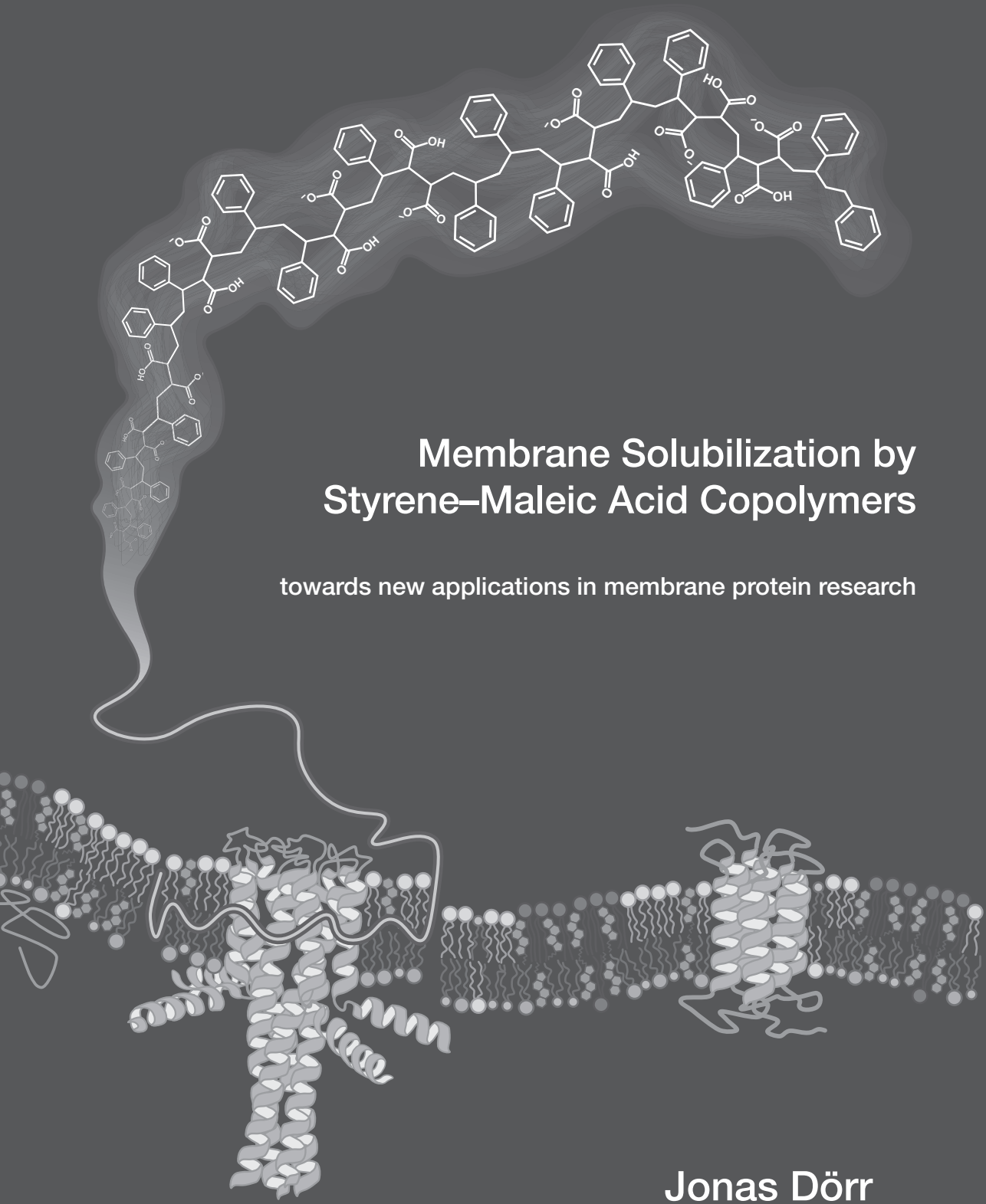

Membrane Solubilization by Styrene–Maleic Acid Copolymers

towards new applications in membrane protein research

Jonas Dörr

Membrane solubilization by styrene–maleic acid copolymers:
towards new applications in membrane protein research

Jonas Dörr

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Solubilisierung von Membranen mit Hilfe von Mischpolymeren aus Styrol und Maleinsäure:

Neue Anwendungen in der Membranproteinforschung

(mit einer Zusammenfassung auf Deutsch)

Solubilisatie van membranen door copolymeren van styreen en maleïnezuur:

toepassingen in onderzoek aan membraaneiwitten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht op gezag van de rector magnificus,
prof.dr. G.J. van der Zwaan, ingevolge het besluit van het
college voor promoties in het openbaar te verdedigen
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door

Jonas Matthias Dörr

geboren op 2 april 1987
te Saarbrücken, Duitsland

Promotor:
Prof. dr. J. A. Killian

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I think you'll find it's a bit more complicated than that.

Ben Goldacre, *not* a bad scientist

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Chapter I

Introduction

This chapter is partly based on:
Jonas M. Dörr, Stefan Scheidelaar, Martijn C. Koorengevel, Juan J. Dominguez Pardo, Marre Schäfer, Cornelis A. van Walree, and J. Antoinette Killian. **2016**. The styrene–maleic acid copolymer: a versatile tool in membrane research. *Eur. Biophys. J.* 45:3–21.

Biomembranes

Cells of any living organism are enveloped by a biomembrane, a complex fluid two-dimensional structure that marks the border between a cell and its environment. Membranes define the very essence of a cell by separating its interior from the outside world, which is essential for cellular integrity. In addition, they form the basis for the spatial separation of biochemical reactions in different compartments which is the underlying principle for any kind of cellular organization. Hence, one of the most crucial steps in early evolution must have been the first event of forming a compartment surrounded by a membrane, defining the first precursor of a cell as an entity. This common ancestor has then evolved over billions of years, with descendants today ranging from "simple" archaeal and bacterial cells to complex eukaryotic cells that contain diverse subcellular compartments with distinct and specific functions, termed organelles, whose interplay determines cellular life (Figure 1).

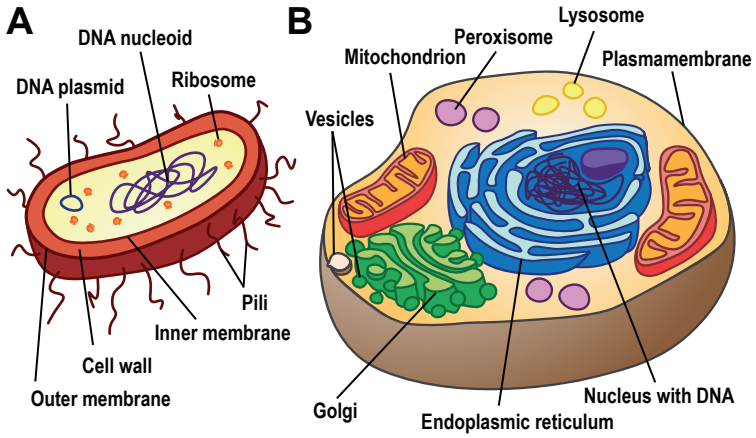

Figure 1: Schematic representation of different cells. (A) A Gram-negative bacterium as an example of a prokaryotic cell. (B) A typical animal cell that shows subcellular organization in organelles as is common in eukaryotes.

Membranes are generally impermeable for most water soluble molecules, thus forming tight seals. This is one of their most essential functions since it allows for establishing gradients of protons, ions and other solutes across the membrane. Such gradients are elementary requirements for life since they provide the driving force for the generation of energy and solute transport and they result in an electrochemical potential that in mammalian cells forms the basis for signal transduction in neurons and thus eventually for every thought in our brains.

Apart from separation of compartments, membranes fulfill a variety of other vital functions including maintaining cellular integrity, the mediation of cell–cell communication and protection from pathogens, to name only a few. So one could really state that membranes make all the difference in biology. With an average thickness of only ~4 nm, membranes

surround cells that in mammals easily reach dimensions of tens of micrometers and that in neurons even can make protrusions of a length that reaches the scale of meters. Thus, in order to maintain cellular integrity, biomembranes need to be very stable structures. Yet, they also need to remain flexible to ensure functional adaptability. Nature has solved this apparent paradox in a genius way by designing an organizational principle in which many small molecules are held together via relatively weak non-covalent interactions ensuring a fluid, adaptable arrangement. At the same time, the summation of the interactions of all its single components provides a high overall stability of the membrane.
In view of the fundamental importance of biomembranes for life, it is not surprising that membrane research has developed into a broad interdisciplinary field in which scientists from diverse backgrounds collaborate to elucidate the principles that govern the functionality of these fascinating structures. But what then defines such a membrane? What is the nature of its components that results in such remarkable properties? To address these questions one has to zoom in on their molecular composition and their major building blocks that are lipids and proteins.

