal muscle diseases coexisted with diabetes compared to RSV treatments.

Synergistic effect of membrane-active peptides SP-A and SP-BN on multidrug-resistant Klebsiella pneumoniae

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The emergence of multi-resistant strains of the respiratory pathogen *Klebsiella pneumoniae* underlines the need to implement new non-antibiotic therapies, including the characterization of natural antibacterial proteins as a paradigm for endogenous defense pathways. We have previously shown that lung surfactant protein (SP-A), acting synergistically with the lung anti-microbial peptide SP-B^N, enhances capsulated *K. pneumoniae* K2 clearance *in vivo*, in part by contributing to bacterial killing. However, the factors that govern SP-A/SP-B^N anti-microbial activity are still unclear. The

aim of this work was to study the mechanism by which SP-A and SP-B^N exert a synergistic microbicidal activity against capsulated *K. pneumoniae*, which is otherwise resistant to either protein alone. Our results indicate that the SP-A/SP-B^N complex, but not the individual proteins, alters the bacterial ultrastructure, forming pores in the membrane that favor the translocation of both proteins to the periplasmic space, where they interact with the inner membrane, inducing its depolarization and fission into small vesicles. *In vitro*studies with model membranes, which mimicked the internal and external bacterial membranes, showed that both SP-A and SP-B^N bound to lipopolysaccharide molecules present in the outer membrane, inducing lipid phase separation and disrupting membrane packing. This effect was stronger for the SP-A/SP-B^N complex, which rendered both the outer and inner bacterial membranes leaky as determined by permeabilization studies. Finally, the SP-A/SP-B^N-induced fission of the inner membrane may be related to the promotion of positive curvature observed by differential scanning calorimetry. Taken together, our results indicate that the antimicrobial activity of the SP-A/SP-B^N complex is related to its capability to alter the integrity of outer and inner bacterial membranes.

Ultrastructure of COPII vesicle formation

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Inside the cell, transport of proteins and lipids between organelles is mediated by membrane carriers known as vesicles. The formation of these vesicles requires the participation of coat proteins which are capable to change the shape of membranes into extremely curved structures, ultimately catalyzing the scission and release of a free vesicle to the cytoplasm. Vesicular transport from the endoplasmic reticulum (ER) to the Golgi apparatus is mediated by Coated Protein complex II (COPII). How this machinery organizes to overcome energy barriers such as membrane bending and protein packing remains poorly understood. Using *S. cerevisiae*, our goal is to study the ultrastructure of both the ER exit sites and vesicle formation process. We use high resolution microscopy methods, such as correlative light and electron tomography to observe membrane shape and localization of COPII vesicles with high spatial resolution.

Interaction of diethylstilbestrol with model membranes and its location in the membrane bilayer

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Diethylstilbestrol (DES) is a synthetic form related to estrogens that can be localized in membranes, with antiestrogenic and antiadrogenic effects., used to treat some conditions, although its mechanism of action is not well understood. Our study indicated that DES tends to disorder the membrane, especially in the polar heads of phospholipids. Using DSC, we observed a decrease in the main transition ΔH and a shift toward lower temperatures of the onset of these transitions. SAXD diffractograms showed that DES tends to increase the thickness of the lipid bilayer of DMPC (MLV), whereas WAXD diffractograms illustrated the existing differences between the lipid phases of the different samples, according to the concentration of DES, which corroborates the data obtained by DSC; with higher concentrations of compound shifting the transition temperature (T_c) towards lower temperatures. ^{31}P-NMR spectroscopy showed different anisotropic spectra of DEPE and DMPC samples depending on the different molar ratios of DMPC/DES used. For samples with DEPE, we obtained a spectrum with two peaks, likely due to the existence of two lipid phases; bilayer phase and H_{II} phase. For DMPC spectra, as the temperature increases there is an increase in intensity of the center region of the spectrum. In the case of samples with DES, the same thing occurs. But comparing with the signals obtained for samples with pure DMPC, it was observed that increasing concentrations of DES caused an increase in the spectrum width. Regarding CSA values; the higher the concentration of DES was, the greater was the decrease in the values of CSA produced, indicating a certain disorder in the polar group of the lipid molecules in the presence of DES. Using ^{1}H-NOESY-NMR-MAS technique, different rates for different proton cross relaxation of DES were calculated. From this information, it was possible to deduce the location of DES in the POPC membrane, that it is most likely anchored to the lipid/water interface.

15 P3 - Protein Folding, Misfolding and Stability

Novel Scenarios of Protein Unfolding in the Presence of an External Force: Experiments and Simulations

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Single molecule force spectroscopy studies of protein folding dynamics have yielded unprecedented insight into the mechanisms of the folding reaction. Still, they usually allow for a very limited description of the process of interest (e.g. based on the reaction coordinate that is probed experimentally). Molecular simulations –even using very simplified models– can give a much-enriched view of the underlying molecular events. I will explain the observations on a prototypical two-state folding protein, the cold shock protein, that in the AFM turns out to exhibit multiple intermediate states [1]. Using a very simple topology based model for the protein we show that in fact, this should not come as a surprise. Indeed, force can selectively stabilize different protein intermediates and act in a very different way compared to that of chemical denaturants[2].

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Multivalent cross-linking of actin filaments and microtubules through the microtubule-associated protein Tau

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Microtubule-associated proteins regulate microtubule dynamics, bundle actin filaments and cross-link actin filaments with microtubules. In addition, aberrant interaction of the microtubule-associated protein Tau with filamentous actin is connected to synaptic impairment in Alzheimer's disease. Here we provide insight into the nature of interaction between actin filaments and microtubule-associated proteins and the actin/microtubule crosstalk. We show that Tau uses several short helical segments to bind in a dynamic, multivalent process to the hydrophobic pocket between subdomains 1 and 3 of actin. Although a single Tau helix is sufficient to bind to filamentous actin, at least two, flexibly linked α-helices are required for actin bundling. In agreement with a structural model of Tau repeat sequences in complex with actin filaments, phosphorylation at serine 262 attenuates binding of Tau to filamentous actin. Taken together the data demonstrate that bundling of filamentous actin and cross-linking of the cellular cytoskeleton depend on the metamorphic and multivalent nature of microtubule-associated proteins.

