Physical Modes of Action of Membrane-Active Compounds

807. WE-Heraeus-Seminar

07 – 10 April 2024

at the Physikzentrum Bad Honnef, Germany

WILHELM UND ELSE
HERAEUS-STIFTUNG

Introduction

The Wilhelm und Else Heraeus-Stiftung is a private foundation that supports research and education in science with an emphasis on physics. It is recognized as Germany's most important private institution funding physics. Some of the activities of the foundation are carried out in close cooperation with the German Physical Society (Deutsche Physikalische Gesellschaft). For detailed information see https://www.we-heraeus-stiftung.de

Aims and scope of the 807. WE-Heraeus-Seminar:

Lipid bilayers are formed by spontaneous self-assembly of amphiphilic lipid molecules. They represent the structure-giving matrix and hydrophobic barrier of cell membranes and other biological membranes. Technically, they may form, for example, liposomes that are being used as smart drug delivery systems.

The seminar aims at discussing the physical effects of amphiphilic or lipophilic peptides, drugs, and other membrane-interacting solutes of natural or synthetic origin on the key properties of lipid membranes. Such key properties are named integrity, permeability, heterogeneity, order-dynamics-hydration, and curvature, which are addressed by respective sessions. It turns out, however, that these properties are closely related to each other and membrane-active substances typically affect several of them. It is one main aim of the seminar to better connect these phenomena and catalyze communication and collaboration between the research communities who study them.

Scientific Organizer:

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Introduction

Venue:

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Registration:

Elisabeth Nowotka (WE Heraeus Foundation)
at the Physikzentrum, reception office
Sunday (16:00 h – 21:00 h) and Monday
morning

Program

Program

Sunday, 07 April 2024

	Early arrivers are welcome to join a small hike to Drachenfels	
16:00 – 21:00	Registration	
18:00	*BUFFET SUPPER and informal get-together*	
20:00	Heiko Heerklotz	**Welcome words**
20:10	**Debate 1**	

Monday, 08 April 2024

07:15	*BREAKFAST*	
08:30 – 08:40	Alfred Blume	**INTRO integrity**
08:40 – 09:20	Daniel Harries	**Insights into the material properties of nanodiscs and other mesophases**
09:20 – 10:00	Sandro Keller	**Native nanodiscs for membrane-protein biophysics**
10:00 – 10:20	Attila Tortorella	**Bacterial model membranes under the harsh subsurface conditions of Mars**
10:20 – 10:50	*COFFEE & POSTERS*	
10:50 – 11:50	**30 poster flashes**	
11:50 – 12:20	**Posters**	
12:20	*LUNCH*	

Program

Monday, 08 April 2024

13:50 – 14:00	**Conference photo** (in the front of the lecture hall)	
14:00 – 14:10	John Seddon	**INTRO curvature**
14:10 – 14:50	Rumiana Dimova	**Membrane remodeling in artificial cells: To bud or not to bud**
14:50 – 15:30	Michael Kozlov	**Model for tension propagation in cell membranes**
15:30 – 15:50	Lisa Hua	**How to relieve asymmetry stress in model membranes**
15:50 – 16:20	*COFFEE & POSTERS*	
16:20 – 17:00	Dimitrios Stamou	**Plasma membrane curvature induces long-lived multimodal activation of G protein coupled receptors.**
17:00 – 17:40	**20 poster flashes**	
17:40 – 19:00	**Posters**	
19:00	*DINNER*	
20:00	**Debate 2**	

Program

Tuesday, 09 April 2024

07:15	*BREAKFAST*	
08:30 – 08:40	Kalina Hristova	**INTRO permeability**
08:40 – 09:20	Paulo Almeida + Antje Pokorny	**A molecular view of peptide binding and translocation across lipid bilayers**
09:20 – 10:00	Lorenzo Stella	**Quantitative studies on peptide interaction with live bacterial cells: lessons learned and new questions**
10:00 – 10:20	Jasmin Schlauch	**Fluorescence quenching reveals the distribution of two synergistic antimicrobial peptides on a lipid membrane surface.**
10:20 – 10:50	*COFFEE & POSTERS*	
10:50 – 11:30	William Wimley	**Self-assembling peptide nanopores are stabilized by a cooperative hydrogen-bond network**
11:30 – 12:10	Claudia Steinem	**Lugdunin: a cyclic peptide that partitions and self-assembles in lipid membranes to form water-filled channels**
12:10 – 12:30	Maria Hoernke	**Membrane permeabilization and other membrane perturbations: a network of mechanisms affecting the significance of model studies**
12:30	*LUNCH*	

Program

Tuesday, 09 April 2024

14:00 – 14:10	Roland Winter	**INTRO order- hydration**
14:10 – 14:50	Daniel Huster	**Membrane thickness can regulate protein function**
14:50 – 15:30	Olaf Andersen	**Bilayer-mediated regulation of membrane protein function by small molecules, friend or foe**
15:30 – 15:50	Guillaume Gilliard	**The elicitor surfactin triggers plant immunity by altering membrane lipid properties**
15:50 – 16:20	*COFFEE & POSTERS*	
16:20 – 17:00	Emma Sparr	**The impact of nonequilibrium conditions in interfacial barrier membranes**
17:00 – 19:00	**Posters**	
19:00	*HERAEUS DINNER* *(social event with cold & warm buffet with complimentary drinks)*	

Program

Wednesday, 10 April 2024

07:15	*BREAKFAST*	
08:30 – 08:40	Georg Pabst	**INTRO heterogeneity**
08:40 – 09:20	Ilya Leventhal	**Novel dimensions of membrane asymmetry: phospholipid and cholesterol abundance imbalances and their remarkable consequences**
09:20 – 10:00	Rainer Böckmann	**Non-universal impact of cholesterol on membranes: Mobility, curvature sensing, and elasticity**
10:00 – 10:20	Zhibo Deng	**Unravelling cholesterol flip-flop dynamics in lipid bilayers under thermal gradients: Insights from coarse-grained molecular dynamics simulations**
10:20 – 10:50	*COFFEE & POSTERS*	
10:50 – 11:30	Tobias Baumgart	**Connecting lipid bilayer asymmetry with membrane shape**
11:30 – 12:10	Thomas Gutsmann	**How pathogenes can kill and be killed by membrane-active peptides**
12:10 – 12:30	Heiko Heerklotz	**Poster awards** **Closing words**
12:30	*LUNCH*	

End of the seminar and departure

NO DINNER for participants leaving on Thursday; however, a self-service breakfast will be provided on Thursday morning

Posters

Posters

Mahmoud Abouelkheir	**Investigating the role of cholesterol on EGFR ligand binding and phosphorylation**
Matilde Accorsi	**Reconciling past and future research: a study on calcium as a protein-free fusogen in negatively-charged cell-sized vesicles**
Christoph Allolio	**Mitochondrial membrane models – from lipids to crista morphology**
Katharina Beck	**Deciphering how membrane active compounds affect membrane lipid order**
Marco Campanile	**Exploring the role of the C-terminus of the cAMP GKY20 in membrane perturbation**
Iulia Carabadjac	**Can TCSPC of tryptophan shed light on concerted effects of cyclic lipopeptides on membranes?**
Federico Carneri	**Pharmacodynamics of antimicrobial peptides: the role of water-membrane partition**
Sarah Crocoll	**D(y)e-coding membrane solubilization: Characterizing lipid-detergent systems with Laurdan and Nile Red**
Ismail Dahmani	**Charged extracellular vehicles (EVs) interfere with Leishmania parasite binding and uptake by phagocytic cells.**
Magali Deleu	**Deciphering the distinct biocontrol activity of fengycin and surfactin, two bacillus lipopeptides, through their differential impact on lipid membranes**
Zhibo Deng	**Unravelling cholesterol flip-flop dynamics in lipid bilayers under thermal gradients: Insights from coarse-grained molecular dynamics simulations**

Posters

Simon Drescher	**Azide- and diazirine-modified membrane lipids: Physicochemistry and applicability to study peptide/lipid interactions via cross-linking/mass spectrometry**
Oskar Engberg	**The impact of tryptophan derivatives on synaptic vesicular exocytosis**
Robert Ernst	**Lipid fingerprints of a stressed membrane**
Noemi Ferrante Carrante	**α-Synuclein cooperative binding to lipid membranes**
Lucas Gewehr	**Monitoring alterations in lipid bilayers in real-time**
Felix M. Goni	**Lateral heterogeneity in phospholipid bilayers containing cardiolipin and ceramide: relevance to autophagy**
Sinja Götz	**Interactions of the antibiotic daptomycin with model membranes compared to a novel cyclic lipopeptide**
Heiko Heerklotz	**Understanding temperature-triggered membrane permeabilization as used in drug delivery**
Nadja Hellmann	**Membrane remodeling by the cyanobacterial protein IM30**
Maria Hoernke	**Membrane permeabilization and other membrane perturbations: a network of mechanisms affecting the significance of model studies**
Andreas Horner	**Monitoring detergent effects in lipid bilayers**
Kalina Hristova	**A membrane model to measure the transducer function of signal propagation along single-pass membrane receptors**
Lisa Hua	**How to relieve asymmetry stress in model membranes**

Posters

Jochen S. Hub	**Lipid composition and lipid-protein interactions greatly modify the free energy landscape of pore formation and fusion**
Michael Kaltenegger	**Shape-based design of bitopic proteins as probes for membrane elastic stress**
Mona Krompers	**Effects of a phase transition in lipid-asymmetric vesicles**
Akanksha Kumari	**Formation of supramolecular structure and phase separation in lipid bilayer upon reconstitution of water-soluble protein hemoglobin**
Melissa Lehnert	**Understanding the fate of mixed micellar drug delivery systems**
Jan Lembeck	**Design of phospholipid-polymer-nanoparticles with phospholipid-dependent drug delivery profiles**
Natalia Markova	**Property-structure-function analysis of complex LNPs using integrative biophysical, molecular-, and cell-based assays**
José C Martins	**Tolaasin structure revisited: On the impact of environment and macrocycle integrity for tolaasin structure and its interaction with membranes**
Annette Meister	**How charges effect the solubilization of artificial and native membranes by amphiphilic copolymers**
Christian Nehls	**Liposomes as model systems for standardized evaluation of antimicrobial peptide membrane permeabilization in microfluidic setups**
Rosario Oliva Roland Winter	**Antimicrobial peptides in action under high pressure conditions**

Posters

Georg Pabst	**Ion-mediated changes of spontaneous monolayer curvature activate the integral enzyme OmpLA.**
Peter Pajtinka	**Can amphipathic helices sense both positive and negative membrane curvatures?**
Kristyna Pluhackova	**Molecular impacts of the drug disulfiram on lipid membranes**
Chetan Poojari	**Mechanistic insights into virus-host interactions**
Garima Rani	**Lipid packing defects in membrane interactions of biomimetic antimicrobial polymers**
Daniela Roversi	**The "Sand in a gearbox" effect of antimicrobial peptides: beyond pore formation**
Alexandre Ahmad Saad	**Solid-State NMR Investigation of Magainin Antimicrobial Peptides in a Realistic Membrane Environment**
Katharina Scherer	**Free energy landscape of membrane topological transitions during fusion and pore formation**
Dirk Schneider	**Membrane shape transitions mediated by prokaryotic members of the ESCRT-III superfamily**
Christian Schwieger	**Adsorption of lipid nanodiscs to monolayers: A new triple layer system for studying membrane proteins**
Enrico Federico Semeraro	**Probing protein-induced local membrane deformation: a small-angle scattering study**
Tsu-Wang Sun	**Photoswitchable lipid dynamics in phase-separated membranes**

Posters

Marius F.W. Trollmann	**mRNA lipid nanoparticle phase transition**
Astrid Walrant	**Arg/Trp cell-penetrating peptides incorporating Trp analogues: internalization and interactions with cell membrane components**
Pablo Zambrona	**Chemically driven self-division in synthetic vesicular systems**
Katja Zieske	**Topographies of lipid membranes are biophysical regulators for the spatial organization of liquid protein condensates**

Abstracts of Talks

(in alphabetical order)

A molecular view of peptide binding and translocation across lipid bilayers

I. Bilayers are not impermeable to charged peptides

P. Almeida and **A. Pokorny**

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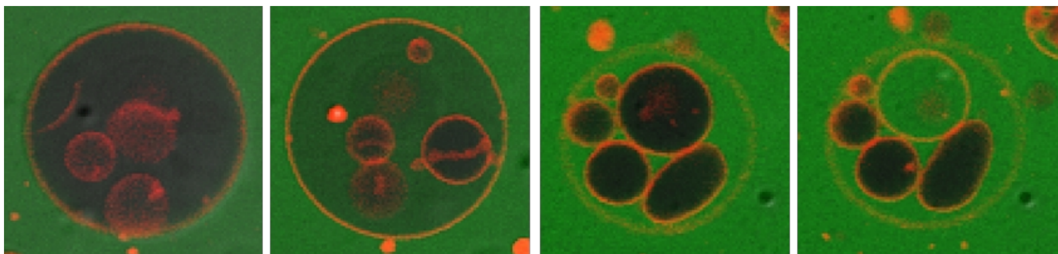
E-mail: almeidap@uncw.edu

When we entered the field of membrane-active peptides, about 20 years ago, the focus was largely on the types of pores formed by antimicrobial peptides. Our approach was to see if those models were consistent with quantitative interpretation of kinetics of peptide binding and flux of water-soluble molecular dyes through membranes. Rigorous use of kinetic models to explain experimental data, led us to propose that dye flux through membranes occurred mainly concomitant with the translocation of peptides across the bilayer.

The possibility of peptide translocation, triggered our interest to examine cell-penetrating peptides containing significant numbers of cationic residues. We found that, in these cases too, the kinetic data were best explained by quantitative models that included peptide translocation. Direct visual examination of peptide interaction with giant unilamellar vesicles (GUVs) demonstrated that indeed these highly charged peptides are able to enter vesicles concomitant with dye flux but without causing membrane disruption. We further suggested that peptide translocation may even be “silent,” in the sense that no dye flux necessarily accompanies peptide translocation. Both predictions have been independently confirmed by other investigators with different peptides and different methods.



Bilayer-mediated regulation of membrane protein function by small molecules, friend or foe?

Olaf S. Andersen, **Radda Rusinova, Thasin A. Peyear**

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1300 York Avenue, New York, NY, USA

Bioactive molecules can alter ion channel (membrane protein) function by at least five non-exclusive mechanisms (Figure 1): 1. binding to the pore (active site); 2. binding to allosteric sites formed by the protein; 3. binding to allosteric sites formed by the protein plus bilayer lipids; 4. non-specific binding/enrichment at the protein/bilayer boundary; 5. and partitioning into the bilayer/solution interface to alter bulk bilayer properties such as curvature, elasticity, interfacial dipole potential and thickness.

The first two pertain to all proteins; the last three result from the energetic penalty of exposing hydrophobic surfaces to water or polar residues, which causes proteins and bilayers to adapt to each other. Membrane protein conformational changes therefore will alter the packing and dynamics of the adjacent lipids, which has an energetic cost, the bilayer deformation energy. The energetic cost of membrane protein conformational changes thus will be the sum of contributions from the protein and from the lipid bilayer leading to a bilayer-mediated regulation of protein function. Amphiphiles (bioactive molecules) that partition into the bilayer/electrolyte interface will alter lipid bilayer properties and the bilayer contribution to the energetic cost of membrane protein conformational changes. The ensuing dysregulation of membrane protein can be quantified using suitable reporters, e.g., channels formed by the linear gramicidins. This allows for demonstrating the generality of bilayer-mediated (dys)regulation of membrane protein and cell function. It further provides a strategy for quantifying the probability that a bioactive molecule alters membrane protein function through bilayer-mediated mechanisms, as opposed to the canonical mechanism, binding to (specific) sites on proteins of interest, e.g., [3, 4].

