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Tagging 3-Hydroxy-4-Pyridinone Iron Chelators with Rhodamine B Derivatives is Essential to Target Mycobacterium Avium Infection

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The increasing development of bacterial resistance to traditional antibiotics has reached alarming levels and a strong effort to develop new antimicrobial agents is imperative. The process of iron acquisition represents a pathway, which can be successfully targeted by novel therapeutic tools and iron restriction has been shown to improve the outcome of a number of infectious diseases.
Our group designed a new type of tripodal iron chelators which incorporate fluorescent labels that allow following its pathways within the cell. The results demonstrate that these chelators can limit the access of iron to bacteria and have a significant inhibitory effect on the intramacrophagic growth of M. avium. Furthermore, it was found that chelation of iron is a determinant but not sufficient property for antimicrobial activity. 1,2.
In order to identify key molecular features essential for biological activity we designed parent hexadentate and bidentate chelators, in which different structural groups are introduced in the molecular framework. The results show that rhodamine B isothiocyanate labeled chelators exhibit the highest biological activity and suggest that the high affinity of rhodamine B towards lipid phases may be determinant to situate the chelator in a favourable position to compete with mycobacterial siderophores.
The work developed in model membranes using fluorescence and electron paramagnetic resonance spectroscopies will be rationalized from the chemical, biophysical and biological points of view.
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Langmuir Monolayers of Bacterial Outer Membranes

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The increase of Gram-negative bacteria super-resistant to antibiotics is a major problem in medicine. These bacteria possess a double membrane system, with the external leaflet of the outer membrane composed of lipopolyssaccharides (LPS) that act as the first barrier to drugs. Thus, bacteria's susceptibility to lipophilic antibiotics is highly correlated with the permeability of this LPS barrier. In this study, we extracted LPS from two isogenic Salmonella sp strains, using the method of Galanos. These strains contain alterations in the lipid A portion of their LPS regulated by the PhoPQ-system. These modifications include palmitoylation of the 3-hydroxyl group in the 3-OH-myristyl residue, addition of 4-aminoarabinose to the 4' phosphate group and addition of a 2-hydroxy group to the myristate residue at the position 3'. Langmuir monolayers were formed of both LPS and compression isotherms indicated that PhoP- (LPS from the strain with no modifications) occupies a limiting area of 150Å2, while for PhoPc (LPS from the strain with the modifications cited above) this value is 175Å2. These are in agreement with previous report for other Salmonella sp LPS. Epifluorescence microscopy reveals PhoP- has a very homogeneous, fluid-like, morphology throughout the compression without discrete domain formation. In contrast, PhoPc displays circular-shaped domains that increase in size and number with the compression. The addition of novobiocin (0.4 μg mL-1) to the subphase promoted slight changes in PhoP- morphology, suggesting that this monolayer was already fluid enough to incorporate this antibiotic. For PhoPc monolayer, changes in morphology were observed only for high concentrations (2.5x) of novobiocin at which the domains started to aggregate generating structures like a ''pearl necklace''.

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Structure of Glycocalyx

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Glycocalyx is a highly charged layer of membrane-bound biological macromolecules attached to a cell membrane. This layer functions as a barrier between a cell and its surrounding. Glycocalyx also serves as a mediator for cell-cell interactions and protects a cell membrane from the direct action of physical forces and stresses allowing the membrane to maintain its integrity. Glycocalyx is also involved in development and progression of many diseases. Glycocalyx is composed of glycosaminoglycans, proteoglycans and other glycoproteins bearing acidic oligosaccharides and terminal sialic acids. Most glycocalyx associated proteins are transmembrane that can be linked to the cytoskeleton. This linkage not only restricts their position and constitutes the foundation of the glycocalyx structure, but it also allows signal transduction from the external to the internal parts of a cell. Here we focus on the endothelial cells' glycocalyx.
The aim of this project is to employ Molecular Dynamics simulations to gain insight into the molecular structure and functions of the glycocalyx. This choice is justified by the fact that experimental studies of the glycocalyx are to a large extent restricted by its dynamic and soft nature driven by thermal fluctuations, implying that the glycocalyx has no unique state or structure but it instead is expected to fluctuate significantly in time. Only recent progress in the development of molecular simulation techniques and the availability of larger computer resources have fostered studies of complex biological systems such the glycolalyx itself.