From single-molecule protein unfolding to protein degradation by the proteasome

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Biological nanopores are frequently found within many biological structures involved in protein folding/unfolding. In the case of the proteasome, protein degradation requires unfolding and translocation of protein substrates through a narrow pore into the internal catalytic chamber. How protein stability relates to protein degradation remains an unsolved question. Single molecule approaches using protein nanopores with dimensions comparable to the size of the proteasome have the potential to provide insight into the underlying physics of the process. Here we measure the kinetics and energy consumption of the bacterial proteasome ClpXP during degradation of a battery of mutants of SsrA-tagged thioredoxins. Single-molecule measurements of protein unfolding during translocation through the α-hemolysin pore were expected to show excellent correlation with protein degradation because both unfold proteins by pulling the polypeptide chain through nanometer scale pores. Our results show a lack of correlation between nanopore induced protein unfolding and ClpXP protein degradation. This suggests that the unfolding observed during protein degradation may be more complicated than a mere one-end pulling unfolding mechanism.

Effects of aggregating agents in protein misfolding. An infrared spectroscopy study

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Protein misfolding, which include the formation of amyloid aggregates, insoluble aggregates resistant to degradation, are related to a large number of different diseases, mostly neurodegenerative. In this work, hen egg white lysozyme has been used as model because it is a good characterized protein with ability to form this kind of aggregates if it is exposed to extreme conditions. Usually, the in vitro studies are done in a diluted medium, and the action of the protein at these concentrations differs from what happens inside the cell, mainly because the internal concentration is crowded by numerous macromolecules. The purpose of this research is to prove what occurs to a protein when it forms amyloid aggregates in a crowded medium by different agents (Dextran 40, Dextran 70 and Ficol 70) and at different crowder concentrations (5%, 10% and 20%). In order to characterize what happens with lysozyme when it is forced to form this kind of aggregates, the infrared spectroscopy has been used not only because it is a useful technique for this kind of studies, but also due to the fact that its analysis time is short. Moreover, little quantity of protein is needed and also this technique has a high sensitivity to β structures which characterize the amyloid fibers. As it is shown, the lysozyme aggregates are not formed in the same way in a diluted medium or in a crowded one because the fiber quantity formed decreases, and the kinetic formation differs. These changes arise from the different effect of the crowders at distinct concentrations.

Nanomechanical phenotypes in hypertrophic cardiomyopathy caused by missense mutations in cardiac myosin-binding protein C

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Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac muscle disease, with a 0.2% of global prevalence. This illness is characterized by left ventricular chamber reduction and left ventricular wall hypertrophy, together with hypercontractility and impaired diastole. Despite of its high prevalence, HCM still has no cure, as its heterogeneity difficults the identification of a single underlying pathogenic mechanism. We investigate the molecular mechanisms that induce HCM in the 10-20% of patients who carry missense mutations in MYBPC3, the gene coding for cardiac myosin-binding protein C (cMyBP-C). This is a sarcomeric protein that tethers the actin and the myosin filaments, braking their gliding and tuning muscle contraction. We have curated a database of missense variants in MYBPC3 according to their clinical presentation and studied potential drivers of the disease. Using bioinformatics tools, we found that most of the pathogenic variants are not predicted to induce gross changes in thermodynamical stability or RNA splicing. Hence, we hypothesized that pathogenic mutations can alter the mechanical properties of cMyBP-C, leading to reduce braking ability and thus to hypercontractility and HCM. To test this, we

have produced several variants of the C3 domain of cMyBP-C, a central domain of the protein without known protein interactors. As expected, most mutants retain close-to-wild-type structure and thermodynamic stability. However, some pathogenic mutants show altered mechanical stability or refolding behaviour, as determined by single-molecule atomic force microscopy experiments in force-clamp mode. This is the first time that pathogenic mutations occurring at C3 are linked to nanomechanical phenotypes, which could affect the tethering function of cMyBP-C and trigger the development of HCM.

16 P4 - Receptors, Channels Transporters and Transporters

IFN-γ Receptor Signaling is Critically Dependent on its Location in Lipid Nanodomains

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Interferon-γ (IFN-γ) is a cytokine that orchestrates many critical cell functions and signaling processes through transcriptional activation and regulation of a vast number of genes. Among others, INF-γ plays crucial roles in controlling host defense, immunopathological processes, and fighting tumors. IFNγ mediates its pleiotropic effects on cells binding to a receptor (IFNGR), a pre-assembled heterodimeric complex expressed on the membrane surface of a large variety of cells. However, understanding how membrane nanoscale organization controls transmembrane receptor signaling activity remains a challenge mainly due to a lack of accurate methodology. Here, we show that IFNGR localizes, in vivo, in lipid nanodomains and that IFN-γ addition induces a conformational change in IFNGR that leads to a specific IFNGR-sphingolipid interaction that could play a role as a docking site for receptor phosphorylation and JAK-STAT signaling cascade activation. Furthermore, the impact of these lipid-proteins interactions in receptor oligomerization by site-specific protein-protein interaction studies between the two subunits of the receptor has been tested. Finally, we described the impact of such proteo-lipid interactions in the modulation of the JAK-STAT signaling pathway using fluorescence microscopy techniques. These experiments of nature established the critical importance of dynamic interactions with lipid nanodomains in IFN-γR signaling. This work shed new light on the role of membrane protein-lipid interaction in the partitioning of transmembrane receptors into lipid nanodomains and how these types of interactions

control transmembrane receptor signaling in vivo.

Comparison of Contraction Parameters in Diabetic Aorta Exposed to 5 Hz or 15 Hz Frequency Pulsed Magnetic field

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Diabetes Mellitus (DM) is an autoimmune disease which is increasingly common in the population without sex difference. Literature were shown that low frequency pulsed magnetic field (PMF) applications induced angiogenesis and affected signal transduction, cellular processes, and specific genes expressions. The aim of our study was to compare the effects of 5 Hz or 15 Hz frequency pulsed magnetic field (5-PMF or 15-PMF) on contraction parameters of diabetic rat aorta rings.