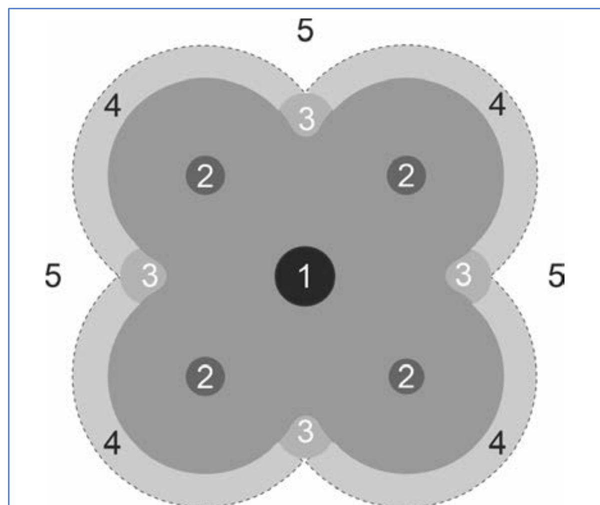

Figure 1: Schematic ion channel with potential sites for drug modulation of function, see text for details. After [1]; see also [2].

1. Andersen, O. S. *J. Gen. Physiol.* **2008,** 131, 395-7.
2. Urban, B. W. *Brit. J. Anaesth.* **2002,** 89, 167-83.
3. Rusinova, R.; et al. *Proc. Natl. Acad. Sci. USA* **2021,** 118, e2113229118.
4. Peyear, T. A.; Andersen, O. S. *J Gen Physiol* **2023,** 155, (4).

Connecting lipid bilayer asymmetry with membrane shape

T. Reagle, Z. Graber, S. Shukla, R. Jin, T. Baumgart[1]

[1]*University of Pennsylvania, Philadelphia, USA*

Lipid asymmetry- that is, a nonuniform lipid distribution between the leaflets of a bilayer- is a ubiquitous feature of biomembranes and is implicated in several cellular phenomena. Differential tension- that is, unequal lateral monolayer tensions comparing the leaflets of a bilayer- is closely associated with lipid asymmetry underlying these varied roles. Because differential tension is not directly measurable in combination with the fact that common methods to adjust this quantity grant only semi-quantitative control over it, a detailed understanding of lipid asymmetry and differential tension are impeded. To overcome these challenges, we leveraged reversible complexation of phospholipid by methyl-β-cyclodextrin (mbCD) to tune the direction and magnitude of lipid asymmetry in synthetic vesicles. Lipid asymmetry generated in our study induced i) vesicle shape changes and ii) gel-liquid phase coexistence in 1-component vesicles. By applying mass-action considerations to interpret our findings, we discuss how this approach provides access to phospholipid thermodynamic potentials in bilayers containing lipid asymmetry (which are coupled to the differential tension of a bilayer). Because lipid asymmetry yielded by our approach is i) tunable and ii) maintained over minute to hour timescales, we anticipate that this approach will be a valuable addition to the experimental toolbox for systematic investigation into the biophysical role(s) of lipid asymmetry (and differential tension).

Non-Universal Impact of Cholesterol on Membranes: Mobility, Curvature Sensing, and Elasticity

Matthias Pöhnl[1], Marius F.W. Trollmann[1,2], and Rainer A. Böckmann[1,2,3]
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Biological membranes, composed mainly of phospholipids and cholesterol, play a vital role as cellular barriers. They undergo localized reshaping in response to environmental cues and protein interactions, with the energetics of deformations crucial for exerting biological functions. This study investigates the non-universal role of cholesterol on the structure and elasticity of saturated and unsaturated lipid membranes. Our study uncovers a highly cooperative relationship between thermal membrane bending and local cholesterol redistribution, with cholesterol showing a strong preference for the compressed membrane leaflet. Remarkably, in unsaturated membranes, increased cholesterol mobility enhances cooperativity, resulting in membrane softening despite membrane thickening and lipid compression caused by cholesterol. These findings elucidate the intricate interplay between thermodynamic forces and local molecular interactions that govern collective properties of membranes.

References:

M. Pöhnl, M.F.W. Trollmann, and R.A. Böckmann. **Nature Comm.** 14:8038 (2023)

Unravelling Cholesterol Flip-Flop Dynamics in Lipid Bilayers under Thermal Gradients: Insights from Coarse-Grained Molecular Dynamics Simulations

Z. Deng[1], F. Carman[1], J. Seddon[1] and F. Bresme[1]

[1]*Imperial College London, London, United Kingdom*

In this work we study cholesterol flip-flop under non-equilibrium conditions. Specifically, we investigate the role of thermal gradients across lipid bilayers in promoting membrane asymmetry by biasing the flip-flop motion. To gain insights into the phenomenon of cholesterol flip-flop in lipid bilayers under non-equilibrium conditions, we conducted coarse-grained (CG-MARTINI), Non-Equilibrium Molecular Dynamics (NEMD) simulations. The focus was on identifying key factors that influence cholesterol flip-flop and the thermal preference of membrane asymmetry.

As the temperature gradient increases, we find an enhancement of cholesterol accumulation on the cold lipid region (thermo-phobicity), leading to an increased asymmetry within the bilayer. We investigate the behaviour of cholesterols in various lipid membranes (DLPC, POPC, DPPC, DBPC) under a stationary temperature gradient.

Our study further explores the nuanced relationship between the length of the hydrocarbon chain and the rate of cholesterol flip-flop and the influence of the saturation of the phospholipids on cholesterol dynamics.

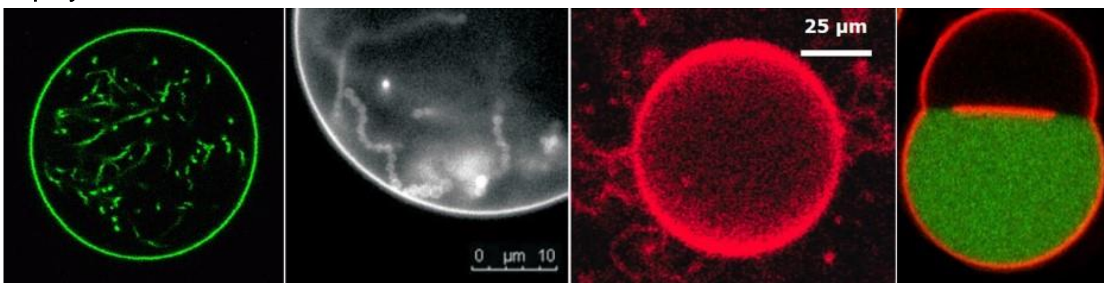
In summary, this research, based on NEMD simulations and coarse-grained phospholipid bilayers, contributes to our understanding of thermal-dependent cholesterol dynamics and the intricate relationship between phospholipids and cholesterol in biological membranes, providing valuable insights into membrane permeability, fluidity, and stability in out of equilibrium conditions.

Membrane remodeling in artificial cells: to bud or not to bud

R. Dimova

Max Planck Institute of Colloids and Interfaces, Potsdam, Germany

Cell membranes exhibit a large variation in curvature. It is a common perception that curvature is caused by the activity of specific protein species. Here, we will demonstrate that it can be readily generated by various other asymmetries across the membrane, which plausibly represent a governing factor for defining shapes of membrane organelles. As a workbench for artificial cells, we employ giant unilamellar vesicles (10-100µm), which represent a suitable model system showing the response of the membrane at the cell-size scale [1], see images below. In this talk, we will introduce approaches employing giant vesicles for the precise quantification of the membrane spontaneous curvature. Several examples for generating curvature will be considered: asymmetric distribution of ions on both sides of the membrane [2], insertion/desorption of the ganglioside GM1 [3], and PEG adsorption [4, 5]. We will also show how spontaneous curvature generation by protein adsorption at low surface density is able to modulate membrane morphology and topology to the extent of inducing vesicle fission [6]. Finally, the process of membrane wetting by molecularly-crowded aqueous phases will be shown to induce vesicle budding and tubulation [7]. Wetting by biomolecular condensates will also be discussed as means of molding the membrane [8], while modulating lipid organization [9] and protection against poration and damage [10]. The presented examples will demonstrate that even in the absence of specific proteins and/or active processes, the membrane is easily remodeled by simple physicochemical factors.



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[9] A. Mangiarotti et al., Nature Com. 14, 6081 (2023)
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The elicitor surfactin triggers plant immunity by altering membrane lipid properties

G. Gilliard[1], J. Pršić[1], M. Ongena[2] and M. Deleu[1]

[1]Laboratory of molecular biophysics at interfaces, Terra Teaching and Research Center, Gembloux Agro-Bio Tech, Université de Liège, Gembloux, Belgium
[2] Laboratory of Microbial Processes and Interactions, Terra Teaching and Research Center, Gembloux Agro-Bio Tech, Université de Liège, Gembloux, Belgium

Surfactin (SRF) is a lipopeptide produced by plant beneficial bacilli able to trigger immunity in plants. Previous data strongly suggest that SRF sensing at the plant plasma membrane (PPM) is mainly driven by the interaction between SRF and membrane lipids, contrasting with detection by protein receptors as commonly described for plant immune stimulation by plant elicitors. Nevertheless, the mechanism underneath the lipid-driven sensing of surfactin by plant cells remains largely unknown. To investigate the role of PPM lipids in SRF sensing in plants, we combined functional assays on *Arabidopsis thaliana* root protoplasts with experimental biophysics on biomimetic membrane models.
Isothermal titration calorimetry first revealed a higher binding of SRF for liposomes containing sphingolipids. This SRF-lipid interaction affects the physical properties of the membrane with a thinning of the membrane, a decrease in membrane fluidity, and an increased curvature. Moreover, the *loh1* mutant, impacted in its PM sphingolipid content, showed a strong reduction in SRF-induced ROS burst, one hallmark of plant early immune event, supporting the importance of sphingolipids in SRF sensing.

The involvement of plant lipids in SRF sensing and the impact of SRF on PPM mechanics lead us to investigate the importance in SRF sensing of the PPM-located mechanosensitive (MS) channels, the Mid1-Complementing Activity (mca) channels and MscS-like (msl) channels. Interestingly, a lower calcium response, another hallmark of early immune event, is observed in root protoplasts from *mca* and *msl* channels-depleted mutants upon SRF treatment compared to Col-0 protoplasts.

Altogether, our results support a SRF eliciting mechanism triggered by an activation of MS channel through a disturbance of PPM lipids organization, which represents a new aspect of plant immune stimulation by beneficial bacterial molecules.

How pathogenes can kill and be killed by membrane-active peptides

Christian Nehls[1], Monika Rangole[1], Susanne Homolka[1], Norbert Reiling[1], Martin Kuhns[1], Stefan Niemann[1], Ulrich Schaible[1], Albert Haas[2], Bernhard Hube[3], Thomas Gutsmann[1,4]

[1] Research Center Borstel, Leibniz Lung Center, Borstel, Germany
[2] University of Bonn, Bonn, Germany
[3] Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie Hans-Knöll-Institut, Jena, Germany
[4] Centre for Structural Systems Biology, Hamburg, Germany

In the era of antibiotic therapy, the development of new active substances seemed less important, but the emergence of antimicrobial resistance (AMR) has become an increasing problem in recent years. The WHO estimates that around 10 million deaths in 2050 will be due to resistant bacteria. Mycobacteria are already one of the leading causes of death worldwide, accounting for 1.3 million deaths. The frequent occurrence of multi-resistant Gram-negative bacteria in hospitals is also a major social problem.
Between microbes and humans there is a permanent battle in which pore-forming molecules play a decisive role. On the one hand microbes attack other microbes, and the cytoplasmic or the phagosome membrane of mammal cells by pore forming proteins or peptides. On the other hand mammals use pore-forming peptides of the innate immune system, the so called Host Defense Peptides (HDP) or Antimicrobial Peptides (AMP), to kill microbes.
All these membrane active peptides and proteins follow a general interaction pathway: binding, intercalation, aggregation, modification of membrane properties, pore formation, and disruption of membranes. However, the specificity to lipid composition, pH, ions, transmembrane potential and other factors is crucial for the respective function. Our goal is to elucidate the molecular interaction mechanisms in order to identify targets to prevent exit of microbes and to improve drug design.
Using various membrane reconstitution systems (liposomes, planar bilayers, solid-supported bilayers and multilayers) composed of phospholipids and bacterial glycolipids we analyzed the basic structure of these membranes, e.g. supramolecular organization of the lipids, phase separation and orientation of fatty acids, and the influence of specific membrane active peptides and proteins.

Insights into the material properties of nanodiscs and other mesophases

D. Harries[1]

[1]*Institute of Chemistry and The Fritz Haber Center, The Hebrew University, Jerusalem, Israel*

Extraction of thermodynamically instructive parameters from molecular dynamics simulations poses a continuing challenge. Methodologies that we have developed enable the accurate determination of the bending and tilt elastic constants of lipid mesophases.[1] Several applications will illustrate the practical implementation of the methodology. Specifically, we resolve the impact of confinement on the properties of lipids in nanodiscs.[2,3]

References

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Membrane permeabilization and other membrane perturbations: a network of mechanisms affecting the significance of model studies

S. Shi[1], K. Beck[1], A. Stulz[1] and M. Hoernke[1,2]

[1] *Pharmaceutical Technology, Albert-Ludwigs-Universität Freiburg i. Br., Germany*
[2] *Physical Chemistry, Martin-Luther-Universität Halle (S.), Germany*

For several decades, the effects of antimicrobial peptides or their synthetic mimics on model membranes have been investigated, for example with the aim to develop much-needed alternatives to classical antibiotics. Lipid membranes are supposed to be the main target of these natural or designed molecules. Therefore, induced membrane permeabilization or leakage is often taken as an indication for activity.
Despite a wealth of natural and designed molecules and high-throughput approaches, a break-through is still missing.
To elucidate this discrepancy here, different mechanisms of membrane permeabilization (leakage) are distinguished [1] and their relation to other membrane perturbations was examined by a range of biophysical methods. For example, to unambiguously establish a mechanistic link of concomitant leakage and fusion, leakage and fusion are studied while aggregation, fusion, and similar effects are inhibited or enhanced [2,3]. We found that a commonly used composition of vesicles (POPG/POPE) is especially prone to leaky fusion, potentially leading to misinterpretation [4]. En route, strategies to recognize, judge, and prevent potential side-effects caused by vesicle aggregation or fusion, or both are compiled.
This additional knowledge is expected to improve model studies of membrane-mediated antimicrobial activity and, in turn, future treatments against increasingly resistant microbes.

References

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How to relieve Asymmetry Stress in Model Membranes

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Asymmetry stress is a regulator in membrane remodeling processes and is discussed as a mode of action of various antimicrobial peptides. It describes the intrinsic area mismatch between the inner and outer leaflet in the membrane, which can result from asymmetric partitioning of molecules that have a low transbilayer diffusion rate, such as lysolipids, maltosides and digitonin.

To deal with asymmetry stress, different mechanisms have been reported or hypothesized. Digitonin and lysolipids are known for “staying out” [1,2], but maltosides are known to “crack in” [3] above a certain threshold. Previous studies have identified vesicle budding as an effective but limited mechanism to relieve asymmetry stress induced by lysolipids [4]. However, it remains unclear whether this mechanism extends to other asymmetrically inserting detergents as well.

Utilizing asymmetric flow field-flow fractionation (AF4), we developed an assay to quantify the fraction of lipid budding off from large unilamellar vesicles (LUVs). Surprisingly, our findings reveal that vesicle budding is not exclusive to lysolipids but also extends to other asymmetrically inserting molecules, even those associated with alternative stress-relieving mechanisms. Remarkably, a minimal amount (2 mol%) of detergent in the outer leaflet is sufficient to trigger budding. This suggests that vesicle budding occurs as a primary response to asymmetry stress, preceding other stress relieving mechanisms. The extent of budding is not solely dependent on detergent quantity, but is constrained by the excess surface stored in the initial LUV.

Our study establishes vesicle budding as a rapid and efficient mechanism of relieving asymmetry stress in model membranes, shedding light on its broader applicability beyond lysolipids and its potential significance in membrane dynamics.