Lipids

The term lipid refers to a large variety of molecules ranging from simple fatty acids to complex structures that contain branched polysaccharides to which several hydrophobic chains are attached. Depending on their specific properties and cellular localization, lipids fulfill a diverse set of essential physiological functions including energy storage in the form of triglycerides as well as signal transduction, for instance as lipid hormones or in processes related to inflammation and apoptosis [1]. However, their most fundamental function is of a structural nature: a large subset of lipids comprises amphipathic molecules that consist of a polar or charged headgroup region and a hydrophobic region, typically two tails (Figure 2), which under physiological conditions causes them to self-assemble into bilayer structures that serve as the scaffold for any biomembrane.
A variety of classes of membrane lipids exist, with their chemical structures and composition differing markedly in species from different domains of life. In addition, the composition of membranes in single species can vary drastically as is the case for the inner and outer membranes of Gram-negative bacteria [2] or for the membranes of different subcellular compartments in eukaryotes [3]. The most abundant class of membrane-forming lipids in bacteria and eukaryotes are glycerophospholipids (Figure 2A). These molecules have a hydrophobic region that consists of two acyl chains that are linked to a glycerol backbone via ester bonds. Together with a small polar or charged molecule that is attached via a phosphate linker this backbone then forms the hydrophilic headgroup. Lipids found in typical bilayer membranes are generally classified according to this headgroup, with bacterial membranes mainly containing glycerophospholipids and, in the case of the outer membrane of Gram-negative bacteria such as *Escherichia coli*, glycolipids termed lipopolysaccharides. Eukaryotes often exhibit a more complex membrane lipid composition with glycerophospholipids, sphingolipids and sterols being the most abundant classes (Figure 2). Important other classes of lipids include glycolipids that have special functions, for instance in photosynthetic membranes or fatty acids that aside from being substrates for lipid metabolism have important signaling functions. Taking into account additional variations in the composition of their acyl chains, this results in hundreds if not thousands

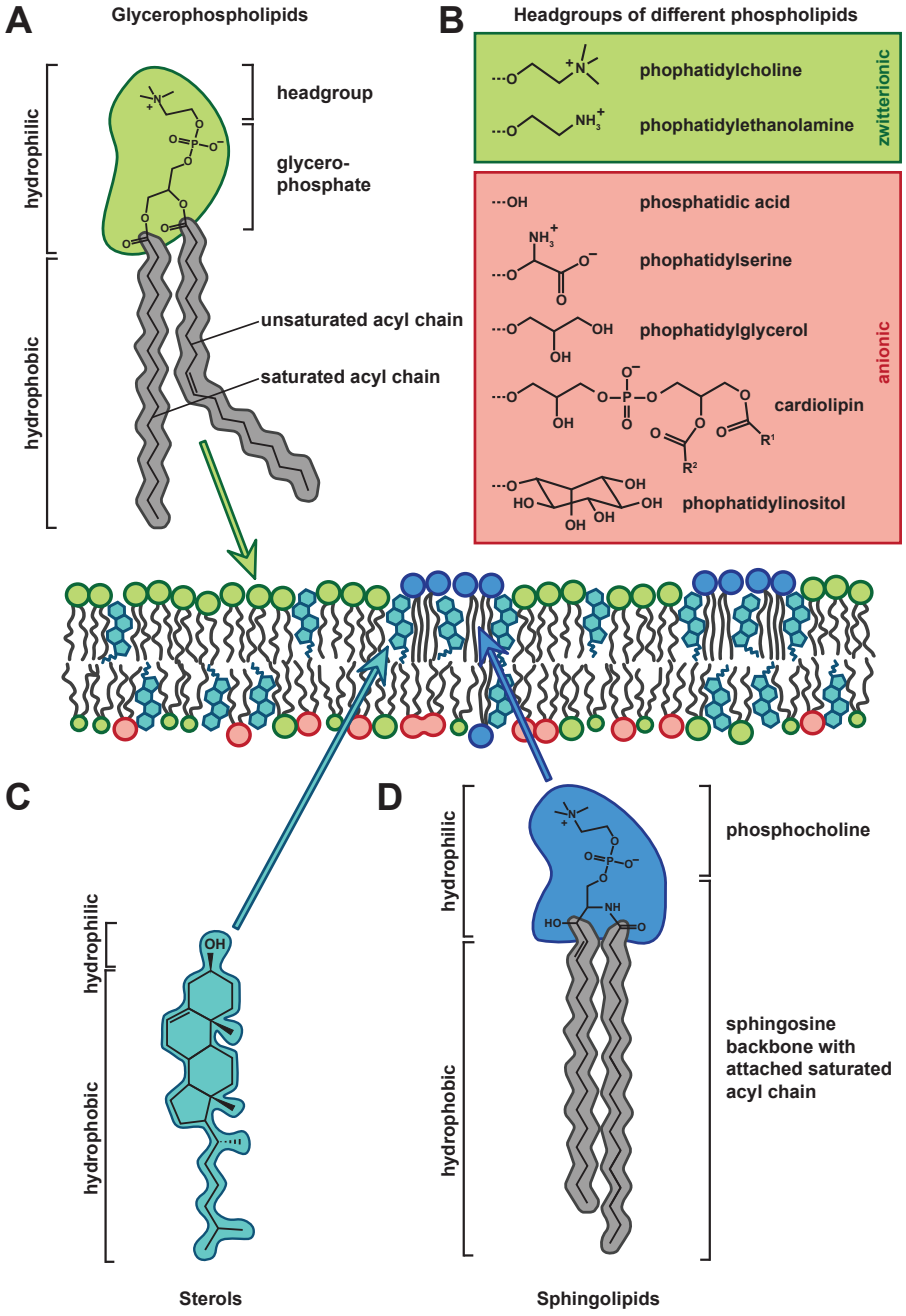

Figure 2: Structures of typical membrane lipids that assemble in a bilayer structure (middle). Chemical structures are shown for a glycerophospholipid (A), for their different headgroups that define overall lipid charge (B) as well as for cholesterol as an example for sterol lipids (C) and of a sphingomyelin as an example for sphingolipids (D).

of different lipid species that represent nature's toolbox for membrane construction. These lipids can be assembled in bilayers with markedly different physico-chemical properties, which leads to a high adaptability for different biological functions.

Bilayer membranes

If lipids are dispersed in an aqueous solution they spontaneously self-assemble into supramolecular structures in which their hydrophobic regions are shielded from the polar water molecules.
This process is energetically driven by a principle known as the hydrophobic effect. If hydrophobic surfaces, such as lipid acyl chains, are exposed to the aqueous surroundings, water molecules at the interface form ordered networks, which drastically reduces their motional freedom and hence their entropy. Burial of hydrophobic surface area leads to an increase of the number of free water molecules, which is energetically very favorable and outbalances the concomitant unfavorable stronger motional restriction of the lipids. Thus, lipids form membranes not because they "like" each other but rather because they are strongly "disliked" by the surrounding water.
While a lamellar bilayer organization is the dominant principle in biological membranes, lipid self-assemblies are not limited to this particular arrangement but can adopt very different kinds of two- or three-dimensional structures [4]. This phenomenon is known as lipid polymorphism, with other forms of lipid self-assembly including micellar arrangements, hexagonal phases and cubic phases (Figure 3). Whether lipids prefer a bilayer or non-bilayer arrangement is determined by thermodynamic principles that depend on conditions such as temperature, ionic strength and pH. However, the dominant factor is the effective shape of the lipid components. Lipids with a cylindrical shape, such as phosphatidylcholine (PC) and phosphatidylglycerol (PG), have a thermodynamic preference for forming lamellar structures, whereas the small headgroup of phosphatidylethanolamine (PE) results in a more cone-shaped molecule making it a "non-bilayer lipid" that exhibits a more complex phase behavior, favoring, among others, hexagonal phases. By contrast, lysophospholipids that only contain one acyl chain deviate from a cylindrical shape towards the other extreme and their inverted cone shape makes them prone to form micelles. All of these phases tolerate a certain amount of lipid that by itself would favor another arrangement and in complex mixtures of lipids the ultimately-adopted phase depends both on lipid composition and environmental conditions.

The dominance of lamellar phases in biology is reflected by the presence of high amounts of bilayer-forming lipid species in eukaryotes [3]. However, virtually every biomembrane also contains non-bilayer lipid components that disturb efficient bilayer packing to some extent and thus increase the plasticity of the membrane, which is vital for proper functionality [5]. One should also note that the dominance of bilayer-forming lipids by no means restricts the diversity of bilayer properties that can vary drastically, e.g., in surface charge or fluidity, depending on the composition of their headgroups and acyl chains.

The lipid behavior in biomembranes is often studied by approximations with lipid-only model membranes of varying degrees of complexity that facilitate investigation by a wide variety of biophysical techniques. Bilayer properties are then described in terms of physico-chemical

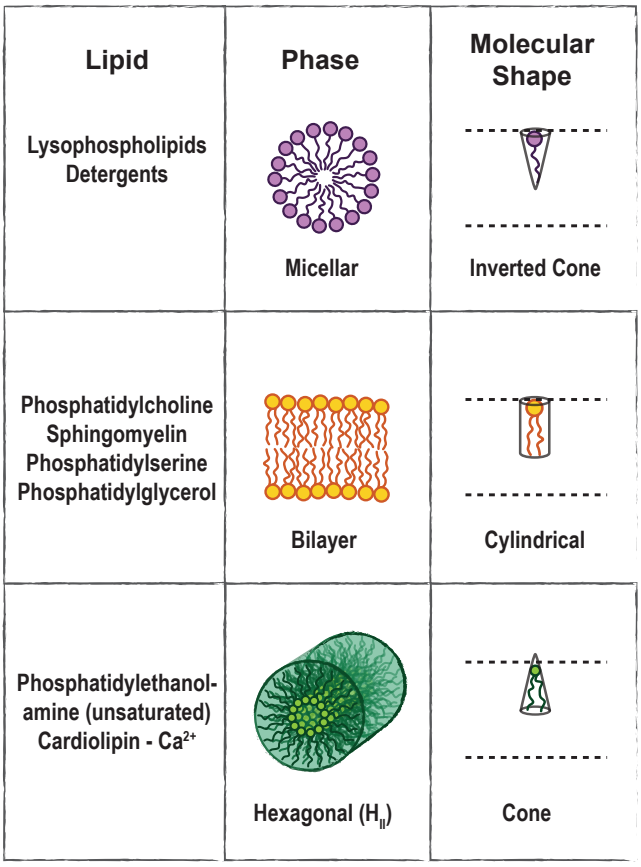

Figure 3: Lipid polymorphism. Lipids of different shape favor different kinds of self-assemblies ranging from micellar aggregates to lamellar bilayers and hexagonal phases. Figure adapted from [4].

principles that among others include considerations of surface/interface phenomena [6], a concept of lateral pressure in the bilayer that depends on packing properties of the lipids [7] and a resulting propensity for the adoption of different thermodynamic phases [8]. In such studies it has been extensively documented that both lipid headgroups and acyl chains have a strong influence on the properties of the bilayer they form (for an overview see e.g. [9]). While the properties of the lipid headgroup mainly determine the overall shape of lipids and thus govern their general tendency to assemble in different arrangements, the saturation and length of acyl chains is the main factor determining the overall fluidity in lamellar bilayers, which is described by a terminology of different bilayer phases (Figure 4). Membranes of a given composition of lipid components upon increasing temperature undergo a highly cooperative transition from the gel phase (L_β) to the liquid-crystalline phase (L_α). This phase transition is often described by the temperature of the transition midpoint, or melting temperature T_m. Bilayers with lipids in the gel phase are characterized by the high degree of order of their acyl chains which causes a relatively high rigidity. This phase is promoted

by the presence of long saturated acyl chains or low temperatures. Increasing the degree of acyl chain unsaturation or increasing temperature leads to the adoption of the more fluid liquid-crystalline phase that is common for biological membranes. The often unsaturated acyl chains of lipids in this phase facilitate relatively fast diffusion of its components.