In this study, rats were divided into four groups; Control (C), control-sham (SC), diabetes (D), diabetes-sham (SD). C and D groups were exposed to 15-PMF or 5-PMF system for eight weeks, 1 hour per day, 5 days a week. Subsequently, the SC and SD groups were also exposed to a non-current magnetic field for eight weeks, 1 hour per day, 5 days a week.

Blood glucose levels and weights of rats were measured once a week for eight weeks. At the end of the eight weeks, rats were blood from their hearts and were measured the levels of insulin, IL-6, IL-2, TNF-α and TGF-beta 1, then the aorta rings were isolated and contraction-relaxation parameters of aorta rings were measured.

In consideration of our data, there was no weight loss and blood glucose levels decreased in group C that was exposed to 15-PMF or 5-PMF for eight weeks. In group D, the weight loss rate and blood glucose levels caused by diabetes decreased ($p>0.05$). Also, the contraction forces of the aorta rings of the rats in the D groups affected by 15-PMF decreased, the percent rate of relaxation increased. Also, relaxation duration increased while the contraction duration decreased ($p>0.05$).

15-PMF applications may have beneficial effects on the biomechanical parameters of rat aorta preparations, that PMFs can be used for juvenile diabetic therapy, considering the intensity, frequency and exposure duration of PMF. Further research is needed to clarify the action mechanism of PMF and to confirm the clinical usability of PMF.

Caveolar Kv1.3 targeting for proper insulin signaling in adipocytes

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The voltage dependent potassium channel Kv1.3 participates in the peripheral insulin sensibility. Because the genetic ablation of Kv1.3 triggers a lean phenotype this protein has been suggested as a pharmacological target for obesity and the associated Type II diabetes. However, this role is under intense debate because the Kv1.3 expression in the adipose tissue raises controversy. We demonstrated that Kv1.3 mRNA, protein and activity are expressed in white adipose tissue from humans and rodents. Moreover, other channels such as Kv1.1, Kv1.2, Kv1.4 and Kv1.5 from the same Shaker family are also present. Although the Kv phenotype during adipogenesis and upon insulin stimulation is remodeled, Kv1.3 is still participating in the insulin-dependent regulation of glucose uptake in mature adipocytes. Adipocyte differentiation increases the expression of Kv1.3 which targets to caveolae by molecular interactions with caveolin 1. By using a caveolin 1 deficient 3T3-L1 adipocyte cell line we demonstrated that the localizacion of Kv1.3 in caveolar raft structures is important for a proper insulin signaling. Insulin phosphorylates the channel being at the onset of the insulin-dependent signaling. However, when Kv1.3 is spatially out of these lipid microdomains exhibited an impaired phosphorylation. Our data bring light to the putative role of Kv1.3 in the weight gain and insulin-dependent responses and points to this channel as a putative target for obesity and related disorders.

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Anterograde traffic of the Kv7.1/KCNE1 complex

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The voltage-gated potassium channel Kv7.1 associates with the KCNE1 β-subunit to form the slowly activating delayed rectifying potassium current, IKs, which modulates the repolarization of cardiac action potential. Mutations in both subunits lead to severe cardiac channelopathies, such as long QT syndrome. Despite the functional effect of KCNE1 onto Kv7.1 and the subsequent specific interaction domains have been widely described, there is still an intense debate about where the assembly of the complex takes place. We demonstrate that Kv7.1-KCNE1 complex is not yet built within the initial stages of the secretory pathway, at the endoplasmic reticulum. Our results prove that both channel subunits can use different routes to reach the cell surface. While KCNE1 relays on COPII-dependent forward trafficking machinery, Kv7.1 can alternatively use a non-conventional secretory pathway that skips the Golgi apparatus. Once associated, Kv7.1 redirects KCNE1 to a COPII-independent route to the membrane surface. Finally, studies in plasma membrane lawn preparations demonstrate that the functional complex is fully assembled at the membrane surface, suggesting that Kv7.1-KCNE1 association takes place in an alternative route late in the secretory pathway.

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Study of access resistance in bacterial channels

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Access resistance (AR) is a well known concept in the nanopore field that accounts for the resistance of the medium outside the pore and is related to the concentration polarization in the vicinity of a nanochannel. Here we show that it may be a crucial component of the measured conductance of ion channels under physiological concentrations. We present a new method of measuring AR. It is based on single channel measurements of conductance in electrolyte solutions containing varying concentrations of a high molecular weight neutral polymer (Polyethylene Glycol) sterically excluded from the pore. With the aid of a simplified theoretical model we have split the measurable conductance into two contributions: one coming from the pore itself and the contribution coming from the access resistance. Although AR is only a moderate contributor to the total resistance in concentrated solutions regardless the membrane charge, it is essential to account for it at low salt concentration in neutral membranes. In diluted solutions the channel charges could pass functionally unnoticed if AR is ignored. Furthermore, our measurements show that lipid charge decreases the AR, thus confirming earlier numerical predictions. Biological channels perform their physiological function in the cellular environment, which has two common features: low ionic strength and macromolecular crowding. Both factors make the AR contribution to channel conductance important. Therefore our findings are relevant to in vivo conductive properties of protein channels.

A 3D Poisson-Nernst-Planck study of the fluctuation driven transport in the OmpF channel

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In this work, the transport through the OmpF ion channel under electric potential fluctuations is analyzed numerically. Our goal is to determine under which conditions it is possible to obtain ion transport against its concentration gradient. To achieve this we use a model based on the 3D Poisson-Nernst-Planck transport equations, which allows us to obtain the flow of each ion under different conditions of concentration and applied potential, from the known three-dimensional structure of the OmpF pore (2OMF PDB access code).

Assuming that the system response under fluctuating potentials is determined by the I/V curve for each ionic species, it is possible to obtain the average current through the system. The results show that particularly high potentials would be required to achieve ion transport against its concentration gradient because of the low channel selectivity of OmpF and the relative inefficiency of the characteristic fluctuating signals of membrane cells [1]. We additionally compare the numerical results to experimental data to validate our approach [2].