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Membrane Thickness Can Regulate Protein Function

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We investigated lipid membrane thinning induced by the intramembrane rhomboid protease GlpG from *E. coli,*[1] which has been proposed to be caused by the "protrusive shape" of the protein. Such thinning has been confirmed for the lipid annulus around GlpG in molecular dynamics simulations. It was hypothesized that this membrane distortion could break the viscosity-imposed limitation of the diffusion velocity of GlpG to allow for optimal substrate catalysis.[2] The effect of GlpG on the surrounding membrane was studied in membranes composed of lipids of various composition. Interestingly, GlpG leaves POPC membranes completely unperturbed while reducing the thickness of mixed POPE/POPG (molar ratio 3:1) by 8%. This is remarkable as all three lipids contained identical chains and glycerol backbones. It is important to note that free standing POPE/POPG membranes are thicker than POPC membranes because of the negative intrinsic curvature of PE. In the presence of GlpG, the thickness difference between the free standing POPC and POPE/POPG membranes of Δ = 3.6 Å was reduced to only 1.0 Å suggesting that GlpG requires a specific hydrophobic thickness of the membrane for optimal function. This was corroborated by measurements of enzyme function in these different membrane environments. Surprisingly, substrate cleavage velocity in the *E. coli* mimicking POPE/POPG membranes was slower than in POPC membranes, which is not a bacterial lipid. Furthermore, in this study, fastest catalysis was recorded in completely artificial DMPC lipids, which have also no relevance for *E. coli* or other biological cells. Clearly, the hydrophobic coupling between protein and surrounding membrane influences protein function. It seems tempting to suggest that the membrane itself can become an allosteric modulator of protein function, a notion that was also suggested recently also by Pabst and coworkers.[3] Being comprised of hundreds of different molecular species, featuring a dynamic domain structure and leaflet asymmetry, the lipid membranes provides the best and most well developed environment for biological function. In this sense, allosteric regulation is generalized to a large assembly of molecules which by varying their mutual interactions can influence the environment to enable or disable specific function. By adjusting membrane thickness, for instance through dynamic domain formation, the cell can regulate membrane protein function.

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Native nanodiscs for membrane-protein biophysics

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Nanodiscs that harbour individual membrane proteins or membrane-protein complexes in a lipid-bilayer environment hold great promise for biophysical and structural investigations under well-controlled yet native-like conditions. Our laboratory focusses on new methods for the direct, detergent-free extraction of proteins from cellular membranes into native nanodiscs, which preserve both the overall bilayer architecture and the local lipid composition of the original cellular membrane. Thus, these native nanodiscs render membrane proteins amenable to *in vitro* biophysical investigations without ever removing the proteins from a lipid-bilayer environment. Recently, we have developed and used novel amphiphilic polymers with improved properties for forming nanodiscs that are compatible with a broad range of ensemble and single-molecule biophysical techniques [1–3]. In this talk, I will present selected examples including antibody–receptor and peptide–lipid interactions studied by microfluidic diffusional sizing, membrane-protein complexes investigated by mass spectrometry, and cell-free translation of membrane proteins with cotranslational insertion into lipid-bilayer nanodiscs.

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Model for tension propagation in cell membranes

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Propagation of membrane tension mediates mechanical signal transduction along surfaces of live cells and sets the time scale of mechanical equilibration of cell membranes. In stark contrast to the earlier expectations, recent studies in several cell types and under different conditions revealed a strikingly wide variation range of the tension propagation speeds including extremely low ones. The latter suggests a possibility of long-living inhomogeneities of membrane tension crucially affecting mechano-sensitive membrane processes. Here, we propose and analyze theoretically a mechanism of tension propagation in membranes crumpled by the contractile cortical cytoskeleton. We predict the pace of the tension propagation to be controlled by the intra-cellular pressure and the degree of the membrane crumpling. The tension spreading is mediated by the membrane flow between the crumples.

Novel dimensions of membrane asymmetry: phospholipid and cholesterol abundance imbalances and their remarkable consequences

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Membranes are molecular interfaces that insulate cells from external stresses, compartmentalize the cytoplasm, and control the flow of nutrients and information. These functions are facilitated by diverse collections of lipids, nearly all of which are distributed asymmetrically between the two leaflets of living bilayers. Previous models of biomembrane structure and function have rested upon the implicit assumption that the two membrane leaflets have similar abundances of phospholipids. Here, we show that this assumption is generally invalid and investigate the consequences of lipid abundance imbalances in mammalian plasma membranes (PM). Using quantitative lipidomics, we discovered that cytoplasmic leaflets of human erythrocyte PMs have >50% overabundance of phospholipids compared to exoplasmic leaflets. We show that this phospholipid imbalance is enabled by an asymmetric interleaflet distribution of cholesterol, which rapidly redistributes to buffer leaflet stresses. Asymmetric phospholipid abundance and composition combine to enrich cholesterol in the exoplasmic PM leaflet. Through a combination of experimental and computational approaches we demonstrate how these lipid distributions impart unique functional characteristics to PMs, including low permeability, surprisingly fast cholesterol diffusion, and resting tension in the cytoplasmic monolayer that regulates protein localization. Our observations of these previously overlooked aspects of membrane asymmetry represent an evolution of classic paradigms of biomembrane structure and physiology.

A molecular view of peptide binding and translocation across lipid bilayers

II. Case in Point: Daptomycin

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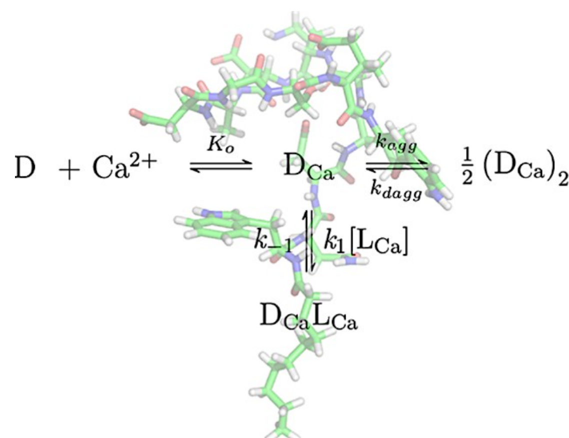
Daptomycin is a lipopeptide of clinical importance in the treatment of multi-drug resistant infections, including those caused by methicillin-resistant *S. aureus* (MRSA) strains. Similar to other antimicrobial peptides, daptomycin binds with preference to the anionic cytoplasmic membranes typically found in prokaryotes; yet, it differs from this class of peptides in several important aspects. Daptomycin is a non-ribosomally synthesized and structurally complex tridecapeptide, with three of its 13 amino acids in the D-configuration (D-Asn-2, D-Ala-8, and D-Ser-11), and three carcinogenicity residues (Orn, 3-MeGlu, and the highly fluorescent Kyn). The 10 C-terminal amino acids are cyclized to form a lactone, and the N-terminal amino acid, tryptophan, is amidated with n-decanoic acid, which enhances binding to lipid bilayers.

In contrast to linear, alpha-helical peptides, daptomycin binds to lipid bilayers only in the presence of calcium ions and its activity is absolutely Ca^{2+}-dependent. Why calcium ions are required for binding of daptomycin to the lipid bilayer is not well established, but, in the presence of calcium ions, binding induces clustering of anionic lipids in the cell membrane of gram-(+) bacteria, which impairs cell division and leads to cell death.

We set out to probe the initial interaction of daptomycin with lipid vesicles containing anionic lipids, starting from first-principles, through kinetic and equilibrium binding experiments. We determined the rate and equilibrium constants for binding of daptomycin to lipid and Ca^{2+} and could show that daptomycin colocalizes with anionic lipid clusters in giant lipid vesicles.



Fluorescence quenching reveals the distribution of two synergistic antimicrobial peptides on a lipid membrane surface

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Antibiotic resistance is seen as one of the most pressing public health crises and must therefore be fought with all means. Antimicrobial peptides represent an effective and promising alternative to conventional antibiotics as they attack bacterial cells via different pathways which are less prone for resistance development. A variety and combination of biophysical methods like fluorescence quenching is used to determine the peptides' distribution on membrane surfaces. The focus of this work lies on two cationic amphipathic peptides from the magainin family: PGLa and magainin2, which interact with biological membranes in a synergistic manner. Understanding their interaction with each other provide new insights in how membrane-active antibiotic peptides function.
Peptide labelling at different positions on the PGLa sequence (1, 10, 16, 21) was performed by introducing the diaminopropionic acid (Dap) which intrinsically carries a fluorophore (nitrobenzoxadiazole, NBD) coupled to the amide of the side chain. By using N-terminal labelled PGLa it has been demonstrated previously [1], that PGLa and magainin2 assemble into defined supramolecular structures along the lipid membrane interface. Here, we confirm the formation of such assemblies, and the data suggests a denser clustering of PGLa at specific positions in the presence of magainin2. Presumably, end-to-end peptide-peptide contacts occur more likely than side-by-side contacts. In summary, the quenching behavior of locally different labeled peptides provides the opportunity to establish a distribution model.

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The Impact of Nonequilibrium Conditions in interfacial barrier membranes

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Land-living organisms face the challenge of adjusting to the relative humidity (RH) of ambient air, which varies from a few percent to saturation at RH=100%, whereas the water chemical potential in living cells corresponds to a RH of above 99%. This difference drives water transport from cells to air, exposing life to a drying-out threat. Different strategies have emerged to counter this threat. In humans, the skin and the eyes are the only surfaces that are exposed to the dry air, whereas interfacial films in the lungs, nose and mouth are exposed to humid air. Water evaporation across the these interfacial barrier films will dynamically modify the concentration of nonvolatile species by forming gradients. In system containing self-assembled molecules, such as lipids, proteins, surfactants and polymers, the concentration gradients will typically trigger the build-up of mesostructure gradients, creating a mass transport feedback loop as drying proceeds [1-3]. Insights into these compositional and structural variations imposed by water evaporation are crucial for our understanding of many biological barrier membranes, but also for a great variety of technologically relevant processes, for example the drying of paints and liquid extraction.

We have designed an experimental set-up that allows for studies of the non-equilibrium time evolution self-assembly at drying interfaces [1]. The composition and structure gradients arising from the evaporation process are characterized using optical microscopy, Raman confocal microscopy, infrared microscopy, and small angle X-ray scattering [1,2]. The experimental characterization is combined with theoretical analysis of the same conditions [3]. We demonstrate that a thin and dry outer layer of the multilayer self-assembled barrier responds to changes in air humidity by increasing its thickness as the air becomes dryer, which decreases its permeability to water, thus counterbalancing the increase in the evaporation driving force. This thin and dry outer phase therefore shields the systems from humidity variations. Such a feedback loop achieves a homeostatic regulation of water evaporation. The findings have implications to the barrier membranes formed by the upper layer of skin [4,5] and the alveolar interface of our lungs [2].

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Plasma membrane curvature induces long-lived multimodal activation of G protein coupled receptors

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G protein-coupled receptors (GPCRs) mediate many physiological functions and are key targets in drug development. A long-held tenet of molecular pharmacology is that GPCRs can spontaneously sample preexisting active conformations. This concept is pivotal to our understanding of ligand pharmacology, however, direct evidence supporting it has only been obtained with reconstituted receptors. Here, we introduce a method for quantitatively imaging the intrinsic activation probability of GPCRs directly at the plasma membrane of live cells, utilizing fluorescent conformational biosensors. Our findings unveil a remarkable spatial multimodality in intrinsic activation probability, with a significant majority (up to 99%) of plasma membrane-expressed receptors showing negligible spontaneous activation. In contrast, the remaining minority of receptors exhibits spontaneous activation up to 22-fold higher than previously estimated. Experiments and theoretical calculations revealed that receptors diffuse into and out of ultralong-lived (~5 minutes) nanodomains where the local membrane curvature allosterically enhances activation in the absence and presence of ligands. Extensive testing across five prototypic GPCRs indicates spatial nanoscale multimodality is ubiquitous, but varying in magnitude depending on the receptor and cell type. Upending conventional wisdom, this study reveals that drug efficacy is not a constant number but a spatiotemporal function *e (x, y, z, t)* whose properties define and multiplex the signaling potency and efficacy of ternary complexes of GPCRs and likely other plasma membrane-receptors.

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Lugdunin: a cyclic peptide that partitions and self-assembles in lipid membranes to form water-filled channels

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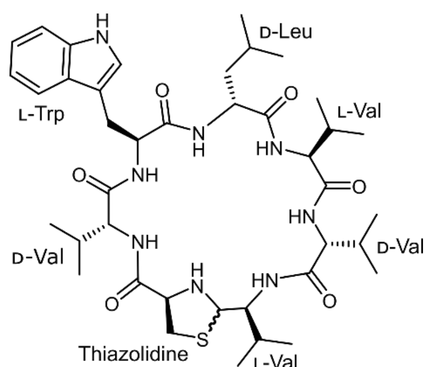

A recently discovered cyclo-heptapeptide, lugdunin, derived from *Staphylococcus lugdunens*is harboring a unique thiazolidine ring, exhibits potent antimicrobial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. In this talk, I will discuss lugdunin's mechanism of action, revealing its rapid destabilization of bacterial membrane potential. Through *in vitro* experiments, we demonstrate that lugdunin preferentially interacts with lipid compositions resembling Gram-positive bacterial membranes, forming hydrogen-bonded antiparallel β-sheets and self-assembles into peptide nanotubes. These nanotubes, observed through ATR-FTIR spectroscopy and molecular dynamics simulations, facilitate the translocation of protons and monovalent cations, as evidenced by voltage-clamp experiments on black lipid membranes [2]. Our findings establish lugdunin as a peptidic channel spontaneously formed by an intricate stacking mechanism, resulting in the dissipation of bacterial cell membrane potential.

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Quantitative studies on peptide interaction with live bacterial cells: lessons learned and new questions.

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Biophysical studies of the interaction of antimicrobial peptides (AMPs) with artificial membranes were pivotal in developing models of the mechanisms of pore formation. However, quantitative data on the behavior of AMPs in real cells are scant. We developed spectroscopic assays allowing the thermodynamic and kinetic characterization of peptide interaction with live bacterial cells [1]. Our studies, with a particular focus on a representative peptide (PMAP-23), showed that AMPs bind to cell membranes in less than one second, and that millions of molecules accumulate on the surface of a single cells, before pore formation occurs. The high coverage of the membrane surface by peptide molecules leads to significant perturbation of the lipid dynamics, which might contribute to the bactericidal mechanism [2]. Bacterial killing then takes place in a few minutes, and peptides translocate to the cell interior, where they associate to cytosolic components. This sequestration of peptide molecules by dead cells can protect the remaining bacteria from AMP activity. Based on cell-binding results, we predicted and observed a specific trend for AMP activity as a function of cell-density (inoculum effect) [3]. As a consequence, AMP activity and selectivity depend on the concentrations of target and host cells; the commonly used activity and selectivity determinations, performed at fixed, standardized cell densities, are questionable. In addition, our model explains why active AMP concentrations never decrease below the micromolar range, even in the presence of low bacterial counts. Overall, our findings clarified some key aspects of AMP behavior but also led to several new questions, which will be addressed during the presentation.