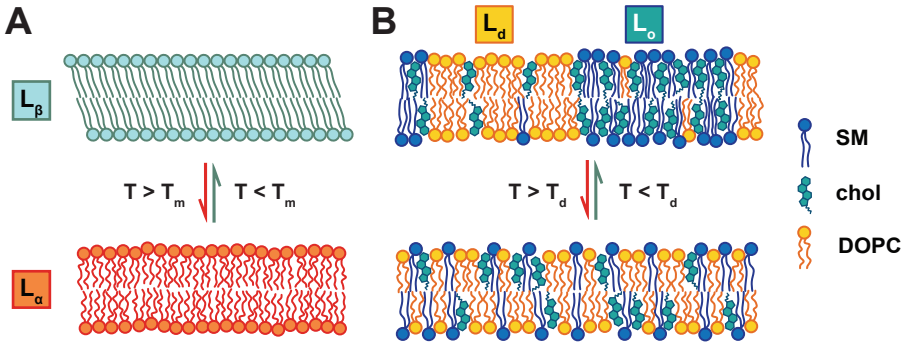

Figure 4: Schematic representations of selected lipid bilayer phase transitions. (A) Temperature-dependent transition between a gel phases (L_β, top) and a fluid liquid-crystalline phase (L_α, bottom). (B) Phase-separating ternary lipid mixture of an unsaturated phosphatidylcholine (DOPC), sphingomyelin (SM) and cholesterol (chol). At temperatures below the demixing temperature (T_d), domains in the liquid-ordered phase (L_o) that are enriched in SM and chol coexist with domains in the liquid-disordered phase (L_d) enriched in DOPC (top). Upon increasing temperature above T_d, homogenous bilayers are formed (bottom).

Due to its high intrinsic rigidity, the presence of cholesterol in phospholipid bilayers can lead to the adoption of a particular phase that is known as liquid-ordered (L_o) phase. L_o phases are characterized by a high degree of order of the acyl chains, while rapid diffusion is still maintained resulting in a seemingly paradox rigid, yet fluid bilayer. Furthermore, ternary mixtures of an L_α-favoring lipid, an L_β-favoring lipid and cholesterol yield bilayers that spontaneously separate into two distinct phases: a liquid-disordered (L_d) phase enriched in the L_α-favoring lipid and an L_o phase enriched in cholesterol and the L_β-favoring lipid. Since the plasma membranes of mammalian cells have a high cholesterol content, this particular phase behavior has gained significant interest in biology and it has been proposed that it may lead to the formation of ordered domains that modify membrane functionality [10,11]. While biophysical considerations of lipid behavior have made important contributions to our understanding of membrane properties, they alone cannot account for all functional aspects of biomembranes. Hence, a second focus of membrane research lies on the other major membrane component: membrane proteins.

Membrane proteins

Proteins account for a substantial fraction of the mass of biomembranes that can reach up to ~75 % in particularly protein-rich membranes such as thylakoid or mitochondrial

membranes [12]. Membrane-associated proteins are generally divided into two classes: integral membrane proteins and peripheral membrane proteins (Figure 5). Integral membrane proteins contain intrinsic sequences that form hydrophobic surfaces in their folded physiologically-active state, which causes their stable embedding in the hydrophobic membrane core. By contrast, so-called peripheral membrane proteins generally exhibit transient interactions with a membrane that depend on environmental conditions or chemical modification. Membrane association of these proteins can be mediated for instance via electrostatic interaction with lipid headgroups, via the transition of an otherwise disordered region to an amphipathic helical structure or via covalent attachment of lipids or fatty acids.

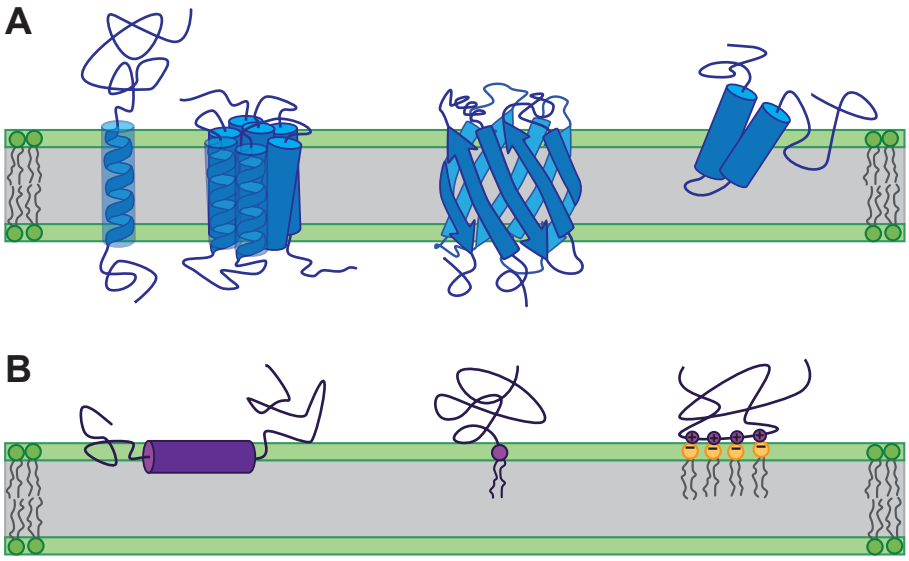

Figure 5: Different types of membrane proteins. (A) Integral membrane proteins (blue) are characterized by a hydrophobic surface area that is embedded in the membrane. Polytopic membrane proteins (left and middle) span the membrane and contain domains on both sides of the membrane. These proteins adopt either α-helical secondary structure (left) or form so-called barrels comprising β-sheets (middle). Monotopic membrane proteins (right) generally interact only with one membrane leaflet. (B) Peripheral membrane proteins (purple) interact transiently with the membrane via the formation of amphipathic helices (left), covalent attachment of lipids (lipid anchors, middle) or electrostatic interactions with lipid headgroups (right).

The membrane-embedded domains of polytopic integral membrane proteins in most eukaryotes or in the inner bacterial membrane mainly adopt an α-helical secondary structure. An alternative β-barrel fold is common in many proteins in the outer membrane of bacteria as well as those of mitochondria and chloroplasts in eukaryotes. Both forms of secondary structure lead to an efficient saturation of the hydrogen-bond donor and acceptor groups of the peptide bond in the protein backbone, which is energetically favorable in a hydrophobic environment like that in the core of a membrane.

Membrane proteins fulfill a variety of functions ranging from selective transport across membranes to enzymatic activity. They contribute to the structural integrity of the membrane and are involved in generation of energy. In addition, signal transduction and cell–cell communication are also often mediated by membrane proteins. Thus, essential membrane functions are often carried out by its protein components but many of these functions are modified by the surrounding lipids. Physiological function of membranes can thus only be achieved by the interplay of lipids and proteins.

Lipid–protein interactions and membrane organization

Membrane lipids affect the structure and function of proteins they interact with on several levels. On the one hand, lipid composition influences global membrane properties such as the lateral pressure profile, which can strongly affect protein stability and organization [7]. In extreme cases, this can even lead to major structural rearrangement of proteins [13]. On the other hand, certain lipid molecules can interact very specifically with membrane proteins and tightly regulate their function [14,15]. This influence of lipids has important consequences for biological function since some proteins can essentially be switched on or off depending on their immediate lipid environment.

Many theoretical concepts for the interplay of lipids and proteins in biomembranes have been developed to explain overall membrane properties. Early models describe the membrane as a “fluid mosaic”, an anisotropic two-dimensional liquid comprising lipids and proteins whose lateral diffusion depends on the bilayer viscosity [16,17]. This approximation of membrane organization can explain many functional aspects of biomembranes but subsequent experimental findings led to its adjustment into more complex models. These include the influence of the interaction of the membrane with the cytoskeleton that can be described as an underlying “fence” scaffold that is attached to the membrane via “pickets” [18,19], resulting in a heterogeneity in lateral diffusion of membrane components.