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17 P5 - Supramolecular Complexes

Spectroscopic characterization of biomimetic light harvesting complexes

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Photosynthesis is the main process by which living beings can take energy from sunlight transforming it into chemical energy. Light harvesting complexes are formed by proteins with chlorophyll molecules inside [1], which capture the light and transfer the excitation to a reaction center with near-perfect quantum efficiency. This fact makes them candidates for development of new renewable energy technologies. Two-dimensional electron spectroscopy techniques have demonstrated in recent years that this high efficiency is due to a quantum coherence energy transfer [2]. Here, we propose the development of simple biomimetic light harvesting complexes assembling chlorophyll molecules as models for spectroscopic studies of this quantum phenomenon, that help us to understand the complexity of the photosynthetic process and its use in the development of new technologies.

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Mechanistic basis for the recognition of a misfolded protein by the molecular chaperone Hsp90

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The critical toxic species in over 40 human diseases are misfolded proteins. Their interaction with molecular chaperones is essential for blocking disease progression, because chaperones select misfolded proteins for refolding or elimination. A key element of the proteostasis network is Hsp90, which preferentially interacts with metastable proteins. Here we determined by NMR spectroscopy the three-dimensional structure of the misfolded cytotoxic monomer of the amyloidogenic protein transthyretin, which is characterized by the release of the C-terminal β-strand and perturbations of the A-B loop. Using a combination of NMR methods optimized for macromolecular machines and small-angle X-ray scattering we show that the transthyretin monomer but not the wild-type protein binds to Hsp90. In the bound state, the Hsp90 dimer predominantly populates an open conformation and transthyretin retains its globular structure. The interaction surface for the transthyretin monomer comprises the N-terminal and the middle domain of Hsp90 and overlaps with that of Alzheimer-related protein Tau. In contrast, Hsp90-clients, whose function depends on the interaction with Hsp90, bind to other Hsp90 regions and cause structural consequences in Hsp90 that are different from those induced by amyloidogenic proteins, indicating that Hsp90 can employ distinct mechanisms for regulating protein misfolding and physiological function.

Self Assembled Designed Proteins For The Organization Of Gold Nanomaterials

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Biotechnology is a powerful tool to develop a wide variety of biomaterials with the added value of new functionalities, which can be achieved using a rational design approach. The bottom-up construction of complex and designed nanostructures allows controlling the organization of different elements based in the principles of modular self-assembly. Repeat proteins are an excellent option as basic construction components for these biomaterials due to their modular assembly properties and their large potential for functionalization with the addition of different reactivities[1]. In particular, we use consensus tetratricopeptide repeat (CTPR) proteins, based on 34 amino acids that form a TPR motif with a helix-turn-helix secondary structure. These proteins are easy to design and can be combined in a modular way in order to build a certain desired structure[2]. In our approach, we use CTPR proteins for templating two different gold nanomaterials: gold nanoparticles (AuNPs) and gold nanorods. This allows us to build conductive structures by organizing their components at nanoscale level in a controlled way, for their uses in nanoelectronics and plasmonics[3]. The main goal of this work is to demonstrate that CTPR proteins can be used as scaffolds for patterning gold elements and that they can provide not only precise order at the nanoscale but also new properties to the gold nanomaterials, such as chirality, which are intrinsic features of proteins.

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Determination of the effects of chronic steroid usage on sugammadex reversal by evaluating biophysical parameters

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Background: Sugammadex is a novel developed reversal agent which encapsulates non-depolarizing steroidal muscle relaxants (e.g. rocuronium) and removes their neuromuscular blocking effects rapidly and effectively. Previously in vitro study showed that dexamethasone (Dex), a steroid, reduced sugammadex-rocuronium efficiency dose-dependently since sugammadex interacts with steroidal structures. However, there was no available data about the effects of long-term steroid usage on sugammadex reversal. The aim of the present study was to determine whether chronic usage of steroids (Dex and Prednisolone (Pred)) affect the sugammadex reversal efficiency on rocuronium induced neuromuscular blockade by evaluating contraction parameters of diaphragm muscle. Methods: Animals (rats) were divided into three groups and injected daily intraperitoneally with; I- Saline (600 µg/kg or 10 mg/kg; as control) II-Dex (600 µg/kg), III- Pred (10 mg/kg) during 14 days. After the completion of injections, rats were decapitalized. Isolated phrenic nerve-hemidiaphragm muscles were mounted by silver electrodes to an organ bath containing Krebs solution. After preparations were allowed to stabilize for 60 min, isometric twitch tensions were measured for muscles and nerves at 1 Hz, for 0.2 ms and 0.05 ms stimulations, respectively. Measurements were repeated with addition of rocuronium (10 µmol/L) and sugammadex (30 µmol/L) to solution. Results: Rocuronium-induced depressed twitch tensions were recovered by sugammadex, with % 81,5 and % 89,8 efficiency in chronic dex and pred group, respectively. When nerve was stimulated, efficacy of sugammadex reversal were obtained as % 96,9 and % 88,9 for dex and pred group, respectively (p>0,05). Conclusion: Our results demonstrated that chronic usage of steroids does not significantly affect the efficacy of sugammadex reversal and may be considered as a negligible factor in effectiveness of sugammadex-rocuronium interaction.

Cytochrome *c* regulates SET-mediated acetylation of the C-terminal domain of p53

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The C-terminal domain (CTD) of p53 is a target for acetylation; which can be negatively regulated by the oncoprotein SET1. The acidic-domain-containing SET binds the CTD of p53 primarily through electrostatic interactions. However, during cell stress CTD-acetylation displaces SET by masking CTD lysine residues. What's more, this mechanism of acetylation-mediated regulation corresponds to a conserved mode of post-translational protein interaction control. In a recent study, we showed that the haemprotein Cytochrome c (Cc) interacts with SET (KD ca. 3.1 μM) during

DNA damage2. Thus, Cc may modulate the SET/p53-CTD interaction under stress conditions. Nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC) experiments were performed to determine the nature of such interaction. ITC studies confirmed un-acetylated (KD ca. 3 μM) but not acetylated p53-CTD binds SET. Next, 1D 1H NMR - tracking Cc Met80-εCH3 – was used to assess the competition between p53-CTD and Cc for SET. Increasing concentrations of p53-CTD led to dissociation of Cc from SET and recovery of the Met80-εCH3 signal, indicating p53-CTD is able to compete with Cc for SET binding. 1D 19F NMR was then used to monitor a 19F-Phe-CTD p53 peptide. Increasing concentrations of Cc caused the signal corresponding to 19F-Phe-CTD to be partially recovered (but not fully due to conformational exchange in the peptide). These data suggested competition between p53-CTD and Cc for SET binding. Further studies will seek to better characterise p53-CTD dynamics in relation to SET binding as well as corroborating biophysical data obtained with whole-cell assays.