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Bacterial model membranes under the harsh subsurface conditions of Mars

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In recent years, the exploration of extremophiles has gained a lot of interest, due to their remarkable adaptability to harsh environmental conditions. The comprehension of life's boundaries and its associated molecular processes from a physicochemical point of view holds crucial implications for both biology and astrobiology [1-3]. While Earth exhibits numerous examples of organisms thriving in conditions of high salinity, extreme temperature, and high pressure, limited attention has been directed towards extraterrestrial environments [2]. Within this context, recognizing the pivotal role of membranes in the development of most life forms, our study focuses on a bacterial model membrane in a potential Martian-like habitat. Mars is postulated to harbour subsurface lakes containing high concentrations of salts, including magnesium perchlorate, sulphate, and chloride [2]. Here we investigated the impact of these salts on the stability and morphology of a model membrane composed of phosphatidylethanolamine and phosphatidylglycerol, mimicking the main composition of bacterial membranes. Moreover, the impact of Martian relevant salts was investigated on the kinetics of the biologically relevant reaction between the PLA2 protein and the lipid bilayer. Employing an array of biophysical techniques including, differential scanning calorimetry, high hydrostatic pressure fluorescence spectroscopy, confocal microscopy, and small-angle X-ray scattering we observed that in the presence of salts and under high pressure stress, lipids are able to form a stable bilayer, while the activity of PLA2 is strongly modulated. Remarkably, our findings indicate that the chaotropic perchlorate ion plays a crucial role in stabilizing the liquid-like bilayer phase essential for cellular function, thereby counteracting the effects of high pressure in the Martian subsurface.

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Self-assembling peptide nanopores are stabilized by a cooperative hydrogen-bond network

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We have been using synthetic molecular evolution (generations of iterative library design and screening) to evolve peptides that self-assemble into large macromolecule-sized (5-10 nm diameter) “nanopores” that are controllable and membrane-selective. Specifically, we have identified the **pHD peptides** that self-assemble into nanopores in lipid bilayers at very low concentration, triggered by mildly acidic pH. We also evolved the closely related **macrolittins,** which have the same activity, but are not pH sensitive. Such peptide nanopore formation is unprecedented. These peptides fold into α-helices that, despite multiple charged and polar residues, insert into membrane-spanning configurations and stabilize the perimeter of large water-filled pores. Classical textbook concepts of protein folding in membranes do not predict this structure because the peptides appear to be too polar, overall, to stably insert across membranes. Since hydrophobicity alone does not account for their stability in membranes, these nanopores must also be stabilized by other interactions. Individual sidechain H-bonds in contact with bulk water, are relatively weak. But our atomistic molecular dynamics (MD) simulations show that charged and polar groups that are densely located along the fully hydrated inner surfaces of the nanopore form dynamical, yet persistent, cooperative H-bond networks between peptides, lipid headgroups, and water. The headgroups of multiple lipid molecules with unusual orientations participate in the H-bond network and stabilize the nanopore. No previously described H-bond network in membranes is as extensive as the one we have identified in the pHD peptide and macrolittin nanopore structures. These nanopore forming peptides may represent a new type of membrane protein structure.

Abstracts of Posters

(in alphabetical order)

Investigating the role of cholesterol on EGFR ligand binding and phosphorylation

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The lipid composition of the plasma membrane is known to strongly influence the dynamics and subsequent biological function of membrane proteins. Cholesterol accounts for roughly a third of the amount of all components in the mammalian plasma membrane and is known to have significant effects on the membrane's biophysical properties. Receptor Tyrosine Kinases (RTKs) are an important class of membrane proteins that serve integral roles in cell signaling. EGFR is one of the most studied RTKs, and previous studies have documented the effect of lipids on EGFR activation and dynamics in cells. Despite these cell studies, it is not understood if and how lipids directly affect receptor activity in the plasma membrane in the absence of cellular feedback loops that respond to lipid perturbations via other indirect mechanisms. To address this gap, our lab has developed a quantitative microscopy technique to study RTK activation using plasma membrane derived vesicles, produced using a chloride salt vesiculation buffer from CHO cells. This model system allows us to simultaneously measure ligand binding to EGFR and phosphorylation of EGFR tyrosine residues (1). By manipulating cholesterol content in the vesicles using methyl-β-cyclodextrin (MβCD), we quantify the effect of cholesterol on ligand binding and on EGFR phosphorylation levels in the plasma membrane.

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Reconciling past and future research: a study on calcium as a protein-free fusogen in negatively-charged cell-sized vesicles

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In the late 20th century, calcium took on a new identity as an independent fusogen, when it was found to induce fusion in negatively-charged nanometric vesicles (large unilamellar vesicles, LUVs) even in the absence of proteins [1]. These studies are still a core part of membrane model fusion research and are frequently cited. While cell-sized lipid vesicles (giant unilamellar vesicles, GUVs) gained popularity in the 1990s as membrane model, they have not been the subject of an in-depth study about calcium-induced fusion. To investigate this matter, we developed an approach based on confocal microscopy and microfluidics (e.g. Fig. 1) [2][3] and explored how different GUV compositions respond to varying calcium concentrations and environmental conditions. We find that calcium can easily induce hemifusion with lipid mixing in negatively-charged GUVs, while full fusion with content mixing is rather rare and requires fine membrane composition and environmental control to attempt to curb membrane instability. Reconciling past research with modern models proves to be difficult in this case, as our results point to calcium as a less ubiquitous and more elusive fusogen than previously thought in pure lipid GUVs. To find a compromise between past and present models, we explore instead calcium as a protein-free fusogen in negatively-charged LUV to GUV fusion, a task that introduces new challenges and questions.

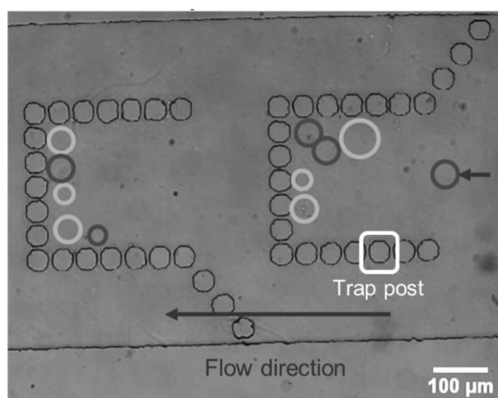

Figure 1: Confocal microscopy image portraying two microfluidic traps formed by PDMS posts. Two populations of GUVs, represented by circles, are transported by the flow and are confined in a trap made of multiple trap posts. This allows prolonged observations on the same vesicle while exchanging the external solution.

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Mitochondrial Membrane Models – From Lipids to Crista Morphology

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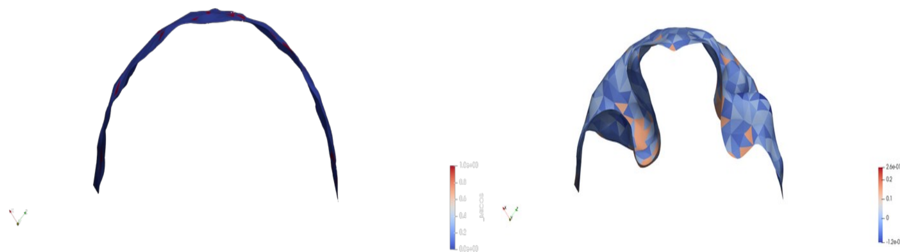

Mitochondrial membranes have a complex composition. They are distinguished by a relative absence of cholesterol, a high degree of asymmetry, and the presence of the enigmatic lipid cardiolipin. We built a set of mitochondrial membrane models, for Mammalia and *Drosophila*, taking into account membrane asymmetry. We then performed MD simulations of these model membranes and extracted curvature elastic properties from them, using the tools we developed for this purpose [1,2]. The resulting study presents a set of model membrane compositions as well as a method to compute bulk properties for any mixture of their components[3]. This is especially relevant when treating local demixing phenomena as e.g. generated by curvature. We also find that in particular Cardiolipin has an ambiguous behavior with respect to curvature and that its properties are highly dependent on counterion distribution and ion specific effects. Finally, we present a new software toolkit, which allows to equilibrate the shapes of arbitrary mitochondrial membrane compositions at organelle scale.[4] We use this toolkit, OrganL to study crista formation and compare results with known cryoelectron microscopy data.[5]

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Deciphering how membrane active compounds affect membrane lipid order

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Lipid systems are ubiquitous in our lives. Every cell is surrounded by a lipid membrane and lipid nanoparticles (LNPs) have enabled us to overcome the corona pandemic. The state of such a synthetic or biological lipid system can be characterized by the order of its lipid molecules. Our aim is to exploit the dependency of application relevant properties on the lipid order such as membrane demixing or permeability and membrane drug interactions. We used membrane embedded dyes such as Laurdan that are sensitive to the surrounding lipid order state.
Laurdan was incorporated either during the preparation of synthetic lipid systems or in case of biological samples by dissolving it into the buffer using an organic solvent. The lipid order was quantified by calculating the generalized polarization GP based on the measured fluorescent emission spectra. Phase transition temperatures of both model and biological membranes were estimated by measuring GP as a function of temperature[1]. This can also detect demixing tendencies.
The phase transition temperatures of model vesicles determined by Laurdan fluorescence and GP analysis are in agreement with DSC data. In this way, it is possible to characterize the interaction of membrane-active peptides with model membranes. We showed the influence of such peptides on a binary POPG/POPE (1:1) model membrane. While there was no significant change in the main phase transition, a second peak was observed after the addition of the peptide. This indicates enhanced phase separation in model membranes after peptide binding and can provide information about the mechanism of action.
Lipid phase transitions within cellular membranes were strongly influenced by short and long-time exposure of the drug tamoxifen. These results open up a new perspective on the mode of action and long-time adaptation effects of membrane targeted drugs.
These observations might inspire researchers across different disciplines to include lipid order measurements in their studies. For this we provide detailed insight into the measurement procedure and introduce a custom-made device that facilitates this kind of studies.

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Exploring the role of the C-terminus of the cAMP GKY20 in membrane perturbation

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Cationic antimicrobial peptides (cAMPs) are natural compounds able to selectively target the negatively charged bacterial plasma membrane, leading to its disruption. [1] In a previous study, the action mechanism of the cAMP GKY20, a sequence derived from the C-terminus of human thrombin, was reported, revealing that this peptide can lead to the disruption of a model bacterial membrane by exerting a detergent-like action. [2] To gain more insights into the molecular determinants responsible for the membrane-perturbing effect, a truncated version of GKY20 corresponding to the last 10 amino acids, named GKY10, was selected. An analysis of the sequence of GKY10 showed in fact that this portion can adopt a perfectly amphipathic helical structure, a feature necessary for an effective interaction with lipid bilayers. Despite its shorter length, we found that GKY10 retains an antimicrobial activity, although with higher minimum inhibitory concentration (MIC) values compared to the parent peptide, consistently with the reduced affinity constant for the bacterial model membrane determined by means of fluorescence spectroscopy. Calorimetric (DSC) and fluorescence experiments, utilizing DPH and Laurdan as probes, consistently indicate that GKY10 binds to the membrane surface where, like the parent peptide, it adopts an α-helical structure and induces anionic lipid segregation resulting in micro-domains formation. Our findings suggest that GKY10 sequence plays a prominent role in GKY20-induced membrane perturbation, providing valuable insights into its action mechanism and relevant information for the design of new membrane-active compounds.

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Can TCSPC of tryptophan shed light on concerted effects of cyclic lipopeptides on membranes?

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Time-resolved (TR) emission spectral shift can provide information about the polarity, order and dynamics of the fluorophore's environment. This information is essential for understanding the mechanism of action of antimicrobial membrane-active peptides, aiming at designing new types of antibiotics in times of antibiotic resistance crisis.

To obtain this information, the usual way is to label the peptide or the membrane with solvatochromic fluorophores. However, the fluorophore itself already alters the properties of its vicinity, changing the very system we want to observe.

An elegant way to solve this problem is to use intrinsic fluorophores - fluorophores that are already present in the system of interest. They often have less-than-ideal, complex fluorescence behavior, making analysis and understanding of the data difficult. The aromatic amino acid tryptophan (Trp) is one of such fluorophores and is commonly found in hydrophobic, transmembrane, or membrane-active peptides.

The aim of this project is therefore to learn more about the TR fluorescence behavior of Trp in peptides. To this purpose, we measure and analyze TR emission spectra (TRES), TR anisotropy (TRFA), TR quenching, and steady-state spectra of four different peptide analogs in which one hydrophobic amino acid of the natural variant is exchanged for Trp, in POPC membrane and compare these data with MD simulations. In this way, we get, interpret and understand data on the impact of the Trp’s position in the peptide, the incorporation of the peptides into the membrane, their incorporation depth and orientation in the membrane and their effects on the membrane structure.

Pharmacodynamics of antimicrobial peptides: the role of water-membrane partition

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Pharmacodynamics describes the relationship between the concentration of antimicrobial agents and their effects on bacteria. Often, a concentration range wherein some bacteria are killed while others survive, is observed. Within this interval (often called “mutant selection window”, i.e., MSW) resistance may develop. Antimicrobial peptides (AMPs) generally act by perturbing the permeability of bacterial membranes. Their MSW is typically smaller than that of traditional antibiotics, but it still encompasses ~one order of magnitude of peptide concentrations. Phenotypic or genetic differences between individual cells could be the origin of this heterogeneous bacterial response to AMPs. However, this explanation cannot be applied for the similar behaviour observed on liposomes, which are extremely homogeneous in terms of size and composition.

Here, we studied the effects of three different AMPs (novicidin, P9-4, Sub3) on artificial vesicles, observing that over three orders of magnitude of peptide to lipid ratios, only a fraction of liposomes released their contents. Membrane perturbation is obviously caused only by peptides bound to the liposomes; by characterizing the water/membrane partition of the three peptides, we were able to report the vesicle-perturbing activity as a function of the membrane-bound peptide concentration. In this case, the curves become essentially step functions, with a well-defined (bound) concentration threshold at which pores are formed in all liposomes. Surprisingly, when taking into account the different lengths of the three peptides (by calculating the number of bound amino acids per lipid) the threshold coincides for all three peptides ($[Residues]_{bound}/[L] \sim 0.4$), suggesting a mostly steric mechanism of pore formation. In conclusion, in the case of liposomes, the MSW is determined by the variation of the fraction of membrane bound peptides under different conditions. Further investigations will clarify if other effects are at play in the case of bacteria.

D(y)e-Coding Membrane Solubilization: Characterizing Lipid-Detergent Systems with Laurdan and Nile Red

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Interactions of detergents with lipid bilayers, and thus membrane solubilization, play a crucial role in numerous fields. For instance, detergents are substantial in various biotechnological applications, such as the isolation and purification of proteins of membranes [1]. Therefore, it is essential to develop a comprehensive understanding of this process. The primary objective of our study is to compare the different information acquired on the membrane solubilization process by two membrane probes, Laurdan and Nile Red, using time-resolved fluorescence studies.
The membrane solubilization itself is a complex process. The well-known three-stage model by Helenius and Simons (1975) simplifies this process and distinguishes between three stages: Stage I (detergent binding), stage II (lamellar-micellar phase transition) and stage III (mixed micelles) [2]. In our experiments, samples of Large Unilamellar Vesicles (LUVs) and Triton® X-100 in all three stages were characterized by time-resolved emission spectra (TRES) and time-resolved anisotropy measurements (TR anisotropy). TRES provides insights into the presence and mobility of water close to the fluorophore, while TR anisotropy elucidates the speed and limitation of its angular motions. Consequently, both methods provide information on the order and dynamics of lipid bilayers.
Our data demonstrate that Laurdan is sensitive to Triton-induced changes in the lipid order and dynamics of bilayers. Conversely, Nile Red primarily focuses on topological changes rather than general disordering. This renders Nile Red superior in detecting the onset and completion of solubilization. However, why do both fluorescence dyes yield different information concerning the solubilization process? Presumably, the different localizations of Laurdan and Nile Red in stage I to stage III are responsible for this observation.

Keywords: Membrane Solubilization, Time-Resolved Fluorescence, Nile Red, Laurdan

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Charged extracellular vehicles (EVs) interfere with Leishmania parasite binding and uptake by phagocytic cells.