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Bacterial Signal Indole Modifies the Physicochemical Properties of Lipid Membranes

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Indole is a biological molecule produced by more than 85 bacterial species. It acts as an inter-cellular signal, influencing multiple aspects of bacterial physiology and has proved to be an important factor in the transition to stationary phase. It also promotes resistance to a range of drugs and toxins and is involved in preventing plasmid instability. Aspects of bacterial ecology and host-pathogen interactions which respond to indole include biofilm formation and the expression of virulence factors.
As indole is widely affecting cellular organisms here we study the effect of indole on the physicochemical properties of the lipid membrane. We use the intrinsic florescence of the indole molecule to show that indole is freely diffusing through the lipid membrane barrier. Using electrophysiology we show that the indole is able to collapse membrane potential. We also show that the rate of mitochondrial oxygen consumption increases when the indole concentration is raised from 0 to 1mM. Therefore our results demonstrate that indole is capable of lowering the energetic barrier for proton permeation across lipid membranes. Concluding from these results to obtain an enhanced protonophoric activity we proposed a point mutation on the indole molecule 4F-indole. Confirming our interpretation on the mechanistic details of indole induced proton transport, 4F-indole is two time more efficient in uncoupling the oxidative phosphorylation in isolate mitochondria. Further using light spectroscopy we find the partition coefficient for indole into lipids in aqueous environment.Then using differential scanning calorimetry we show that indole modifies the phase transition of *Escherichia coli* cell membranes. These findings offer an explanation for the multiple roles that indole has in membrane biology, bacterial cell cycle control and potentially for the design of antibiotics that target the cell membrane.

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Critical Temperatures in Plasma Membrane Vesicles are Dependent on the Cell Cycle

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Giant plasma membrane vesicles (GPMVs) isolated from RBL-2H3 cells appear uniform at physiological temperatures, contain coexisting liquid-ordered and liquid-disordered phases at low temperatures, and experience micron-sized critical fluctuations close to their critical temperature. We observe a broad distribution of critical temperatures in GPMVs isolated from a dish of seemingly identical cells even though each individual vesicle has a well defined critical temperature. In addition, we observe that the average transition temperature of GPMVs isolated from a population of cells is inversely proportional to the surface density of cells growing in the culture dish. Since it is known that cellular doubling times are reduced in more densely plated cells due to contact inhibition, we hypothesized that critical temperatures are linked

to the cell cycle. To test this hypothesis, we isolated GPMVs from populations of cells synchronized using a double Thymidine block that arrests cells at the border between G1 and S phases. After Thymidine is removed from the culture media, cells proceed synchronously through one cell cycle and GPMVs are isolated at time-points corresponding to S, G2, M, and G1 phases. We find that critical temperatures are significantly elevated in the cell cycle phases that immediately preceding cell division (G2 and M) compared to the remainder of the cell cycle phases (G1 and S), consistent with our observations of higher overall transition temperatures in more rapidly dividing cells. We speculate that membrane heterogeneity arising from this critical point may play a role in cytokinesis or other processes vital for cell division. If this hypothesis is correct, it may suggest that biochemical perturbations that lower critical temperatures in GPMVs can be used to inhibit cellular proliferation, possibly providing a novel treatment strategy for some cancers.

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Cytoskeletal Pinning Prevents Large-Scale Phase Separation in Model Membranes

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During the last decades, artificial lipid bilayers have become an important tool in studies of properties of the plasma membrane of cells. One important feature of cell membranes, which has been difficult to recapitulate in the artificial bilayer systems, is the membrane-associated cytoskeleton. The cytoskeleton is believed to affect diffusion of lipid and protein molecules in the plasma membrane, and is considered to be one of the reasons for the sub-resolution size of membrane domains by preventing large-scale phase separation. Mimicking the eukaryotic actin-based cytoskeleton in vitro is inefficient and complicated, owing to the number of components involved and the nature of membrane binding of the actin-network complex. Here we describe a minimal cytoskeletal network formed by the prokaryotic tubulin homologue, FtsZ. FtsZ has been modified to interact with the membrane through a membrane targeting sequence (MTS) from MinD, another prokaryotic protein. FtsZ-MTS efficiently forms a highly interconnected network on the membrane with a concentration-dependent characteristic mesh size, much similar to the eukaryotic network underlying the plasma membrane. Using giant unilamellar vesicles formed from a quaternary lipid mixture, we demonstrate that, on the one hand, the artificial membrane-associated cytoskeleton suppresses large-scale phase separation below the phase transition temperature, and, on the other hand, preserves phase separation above transition temperature. Our experimental observations support the ideas put forward in our previous simulation study [1]: In particular, the picket-fence effect on phase separation explains why micrometer-scale membrane domains are observed in isolated, cytoskeleton-free giant plasma membrane vesicles, but not in intact cell membranes. The experimentally observed suppression of large-scale phase separation much below the transition temperatures also serves as an argument in favor of the cryoprotective role of the cytoskeleton.