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Protein Hydrogels to Examine Emergent Mechanical Properties of Striated Muscle

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Striated muscle is a natural biomaterial with remarkable properties: it is strong, elastic and capable of self-healing. Proper function of striated muscle depends on its mechanical properties. Single-molecule approaches have shown that the passive elasticity of muscle is determined by the mechanics of specific cytoskeletal proteins in cardiomyocytes, such as titin. Due to the complexity of muscle architecture, these characteristics have proven tough to recapitulate, and there are no models that can predict macroscopic mechanic behaviour directly from the mechanical properties of constituent proteins. Therefore, it remains a challenge to synthesize materials that possess the properties of biological muscles. In this work, we propose a bottom-up approach to produce protein hydrogels that recapitulate the basic passive elastic properties of muscle. This approach will allow to examine direct correlations between the mechanical properties of the constituent proteins and those of the resulting hydrogels. To generate the hydrogels, we use engineered polyproteins based on titin domains whose mechanical properties can be probed by single-molecule Atomic Force Microscopy. Proteins are subsequently crosslinked through photocatalytic tyrosine oxidation. Hydrogel stiffness is measured using a custom-built stretching device. Working with single-point mutants of the constituent polyproteins, we will be able to determine how changes in the nanomechanical properties of the protein building blocks translate into macroscopic changes in hydrogel stiffness. Thanks to this strategy, it will be possible to translate the mechanical properties of proteins to higher order arrangements. In addition, these protein hydrogels are an initial step in the establishment of versatile biomaterials that mimic the mechanical properties of muscle, with promising potential in tissue engineering.

18 P6 - Cellular Biophysics

Mechanical softening of lipid membranes by the rotating motor protein F1F0 ATP synthase

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ATP synthase is a rotating membrane protein that synthesizes adenosine triphosphate (ATP), the chemical energy source of the cell. To unveil the mechanical impact of this molecular motor protein on the bending properties of its lipid environment, we have functionally reconstituted the ATP synthase in giant unilamellar vesicles and tracked the membrane fluctuations by means of flickering spectroscopy. We find that ATP synthase rotates at a frequency of about 20 Hz, promoting large out-of-equilibrium deformations at discrete hot-spots in lipid vesicles and thus inducing an overall membrane softening. Therefore, the rotation of ATP synthases promote mechanically adapted membranes with a high bending compliance and able to support high local curvatures. Our results evidence a mechanical functionality of the ATP synthase for biomembrane re-structuring and shaping.

Iron limitation promotes partial inhibition of the photosynthetic electron transport in the diatom Phaeodactylum tricornutum

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Iron limitation is the major factor controlling phytoplankton growth in vast regions of the oceans. We have used thermoluminescence (TL), chlorophyll fluorescence and P700 absorbance measurements to elucidate the effects of iron deficiency in the photosynthetic electron transport of the marine diatom *Phaeodactylum tricornutum*. First, the effects of iron limitation on photosystem II (PSII) activity were determined by TL and chlorophyll fluorescence measurements. Excitation of iron-replete *P. tricornutum* cells with single turn-over flashes induced the appearance of TL glow curves, with two components with different peaks of temperature and contributions to the total signal intensity: the B band (23°C, 63%), and the AG band (40°C, 37%). Iron limitation did not significantly alter these bands, but induced a decrease of the total TL signal. Far-red excitation did not increase the amount of the AG band in iron-limited cells, as observed for iron-replete cells. Measurements of Chl *a* fluorescence and steady-state light curves showed lower values of F_v/F_m (maximum quantum yield of PSII), Y(II) (effective quantum yield of PSII), and rETR (relative electron transport rates) in Fe-deficient cultures as compared with Fe-replete conditions at all irradiance levels tested. The effect of iron deficiency on the photosystem I (PSI) activity was also examined by measuring the changes in P700 redox state during illumination. The electron donation to PSI was substantially reduced in iron-deficient cells, probably related with the important decline in the content of the soluble cytochrome c_6 carrier observed in these cells. Our results suggest that iron deficiency induces partial blocking of the electron transfer between PSII and PSI, due to a lower concentration of cytochrome c_6. This decreased electron transfer could induce the over-reduction of the plastoquinone pool and, consequently, the appearance of acceptor side photoinhibition in PSII, even at low light intensities.

Efficient transport and delivery of mfn1 by Gemini/DOPE nanovehicles in mfn1-knockout fibroblasts

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Mitochondria form a highly dynamic network of organelles which constantly fuse and divide.[1] The balance between these antagonistic processes of fusion and fission is extremely important for normal cellular function.[1],[2] In mammalian cells, there are three central players involved in the mitochondrial fusion: mitofusin 1 and mitofusin 2 (Mfn1/Mfn2) (outer mitochondrial membrane fusion) and OPA1 (inner mitochondrial membrane fusion). Deletion of either the MFN1 or the MFN2 gene in mouse embryonic fibroblasts (MEFs) leads to fragmented mitochondria due to a lack of mitochondrial fusion. More importantly, mutations of any of these putative functional domains impair mitochondrial fusion and lead to mitochondrial diseases (MD), to which there is no cure.[3] Here, we have conceived lipoplexes as efficient therapeutic agents against MD. Lipoplexes consist on

a lipid/DNA highly packed complex that transport and efficiently deliver DNA into the cytoplasm. We have tested different mixed lipoplexes made of Gemini/DOPE[4] and a p-MFN1 plasmid coding for the Mitofusin1 protein in eukaryotic cells. Our results show that lipoplexes recover the normal mitochondrial dynamics phenotype in MFN1-Knockout MEFs. Moreover, we show a good viability and high transfection efficiencies as compared with other canonical transfer agents.