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Leishmania donovani is an intracellular protozoan parasite that causes visceral leishmaniasis. It interacts and infect a variety of host cell types, macrophages and dendritic cells (DCs). In this study, we studied the role of cationic and anionic extracellular vesicles (EVs) in interacting with Leishmania uptake by phagocytic cells. Flow cytometry and confocal microscopy analysis showed that mucin coated EVs have a strong binding affinity with Leishmania parasite. The cationic EVs, on the other hand, showed a slightly weaker interaction. We also confirmed that by binding to the parasite, mucin coated EVs reduce its uptake by macrophages and dendritic cells. Furthermore, by using Surface plasmon resonance (SPR) we demonstrate that mucin coated EVs exhibits strong electrostatic interactions with a model membrane that mimic the parasite plasma membrane. This study sheds light on the role of lipids of host cell membranes in parasite interaction and binding, while also supporting previous results implicating the role of electrostatic interaction in parasite entry.

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Deciphering the Distinct Biocontrol Activity of Fengycin and Surfactin, Two *Bacillus* Lipopeptides, through Their Differential Impact on Lipid Membranes

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Lipopeptides produced by beneficial bacilli are interesting candidates as biocontrol agents to reduce the use of chemical pesticides. These molecules present dual properties, i.e, some of them can have a direct antagonism against plant pathogens while others are able to elicit plant immunity. Their biological activities rely on their interaction with plasma membrane lipids but their exact mode of action remains to be elucidated. In this study we conducted a comparative analysis of two bacilli lipopeptides, namely surfactin (SRF) and fengycin (FGC) in terms of biocontrol activity. We examined both their antagonistic property against *Botrytis cinerea* and their capacity to elicit early immune responses in *Arabidopsis thaliana*. In parallel, we investigated their effects on membranes using biomimetic liposomes. FGC exhibits a direct antagonistic activity and a very slight plant immune-eliciting activity while SRF only demonstrates an ability to stimulate plant immunity. Our analysis of membrane permeability, lipid packing, and liposome size revealed that FGC interacts with lipids through a solubilizing/permeabilizing mechanism while SRF interacts by disturbing the lipid packing within the membrane. By correlating the findings from both studies, we can suggest that the direct antagonistic activity of CLPs is linked to their capacity to disrupt lipid membrane (through pore formation or solubilization) while the stimulation of plant immunity is more likely the result of their ability to alter the mechanical properties of the membrane. Hence both aspects, membrane disruption and membrane disturbance, play a pivotal role in determining the effectiveness of lipopeptide or other membrane-active molecules as biocontrol agents.

Unravelling Cholesterol Flip-Flop Dynamics in Lipid Bilayers under Thermal Gradients: Insights from Coarse-Grained Molecular Dynamics Simulations

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In this work we study cholesterol flip-flop under non-equilibrium conditions. Specifically, we investigate the role of thermal gradients across lipid bilayers in promoting membrane asymmetry by biasing the flip-flop motion. To gain insights into the phenomenon of cholesterol flip-flop in lipid bilayers under non-equilibrium conditions, we conducted coarse-grained (CG-MARTINI), Non-Equilibrium Molecular Dynamics (NEMD) simulations. The focus was on identifying key factors that influence cholesterol flip-flop and the thermal preference of membrane asymmetry.

As the temperature gradient increases, we find an enhancement of cholesterol accumulation on the cold lipid region (thermo-phobicity), leading to an increased asymmetry within the bilayer. We investigate the behaviour of cholesterols in various lipid membranes (DLPC, POPC, DPPC, DBPC) under a stationary temperature gradient.

Our study further explores the nuanced relationship between the length of the hydrocarbon chain and the rate of cholesterol flip-flop and the influence of the saturation of the phospholipids on cholesterol dynamics.

In summary, this research, based on NEMD simulations and coarse-grained phospholipid bilayers, contributes to our understanding of thermal-dependent cholesterol dynamics and the intricate relationship between phospholipids and cholesterol in biological membranes, providing valuable insights into membrane permeability, fluidity, and stability in out of equilibrium conditions.

Azide- and diazirine-modified membrane lipids: Physicochemistry and applicability to study peptide/lipid interactions via cross-linking/mass spectrometry

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Although the incorporation of photo-activatable lipids into membranes potentially opens new avenues for studying interactions with peptides and proteins, the question of whether azide- or diazirine-modified lipids are suitable for such studies remains controversial. We have recently shown that diazirine-modified lipids can indeed form cross-links to membrane peptides after UV activation and that these cross-links can be precisely determined in their position by mass spectrometry (MS) [1]. However, we also observed an unexpected backfolding of the lipid's diazirine-containing stearoyl chain to the membrane interface challenging the potential application of this modified lipid for future cross-linking (XL)-MS studies of protein/lipid interactions.

In this work, we compared an azide- (AzidoPC) [2] and a diazirine-modified (DiazPC) [1] membrane lipid regarding their self-assembly properties, their mixing behavior with saturated bilayer-forming phospholipids, and their reactivity upon UV activation using differential scanning calorimetry (DSC), dynamic light scattering (DLS), small-angle X-ray scattering (SAXS), transmission electron microscopy (TEM), and MS. Mixtures of both modified lipids with DMPC were further used for photo-chemically induced XL experiments with a transmembrane model peptide to elucidate similarities and differences between the azide and the diazirine moiety.

We showed that both photoreactive lipids can be used to study lipid/peptide and lipid/protein interactions [3]. The AzidoPC proved easier to handle, whereas the DiazPC had fewer degradation products and a higher cross-linking yield. However, the problem of backfolding occurs in both lipids; thus, it seems to be a general phenomenon.

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The impact of tryptophan derivatives on synaptic vesicular exocytosis

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The neurotransmitter serotonin is involved in physiological processes like appetite regulation, sleep and mood, as well as pathophysiological conditions like anxiety and depression [1]. Traditionally, all effects of serotonin were thought to be mediated by binding to its target transmembrane receptors [2]. It is now known that serotonin can also bind to the membrane with high affinity [3] and modulate both lateral segregation of lipids [4] and membrane protein activity [5]. We wanted to further investigate if the molecules in the metabolic pathway of serotonin can affect synaptic vesicular fusion. Using total internal reflection fluorescence microscope microscopy in neural cells, it was found that serotonin and its metabolite N-acetyl serotonin (NAS), but not the other tryptophan derivatives, can increase synaptic vesicular exocytosis by stabilizing the synaptic vesicular association to the synaptic plasma membrane. Furthermore, we wanted to distinguish why only some of the metabolites affected the synaptic vesicle exocytosis. Therefore, we employed solid state ^{1}H NOESY magic angle spinning NMR to examine the distribution of the metabolite's tryptophan, 5-hydroxytryptophan, serotonin, NAS, and melatonin within 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) multilamellar vesicles. Slight differences in membrane distribution were found for the tryptophan derivatives. In addition, utilizing ^{2}H NMR, we observed that all tryptophan derivates disordered perdeuterated POPC-d_{31} within a synaptic vesicle model membrane. The largest disordering effect was observed for NAS and the lowest for tryptophan. In conclusion, a combination of factors like disruption of acyl chain order, membrane distribution and potential H-bonding of the different metabolites could lead to different effects on synaptic exocytosis. However, further examination is required to fully understand the connection between the molecular properties of the tryptophan derivatives and the biological consequences.

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Lipid fingerprints of a stressed membrane

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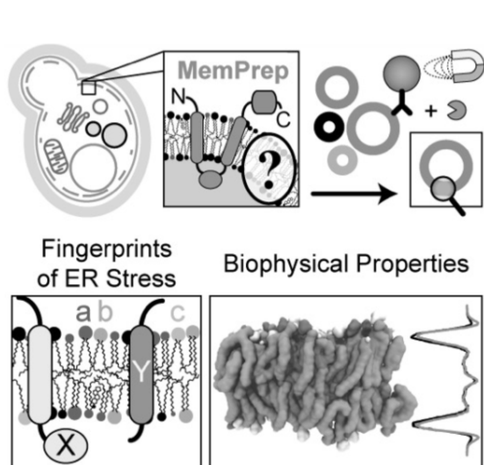
Biological Membranes are complex and dynamic assemblies comprising thousands of different lipid and protein species. Collective physicochemical bilayer properties determine the identity and function of biological membranes in the cellular context. The endoplasmic reticulum (ER) is crucial for membrane biogenesis and membrane protein folding. Imbalances in protein folding and lipid metabolism can cause ER stress and trigger a large-scale transcriptional program to reestablish ER homeostasis. ER stress can be triggered either by unfolded proteins or aberrant properties of the ER membrane. Unmitigated ER stress has been linked to diseases like type II diabetes and cancer. Using baker's yeast as a model, we dissect the molecular basis of the membrane-based ER stress.



We established an approach for immuno-isolating subcellular membranes from stressed and unstressed cells: MemPrep. Quantitative lipidomics reveals a substantial remodeling of the ER membrane upon ER stress. Using these data and molecular dynamics simulations, we suggest a simple ER-like membrane composition that mimic more faithfully the properties of the natural, cellular ER membrane. Label-free, quantitative proteomics provide holistic insight into the changes of the ER membrane during stress. We identify characteristic fingerprints of the stressed ER and provide evidence that anionic lipid can dampen UPR signaling. We show that MemPrep can be used to study the composition of ER subdomains and other organelles such as the vacuole. We are convinced that this technology will prove essential to study the subcellular organization of lipids and proteins between co-existing organelles during stress and adaptation.

α-Synuclein Cooperative Binding to Lipid Membranes

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α-Synuclein is a 140 a.a. long and intrinsically disordered protein localized in vivo in synapses. As far as it is currently known, it is implied in still-poorly-understood healthy functions and also found as the main component of aggregates in Lewy bodies and Lewy neurites, hallmarks of the Parkinson's disease. In both cases, the interaction with lipid membranes is crucial. In this study we investigate the interaction between α-Synuclein and model systems made of DOPC and DOPS, either in the form of giant and small unilamellar vesicles. We systematically changed parameters such as pH, ionic strength, salt composition and α-Synuclein amino acidic sequence (mutant α- Synuclein H50Q) in order to evaluate the cooperativity with the final goal to understand the driving forces leading to this feature. To do so, we took advantage of Confocal Laser Scanning Microscopy, Circular Dichroism and Fluorescence Cross Correlation Spectroscopy and we characterized the binding as cooperative for all the conditions investigated.

Monitoring alterations in lipid bilayers in real-time

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Biological cells are surrounded by lipid bilayers that act as physical barriers to separate the inside of a cell from its environment. However, lipid bilayers are not rigid structures, yet are continuously remodeled. While this enables biological cells to adapt quickly to changing environmental conditions, changing lipid environments will affect the biophysical properties of the lipid bilayer system as well as the structure, stability, and activity of membrane-integrated proteins.

We use the activity of a membrane protein reconstituted into proteoliposomes to establish a system allowing controlled alterations of membrane properties induced by an external stimulus. The prokaryotic diacylglycerol kinase (DAGK) is a membrane protein that catalyzes the phosphorylation of the glycerolipid diacylglycerol (DAG) to phosphatidic acid (PA) using ATP as phosphate and energy source. The conversion of DAG, which prefers the formation of inverse hexagonal (H_{II}) lipid phases over lamellar bilayers, into PA in defined model membranes clearly has a large impact on the membrane lipid order.

The DAGK of *E. coli* is a well-studied membrane protein that can be purified in large quantities upon overexpression [1]. We recombinantly expressed and purified functional DAGK of *E. coli* in large quantities. We are currently investigating the effect of the DAGK activity on model membranes having a defined lipid composition. Furthermore, the effect of DAGK-induced lipid bilayer alterations on the stability of transmembrane proteins is studied in model membranes via monitoring the stability of a transmembrane helix dimer. Our analyzes allow real-time monitoring the impact of triggered lipid modification on the structure and properties of lipid bilayer systems as well as of a membrane protein’s structure.

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Lateral heterogeneity in phospholipid bilayers containing cardiolipin and ceramide: relevance to autophagy

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LC3/GABARAP human autophagy proteins are key in the recognition and removal of damaged mitochondria. The role of cardiolipin (CL) and ceramide (Cer) in this process is unclear, but both lipids have been proposed to coexist in mitochondria under certain conditions. Varela et al. [1] showed that in model membranes composed of egg sphingomyelin (eSM), dioleoyl phosphatidylethanolamine (DOPE), and CL, the addition of Cer enhanced the binding of LC3/GABARAP proteins to bilayers. Cer gave rise to lateral phase separation of Cer-rich rigid domains but protein binding took place mainly in the fluid continuous phase. In the present study, a biophysical analysis of bilayers composed of eSM, DOPE, CL, and/or Cer was attempted to understand the relevance of this lipid coexistence. Bilayers were studied by differential scanning calorimetry, confocal fluorescence microscopy, and atomic force microscopy. Upon the addition of CL and Cer, one continuous phase and two segregated ones were formed. In bilayers with egg phosphatidylcholine instead of eSM, in which the binding of LC3/GABARAP proteins hardly increased with Cer in the former study, a single segregated phase was formed [2]. Assuming that phase separation at the nanoscale is ruled by the same principles acting at the micrometer scale, it is proposed that Cer-enriched rigid nanodomains, stabilized by eSM:Cer interactions formed within the DOPE- and CL-enriched fluid phase, result in structural defects at the rigid/fluid nanointerfaces, thus hypothetically facilitatingLC3/GABARAP protein interaction.

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Interactions of the Antibiotic Daptomycin with Model Membranes compared to a Novel Cyclic Lipopeptide

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The challenge of antimicrobial resistance is increasing, leading to decreased clinical efficacy of numerous antibiotics. This highlights the constant need for new antibiotic agents. Cyclic lipopeptides (CLiPs) are a promising group. Various genera of bacteria, such as *Streptomyces*, *Bacillus*, or *Pseudomonas*, produce CLiPs as secondary metabolites of non-ribosomal peptide synthetases. Daptomycin, a CLiP from the soil bacterium *Streptomyces roseosporus*, is serving as a prime example. It is approved as a last-resort antibiotic to treat infections caused by Gram-positive bacteria.
Here, we introduce a new daptomycin analog, which is also a CLiP from *Streptomyces*. Our aim is to provide preliminary insights into mode of action of the analog and compare it to daptomycin. Our focus is on the membrane binding and membrane permeabilization.
We use isothermal titration calorimetry (ITC) to characterize the membrane binding. The binding of daptomycin to the membrane is a complex process and depends on interactions between: Daptomycin, Ca^{2+}, PG-containing lipid membranes, various intermediates, and their corresponding concentrations [1]. A simple fitting model is insufficient to represent the complex interactions. Therefore, we are developing a comprehensive fitting model for ITC data to estimate and quantify the intricate binding model. In a subsequent step, we determined the extent to which the mode of action of daptomycin and its analog is comparable. After membrane binding, membrane permeabilization induced by daptomycin is discussed as a part of its mode of action. We performed conductivity measurements on planar lipid membranes to investigate whether the analog also permeabilizes the membrane.
Our studies revealed that the analog binds to lipid membranes in a calcium-dependent manner, like daptomycin. Therefore, lipid membranes containing PG are required. However, the analog differs significantly in terms of its stoichiometric binding. We could also demonstrate that the novel daptomycin analog is able to form pores in membranes.

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Understanding temperature-triggered membrane permeabilization as used in drug delivery

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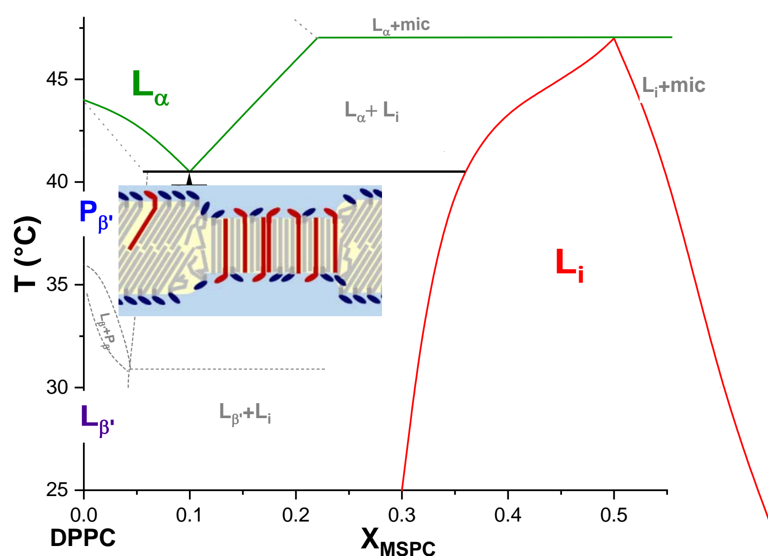
Mixtures of DPPC with 10 mol% monostearoyl-lysolphosphatidylcholine (MSPC) show a strong, transient leakage when heated up to the "melting" temperature, 41°C. This is utilized by so-called thermoresponsive liposomes used to target an enclosed drug to a hyperthermal tissue. The traditional explanation, the lysolipid would induce defects in fluid membranes but mix ideally with gel membranes is in conflict with what has been found for other detergents: They destroy gel phase liposomes already at concentrations that are well below the defect-formation threshold in fluid membranes.