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Extracellular, Membrane and Intracellular Proteins that Alter Integrin Cell Membrane Diffusion and Clustering

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Integrins are ubiquitous membrane proteins that are involved in cell adhesion and signaling across the cell membrane. We are elucidating the role of other membrane proteins, extracellular ligand, and intracellular proteins in altering integrin clustering and diffusion using several fluorescence microscopy techniques. Our work provides vital information on the molecular mechanism of integrin function through altered dynamics and membrane organization. Clustering is measured using fluorescence resonance energy transfer (FRET) and stimulated emission depletion imaging (STED). In order to noninvasively measure membrane structure and dynamics, we have developed a novel STED instrument for fluorescence lifetime imaging with 40-nm spatial resolution, which is below the diffraction limit of light. Diffusion is measured using fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT). Select membrane proteins that we study include focal adhesion kinase and epidermal growth factor receptor, while select cytoplasmic proteins that we study include paxillin, vinculin, actin and focal adhesion kinase. Among our interesting findings, we have determined that other membrane proteins do not significantly alter the percentage of mobile integrins, but significantly constrain integrin diffusion; the role of cytoplasmic proteins in altering integrin clustering depends on the concentration of extracellular ligand available for binding; and increasing integrins' ligand affinity reduces the population of mobile integrins and also reduces the diffusion coefficient of integrins that remain mobile. We hypothesize that altered partitioning into membrane nanodomains is the main mechanism for altered integrin clustering and diffusion when the concentration of select cytoplasmic, membrane or extracellular protein is altered; and we are further testing this hypothesis using STED lifetime imaging.

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Ligand Receptor Binding Rate Kinetics via K-Space Image Correlation Spectroscopy: An in Silico Study

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To achieve a fundamental understanding of intra-cellular signalling pathways, it will be necessary to measure the rates of reaction between chemically reacting and interacting macromolecules. The magnitude of binding rates plays a very important molecular regulatory role in a range of systems including the binding of antigens to T-cell receptors, which can lead to differing immune-responses, as well as rates of hydrolysis of GTPs with Rac or Rho proteins, which lead to temporal changes in actin filament lengths and cell motility. Here, we develop an image correlation technique which may be used to study such systems in conjunction with fluorescence microscopy. We simulate receptors diffusing in 2D with diffusion coefficients varying from 0 to 10 pixels^2/frame. Receptors are only visible as point emitters when a fluorescent ligand is bound to the receptor; ligand binding kinetics are created by turning receptors 'on' or 'off' with average life-times varying from 3 to 20 frames drawn from a negative exponential distribution. Photobleaching is similarly incorporated by permanently turning diffusing receptors 'off' after average times varying from 20 to 400 frames. The image time-series is generated by convolving a Gaussian function of fixed radius with the point emitter distribution to create 128x128 pixel images with background noise added to give a signal to noise ratio of 3. We use k-space image correlation spectroscopy (kICS) and develop a two-state kinetic binding model with freely diffusing receptors on a 2D membrane to capture the membrane binding kinetics of the image time-series. We show that kICS can accurately recover on/off binding rates at the 95% confidence level for over 75% of simulations. Typically, percentage errors are less than 30% for weak photobleaching and within 60% for strong photobleaching effects.

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Single Molecule Tracking of Annexin V in Cushioned DMPC Assemblies

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Using single molecule imaging, this study describes the phase behavior and mobility of individual transmembrane (TM) proteins and compares those results with the bulk phase behavior of the biomimetic membrane in which they have been incorporated. To accomplish this a TM protein, Annexin V, was incorporated into a cushioned planar supported 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) / L-α-lysophosphatidyl-serine (Brain-PS) biomimetic assembly and its mobility between 30 and 16 C was measured. Fluorescence microscopy and Fluorescence Recovery After Photobleaching (FRAP) were used to verify the structural integrity and the phase behavior (median melting temperature, TC = 22 C) of the lipid assembly. The spatial confinement of individual Annexin V molecules was measured in three distinct phase regions: (1) a homogenous liquid crystalline phase region (Lα) in which Annexin V was unconfined ($\geq$ 25 C), (2) a two-phase region (Lα + gel-phase-Pβ') in which Annexin V displayed intermediate confinement (24 - 20 C), and (3) a gel-phase region (Pβ') with included nanoscopic domains that are enriched with PS and surround a single Annexin V TM protein (19 - 16 C); the mobility of Annexin V in these domains is highly confined. At early time lags, Annexin V moves with apparent Brownian-like behavior at all temperatures but the diffusion coefficients have very different magnitudes and temperature dependence. A possible mechanism for nanoscopic domain formation will be discussed.

Protein-Nucleic Acid Interactions I

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A SAXS Study of the CRISPR Associated Gene (Cas) Csn2 in the Presence and Absence of Calcium Ions

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Small angle X-ray scattering (SAXS) was used to study the solution structures of the *E. Faecalis* Csn2 protein, a *cas* gene required for DNA spacer acquisition