Another interesting concept is the introduction of lateral heterogeneity by the formation of membrane domains as a result of preferential lipid–lipid (and lipid–protein) interactions (Figure 6). Such domains readily form on a macroscopic level under a range of conditions in both artificial model membranes [20] and plasma membrane-derived vesicles [21]. However, the existence of domains in the plasma membrane of living cells at physiological temperature has neither been confirmed nor ruled out to date [22]. Although it is clear that membranes have a capacity for lateral organization, the properties of hypothetical membrane domains remain a controversial subject. While they have initially been proposed to be long-lived stable structures termed “lipid rafts” that modulate the function of proteins that partition into them [10,11], more recent experimental findings suggest that membrane domains exist only on short time scales and exhibit rather small dimensions of 10–100 nm [22]. Thus, their visualization is challenging even with modern super resolution microscopy [23]. Difficulties in detecting membrane domains may be due to the immense compositional complexity of membranes, particularly if the asymmetric distribution of lipids in the inner and outer membrane leaflet is taken into account [24,25]. Furthermore, one should appreciate that very different kinds of membrane domains may exist for different cell types owing to variations in the protein component of the domains. This can result in very diverse assemblies, ranging from structures such as caveolae to signaling platforms like the so-called “immunological synapse”. One of the remaining questions in terms of the interplay

of lipids and proteins in these structures is the “who recruits whom?” that likely needs to be answered independently for every form of domain.
In summary, the interplay of lipids and proteins leads to the formation of complex biomembranes that are difficult to approximate with simple models [26,27].

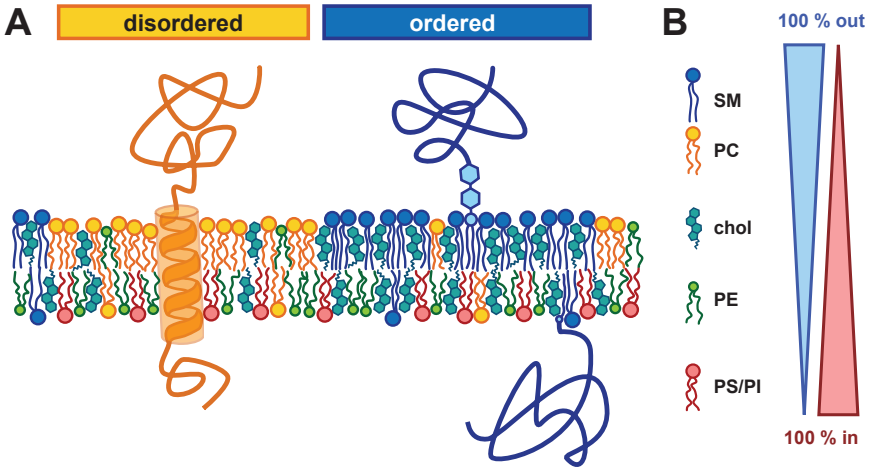

Figure 6: Model for the lateral organization of the mammalian plasma membrane. (A) Whereas integral membrane proteins (orange) tend to partition into disordered domains in the fluid phase (left), many lipid-anchored proteins (dark blue) are thought to have a preference for ordered domains (right). (B) Schematic representation of the distribution of lipids in the two bilayer leaflets. The compositional variety of biological membranes and in particular their high degree of lipid asymmetry leads to a more complex behavior compared to simple model systems (see Figure 4B).

Studying Integral Membrane Proteins

The study of integral membrane proteins is one of the major challenges in current research in molecular life sciences. Membrane proteins represent a substantial fraction of 20–30 % of protein-encoding genes [28], they fulfill a variety of essential functions in all organisms [29] and they are of high pharmacological relevance [30]. Despite the evident importance of these molecules, our understanding of the principles that govern folding, stability, and function of membrane proteins remains poor as compared to water-soluble proteins. Indeed, structures of membrane proteins are largely underrepresented in the protein database where they account for less than 2 % of all deposited protein structures and only 671 unique membrane proteins structures are known to date [31]. This discrepancy is not due to a lower biological abundance or relevance of membrane proteins, but is mainly caused by difficulties in experimental approaches to study these hydrophobic molecules. These difficulties are mainly due to the hydrophobic surface area of membrane proteins that under physiological conditions is shielded by the membrane. For detailed structural and functional studies however, membrane proteins need to be isolated from this complex environment and purified while maintaining both their stability and activity. This has proven to be a far more demanding task than the

isolation and purification of soluble proteins and thus much effort has been focused on new methodologies for improved membrane protein solubilization and stabilization.

Membrane protein solubilization and stabilization

One of the largest challenges in membrane protein solubilization lies in finding an environment with optimal properties to allow a variety of downstream studies. Ideally, this environment should stabilize the protein, allow for its purification and enable the study of its structural and functional properties while the protein displays native behavior. Figure 7 illustrates some of the membrane-mimetic systems that are commonly used in membrane protein research. The various approaches include the use of detergents for solubilization into micelles (Figure 7A) and replacement of detergent by more stabilizing agents, such as amphipols (Figure 7B). In addition, MPs can be reconstituted into a lipid bilayer-forming environment such as bicelles (Figure 7C), lipid vesicles (not shown), or so-called nanodiscs that are stabilized by membrane scaffold proteins (MSPs) (Figure 7D). A recently-developed alternative approach is the use of amphipathic styrene–maleic acid (SMA) copolymers to directly solubilize membranes in the form of nanodiscs (Figure 7E), which is the main focus of this thesis. In this section a brief overview of these different approaches will be given and some of their advantages and disadvantages will be discussed.

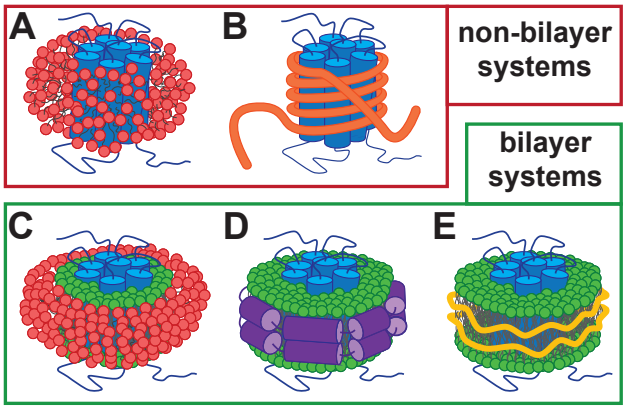

Figure 7: Membrane-mimetic systems for membrane protein stabilization. The protein is indicated in blue, lipids in bilayers are indicated in green. (A) Protein in detergent micelle (red). (B) Protein stabilized by amphipol (orange). (C) Protein in bicelle (detergent in red). (D) Protein in nanodisc stabilized by MSPs (purple). (E) Protein in nanodisc stabilized by SMA (yellow).

Detergents

A common strategy for MP isolation is the solubilization of the lipid bilayer matrix with detergents [32], which generally leads to the formation of spherical micelles, comprising MPs, detergent molecules and possibly some remaining lipids [33,34]. To achieve extraction of different MPs from membranes with varying properties, a wide range of detergents with high solubilizing efficiency has been utilized (for reviews see [35,36,37]). Although this

approach without any doubt has contributed much to our understanding of MPs, detergent solubilization has some inherent disadvantages. First, working with an MP with unknown properties requires an extensive, mainly empirical screening to find a suitable detergent (mix) for each specific case [36,38]. Second, detergent addition strips the protein of its native lipid environment and thus generally leads to a loss of native interactions with both lipids and other proteins. Third, and probably most importantly, detergent micelles are a rather poor mimic of a lipid bilayer since they exhibit very different physico-chemical properties [39,40]. Micelles have a single hydrophilic surface that is highly curved and their hydrophobic parts show a low degree of order. Furthermore, detergent molecules are subject to a monomer–micelle equilibrium causing monomers to rapidly exchange between the micellar and soluble pool, unfavorably increasing the dynamics of the protein environment. In addition, water permeability and lateral pressure profiles differ extensively in micelles and bilayers. As a result, MPs generally show a lower stability in micelles and transient solvent exposure of the hydrophobic MP surface can lead to inactivation or aggregation of the protein. Furthermore, the use of detergents may interfere with MP function [41] or cause the protein to adopt a non-physiological conformation [42,43]. Because of these problems, much effort is being directed towards the development of new detergents with improved properties. In particular, fluorinated compounds [44] and maltose–neopentyl glycol detergents [45] have proven to be powerful with respect to their ability to stabilize MPs and hence may develop into more general tools in membrane research.

Amphipols

An alternative option to achieve a more stabilizing environment for MPs is the replacement of detergent by other classes of specially-designed molecules. Promising approaches include facial amphiphiles [46] and a variety of peptide surfactants ([47] and references therein; [48]), but perhaps the most notable and generally applicable approach at this moment is the use of amphipols. This class of amphipathic polymers, developed by Popot and coworkers, comprises a polyacrylate backbone that is equipped with pendant hydrophobic and hydrophilic sidechains [49]. Amphipols provide a versatile platform for investigations of MPs since they significantly improve the stability of MPs in general and since MP–amphipol complexes are amenable to a plethora of biophysical studies (for reviews see [50,51]). These complexes are characterized by low exchange rates of protein-bound amphipols and free monomers in solution, which results in a high stability and allows the use of relatively low concentrations of free amphipols as compared to detergent. A further advantage of the use of amphipols is that the variety of sidechains with different functional groups allows for optimization of these polymers for specific applications as well as for chemical modification and introduction of labels such as fluorophores.