The specific behavior of the lysolipid is due to the fact that it forms an interdigitated gel phase that incorporates very high lysolipid contents without membrane permeabilization. The trigger temperature of the drug delivery system is just at a eutectic point where a lysolipid-depleted gel bilayer containing small lysolipid-enriched interdigitated domains melt all at once. Membrane regions with very high local lysolipid contents then cause a transient leakage of the membrane [1].

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Membrane remodeling by the cyanobacterial protein IM30

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The inner membrane-associated protein of 30 kDa (IM30) is essential for the development and maintenance of the thylakoid membrane (TM) in chloroplasts and cyanobacteria. The structural details of its large (>2 MDa) ring-shaped assemblies have been solved recently (1). However, how it fulfils its task *in vivo* is still an open question. Detailed functional studies *in vitro* revealed, that the protein binds to lipid membranes containing anionic lipids. We could also show that upon binding the ring structure disassembles, altering the properties of the membrane and potentially stabilizing it (2). However, in presence of magnesium ions, membrane fusion is observed, in particular if the membrane additionally contains non-bilayer forming galactolipids, resembling the thylakoid membrane (3). Thus, IM30 seems to have two controversary functions, controlled by the concentration of magnesium ions. Recently we observed, that NBD-PE located in the inner leaflet of liposomes containing anionic lipids becomes accessible to dithionite after incubation with IM30. This effect was even more pronounced when a mutant which forms dimers and tetramers, but not rings (C7EE, (2)), is used. In this case, even disintegration of liposomes occurs, as indicated by dynamic light scattering. Indications for this fragmentation was also found for wt IM30. With liposomes containing only 40% anionic lipids, and 60% zwitterionic lipids, also an increased accessibility of NBD-PE was observed, albeit higher protein concentrations were required. However, under the same conditions fragmentation was not observed, demonstrating that the increase in accessibility of inner-leaflet NBD-PE is not the result of fragmentation. Thus, also in absence of magnesium ions binding of IM30 to liposomes can lead to destabilization of the membrane and even liposome fragmentation under certain conditions.

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Membrane permeabilization and other membrane perturbations: a network of mechanisms affecting the significance of model studies

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For several decades, the effects of antimicrobial peptides or their synthetic mimics on model membranes have been investigated, for example with the aim to develop much-needed alternatives to classical antibiotics. Lipid membranes are supposed to be the main target of these natural or designed molecules. Therefore, induced membrane permeabilization or leakage is often taken as an indication for activity.
Despite a wealth of natural and designed molecules and high-throughput approaches, a break-through is still missing.
To elucidate this discrepancy here, different mechanisms of membrane permeabilization (leakage) are distinguished [1] and their relation to other membrane perturbations was examined by a range of biophysical methods. For example, to unambiguously establish a mechanistic link of concomitant leakage and fusion, leakage and fusion are studied while aggregation, fusion, and similar effects are inhibited or enhanced [2,3]. We found that a commonly used composition of vesicles (POPG/POPE) is especially prone to leaky fusion, potentially leading to misinterpretation [4]. En route, strategies to recognize, judge, and prevent potential side-effects caused by vesicle aggregation or fusion, or both are compiled.
This additional knowledge is expected to improve model studies of membrane-mediated antimicrobial activity and, in turn, future treatments against increasingly resistant microbes.

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Monitoring detergent effects in lipid bilayers

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Reconstituting membrane proteins into lipid vesicles typically involves the use of detergent molecules. However, completely removing these detergent molecules from the system and detecting any residual molecules in the membrane can be difficult. Additionally, the impact of residual detergent on experiments, such as water permeability measurements, is unclear. To address this issue, we investigated the effect of residual octyl glucoside (OG) using the stopped-flow methodology. Thereby, large unilamellar vesicles from *E.coli* polar lipid extract with various concentrations of OG were challenged with a hyperosmotic gradient to induce water efflux from the vesicles. Analysis of time-dependent scattering and fluorescence self-quenching signals revealed that OG causes a maximal relative increase in membrane water permeability of 60-70% at room temperature, depending on the osmolyte used. Interestingly, in contrast to self-quenching experiments, an OG-dependent second kinetic appeared in scattering mode. Using hyperosmotic shrinkage simulations, we found that the dilution of OG in the measurement cuvette during the application of the hyperosmotic buffer led to a temperature-dependent extraction of OG out of the lipid bilayer into the buffer. The kinetic was only visible in the scattering data due to its sensitivity to the refractive index of the vesicle defined by the refractive indices and the respective volume fractions of the membrane and the vesicle interior. The application of a hyperosmotic gradient did not influence fluorescence self-quenching experiments as the process of detergent extraction, in turn decreasing the vesicle membrane area, only changes the vesicle shape towards a sphere but doesn't affect the volume change, which is solely defined by the osmotic gradient. It is unclear whether the rapid withdrawal of OG from the outer vesicle leaflet creates a mass imbalance between both leaflets, resulting in a destabilized inner leaflet forming mixed micellar structures within the inner monolayer, or if a flip-flop of OG between both monolayers occurs on a similar timescale as the detergent partitioning into the aqueous phase. Thus, stopped-flow light scattering experiments are a reliable method for monitoring detergent removal from lipid vesicles and proving the presence of detergent in vesicular membrane systems.

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A membrane model to measure the transducer function of signal propagation along single-pass membrane receptors

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The extracellular environment is sensed by a multitude of receptors embedded in the plasma membrane, some of which initiate cytoplasmic signaling cascades involving phosphorylation: the addition of a phosphate group to a specific amino acid, such as tyrosine, in a protein. Receptor Tyrosine Kinases (RTKs) are one large class of membrane receptors that can directly initiate signaling cascades through their intracellular kinase domains, which both catalyze tyrosine phosphorylation and get phosphorylated. In the first step of signaling, the ligands stabilize phosphorylation-competent RTK dimers and oligomers, which leads to the phosphorylation on specific tyrosine residues in the activation loop of the kinases. We have performed quantitative measurements of RTK phosphorylation efficiencies, as given by the so-called "transducer function", which links the phosphorylation (the response) and the ligand-receptor binding events (the stimulus). We developed a methodology and a membrane model that allow such measurements for Epidermal Growth Factor Receptor (EGFR) in direct response to ligand binding. Our experiments demonstrated that EGF is a partial agonist of EGFR, and that two tyrosines in the intracellular domain of EGFR, Y1068 and Y1173, are differentially phosphorylated in the EGF-bound EGFR dimers.

How to relieve Asymmetry Stress in Model Membranes

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Asymmetry stress is a regulator in membrane remodeling processes and is discussed as a mode of action of various antimicrobial peptides. It describes the intrinsic area mismatch between the inner and outer leaflet in the membrane, which can result from asymmetric partitioning of molecules that have a low transbilayer diffusion rate, such as lysolipids, maltosides and digitonin.

To deal with asymmetry stress, different mechanisms have been reported or hypothesized. Digitonin and lysolipids are known for “staying out” [1,2], but maltosides are known to “crack in” [3] above a certain threshold. Previous studies have identified vesicle budding as an effective but limited mechanism to relieve asymmetry stress induced by lysolipids [4]. However, it remains unclear whether this mechanism extends to other asymmetrically inserting detergents as well.

Utilizing asymmetric flow field-flow fractionation (AF4), we developed an assay to quantify the fraction of lipid budding off from large unilamellar vesicles (LUVs). Surprisingly, our findings reveal that vesicle budding is not exclusive to lysolipids but also extends to other asymmetrically inserting molecules, even those associated with alternative stress-relieving mechanisms. Remarkably, a minimal amount (2 mol%) of detergent in the outer leaflet is sufficient to trigger budding. This suggests that vesicle budding occurs as a primary response to asymmetry stress, preceding other stress relieving mechanisms. The extent of budding is not solely dependent on detergent quantity, but is constrained by the excess surface stored in the initial LUV.

Our study establishes vesicle budding as a rapid and efficient mechanism of relieving asymmetry stress in model membranes, shedding light on its broader applicability beyond lysolipids and its potential significance in membrane dynamics.

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Lipid composition and lipid-protein interactions greatly modify the free energy landscape of pore formation and fusion

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Membrane topological transitions, including pore formation and membrane fusion, are pivotal in various biophysical processes such as exocytosis, viral infection fertilization, intracellular trafficking, and proton transport. These transitions occur in complex, asymmetric membranes, composed of hundreds of lipid species and membrane proteins. Understanding how lipid composition and lipid-protein interactions influence the free energy landscape of these transitions remains challenging.

We present computationally efficient methods to assess the impact of lipids and membrane-associated proteins on the energetics of the formation of pores and stalks or on stalk widening, based on both atomic and coarse-grained models. Our simulations reveal that the membrane composition may facilitate or hinder such topological transitions by tens or even hundreds of kilojoules per mole, suggesting that cells utilize membrane complexity to control the kinetics of such events. We find that the inner leaflet of a typical plasma membrane exhibits by far higher fusogenicity compared to the outer leaflet, potentially facilitating efficient exocytosis while providing resistance against viral infection. Furthermore, our recent work challenges accepted continuum models for membrane electroporation and elucidates how membrane anchors of fusion proteins play active roles in driving membrane fusion.

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Shape-based design of bitopic proteins as probes for membrane elastic stress

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The function of integral membrane proteins is influenced by their hosting lipid membrane environment. Apart from specific lipid- protein interactions, also the properties of the bulk membrane affect the structure and function of transmembrane proteins (TMPs).

We hypothesize that TMPs respond to the elastic stress distribution of the lipid bilayer. To this end we designed bitopic proteins considering their geometrical shape along the lipid bilayer normal while matching the overall membrane thickness. The transmembrane sequence was chosen in a way to rule out specific interactions between amino acids by exclusively using hydrophobic residues. The self-association of these "probes" is then to be measured by single molecular Förster resonance energy transfer microscopy in large unilamellar vesicles of varying lipid composition to simulate different membrane stress environments.

The artificial proteins were expressed in E. coli using a maltose binding protein (MPB) solubility tag and purified the proteins in the presence of detergent.

The current iteration of our TMP sequence is of asymmetric shape and was designed to improve purification with detergent as well as the interaction with lipid bilayers.

Currently, we are experimenting on the reconstitution into lipid vesicles. In particular, we are exploring the possibility of the MBP-tag as hydrophilic "anchor" on the extravesicular side of the liposomes by side-specific phosphine-quenching of cyanine dyes. This would enable the unidirectional insertion of the TMP.

Effects of a Phase Transition in Lipid-Asymmetric Vesicles

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Membrane asymmetry (in terms of lipid and area asymmetry) is associated with several physiological processes such as apoptosis or intra- and extracellular transport [1]. Lipid asymmetry occurs in most biological membranes in the sense that the outer leaflet contains different lipids than the inner leaflet [2].
The aim of our studies is, first, to establish a two-component, lipid-asymmetric membrane model system in a gel-fluid coexistence, and second, to induce and investigate asymmetry stress without adding additional substances. To prepare lipid-asymmetric large unilamellar vesicles (aLUVs), we use a method based on the exchange of outer leaflet lipids with complexes in an aqueous solution [3]. Pressure perturbation calorimetry (PPC) reveals, that aLUVs made of SOPC and DPPG show a different melting behavior compared to lipid-symmetric vesicles. We assume that these differences occur due to coupling of the inner and outer leaflet of aLUVs [4], nevertheless, our results indicate partial coupling, which we hypothesize is not an all-or-nothing behavior. In addition, we examined phase transitions of aLUVs prepared at different T_{ex} via PPC. Regarding a T_{ex} of 30°C, we observe a shift to lower temperatures, compared to 5 or 15°C. We have evidence to assume that this effect is related to the amount of DPPG incorporated to the outer leaflet.
Based on a phase diagram of SOPC and DPPG prepared using DSC, we created a theoretical prediction of gel-fluid coexistence and amount of PG, as well as extent of membrane curvature. We assume, that depending on T_{ex}, asymmetry stress can be induced in the initially relaxed membrane by increasing the temperature as asymmetrically inserted PG melts. This asymmetry stress can be relieved by budding of small daughter vesicles [5], [6]. Using asymmetric flow field-flow fractionation (AF4), we observe budding in aLUVs prepared at a T_{ex} of 5 and 15°C, whereas at 30°C there is no asymmetry stress induced and therefore no daughter vesicles detected.
In future research, we will continue to investigate phase transitions in lipid- and area-asymmetric liposomes and the effects on membrane structure and dynamics.

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Formation of supramolecular structure and phase separation in lipid bilayer upon reconstitution of water-soluble protein hemoglobin

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Comprehensive study of lipid membrane is incomplete without proteins. Studying the insertion mechanisms, and conformational changes in protein while adapting in lipid environment contribute not only to fundamental biophysical fields but also hold potential applications in therapeutics and synthetic biology. We studied the structural behaviour of a non-membranous protein hemoglobin after insertion in a lipid environment. The protein was reconstituted in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposome via detergent-mediated reconstitution method, using non-ionic detergent n-octyl-β-d-glucoside (NOG) [1]. Supported lipid bilayer was prepared on a glass substrate for further investigations. Microscopic studies suggested that the protein could be visualized without any external labelling agents on the lipid bed. Interestingly, this water-soluble protein molecules assembled themselves spontaneously to form supramolecular structures to confront the high hydrophobic stress. Spectroscopic analysis pointed to slight structural alterations, revealing the exposure of hydrophobic regions in the protein to accommodate the hydrophobic stress. Meanwhile, findings from neutron scattering techniques indicated that hemoglobin molecules retained their tetrameric structure within the system. The presence of a hydrophobic atmosphere induced such protein-protein self-assembly to adapt in the given environment. The expansion of the system components up to fourfold concentration on the limited surface triggered phase separation and membrane deformations. The formation of large stable domains due to this phase separation were observed, exhibiting slow kinetics with correlation times in the order of minutes [2]. These initial observations of hemoglobin behaviour within a lipid environment, driven us to include various concentrations of cholesterol and sphingomyelin in the lipid composition, allowing us to study their impact on this lipid-protein system.

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Understanding the fate of mixed micellar drug delivery systems

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Multicomponent surfactants are widely used in pharmaceutical formulations to solubilize poorly soluble drugs for oral and parenteral administration. Mixed micellar drug delivery systems (MM-DDS) in particular show several advantages, among them easy production and high storage stability. Nonetheless, the number of mixed micellar drug delivery systems for intravenous injection on the market has remained limited. The key problem pointed out by an EMA reflection paper issued in 2012 is the susceptibility of these systems to transformation after administration.

To establish a better mechanistic and physico-chemical understanding of MM-DDS and their fate after i.v. administration, we have established a pseudo-phase model of a surfactant-lipid-plasma protein-buffer system using isothermal titration calorimetry (ITC).[1] However, we have not investigated the transitions of MM systems induced by temperature shifts, e.g. between storage temperature and body temperature, yet. Furthermore, the temperature effect on the maximal additive concentration of active pharmaceutical ingredients (API) in our system remains unknown. The risk of precipitation of the API can represent a serious obstacle in the development of MM-DDS. We therefore want to explore whether differential scanning calorimetry (DSC) is a suitable method to measure precipitation of possible APIs in MM-DDS. Extending the pseudo-phase model established with the information gained on temperature effects shall benefit the field of lipid-based drug delivery systems and, on a more general level, understanding the behavior of lipid-based systems when subjected to different conditions will lead to a deeper understanding of their integrity.