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Selective inhibition of carotid body oxigen sensing by genetic MCI disruption. Effect of NAD+ regeneration

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The carotid body (CB) is essential for systemic acute O_2 sensing. The CB contains O_2 sensitive glomus cells, which have O_2-regulated K^+ channels that mediate transmitter release during hypoxia to elicit compensatory cardiorespiratory reflexes. How variations in O_2 tension (PO_2) are detected and the mechanisms whereby these changes are conveyed to membrane ion channels have remained unknown. We have recently reported the involvement of mitochondrial complex I (MCI) in acute O_2 sensing (Fernández-Agüera et al. Cell Metab 2015). Knockout mice lacking Ndufs2 (a MCI subunit required for ubiquinone binding) in catecholaminergic cells (TH-NDUFS2 mice) lost the hypoxic ventilatory response (HVR). Hypoxia-induced cellular responses (increase in NADH and reactive oxygen species (ROS), inhibition of K^+ channels, and increase in cytosolic Ca^{2+}) were also selectively abolished in Ndufs2-deficient glomus cell. Glomus cells from TH-NDUFS2 mice showed accumulation of NADH and a more oxidized state relative to control cells. To determine whether responsiveness to hypoxia result from a general metabolic disarrangement or a direct consequence of MCI dysfunction, we generated a conditional mouse model (ESR-NDUFS2), in which the *Ndufs2* gene was ablated during adulthood. In these mice the loss of the HVR occurred in parallel to the decrease in MCI activity. Glomus cells from ESR-NDUFS2 mice showed normal electrophysiology. Their basal NADH levels and redox state were close to those of wild type mice. However, Ndufs2-deficient glomus cells were unresponsive to hypoxia, although they were activated by hypercapnia and high potassium. The lack of responsiveness to hypoxia was maintained in Ndufs2-deficient cells treated with α-ketobutyrate, an agent that consumes NADH to regenerate NAD^+. These data support the notion that NADH and ROS produced in MCI mediates acute responsiveness to hypoxia in glomus cells.

Identifying synaptic molecular components that contribute to motor nerve terminals vulnerability in a mouse model of Spinal Muscular Atrophy

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Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease characterized by loss of spinal cord motoneurons, muscle weakness, and paralysis. It is caused by defects in the Survival of Motoneuron 1 gene (*SMN1*). In SMA mouse models, neurotransmitter release is severely impaired, but its cause is unknown. We investigated the molecular mechanisms potentially responsible for this functional deficit, and the molecular basis of the selective muscle vulnerability observed in SMA. We combined electrophysiological experiments with confocal microscopy to investigate the calcium dependence of neurotransmitter release and the expression of distinct synaptic proteins that play a key role in neurotransmission. These experiments were done in control and SMA (SMNΔ7) muscles affected in different degree. Our results show that SMNΔ7 terminals present a reduction in P/Q-type voltage-dependent Ca^{2+} channels density, without alteration in the Ca^{2+} cooperativity and sensitivity of the secretory machinery. Besides this, SMN-deficient terminals present a low release probability (p_r), and are unable to normally increase the number of functional release sites (n) upon high frequency stimulation, even when vesicles were available. Additionally, we found that highly vulnerable muscles show a large reduction of synaptotagmin-2 (Syt2) and synaptic vesicle protein 2 (SV2) B, while other synaptic proteins, such as syntaxin-1B and synaptotagmin-7, were not decreased. The results also show that synaptotagmin-1 (Syt1) undergoes a process of physiological downregulation during the postnatal synaptic maturation period. This process occurs earlier in more vulnerable muscles. In conclusion, we propose that the reduction of Syt2 and SV2B are key factors for the synaptic dysfunction in SMA and that the rate at which the physiological downregulation of Syt1 occurs is important to muscle vulnerability in the disease.

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Nifedipine enhances neurotransmitter release in a mouse model of Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA), an autosomal recessive neurodegenerative disease, is caused by mutations in or loss of the Survival of Motor Neuron (*SMN1*) gene that reduces the level of the Survival of Motor Neuron protein. It is characterized by degeneration of spinal cord α-motoneurons, resulting in weakness and muscle atrophy. Furthermore, the quantal content at the neuromuscular junction (NMJ) is reduced about 50% in the SMA (SMNΔ7) mouse model. We investigated the ability to modulate synaptic release in SMA motor nerve terminals by nifedipine (50 μM), a L-type voltage-dependent calcium channels antagonist that enhances neurotransmission in central synapses, as well as NMJ. Likewise, we studied its effect on the parameters that determine the quantum content: the probability of release(p_r) and the number of release sites(n). To this end,

we performed electrophysiological intracellular recordings in the *Transversus abdominis* muscle in control and SMNΔ7 mice.

We found that nifedipine significantly increased the amplitude of the evoked end-plate potentials at low-frequency of stimulation (0.5 Hz) in both genotypes. In contrast, the amplitude of the spontaneous release was not affected although its frequency was increased. Quantal content was significantly increased after the application of the drug. Additionally, *p_r significantly increased in the control terminals (≈170%), and n* in the mutants (≈300%). At high-frequency of stimulation (20 Hz), nifedipine produced a great facilitation during the first phase of the train and a significant increase in the total number of released quanta in controls. However, the effect was much lower in mutants. We are now studying the mechanism by which nifedipine enhances neurotransmission. Thus, we propose that neurotransmitter release in SMA terminals can be positively modulated through an increase in the number of active release sites after application of nifedipine.