Vesicles and bicelles

A drawback of the membrane-mimetic systems discussed above is the lack of an actual lipid bilayer environment. Such an environment is important because its particular physico-chemical properties may be essential for structure, function and stability of membrane proteins [40]. One way to overcome this problem is the reconstitution of membrane proteins into systems of synthetic lipids such as planar lipid bilayers or lipid vesicles [52,53,54]. These systems cause the separation of compartments and hence membrane protein-mediated vectorial transport can be studied. In addition, they enable systematic investigation of the

effect of the membrane lipid composition on structural and functional properties of membrane proteins, e.g., by light microscopy or by atomic force microscopy. However, these systems also have limitations: planar and supported bilayers for example are immobilized systems and are thus not suitable for solution-based methods, whereas vesicles have a relatively large size that may impede spectroscopic techniques due to light scattering.
An alternative is the use of bicelles, which are discoidal structures obtained by mixing phospholipids with detergents (often short-chain phospholipids) in a defined ratio (for a review see [55]). Depending on their composition, bicelles can have different sizes ranging from 8–50 nm in diameter [56], which gives rise to their application in diverse nuclear magnetic resonance (NMR) spectroscopy methods. While the fast tumbling of small bicelles favors investigation by solution NMR, the larger speciems orient in the magnetic field, which facilitates solid-state NMR investigation [57]. However, it should be noted that bicelles are limited to certain lipid compositions and stability is often a problem.

Nanodiscs bounded by membrane scaffold proteins
A relatively new approach to incorporate MPs in a lipid bilayer environment was developed by Sligar and coworkers. They designed a method to transfer MPs from detergent micelles into lipid nanodiscs: small patches of a lipid bilayer, bounded by membrane scaffold proteins (MSPs) [58]. To achieve this, they engineered amphipathic helical proteins derived from human apolipoprotein A-1 that serve to shield the hydrophobic core of the lipids from the aqueous phase. Reconstitution of MPs into these soluble particles seems to be generically applicable irrespective of the type of protein and they convey a relatively high protein stability (for reviews see [59,60]). The diameter of nanodiscs typically is in the order of ~10 nm but generation of specific MSP variants allows the formation of smaller (6–7 nm) [61,62] and larger (16–17 nm) [63] nanodiscs. Furthermore, the use of different apolipoproteins or derived peptides and the variation of the peptide/protein–lipid ratio enable the preparation of larger particles [64,65]. This control over size renders them excellent tools in many biophysical methods for structural and functional characterization of MPs. In addition, MSPs can be modified by genetic engineering which allows for functionalization and for the introduction of labels or affinity tags. Like in other bilayer systems, the lipid composition in nanodiscs can be controlled, enabling systematic studies [59]. MPs can even be incorporated into nanodiscs with exclusively native lipid material from detergent-solubilized membranes [66]. An additional advantage of nanodiscs is that, in contrast to other bilayer systems, MPs can be trapped in a defined oligomeric state, allowing studies on how oligomerization influences protein function [67,68]. MSP nanodiscs thus are a particularly promising system for membrane protein research and they are being used in a growing number of studies. More recently, alternative amphipathic proteins have also been used to generate lipid nanoparticles with similar beneficial properties [69].

Nanodiscs bounded by styrene–maleic acid copolymers
It is clear that much progress has been made in optimizing different environments to stabilize MPs. However, all systems described above have one common disadvantage: they all rely on detergents for the extraction of native MPs from cellular membranes. Therefore, the problem of transient protein destabilization by detergent persists for MP reconstitution into both membrane-mimetic and bilayer systems. In order to attenuate this problem, alternative approaches are being developed, such as cell-free protein production [70,71],

membrane protein-enriched cell-derived extracelluar vesicles [72], genetic engineering of the membrane protein by fusion [73] and minimization of the exposure time with detergent [74]. However, arguably the most promising new method as an alternative to detergent extraction has become available by the recent discovery of the membrane-solubilizing effect of amphipathic SMA copolymers [75,76,77].

SMA molecules exhibit a distinctly different mode of action than detergents: addition of the polymer to synthetic or biological lipid membranes leads to the spontaneous formation of discoidal particles with diameters of ~10 nm. In this new type of polymer-bounded nanodiscs the bilayer organization of the incorporated lipid molecules is conserved [78,79]. In different studies, these particles are often referred to as SMA–lipid particles (SMALPs) [75], Lipodisq particles [79] or native nanodiscs [80]. Depending on the origin of the lipid material, here the term SMALPs is used for particles derived from synthetic liposomes and native nanodiscs is used for isolations from biological membranes.

The most striking feature of this novel system is the possibility to directly extract MPs from cells without an intermediate step of conventional detergent solubilization ([81], Figure 8). Thus, the native nanodisc system combines a solubilizing power similar to detergents with the small particle size of nanodiscs, while conserving a minimally-perturbed native lipid environment that stabilizes the protein. To date, this method has been used in a number of reports employing various biochemical and biophysical techniques to study MPs, as will be discussed in more detail later (see Chapter VI). In the following, background information will be provided on the properties of SMA copolymers and on their interactions with lipid membranes.

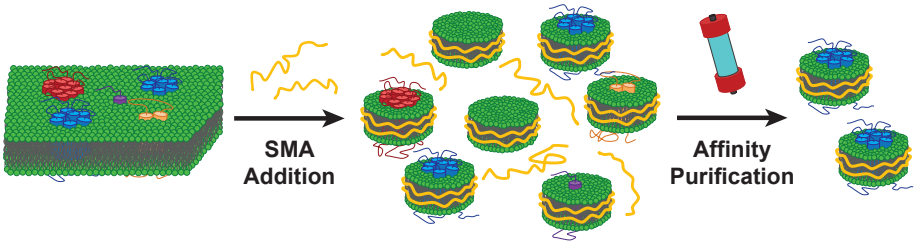

Figure 8: Extraction of membrane proteins with native lipid environment by SMA. SMA addition leads to the formation of native nanodiscs containing different membrane proteins or only lipid material. Subsequent affinity purification allows for the isolation of native nanodiscs with the protein of interest.

The Styrene–Maleic Acid Copolymer

Copolymers of styrene and maleic acid/anhydride: chemical structure, applications and availability

Styrene maleic acid (SMA) is the hydrolyzed form of the styrene–maleic anhydride (SMAnh) copolymer, which is synthesized by the copolymerization of styrene and maleic anhydride monomers (Figure 9, Reaction 1). Both forms of the polymer are widely used in industry and they have many different applications. For instance, SMAnh is commonly used as

thermal stabilizer in plastic blends, while SMA can be used as a dispersing agent for ink formulations and coatings. The SMA/SMAnh copolymers are produced by several suppliers worldwide. The major ones are TOTAL Cray Valley (Beaufort, TX, USA) and Polyscope (Geleen, NL), the latter using the brand name "Xiran" for their SMA/SMAnh copolymers. The products are typically sold in large quantities to companies that process the polymers for downstream products.

SMA copolymers also have a long-standing history in life sciences, originally being described as conjugates for drugs in cancer therapy [82,83]. Later, it was found that SMA can interact with phospholipids to form discoidal structures that can incorporate hydrophobic molecules and therefore would be useful as a drug delivery system [76,84]. Based on this observation, new applications using SMA for the solubilization of lipid bilayers were developed and commercialized, as described in a patent by Malvern Cosmeceutics (Worcester, UK) [77]. In particular, the application of SMA to solubilize membrane proteins, as first reported by the groups of Dafforn and Overduin [75], has led to a rapidly increasing interest in SMA as a novel tool for membrane research. Following these developments, SMA/SMAnh copolymers are now also commercially available in small quantities from Sigma Aldrich (St. Louis, MO, USA). Both SMA and SMAnh copolymers can be obtained in different commercial grades that vary in styrene-to-maleic anhydride/acid ratio and in average molecular weight. However, even within a single preparation of SMA/SMAnh copolymers there are large variations in molecular weight and in composition. The reason for this lies in the synthesis of SMAnh, as will be discussed next.

Synthesis and composition of styrene–maleic anhydride copolymers

The polymerization of styrene and maleic anhydride (MAnh) monomers (Figure 9, Reaction 1) is a radical chain reaction that leads to the formation of SMAnh copolymers with a wide distribution in molecular weights. This distribution is characterized by the so-called polydispersity index (PDI), which for SMA is typically in the range of 2.0–2.5. The PDI is defined as the ratio of the weight-average molecular weight (M_w) and the number-average molecular weight (M_n) as:

$$\mathrm{PDI} \equiv \frac{M_\mathrm{w}}{M_\mathrm{n}};\ M_\mathrm{w} \equiv \frac{\sum M_i^2 n_i}{\sum M_i n_i};\ M_\mathrm{n} \equiv \frac{\sum M_i n_i}{\sum n_i}\ ,$$

where n_i is the number of polymer molecules of a molecular weight M_i. The concept of PDI can be illustrated by a simple example calculation. If one considers a distribution of 3 polymer molecules with molecular weights of 500, 1000 and 10000 Da, M_w is ~8800 Da, and M_n is ~3900 Da, resulting in a PDI of ~2.25. For a typical SMAnh polymer with a PDI of ~2.5 this means that the polymer chains have a broad size distribution with the smallest and largest chains differing by more than at least one order of magnitude in molecular weight and thus length.