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Design of Phospholipid-Polymer-Nanoparticles with phospholipid-dependent drug delivery profiles

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The development of effective drug delivery systems (DDS) that target specifically intracellular compartments needs research beyond the biophysical characterization of nanoparticular systems including size, shape, hydrophobicity, surface charge determining the biofate of nanoparticles (NPs). Only the collective consideration of the nanomaterial interactions with cell biology enables high precision drug delivery [1].

In response to this need, we propose the development of Phospholipid-Polymer-Hybrid-NPs (PPH-NPs) with phospholipid-dependent drug release and delivery profiles that may benefit from intracellular phospholipid dynamics [2] using dual centrifugation (DC) for high throughput screening.

PPH-NPs are promising vehicles that may provide desirable time-dependent changes in particle size, shape, surface properties inducing phospholipid and drug release. Thereby, physicochemical interactions between phospholipid and drug monomers might control the drug release profiles and contribute to high precision drug delivery by exploiting intracellular phospholipid trafficking mechanisms.

In order to establish comprehension for the rational design of PPH-NPs, we aim first at clarifying the phospholipid integrity and heterogeneity on the polymer by differential scanning calorimetry (DSC) and Zeta-Potential measurements. Secondly, at the physicochemical and thermodynamic interaction with the polymer by DSC and isothermal titration calorimetry (ITC) and thirdly the interaction with the drug (ITC) [3].

Further, we aim at clarifying how these physicochemical interactions within PPH-NPs can be used for high precision drug release and delivery by verifying the drug and phospholipid direction not only in buffer but also in dispersions of human serum albumin using methods of time-resolved fluorescence measurements (TRF). Finally, the drug release and delivery will be tracked *in vitro* by confocal-laser-scanning-microscopy (CLSM) and correlated with intracellular phospholipid trafficking pathways.

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Property-structure-function analysis of complex LNPs using integrative biophysical, molecular-, and cell-based assays

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BACKGROUND. The complexity of lipid nanoparticles (LNPs) for delivery of nucleic acids presents a developability challenge. Fit-for-purpose and complementary analytical tools are required to design successful formulations, and to inform robust production of stable, safe, and efficacious products.

METHODS. In this study, different mRNA-LNP formulations and batches were compared using biophysical and cell-based assessments. Fluc mRNA-LNP and eGFP RNA-LNP formulations were compared based on size, particle concentration and zeta-potential with nanoparticle tracking analysis (NTA), dynamic and electrophoretic light scattering (DLS, ELS) [1]. Differential scanning calorimetry (DSC) was used along with cell-based transfection efficiency assay to compare higher order structure (HOS) and functional activity of the two batches of Fluc RNA-LNP2 formulation [2].

RESULTS. Differences in physicochemical profiles of Fluc mRNA-LNPs were well-correlated and robustly resolved with DLS, NTA and ESL. Poor HOS comparability of LNP2 batches was corroborated in the functional assay. For the eGFP mRNA-LNPs, Batch 2 and 4 did not show significant differences in size, while Batch 1 and 3 exhibited a shift to larger sizes, with the aggregation apparent in Batch 1 explaining the poor mRNA encapsulation efficiency (<20%). Batch 2 – 4 were smaller and similar in size, which correlated well with encapsulation efficiency data (>90%). There was also exceptional agreement with cell-based transfection efficiency data showing that LNP aggregation and poor encapsulation efficiency of Batch 1 led to low transfection efficiency compared to Batches 2 – 4.

CONCLUSIONS. Taken together, these data suggest that biophysical characterization of LNP vectors combined with cell-based assays to assess LNP transfection efficiency are key for informing association between particle properties, HOS and functional performance.

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Tolaasin structure revisited: On the impact of environment and macrocycle integrity for tolaasin structure and its interaction with membranes

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Pseudomonas lipopeptides come in many varieties (more than 130 have been described) but all have in common the same chemical blueprint which creates amphipathic 3D structures capable of adsorbing and perturbing membrane bilayers. Over the past decade, we have determined their 3D structures, performed structure activity relationships and investigated their interactions with model membranes. This contribution will mainly focus on tolaasin, the causative agent of brown blotch disease in mushrooms, amongst others. Its structure, determined previously by solution NMR methods in SDS micelles[1], features a 13 residue amphipathic helix with non-canonical amino acids, N-capped by a 3-hydroxy-octanoic acid, and crowned at the C-terminus by a five amino acid residue macrocycle that is closed through ester bond formation. This creates a structural motif not unlike a golf-club fold. The opportunity to obtain tolaasin in ^{15}N or $^{13}C,^{15}N$ labelled form has fostered new investigations using methods allowing to access the phi torsion angle of the backbone, to identify hydrogen bonds and their strength, and methods to define the location and orientation with respect to the micellar structure. Insights into how DMSO, water, water/TFE and SDS/DPC micelles influence tolaasin structure will be reported. Also, in nature, tolaasin can be detoxified by enzymatic hydrolysis of the ester bond, creating a linear structure. We report our comparative investigation of how loss of the macrocycle affects overall structure and its interaction with micellar model membrane systems.

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How charges effect the solubilization of artificial and native membranes by amphiphilic copolymers

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Certain amphiphilic copolymers form lipid-bilayer nanodiscs from artificial and natural membranes, thereby rendering incorporated membrane proteins optimal for structural analysis. Recent studies have shown that the amphiphilicity of a copolymer strongly determines its solubilization efficiency. This is especially true for highly negatively charged membranes, which experience pronounced Coulombic repulsion with polyanionic polymers. Here, we present a systematic study on the solubilization of artificial multicomponent lipid vesicles that mimic inner mitochondrial and fungal membranes, which harbor essential membrane-protein complexes. In particular, we compared the lipid-solubilization efficiencies of established anionic with less densely charged or zwitterionic and even cationic copolymers in low- and high-salt concentrations. The nanodiscs formed under these conditions were characterized by dynamic light scattering and negative-stain electron microscopy. Overall, our results show that some recent, zwitterionic copolymers are best suited to solubilize negatively charged membranes at high ionic strengths even at low polymer/lipid ratios [1].

As a proof of principle, we describe an efficient recovery of protein-encapsulating nanodiscs from membranes of *Chaetomium thermophilum*, a thermophilic fungus. We identified ~1100 proteins by mass spectrometry and obtained two 3D reconstructions from cryo-EM for the nanodisc-containing cell extract. With this combined methodological approach, we provide a deeper understanding of eukaryotic membrane proteomes [2].

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Liposomes as model systems for standardized evaluation of antimicrobial peptide membrane permeabilization in microfluidic setups

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Antimicrobial resistance is one of the major challenges of the 21st century. There is an urgent need on the one hand for new alternative compounds and on the other hand for platforms for compound identification and analysis. In our project, we characterize and compare the membrane permeabilization of a set of antimicrobial peptides (AMPs) that were optimized by a genetic algorithm. As a model system, we use microfluidically generated liposomes, which we trap individually in microfluidic devices [1]. This allows us to monitor the individual permeabilization of many liposomes simultaneously. With this approach, we a) evaluate the permeabilizing efficacy of AMPs, b) classify the mode of action of AMPs in combination with other methods, and c) develop a microfluidic platform that allows standardized and routine compound testing. Therefore, we compare our results with microbiologically determined fitness values of AMPs.

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Antimicrobial peptides in action under high pressure conditions

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Antimicrobial peptides (AMPs) are short peptides active against different pathogens, including bacteria and fungi. In general, they perturb the lipid matrix of the pathogens' membrane. The selectivity against pathogens is due to the presence of anionic lipids, such as phosphatidylglycerol, in the outer leaflet of the membrane, which is absent in eukaryotic cells. Moreover, the lack of specific receptors is at the basis for the low resistance to AMPs in pathogens. Therefore, AMPs can be used in the treatment of infections and in food preservation against food spoilage microorganisms. The food industry relies on the use of chemicals to increase the shelf-life of foodstuff. Unfortunately, such chemicals often affect the nutritional value of food in addition to its taste and colour. Other preservation methods are available, such as high hydrostatic pressure (HHP), in which foodstuff is subjected to a pressure of 3-7 kbar, leading to membrane disruption and protein denaturation, thereby affecting the pathogens' viability. However, a full microbial inactivation is hard to achieve by HHP treatment. Therefore, a synergistic effect using AMPs may be a way to inactivate the fraction of HHP resistant pathogens. We combined HHP preservation with the efficacy of AMPs to kill bacteria and investigated the interaction of GKY20 with bacterial model membranes composed of phosphoethanolamine and phosphatidylglycerol up to the kbar regime. [1] We found that GKY20 interacts with membranes by adopting a helical conformation and inducing the formation of lipid domains. Remarkably, the ability of GKY20 to disrupt lipid bilayers is not affected by HHP. We found that HHP promotes the conformational change of GKY20 from random coil to a helical conformation also in bulk solution. Since the helical fold of the peptide is a prerequisite for its biological activity, the application of HHP can booster its effect, opening the possibility of using AMPs in conjunction with HHP for food preservation.

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Ion-mediated changes of spontaneous monolayer curvature activate the integral enzyme OmpLA

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Transbilayer asymmetry of lipids and proteins is a poorly understood distinct feature across all plasma membranes. In our pursuit to elucidate the biophysics underlying plasma membrane asymmetry, we have devised protocols for generating synthetic asymmetric lipid membranes, incorporating reconstituted integral membrane proteins. Specifically, we interrogated the behavior of outer membrane phospholipase A (OmpLA), an integral enzyme found in Gram-negative bacteria, in diverse asymmetric lipid membranes composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. Our experiments reveal a notable reduction in the enzyme's hydrolytic activity in compositionally asymmetric charge-neutral lipid bilayers. In charged asymmetric membranes, we observed an overall enhancement of protein activity, further influenced by mono and divalent ions. All observations can be primarily explained by an allosteric coupling between protein activity and lateral asymmetry stress, which can be modulated by compositional asymmetry and electrostatic interactions between ions and lipid headgroups. The generic nature of our findings suggests that analogous mechanisms likely govern the behavior of many other plasma membrane.

Can amphipathic helices sense both positive and negative membrane curvatures?

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The precise regulation of biological membrane curvature is essential for fundamental cellular processes, including endocytosis, exocytosis, vesicle trafficking, and cellular signaling[1]. This regulation relies on specialized proteins and peptides, which recognize and modulate membrane curvature. Many amphipathic helices (AHs), helical peptides with opposing polar and nonpolar faces, were shown to have such curvature sensing and modulation ability[2]. While AHs are traditionally associated with sensing positive curvature due to the presence of lipid-packing defects[3], an alternative view of AHs as sensors of intramembrane stresses[4] implies a possibility of AHs sensing negative membrane curvature.

In this research, we employed molecular dynamics simulations to explore the capacity of AHs to sense negative membrane curvature. Our investigations focused on a set of amphipathic peptides composed of leucine and serine residues. We systematically modified peptide properties, observing that increasing hydrophobicity resulted in deeper peptide insertion and a shift towards a preference for negative curvature.

Our findings challenge the conventional understanding of AHs as positive curvature sensors and highlight the correlation between peptide insertion depth and curvature preference. These insights pave the way for the identification and design of novel membrane curvature-sensing molecules.

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Molecular impacts of the drug disulfiram on lipid membranes

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Disulfiram, an FDA-approved drug for the treatment of alcohol abuse, was recently shown to influence a broad spectrum of cellular processes. To name a few examples, next to the inhibition of aldehyde dehydrogenase disulfiram (i) inhibits dopamine β-hydroxylase and thus reduces cocaine consumption [1], (ii) regulates cell growth, differentiation and apoptosis of cancer cells by influencing the human epidermal growth factor receptor-2 [2], and (iii) inhibits pyroptosis by binding to gasdermin D [3]. Thereby disulfiram can either directly bind to the proteins by disulfide bond formation or chelate copper, an essential cofactor for key cellular enzymes e.g. cytochrome c oxidase and superoxide dismutase 1. Disulfiram is also known to function as an ionophore, a substance that shuttles ions through membranes [4]. But due to its hydrophobic nature which is driving disulfiram to the hydrophobic core of the lipid bilayer, disulfiram could also directly alter membrane properties like other membrane intercalators, the most prominent example thereof being cholesterol.

Here, we study the concentration-dependent impacts of disulfiram on a model E. coli polar lipids extract membrane [5], consisting of 14 different lipid types, by molecular dynamics simulations. Next to determination of typical membrane properties like membrane density profiles, membrane area compressibility, membrane curvature, lipid self-diffusion and intrinsic permeability to water, we also estimate the impact of disulfiram on the energy profiles of membrane passage of individual amino acid side chain analogs.

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Mechanistic insights into virus-host interactions

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Viral infection relies on the stable binding of viral fusion proteins to host membranes, which comprise numerous lipid species. While the structures of fusion proteins have been captured, our understanding of how protein-lipid interactions at the host membrane drives formation of fusogenic trimers and membrane fusion remains incomplete. Through multi-scale molecular simulations, we calculated membrane binding free energies of fusion proteins spanning classes I–III, considering various lipid compositions. Our findings reveal membrane binding affinities follow the order: class I > class II > class III. This disparity in affinities implies different mechanisms followed by fusion proteins during membrane fusion. Class II and III proteins drive membrane fusion by specifically bind to polyunsaturated lipids at designated lipid binding pockets, aligning with their respective membrane fusion pathways. To assess the relevance of selective binding to polyunsaturated lipids, we conducted membrane fusion simulations. The stalks formed during membrane fusion exhibited an enrichment of polyunsaturated lipids, indicating that fusion proteins binding to such lipids prime the membrane for the initial stage of fusion through stalk formation. Considering the non-specific binding of lipids by class I proteins, we delved into the role of fusion peptides employed by class I to target the host membrane. Membrane-fusion simulations unveiled that fusion peptides from class I significantly reduce the free energy required to form stable stalks. Thus, beyond serving as host-membrane anchors, fusion peptides from class I actively drive membrane fusion. Our results underscore the significance of lipid recognition by class II and III fusion proteins as a key feature for selective binding and formation of fusogenic trimers at the host membrane. Class I fusion protein which lack lipid binding pockets, utilize fusion peptides to anchor and drive membrane fusion.

Lipid packing defects in membrane interactions of biomimetic antimicrobial polymers

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Antibiotic resistant bacteria present a daunting challenge for modern medicine and development of new antibacterial agents is urgently needed. Although antimicrobial peptides (AMPs) are promising antibiotic candidates, it has been a significant challenge to design and develop them. One strategy then is designing synthetic polymers which mimic essential physiochemical features of AMPs, displaying similar levels of antibacterial activity. Using detailed atomistic simulations, we studied the effects of inclusion of various functional groups in the biomimetic antimicrobial polymer design on the aspects of lipid packing defects in model bacterial membranes. Understanding the emergence and role of lipid packing defects in the detection and subsequent partitioning of such synthetic antimicrobial agents into bacterial membranes is essential for gaining insights into general antimicrobial mechanism. Two antimicrobial polymers are considered: ternary polymers composed of cationic, hydrophobic, and polar moieties and binary polymers with only cationic and hydrophobic moieties. We find that differing modes of insertion of these two polymers lead to different packing defects in the bacterial membrane. While insertion of both binary and ternary polymers leads to an enhanced number of deep defects in the upper leaflet, shallow defects are moderately enhanced upon interaction with ternary polymers only. We provide conclusive evidence that insertion of antimicrobial polymers in bacterial membrane is preceded by sensing of interfacial lipid packing defects. Our simulation results show that the hydrophobic groups are inserted at a single colocalized deep defect site for both binary and ternary polymers. However, the presence of polar groups in the ternary polymers use the shallow defects close to the lipid–water interface, in addition, to insert into the membrane, which leads to a more folded conformation of the ternary polymer in the membrane environment, and hence a different membrane partitioning mechanism compared to the binary polymer, which acquires an amphiphilic conformation.