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Calcium homeostasis in motor nerve terminals of Smn-deficient mice

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Calcium ions play a key role in the regulation of neurotransmission. One of the most important organelles in charge of cytosolic calcium clearance in motor nerve terminals is the mitochondria. Spinal muscular atrophy (SMA) is a genetic disease caused by loss or mutations of the Survival of Motor Neuron (SMN1) gene, characterized by motor impairment of axial and proximal limb muscles. In mouse models of the disease, synchronous neurotransmitter release is half reduced while asynchronous release increases by 300%. Moreover, studies of the mitochondria shown alterations both at the structural and metabolic level. Together, these data suggest possible alterations in the calcium homeostasis of motor nerve terminals in SMA. In this work, we used an SMA mouse model, which severity can be controlled by changing the number of copies of the SMN2 transgene, representing different degrees of the disease (SMA type 1 and 2). We used a fluorescent calcium indicator (Rhod 2-AM) and live imaging to record the changes in mitochondrial calcium upon electrical stimulation in nerve terminals at different postnatal ages (P7-P14). In controls, we found that the calcium signal rises very fast until reaching a plateau. After stimulation, the decrease in calcium is slow (half decay time, one minute). In SMA mutants, we found an apparent correlation between the mouse phenotype and the amplitude of the mitochondria calcium signals, with almost normal parameters in less affected mice. For example, in SMA type 2 mice, at P7, the kinetics of the calcium signals are not altered, although basal fluorescence showed a tendency of being higher than in control animals. We also examined the number of active calcium spots and their mean area per nerve terminal, finding no differences between controls and SMA type 2 mice.

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Estructural analysis of SMN granules in motor axons and presynaptic nerve terminals

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Spinal Muscular Atrophy (SMA) is a neurodegenerative disease characterized by the loss of spinal cord α-motoneurons, muscle weakness and progressive paralysis. SMN, the defective protein in the disease, participates in mRNA metabolism through snRNPs and mRNPs assembly. In addition, SMN regulates mRNP axonal localization in motoneurons, and fast axonal transport of SMN granules has been observed in cell culture. To get a deeper insight into the axonal and presynaptic role of SMN, we investigated the distribution and properties of axonal and presynaptic SMN granules and their association with the cytoskeleton in control and SMA mouse models. Specifically, we are exploring the association of SMN granules with NFs, and MAP1B, a microtubule associated protein involved in neural development. The study was performed at different stages of postnatal maturation, in FVB mice and two types of SMA mouse models, which express full-length and truncated SMN proteins in different amounts. SMN expression was studied by quantitative confocal microscopy immunofluorescence. We quantified SMN granules in both motor axons and nerve terminals, and determine their relationship with the cytoskeletal elements. In addition, we found that SMN granules are down-regulated in an age-dependent manner in these two compartment, supporting a role for SMN granules in the NMJ maturation.

Biophysical Evaluation of Phosphatidylglicerol and Cardiolipin Systems to Predict Peptide Antimicrobial Activity

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Staphylococcus aureus is considered one of the most dangerous bacteria for humans due to its ability to penetrate the epithelial barriers producing sepsis, osteomyelitis, pulmonary, skin and soft tissue infections [1]. It is also the most common cause of postoperative wound infections. The World Health Organization (WHO) report of 2014 emphasizes that over the past decade, was significant the number of cases of methicillin-resistant *Staphylococcus aureus* (MRSA) patients in hospitals and some to vancomycin are now recognized [2]. Antimicrobial peptides (AMPs) are essential components of the innate immunity of several organisms, they have been isolated from animals, plants, fungi, and bacteria. AMPs are considered promising alternatives to conventional antibiotics [3]. Based on the need to evaluate new potential therapeutic agents to combat the antimicrobial resistance, we evaluate four different systems built of phosphatydilglycerol and cardiolipin, the principal lipids present in the *S. aureus* membrane. Lipid membrane composition in different stages of bacterial growth affect the activity of the peptides. Calcein leakage experiments were performed to evaluate the disruption of Daptomycin, LL37 and DM2 peptides on the lipid bilayers. These results were compared with antimicrobial activity obtained by flow citometry experiments. We observed that increasing concentrations of cardiolipin reduced significantly the disrupting mechanism associated with the three membrane active peptides.

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Investigation of the effects of Pulsed Magnetic Field on Apoptosis at Rat Vascular Smooth Muscle Cells

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The therapeutic effects of pulsed magnetic field (PMF) application have been shown in many studies. Although the effects of PMF were investigated on some key players such as voltage-gated Ca^{2+} channels, its cellular action mechanism is not been fully understood. Furthermore, it is unclear how in vivo applied magnetic fields affect the vascular system with electrical signaling mechanism. Therefore, the primary question of this study was the understanding of the effect of PMF on apoptosis through cytosolic Ca^{2+} signals. In our study PMF applied to the rats in vivo for 30 days (40 Hz-1.5 mT/day). Then thoracic aorta of the animals removed and vascular smooth muscle cells (VSMCs) were isolated enzymatically. Primary cells were used for the experiment after 5-6 th day of isolation.

Effects of PMF were tested with an apoptosis inducer H_2O_2 (1 mM). H_2O_2 induced Ca^{2+} responses were measured in Fura 2-AM loaded cells with a CCDI camera system. Measurements were analyzed according to the fluorescence intensity increase. We observed that in PMF treated group 1 mM H_2O_2 application caused a statistically significant increase in the cytoplasmic Ca^{2+} responses according to the control group. We also checked caspase-3 activity of the VSMCs. We did not measure any difference between PMF treated vs. control group. However, H_2O_2 (1 mM, 1 hour) incubation increased caspase-3 activity in PMF group vs. control. These differences in Ca^{2+} responses and caspase-3 activities may reflect that PMF effect is not sufficient to induce apoptosis alone. However, more experiments needed for a full understanding.

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19 P7 - Biophysics of Carbohydrates and Nucleic Acids

Lateral Magnetic Tweezers

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Combining single-molecule techniques with fluorescence microscopy has attracted much interest because mechanical measurements can eventually be correlated with directly visualized DNA:protein interactions. Lateral Magnetic Tweezers (MT) is an advantageous technique because it permits applying an accurate constant force to several tethered DNA molecules tracked in parallel, while fluorescence on the surface can be monitored. In this work, we propose an implementation of a laterally pulling MT modulus and its subsequent calibration in commonly employed microfluidic devices such as flow cells and capillaries using micrometer size superparamagnetic beads. Resulting forces and consistency of results with the Worm Like Chain (WLC) model are discussed for the different scenarios. Finally, a method for estimating forces in flow-stretch experiments is proposed, and the results of the method are compared with those of lateral MT, confirming its validity and superior suitability for performing fluorescence experiments on DNA molecules stretched on a surface.