The styrene-to-maleic anhydride/acid monomer ratio in the polymer can simply be varied by changing the feed monomer ratio used in the polymerization process. However, the resulting polymer generally does not consist of regular repeating building blocks of styrene and MAnh units with this feed ratio, nor does it exhibit a completely random distribution of the monomers along the chain. This is because of the differences in reactivity between chains with end

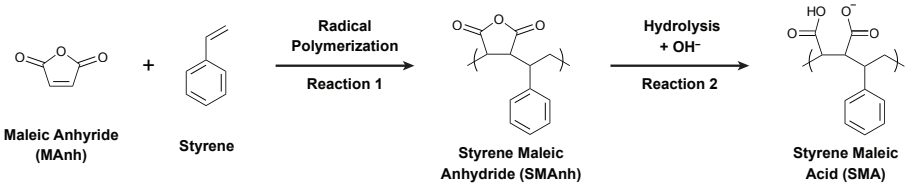

Figure 9: Schematic representation of the synthesis of styrene maleic anhydride (Reaction 1) and the preparation of styrene maleic acid (Reaction 2) as illustrated here for a molar styrene-to-maleic anhydride/acid ratio of 1:1. When styrene is present in excess, the monomer sequence distribution in the polymer becomes more complex (see text for details).

radicals of styrene and MAnh. MAnh-terminated growing chains do not react with MAnh monomers, but they almost exclusively react with styrene monomers [85,86]. Therefore, the maximum content of MAnh units that can potentially be reached in SMAnh copolymers is 50 mol%. Only in this particular case, a polymer with almost perfectly alternating building blocks can be obtained, by mixing styrene and MAnh monomers in a 1:1 molar ratio. This is not possible when styrene is present in excess, because styrene-terminated chains are capable of reacting with both styrene and MAnh monomers, the reaction with MAnh monomers being strongly favored [85,87]. If synthesis is performed in a batch-wise manner, this would result in polymers in which the overall ratio of styrene to maleic anhydride will be the same as the starting ratio, but in which the sequence distribution along the polymer chain may vary significantly: some segments will almost completely consist of alternating styrene and MAnh units, and others will have a high polystyrene content [88,89]. In order to minimize this heterogeneity, the polymerization of SMAnh is typically performed in a continuous manner in which the monomer ratio is controlled by the continuous feed of monomers and the simultaneous collection of polymer product to create a steady state condition during polymerization [90]. In this way, the composition of the collected polymer reflects the monomer composition in the reactor and SMAnh polymerizes in a statistical manner, yielding a much more homogenous distribution of monomer units along the polymer chain [89,91]. However, even under such steady state conditions the synthesis of SMAnh leads to a rather inhomogeneous distribution of polymer chains differing in length and composition instead of well-defined molecules with a unique architecture and molecular weight. It is not clear yet whether or how this heterogeneity affects the membrane-solubilizing properties of SMA.

Hydrolysis of styrene maleic anhydride to form styrene maleic acid

The use of SMA in the solubilization of lipid membranes and formation of nanodiscs is not based on the anhydride form SMAnh, but on the hydrolyzed acid form SMA (Figure 9, Reaction 2). When SMAnh is mixed with water or alkaline solution its anhydride units will be converted to the acid form with two carboxyl groups that become partly deprotonated, yielding water-soluble SMA. Hydrolysis of SMAnh is relatively slow due to the hydrophobic character of the polymer, but it may be accelerated by (1) using the anhydride as powder instead of granulate, (2) elevating the temperature, and (3) adding base (KOH or NaOH) during the reaction.

After hydrolysis, the SMA solution can be processed or purified in different ways. If a minimal amount of base has been used, the required pH can usually be obtained just by addition of extra base or acid. When an excess of base has been used, one can bring the SMA solution into a desired buffering environment either by using dialysis [75] or by making use of their insolubility at low pH [92]. In the latter case, SMA can be precipitated by addition of excess hydrochloric acid (HCl). After several washing steps with diluted HCl, the polymer can then be dried under nitrogen flow causing the residual HCl to evaporate and thus yielding fully-protonated SMA. This can be dissolved in water and the pH can be readily adjusted by addition of KOH or NaOH.

pH-dependent properties of the styrene–maleic acid copolymer

SMA has amphipathic properties due to the hydrophobic styrene units and the hydrophilic carboxyl/carboxylate ([COOH]/[COO^-]) groups. The degree of hydrophobicity depends not only on the ratio of styrene and maleic acid units in the polymer itself, but also strongly on pH. The two carboxyl groups in a maleic acid unit have different pK_a values: the first pK_a is close to 6, whereas the second one is close to 10 [93,94]. This implies that at low pH SMA essentially is non-charged, at neutral pH most of the maleic acid units will carry a single negative charge, and at high pH the maleic acid units will be doubly charged with two carboxylate groups.

This pH dependence has major consequences for the conformation and solubility of SMA, as has been described for several other amphipathic polymers [84,95]. In the case of SMA, at neutral and high pH, electrostatic repulsions between the carboxylate groups are the dominating factor and the polymer adopts a random coil conformation that dissolves relatively easily in aqueous solution. A decrease of the pH well below the lower pK_a of the maleic acid unit will lead to complete protonation of SMA. Charge repulsion is then lost and the hydrophobic effect causes SMA to adopt a globular conformation and eventually to precipitate as aggregates [84,96,97]. The exact pH range that mediates this structural transition depends on the composition of the polymer and also on the ionic strength in the solution [94].

SMA variants in membrane research

In a large majority of available studies on SMA and lipid membranes, the copolymers used had a ratio of styrene to maleic acid of 2:1 or 3:1, with an M_w in the range of 7.5–10 kDa. These polymer variants have been shown to have the most favorable solubilization properties in both model membranes [94] and *E. coli* membranes [98]. For membrane solubilization, the polymers are commonly used at a pH between 7 and 8, at which values they will adopt a random coil conformation and the balance between the hydrophobic effect and electrostatic interactions will be optimal for interactions with lipid membranes.

For particular applications, SMAnh copolymers can also be covalently modified at the highly reactive anhydride moiety. In addition to hydrolysis to form SMA, this enables the introduction of different functional groups, including thiols [99], esters, amides and imides (see e.g. [95]). In this way, a large variety of SMAnh-based copolymers can be realized, each with its own properties and potential applications in membrane research.

Interactions of SMA Copolymers with Lipid Model Membranes

In order to optimize the use of SMA copolymers in membrane research, different studies have focused on the physico-chemical characterization of SMALPs and on the solubilization of lipid vesicles by SMA. Model membranes are useful tools for such studies, because they allow systematic variation of a wide range of lipid parameters, and they allow the formation of SMA–lipid particles with a well-defined lipid composition for detailed biophysical characterization. Here, an overview will be given of studies that investigated the interaction of SMA with model membranes, starting with the characterization of SMALPs.

Molecular structure and properties of styrene maleic acid–lipid particles

The properties of SMALPs have been studied with a variety of biophysical techniques. Most common approaches to analyze the size of these nanodiscs include electron microscopy [75,78,79,92], size exclusion chromatography [78,92] and dynamic light scattering (DLS) [75,79,92,100,101]. Reported sizes are in the order of 10 nm, with minor variations, but it has not yet been investigated in detail what determines the size of SMALPs. Lipid composition does not appear to be a critical factor [92], but it is possible that different sizes result from variations in environmental conditions such as pH, salt concentration or from the use of SMA polymers with differing composition or length. It has also been reported that using relatively low SMA-to-lipid ratios may result in an increase of the size of the nanodiscs [100,101]. It should be noted however, that virtually all examples of membrane protein isolation described in the literature have been performed with an excess of SMA, under which conditions the final size of nanodiscs is expected to be independent of SMA concentration. It is under these conditions that the physico-chemical properties of SMALPs have been characterized, as described below.
Small angle neutron scattering experiments on SMALPs derived from DMPC vesicles revealed that the particles are discoidal with a diameter of ~10 nm and a thickness of ~4.6 nm (Figure 10, [78]), which corresponds well to the thickness reported for pure DMPC bilayers in the fluid phase [102]. The SMA copolymer belt surrounding the lipids was estimated to be ~0.9 nm thick, suggesting the presence of only one layer of SMA molecules. The number of polymer rings required to cover the thickness of the hydrophobic core of the bilayer is not known. It is also not yet clear whether all of the associated polymer material is involved in the stabilization of the disc or whether some parts are forming “floppy ends” that stick out into the solvent or perhaps transiently associate with the headgroup area.

The interactions between lipids and SMA in SMALPs have also been investigated by Fourier transform infrared spectroscopy and NMR [78,79]. It was found that the phenyl groups of SMA intercalate between the lipid acyl chains perpendicular to the plane of the lipids, and that the carboxyl groups interact electrostatically with the head groups of lipids that reside in the outer layer of the nanodisc. In addition, electron paramagnetic resonance (EPR) experiments [79] revealed that carbons at certain positions in the acyl chains are restricted in their motion, consistent with insertion of the polymer phenyl groups. More information on the bilayer character of the nanodiscs was obtained by differential scanning calorimetry (DSC). These experiments showed that the lipids display a typical melting behavior of lipid bilayers. However, the phase transition is broadened and the transition

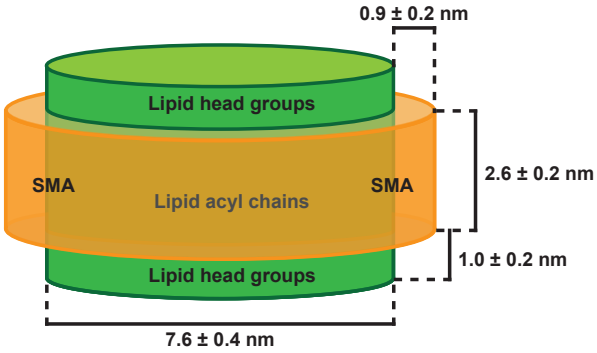

Figure 10: Dimensions of styrene maleic acid–lipid particles (SMALPs) consisting of DMPC lipid and a SMA copolymer with a styrene-to-maleic acid ratio of 2:1, as determined from SANS experiments. Drawn to scale (figure adapted from [78]).

temperature is somewhat shifted [78,79], presumably due to the relatively small number of lipids participating in the transition and their interaction with SMA. This shift in transition temperature may be dependent on the copolymer variant used as indicated by comparing the effects of a copolymer with a ratio of styrene to maleic acid of 2:1 [78] with a more hydrophobic 3:1 copolymer [79]. However, this has not been systematically studied yet. Notably, multiple DSC scans could be recorded of SMALPs, which was not possible for MSP nanodiscs, demonstrating the high temperature stability of SMALPs [78].
The findings described above indicate that the SMA copolymer indeed stabilizes a small patch of lipid bilayer by associating with its hydrophobic core thereby justifying the term nanodisc as had been introduced previously for lipid bilayers bounded by MSP.