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The "Sand in a gearbox" effect of antimicrobial peptides: beyond pore formation

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Antimicrobial peptides (AMPs) are promising compounds to fight resistant infections. They are bactericidal towards a wide range of microbes, acting by perturbing the permeability of the bacterial membrane, thus leading to cell death.
Bacterial killing requires a high coverage of the cell surface by bound peptides.[1] It is thus conceivable that peptide accumulation on the membrane might also interfere with vital cellular functions by perturbing bilayer dynamics, a hypothesis called "sand in the gearbox".
We recently demonstrated through fluorescence spectroscopy studies that the activity of two natural AMPs, alamethicin and PMAP-23, is not limited to membrane permeability, but induces also variations in bilayer dynamics, water penetration and lipid lateral diffusion.[2] To investigate if this behavior could be a general feature of AMPs, we systematically investigated the effects on membrane dynamics of a set of well-characterized but very diverse peptides: the natural AMP magainin, the toxin melittin, the artificial peptides LAH4 and Killer-FLIP, and small-molecule peptidomimetics.
The results indicate that perturbation of membrane dynamics is a shared behavior of all the system analyzed: the membrane-active peptides induced a stiffening of the phospholipids bilayer by reducing the lipid lateral mobility and the degree of water penetration, at least at the ns time-scale observed by fluorescence.
Interestingly, PMAP-23 effects on the membrane dynamics of live E. coli cells are comparable to those observed in model membranes,[2] demonstrating that the AMPs activity goes beyond pore formation and affects the bilayer dynamic also in real cells.

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Solid-State NMR Investigation of Magainin Antimicrobial Peptides in a Realistic Membrane Environment

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Abstract:

Magainin and PGLa are cationic, amphipathic antimicrobial peptides isolated from the skin of *Xenopus Laevis* African frog, known to interfere with the barrier function of the bacterial membrane and cause bacterial killing. They adopt alpha-helical structures in membrane environments and can disrupt the lipid bilayer organization and ordering. When added as a mixture, they show enhanced synergistic activities in both antimicrobial assays as well as biophysical experiments[1,2]. Several models have been proposed to understand the synergistic behavior between PGLa and magainin, however, the mechanism behind this is still elusive. The objective is to elaborate a high-resolution study at molecular level of the peptide-lipid assemblies to decipher the mechanism of synergism between the two peptides.

In the present work, we show a structural analysis of the interaction between magainin's and a liposome model that mimics a bacterial membrane composed of POPE:POPG (3/1 molar) lipids using MAS solid-state NMR. We study the secondary structure, insertion, and dynamic of the ^{13}C-^{15}N labeled peptide on specific positions in the lipid bilayer. Our findings indicate that it is possible to carry out high-resolution ssNMR measurements on realistic membrane models with a low P/L ratio effectively using the inherent nanoscale properties of lipid bilayers. Moreover, the peptides adopt a folded alpha helical structure within the membrane environment and inserts at the water-lipid interface. Over time, these peptides tend to assemble into aggregates. The formation of large peptide clusters on the membrane surface leads to membrane lysis and the subsequent disintegration of the bacterial membrane, ultimately resulting in bacterial death.

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Free energy landscape of membrane topological transitions during fusion and pore formation

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Biological membranes undergo significant topological transitions during essential processes like membrane fusion or pore formation. These transitions lead to the reshaping of membranes into non-bilayer conformations. While the general rearrangements of membranes are known, capturing the free energy landscape of these topological transitions remains a substantial challenge. We use MD simulations to calculate the potential of mean force along a reaction coordinate, serving as a measure of connectivity between two compartments. This computationally efficient approach allows us to explore the energetics associated with membrane fusion and pore formation and expansion, not only at coarse-grained level but also with atomistic resolution. [1][2] With this we investigate the impact of lipid composition and membrane-associated proteins, such as transmembrane domains of fusion proteins, on the energetics of membrane reshaping. Additionally, we learn in which scenarios a coarse-grained force field accurately represents the free energy of membrane topological transitions, while also addressing and explaining crucial discrepancies between coarse-grained and atomistic simulations.

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Membrane shape transitions mediated by prokaryotic members of the ESCRT-III superfamily

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Recently, a structural and functional homology of the prokaryotic proteins PspA and IM30 to eukaryotic ESCRT-III proteins has been established. While the prokaryotic proteins form homo- rather than hetero-oligomers, the structures of the protomers as well as the large oligomeric complexes are remarkably similar to the eukaryotic proteins. While the precise physiological function of the prokaryotic proteins is still under debate, a membrane remodeling activity has been observed for IM30 *in vitro*, and both PspA and IM30 appear to be involved in membrane stabilization and/or repair. IM30 forms large, homo-oligomeric barrel structures in solution, and IM30-mediated membrane remodeling likely involves membrane tubulation via membrane internalization into the central core of such rings. In addition to such barrel structures, rod structures and membrane-covering carpets have also been observed for IM30, the later are probably crucial for the membrane-stabilizing activity of IM30. The oligomeric structures of both IM30 and PspA exhibit remarkable plasticity, which is rarely observed. Furthermore, IM30 ring structures disassemble into smaller oligomers upon binding to negatively charged membranes. Ring disassembly involves partial unfolding of the monomers, and about half of the protein is intrinsically disordered when not part of the homo-oligomeric rings. We could show that such partially unfolded IM30 monomers can form condensates via liquid-liquid phase separation, *in vitro* and *in vivo*. The finding of IM30s capability to form condensates under physiologically relevant conditions is further expanding the landscape of IM30 assembly states and expands our view on how IM30 might be involved in membrane shaping.

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Adsorption of Lipid Nanodiscs to Monolayers: A New Triple Layer System for Studying Membrane Proteins

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Lipid monolayers can be used as very simple model system for one membrane leaflet. They have the advantages of being perfectly oriented, completely hydrated, and accessible through the aqueous phase. Thus, they are well suited for interaction studies with membrane binding compound, such as proteins or peptides. A variety of methods can be used in these studies, e.g. film balance measurements, fluorescence microscopy, infrared reflection-absorption spectroscopy (IRRAS) as well as X-ray reflectometry or scattering.

However, no membrane spanning molecules can be studied in this system until now. Therefore, we are aiming to design a new membrane model system that makes the many advantages of established monolayer methods accessible for more complex samples.

We are exploring methods to assemble lipid bilayers underneath lipid monolayers at the air water interface. These bilayers shall host membrane spanning molecules such as integral membrane proteins or other pore forming molecules. The bilayers will be oriented, allowing to deduce molecular orientations from experiments, while still being in a well-hydrated and natural environment. We will present first successful attempts in constructing such a model system, i.e. the adsorption of lipid nanodiscs (SMAPLS) to lipid monolayer at the air water interface. We will show that these model systems combine the advantages of several well-established membrane models and use the workshop to discuss further perspectives and applications.

Probing protein-induced local membrane deformation: a small-angle scattering study

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The complex interplay between integral membrane proteins and lipid bilayers includes the so called hydrophobic matching, which describes the balancing between the hydrophobic thickness of membrane proteins and the acyl-chain region of the surrounding lipids. Deviations in this matching can introduce a free energy penalty, alleviated by membrane deformations or protein oligomerization. So far experimental measurements of lipid thickness near membrane proteins are scarce and have only recently been explored through NMR [1], to the best of our knowledge. Here we propose an approach combining small-angle X-ray scattering (SAXS) and all-atom molecular dynamics (MD) simulations to probe local membrane deformations induced by the outer membrane protein phospholipase A (OmpLA). OmpLA, an integral enzyme that hydrolyses phospholipids upon dimerization, is reconstituted in large unilamellar vesicles (LUVs), referred to as proteoliposomes [2]. LUVs composed of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) or 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC) are selected for their differing hydrophobic thicknesses, exceeding (28.4 Å) or falling short (21.3 Å) of the OmpLA hydrophobic patch (24 Å). A multi-scale scattering model for proteoliposomes is developed, encompassing nanometric transbilayer structure and protein spatial distribution within the spherical frame of LUVs. According to theoretical expectations, we observed thinning of POPC membranes and thickening in DLPC systems. Specifically, MD simulations reveal lipid deformation within a distance of less than 30 Å from OmpLA for both POPC and DLPC, and support the quantitative SAXS analysis demonstrating average thickness deformations of a few angstroms. Additionally, results highlight that OmpLA maintains its monomeric state in both systems, suggesting that POPC and DLPC model proteoliposomes do not favor the physiological dimerization.

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Photoswitchable lipid dynamics in phase-separated membranes

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Giant unilamellar vesicles (GUVs) serve as cell-sized model systems widely studied to mimic the behavior of natural cell membranes and synthetic cells. The localization and segregation of specific elements, such as functional lipids and proteins, play a pivotal role in membrane regulation and function. Utilizing photo-responsive molecules presents a compelling strategy for achieving precise spatiotemporal regulation. In this context, azobenzene-phosphatidylcholine (azoPC) assumes significance as a well-established photoswitchable lipid with an incorporated azobenzene moiety in one of its lipid tails. AzoPC's ability to respond reversibly to ultraviolet A and blue light holds the potential for inducing shape or phase changes in GUVs, making it a compelling avenue for investigating controlled reactions and mechanical energy. The photoisomerization of azoPC in the membrane significantly increases surface area and alterations in elastic properties [1]. Our study delves into the phase dynamics of membranes incorporating azoPC and gel-phase phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), with a melting point of 41°C. Our primary objective is to elucidate the behavior of azoPC within a phase-separated membrane and explore the potential for domain-specific photo-response. At room temperature, gel domains display irregular patch patterns following electroformation at 60°C and a gradual cooling process (~0.3K/min). Despite a phase transition at 31.8°C identified by differential scanning calorimetry (DSC) in the equimolar mixture, microscopy observations intriguingly indicate that domain coalescence and homogenization take place above 45°C. Subsequently, we delve into the light-responsive behavior of GUVs featuring coarsened azoPC domains. These GUVs display localized membrane protrusions and expansion upon UV irradiation while initially exhibiting twisting or crumpling before coarsening. This investigation sheds light on the dynamic behaviors of photoswitchable lipids within lipid membranes, enhancing our understanding of cell membrane mechanics and controlled reactions.

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mRNA lipid nanoparticle phase transition

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mRNA-based vaccines have recently gained attention for their promising therapeutic potential in the prevention of a severe SARS-CoV-2 infection [1]. An important part in their mode of action are lipid nanoparticles (LNPs) which act as a carrier system to deliver the bioactive mRNA into the target cells. The nanoparticles protect the nucleotides against a premature degradation leading to an increased expression of the encoded protein [2]. In addition, the nanoparticles increase the transfection rate of the vaccine through an enhanced interaction with the membrane of the target cells.

Unfortunately, less is known about the molecular organization of the nanoparticles at molecular and atomistic scales. Here, we characterize the spatial composition and the physico-chemical properties of the lipids used in the BioNTech & Pfizer vaccine employing molecular dynamics (MD) simulations of both lipid bilayer systems and full LNPs at atomistic resolution [3]. At physiological pH, the LNP is characterized by an oil-like core that is surrounded by a lipid monolayer formed by DSPC lipids, cholesterol and PEGylated lipids creating an external PEG layer around the LNP. Self-assembly simulations with nucleoside-modified mRNA strands further corroborate the necessity of protonated cationic aminolipids to envelope the negatively charged poly-nucleotides within the core of the LNPs. Such inverted micellar structures stabilize the mRNA within the LNPs and provide a shielding and likely protection from environmental factors. At low pH, in contrast, the lipid composition used in the Comirnaty vaccine spontaneously forms lipid bilayers that display a high degree of elasticity. Thus, a change in pH of the environment as occurring upon LNP transfer to the endosome, likely acts as a trigger for lipid reorganization followed by LNP destabilization and subsequent mRNA cargo release from the LNP core.

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Arg/Trp cell-penetrating peptides incorporating Trp analogues: internalization and interactions with cell membrane components

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Cell Penetrating Peptides (CPPs) are small peptides able to cross cell membranes in a receptor-independent way. They are generally cationic and often amphipathic, which leads them to interact favorably with membrane lipids and glycosaminoglycans (GAGs) of the cell surface. Their internalization can occur according to two pathways: energy-dependent endocytosis and energy-independent direct translocation. In both mechanisms, the first step involves interactions between the CPP and cell membrane components. Most of CPPs contain Arg or Lys residues able to engage in electrostatic interactions and H-bonds. Some sequences also contain Trp residues, which are essential for penetration properties. Trp may engage in various interactions with both lipids and GAGs, including hydrophobic contacts [1]. Recently, we suggested that ionpair-π interactions involving the guanidinium moiety of Arg, a carboxylate group of a GAG uronic acid and the indole ring of Trp could positively contribute to the internalization of the model CPP (R/W)9 (RRWWRRWRR) [2].
The aim of the present study is to further investigate the role of Trp by modulating its physico-chemical properties in the (R/W)9 peptide sequence. A small peptide library, where Trp was substituted by other natural amino acids or by non-natural Trp analogs was designed and synthesized. The quantification of the internalization of these peptides was performed by MALDI-TOF mass spectrometry. Isothermal calorimetry and differential scanning calorimetry was used to characterize their interactions with model membranes. Density functional theory analysis allowed us to highlight possible salt bridge-π interactions with lipid polar heads and GAGs.
All these results provide precious information on the role of Trp in the internalization mechanism and lead us to design new peptides incorporating non-natural amino acids with improved cell penetration properties.

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Chemically Driven Self-Division in Synthetic Vesicular Systems

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Life's essence is continual renewal and replication [1]. Pursuing simplicity in self-division, we introduce an innovative exploration of self-replication in chemically fuelled vesicular systems.

We demonstrate that carbodiimide (EDC), a compound that may have played a role in prebiotic reactions, serves as a catalyst, inducing remarkable morphological changes in succinic acid derivative vesicles. These alterations promote membrane deformations, leading to vesicle fission. This research reveals a novel fuel-driven self-division mechanism for building synthetic cells with self-replication capacities.

Using confocal microscopy, we monitored vesicular morphological changes after EDC fuelling, with kinetic analyses supported by absorbance assays and HPLC, describing multilamellar vesicles (MLVs) transformation into, mainly, “daughter” giant unilamellar vesicles (GUVs). Complementary cryo-TEM and dynamic light scattering (DLS) experiments corroborated structural and size variances, confirming the formation of a new generation of vesicles. We postulate that this process can occur by two mechanisms: (i) partial conversion of the precursor to anhydride, in which drastic morphological changes in the vesicles can be observed, and (ii) rapid hydrolysis of spherical anhydride droplets and their conversion into new membranous compartments.

This chemically driven vesicular transformation significantly enriches our mechanistic comprehension of self-division processes. Currently, our research is focused on encapsulating materials that not only define vesicular identity but also possess the inherent capacity for self-replication through analogous processes.

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Topographies of lipid membranes are biophysical regulators for the spatial organization of liquid protein condensates

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Liquid protein condensates are assembled by proteins or nucleic acids and form through the process of phase separation. They serve as specialized membrane-less compartments, which regulate biochemical reactions in a confined space and rrecent studies have established associations between lipid membranes and proteins capable of forming liquid condensates. However, little is known about how biophysical parameters of lipid membranes affect liquid condensates.

Here, we explored how the topography of lipid membranes affects the organization of liquid protein condensates. We implemented an assay to assemble liquid protein condensates on supported lipid membranes with microstructured topographies.
By employing membrane surfaces designed with various microstructures, we found that capillary forces, mediated by membrane topographies, lead to the directed fusion of liquid condensates. In addition, we showed that liquid condensates can assemble into orderly patterns or defined shapes on topographically structured membranes. Our results demonstrate that membrane topography is a potent biophysical regulator for the organization of mesoscale liquid protein condensates.