The folding complexity of TERRA G-quadruplexes unveiled at the single-molecule level

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Guanine-rich sequences, which can be found in telomeres and the regulatory regions of many genes in the human genome, have the potential to self-associate into flat guanine quartets, giving rise to four-stranded arrangements known as G-quadruplexes. The presence of these sequences in vivo, along with the fact that their unique conformation provides selective recognition sites for small molecules, have enabled G-quadruplexes as important drug-design targets for the treatment of various human disorders, including cancer. We and others previously studied single-molecule mechanical folding and unfolding processes of long human telomeric RNA (TERRA) [1,2]. Here, we report on the unfolding dynamics of short TERRA molecules by optical tweezers. Specifically, molecules with five repeats of the telomeric sequence GGGUUA, thus, capable of assembling into a maximum of one G-quadruplex. We find that the inherent capacity of single-stranded RNA to self-interact and fold into a condensate blocks the formation of the G-quadruplex with a significant probability. The unfolding dynamics observed in TERRA molecules with more telomeric repeats or with more random extra single-stranded RNA confirm this stochastic bistability with coherent conformational probability. In contrast, DNA analogue molecules did not show this condensation blockage. Therefore, the strong conformational competition exhibited by RNA telomeric sequences confers on these short molecules a unique bistable folding complexity that is not found in proteins, which normally fold into a unique conformation for fixed external conditions. This knowlegde is important to understand G-quadruplex-binding drugs and the mechanical activity of the telomerase.

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Understanding the molecular basis of damaged DNA recognition by the protein MutS

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For any living organism it is of vital importance to safeguard the code stored in its DNA, where the instructions for making life possible are written. But damage occurs constitutively, as single point mutations appear naturally during DNA replication. At the molecular level the change produces a DNA mismatch (MM), a DNA defect occurring when two non-complementary bases are aligned in the same base pair of a duplex. MMs are quickly repaired in DNA by the highly conserved DNA MisMatch Repair mechanism (MMR), which ensures DNA integrity and genome stability. The first step in the MMR pathway is the recognition of the MM by the protein MutS. This first recognition step is crucial as it determine the repair efficiency of the whole pathway. MutS is able to recognize all possible mismatches using two key residues: PHE36, which stabilize the MM base on the Crick strand through stacking interaction; and GLU38, which is thought to form hydrogen

bonds with the same MM base. The interaction with PHE36 is non-specific regardless of the MM base. But according to the chemical nature of each bases, and the x-ray structures analyzed, GLU38 should exist in at least two different protoned states to always satisfy, regardless of the MM, the requirements for the formation of a hydrogen bond, acting as a "recognition switch". In this work we used pKa calculations, Molecular Dynamics simulations, and Umbrella Sampling techniques to study the role of GLU38 with different MMs. Using high resolution x-ray structures we reconstructed by symmetry a complete MutS protein, which was used as a template to study 6 different MMs. Our initial results seem to suggest that GLU38 must always be protonated in order to maintain the hydrogen bond interaction with the MM base, rejecting the "recognition switch" hypothesis. Furthermore, our results challenge the validity of some of the model deposited in the PDB that were refined from diffraction data using the deprotonated state of Glu38.

An RNA Binding Protein from Plasmodium vivax apicoplast

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Malaria parasites harbor an essential vestigial plastid-like organelle, the apicoplast. This organelle contains a ~35 kb circular genome; however, the mechanisms by which transcription and RNA translation are regulated remain poorly understood. The annotation of the *Plasmodium vivax* genome reveals a putative nuclear encoded RNA Binding Protein (RBP), namely apiRBP, that was predicted to be trafficked into the apicoplast. Although the 3D-structural model of apiRBP corresponds to a canonical RNA Recognition Motif fold, protein production trials were nevertheless unsuccessful due to protein aggregation. Theoretical solvation analysis of an apiRBP model supported these findings, highlighting an exposed hydrophobic region close to the C-terminus. Hence, in this work, we use a C-term-GFP-fused chimera to stabilize the highly insoluble apiRBP and determined its ability to bind 10-mer and 25-mer U-rich RNA stretches. The affinity (K_D in the μM range) of apiRBP towards such RNAs is highly dependent on ionic strength, suggesting that the apiRBP-RNA complex is driven by electrostatic interactions rather than π-stacking. Altogether, apiRBP represents a very attractive tool for apicoplast transcriptional studies and anti-malarial drug design.

20 P8 - Biointerfaces, Biofilms and Nanobiophysics

High-Speed force spectroscopy of lipid bilayer rupture

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Supported Lipid Bilayers (SLBs) are simple biological membrane models widely used for fundamental studies. Atomic Force Microscopy (AFM)-based Force Spectroscopy is an ideal technique to investigate the mechanical properties of SLBs at the nanoscale, their elastic constants but also their resistance to failure. The mechanical rupture of the lipid bilayer by the AFM tip has been used extensively as a hallmark of its mechanical stability, usually by measuring the forces required to break the bilayer probed at different velocities. Being a stochastic process, rupture forces generally increases linearly with the logarithm of the loading rate, defining a characteristic dynamic force spectrum. Thanks to the miniaturization of the cantilever and piezoelectric elements, High-Speed AFM (HS-AFM) allows reaching tip velocities in the millimeter per second range with microsecond time resolution (1), covering a wider dynamic force spectrum. Here, we apply HS-AFM Force Spectroscopy to access six decades of indentation velocities on supported lipid bilayers. We also assess the possible contribution of the cantilever resonance frequency to the force spectrum. The preliminary dynamic force spectrum of DOPC SLB can be interpreted with a single energy barrier although possibly followed by an outer state. The outcomes allow us to evaluate the validity of general theoretical developments applied in the field (2).

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