Kinetics of membrane solubilization by SMA

The formation of SMA-bounded nanodiscs requires the solubilization of lipid membranes by the polymer. A simple and convenient way to monitor the kinetics of this process is turbidimetry [92]. Lipid vesicles are large particles (hundreds of nanometers up to micrometers in size) and thus efficiently scatter UV light, whereas SMALPs are much smaller and scatter almost no light. Therefore, the solubilization process can generally be followed as a decrease in light scattering in time by using a spectrophotometer (see Figure 11). This allows systematic studies on the effect of e.g. lipid composition, salt or SMA concentration on the kinetics of solubilization.
Using this approach, it was found that many different physical parameters affect membrane solubilization by SMA and a three-step model was developed to describe its mode of action (Figure 12; [92]). The first step consists of the binding of SMA to the surface of the lipid bilayer. This process can be promoted by increasing the amount of SMA and can be further modulated by electrostatic interactions: the presence of anionic lipids causes repulsion and thus impairs binding of the negatively charged polymer, while increasing the ionic strength promotes binding. In the second step, SMA inserts into the hydrophobic core of the membrane. This is strongly affected by lipid packing (e.g. membrane fluidity

and lateral pressure) and bilayer thickness, with both tight packing and thick membranes impairing penetration of SMA into the hydrophobic core of the lipid bilayer. The final step is the actual solubilization of the bilayer and the simultaneous formation of nanodiscs. The second and third step are closely connected since nanodisc formation also is influenced by lipid packing and bilayer thickness, although in a different way. For example, for thicker membranes the free energy cost of breaking up the bilayer is larger, and lipid packing plays a role because in the nanodiscs the hydrophobic groups of the polymer will have to insert in between the hydrophobic chains of the lipids, which will be more difficult when the lipids are more tightly packed.

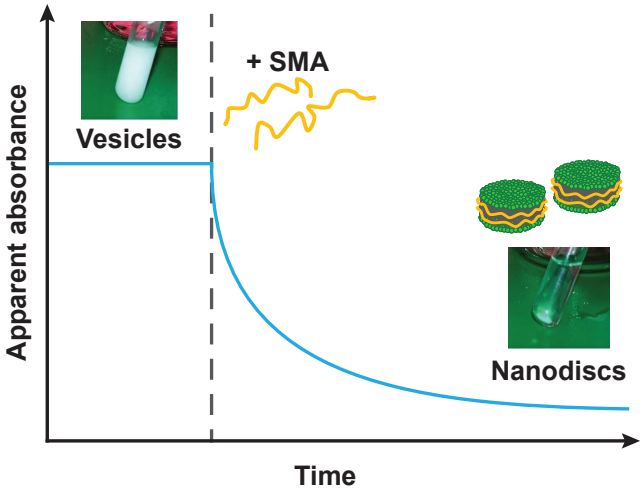

Figure 11: Schematic representation of a turbidimetry experiment. Suspensions of large lipid vesicles show a high degree of light scattering and appear milky. The addition of SMA leads to a clearing of the suspension due the formation of smaller nanodiscs. The concomitant rapid decrease in light scattering can be followed by measuring the apparent absorbance of the sample. For model membranes containing lipids in a fluid phase this process typically occurs on time scales of minutes or tens of minutes [92].

When sufficient polymer is bound to the membrane surface, the kinetics of the solubilization process are mainly determined by the second step. A subsequent insertion into the hydrophobic core of the bilayer then bears a large enthalpic penalty for the polar/charged carboxyl groups. Therefore, as soon as anionic SMA reaches the hydrophobic core of the bilayer, the thermodynamically highly favorable formation of nanodiscs will occur as a downhill process. More detailed thermodynamic considerations can however not be derived based on turbidimetry, because it relies on size as sole observable feature. Nevertheless, this assay led to the identification of major parameters that influence the kinetics of membrane solubilization by SMA.

Consistent with this model, it was found that the phase state of the lipids is an important factor for the kinetics of solubilization [92]. In general, lipids in the gel phase are solubilized much more slowly than the more loosely-packed lipids in the liquid-crystalline phase.

Solubilization of phosphatidylcholine bilayers with saturated acyl chains of a certain length thus shows a distinct temperature dependence that is strongly linked to the gel-to-liquid crystalline phase transition temperature of the lipid. The most rapid and efficient solubilization is generally obtained at this phase transition temperature, where large packing defects help the polymer to enter the hydrophobic core of the membrane.

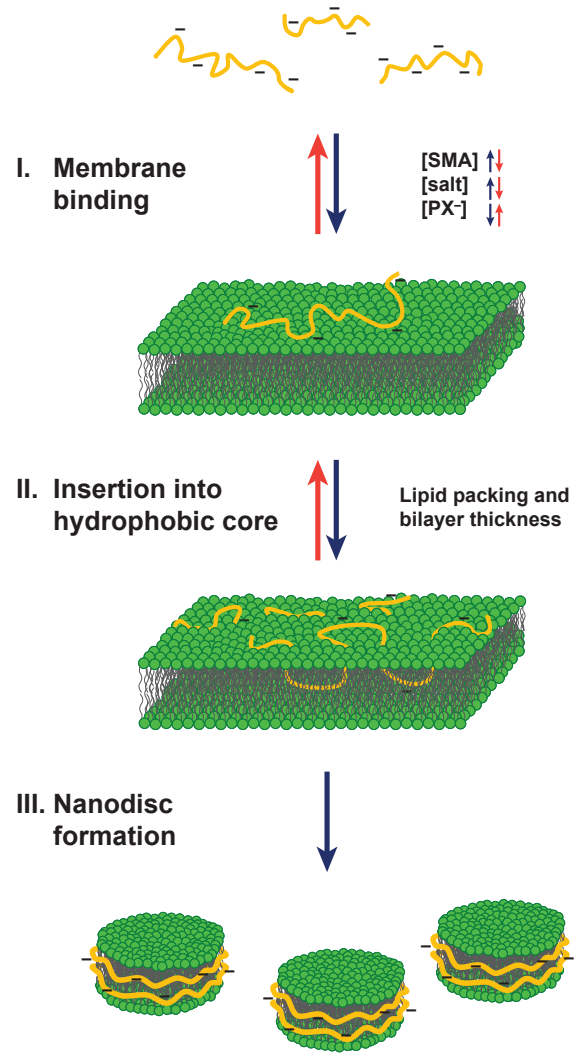

Figure 12: Three-step model for the solubilization of lipid membranes by SMA copolymers. Initially, SMA binds to the surface of the membrane (I), which is modulated by SMA and salt concentration and the presence of negatively charged lipids (PX^-). The next step consists of the insertion of the polymers into the hydrophobic core of the membrane (II), driven by the hydrophobic effect. This process is modulated by the lipid packing and bilayer thickness. Finally, the membrane is solubilized and nanodiscs are formed (III). The kinetics of the solubilization are determined mainly by the second step and after SMA has penetrated into the hydrophobic core of the bilayer, the formation of nanodiscs is a downhill process (see text for details, figure adapted from [92]).

For lipids in the fluid phase, it has been shown that the efficiency of solubilization can be further modulated by changes in lipid packing. For example, it was found that introduction of unsaturated bonds leads to a decrease in the solubilization efficiency. This somewhat counterintuitive and unexpected observation was attributed mainly to an increase in lateral pressure in the hydrophobic core of the bilayer that hinders SMA insertion and thus membrane solubilization, as supported by experiments involving non-bilayer-forming lipids [92]. Despite the individual contributions of different physical properties of the lipids to the kinetics of solubilization, SMA does not appear to preferentially solubilize certain lipid species, i.e. nanodiscs bounded by SMA maintain the overall lipid composition of the vesicles as exemplified for a homogeneous lipid mixture that reflects the composition of *E. coli* inner membranes [92] or an equimolar mixture of PC and PE [103]. This result implies that solubilization is mainly determined by the physical properties of the membrane as a whole rather than by the properties of its individual lipid components.

Thermodynamics of membrane solubilization by SMA

More recent studies have employed ^{31}P NMR as a complementary technique to rationalize membrane solubilization of model membranes in a more quantitative way [101,103]. The authors obtained phase diagrams for mixtures of different amounts of lipid and a 3:1 SMA variant at equilibrium conditions that can be used to approximate transfer free energies for SMA and lipids from vesicular to nanodisc structures. Extrapolations of saturation and solubilization boundaries in these diagrams revealed a vanishing y-axis intercept, which indicates an absence of free polymer in the vesicular and coexistence ranges and thus suggest a very high affinity of SMA for membranes.
Interestingly, it has also been found with such experiments under equilibrium conditions that from a thermodynamics point of view, the solubilization of gel phase lipids requires less SMA than lipids in the fluid phase [103], which was explained by the low tolerance of gel-phase membranes to introduced packing defects by SMA insertion. Thus, the threshold concentration of SMA that triggers nanodisc formation would be lower and nanodiscs would be expelled at lower concentrations in order to avoid unfavorable packing defects. Note that this thermodynamically more favorable solubilization of gel phase lipids does not contradict the observed slow solubilization kinetics in turbidity experiments. This is because in the latter case excess amounts of SMA were used and the solubilization process was followed over time scales in the order of tens of minutes, which does not represent thermodynamic equilibrium but renders information about the kinetics of the process. That is, slow solubilization does not necessarily mean the process in energetically unfavorable and vice versa.

Comparison of the mode of action of different solubilizing agents

The driving force for membrane solubilization by SMA and the formation of nanodiscs lies in the amphipathic properties of the polymer. The hydrophobic effect promotes the insertion of its apolar parts into membranes, while the carboxyl groups render the nanodisc soluble in an aqueous environment. These amphipathic properties are not unique to SMA copolymers however. Detergents, MSPs and amphipols all exhibit a similar amphiphilicity. Yet, these molecules act very differently when mixed with lipid membranes. Detergents, for

instance, dissolve bilayers completely and generally form micelles instead of nanodiscs. MSPs can form nanodiscs together with lipids, but they generally need to be reconstituted from mixtures with detergent [58]. This is because MSPs are α-helical proteins and therefore relatively bulky. As a result, they only can insert into membranes and form nanodiscs when the lipids are at the gel-to-liquid crystalline phase transition, where lipid packing defects exist [92,104]. Amphipols on the other hand generally have large hydrophobic groups that help to stabilize membrane proteins, but that make nanodisc formation unfavorable due to steric hindrance and loss of conformational entropy. The efficient formation of nanodiscs by SMA has been attributed mainly to the small size and rigidity of its phenyl groups [92]. These allow the polymer to efficiently insert into lipid bilayers with a minimal loss in conformational entropy and minimal intrinsic steric hindrance when wrapped around a nanodisc. This is also reflected in a recent more quantitative comparison of the properties of different solubilizing agents [101], where SMA has been characterized as a strong (in terms of requiring low concentrations for complete solubilization) yet mild (in terms of conserving the lipid bilayer structure to a high degree) solubilizing agent.

Incorporation of membrane proteins in SMALPs

The structure of SMALPs and vesicle solubilization by SMA as described above have been studied in detail for SMALPs that consist of lipid material only. However, it is also possible to insert membrane proteins into SMALPs, as was initially shown for PagP and bacteriorhodopsin [75,105] and later for the potassium channel modulator protein KCNE1 [106]. To achieve this, proteins can either first be solubilized with detergent and then reconstituted into liposomes by conventional techniques or native membranes containing the protein can be supplemented with synthetic lipid after which SMA is added to obtain SMALPs (see e. g. [107]). Importantly, it was found that the structure and activity of the proteins in SMALPs remain intact during the solubilization process and that, once incorporated into SMALPs, the proteins can be studied by a variety of methods [108].

Developments in SMA research in recent years

Since the start of the research project underlying this thesis, much progress has been made in the field of SMA solubilization of membrane proteins. With only 3 original research papers published by early 2013, the field has picked up significant momentum in a short period of time with numbers of publications today ranging in the 30s and counting. Relatively little was known at the point when the project was initiated and it was for instance not yet established that membrane proteins could be isolated and purified in native nanodiscs with standard protocols. It was then that investigations were started with KcsA as a model protein to explore the potential of SMA-bounded nanodiscs for the study of membrane proteins as described in this thesis.

KcsA as a Model Protein

Novel methodological approaches, such as SMA solubilization of membrane proteins, initially require a validation with established systems to show their general applicability. To

this end, (simple) model proteins are often used to generate proof-of-principle examples that can then be extended to more challenging specimen. One such model protein for polytopic integral membrane proteins is the tetrameric potassium channel KcsA (Figure 13) from the soil bacterium *Streptomyces lividans* [109]. KcsA is a 17.7-kDa protein with two transmembrane helices that spontaneously forms a tetrameric quaternary structure upon insertion into membranes [110]. In such tetramers, monomer subunits align around a central pore that on the extracellular side terminates in an arrangement of an additional shorter helix (pore helix) and a loop per monomer, forming a filter that is selective for potassium ions. Both, the N- and C-terminal extensions of the transmembrane helices are located intracellularly and are relatively flexible. The N-terminal residues form an amphipathic helix that attaches to the membrane surface depending on environmental conditions, while the C-terminal extensions can assemble in a bundle of 4 α-helices.

The tetrameric form of KcsA exhibits a remarkable stability and even withstands treatment with harsh detergents and thus can be analyzed in its oligomeric state in standard gel electrophoresis [109,111]. In addition, heterologous production of KcsA in e.g. *E. coli* is well established and yields relatively high amounts of protein. Both these properties are important for many downstream processes and thus make KcsA an ideal subject for experimental investigation.

The overall architecture of the transmembrane region of KcsA and its extracellular "selectivity filter" is well conserved in the family of voltage-gated potassium channels, including members in mammals and humans that are essential for neuronal transmission. This high degree of conservation led to extensive study of KcsA as a simple model for general properties of potassium channels by electrophysiology and structural biology, culminating in the elucidation of the structure of KcsA at atomic resolution that revealed that mechanism of selective K^+ transport [112]. This had been a long-standing question since selectivity for potassium, the bigger of the two major physiologically-relevant monovalent cations K^+ and Na^+, excluded a mechanism based solely on ion size. The structure of the selectivity filter revealed that the flanking sidechains closely mimic the hydration shell of K^+ ions, thus solving the conundrum and showing that smaller Na^+ ions are not transported due to insufficient stabilization. Subsequently, many more structures have revealed the universality of this mechanism of K^+ selectivity.

Aside from being used as an archetypical model for ion transport, KcsA has also been of significant interest to the more fundamental research field of lipid–protein interactions since many of its structural and functional properties show a prominent lipid dependence. For instance, KcsA contains several surface exposed tryptophan residues that are flanking the hydrophobic transmembrane region towards both intra- and extracellular side (Figure 13B). This structural feature can be found in many membrane proteins and has been shown to lead to favorable interactions with the interface region between lipid headgroups and acyl chains thus anchoring the protein in the membrane. As a result, KcsA has been extensively studied to address consequences of hydrophobic mismatch between proteins and lipids (see e.g. [114]). In further studies, it has been shown that different lipids affect the stability of the channel via changes in the lateral pressure profile [110,115] or via specific electrostatic interactions with the headgroup of anionic lipids (Figure 4C, [14,110,116,117,118]). In addition, anionic lipids have been shown to greatly enhance open probability and amplitude of the conducted current [119,120]. Another factor in modulating KcsA conductivity is the assembly of single channels into supramolecular clusters under specific conditions, which can lead to a cooperativity of gating events of singe channels [121].

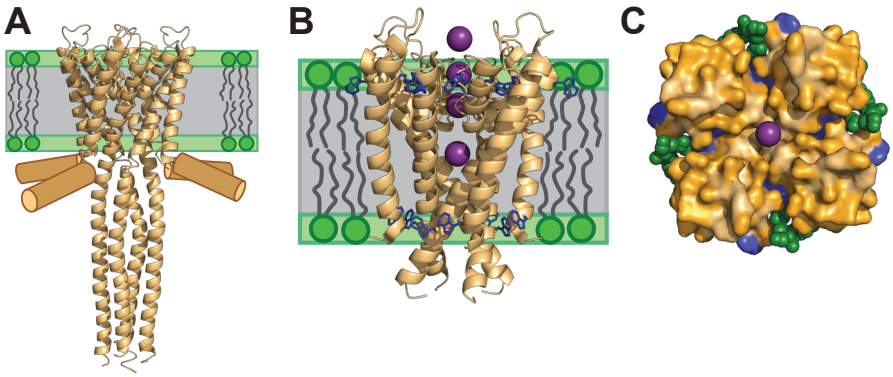

Figure 13: (A) Overall structure of a KcsA tetramer in a side view (PDB entry 3EFF [113]; missing N-terminal helices are depicted as cylinders). (B) Transmembrane segment of KcsA with highlighted potassium ions (purple). Tryptophan sidechains (blue) anchor the protein in the membrane via interaction with at interface between lipid headgroups and acyl chains. (C) Top view on the surface structure of KcsA. Lipid fragments (green) are present in the crystal structure and mediate interactions between monomers (B and C based on PDB entry 1K4C [14]).

Scope of this Thesis

In this thesis, several studies are described that aim to contribute to a better understanding of the mode of action of SMA polymers and to provide proof-of-principle examples for their applications to study membrane proteins.

Chapter II provides a protocol for using SMA to solubilize and purify the potassium channel KcsA from bacterial membranes. We demonstrate that the resulting native nanodiscs readily allow characterization of structural properties of the protein by spectroscopic analysis and thus show that they provide a more stable environment than detergent micelles. In addition, we establish a method to directly study native lipid–protein interactions by analyzing the lipid material that is copurified in the nanodiscs. Finally, a proof-of-principle example is provided for the functional reconstitution of the channel into planar bilayers without addition of detergent in any step of the process.

In **Chapter III** it is assessed whether SMA has an intrinsic preference for certain lipid species or for membrane domains. Such preferences would have implications for the general applicability of the approach to different types of membranes as well as for the study of (native) lipid–protein interactions. We show that SMA has no preference for specific lipids in homogenous bilayers in a fluid phase but that in phase-separated bilayers that contain domains in the fluid phase together with either those in a gel or in a liquid-ordered phase, the fluid phase is solubilized with a strong preference.

Chapter IV describes a live-cell fluorescence imaging approach that focuses on the question whether SMA solubilization is generally applicable to membrane proteins from the membranes of different organelles in human cells. We show that SMA affects the membranes

of all (sub-)cellular compartments and that cellular localization is not a dominant determinant for proteins residing in organelle membranes. Plasma membrane proteins, however, exhibited differential solubilization kinetics which suggests an influence of membrane domains in accordance with the findings described in Chapter III.

Chapter V attempts at rationalizing SMA solubilization of proteins in bacterial membranes using KcsA as a model protein. The influence of different parameters on solubilization yield is investigated, showing that particularly the ionic strength and the pH of the solution are dominant determinants for successful solubilization. In addition, the findings suggest that care should be taken in translating principles derived from lipid-only model membrane studies to the solubilization of proteins from biomembranes, particularly in case their lipid compositions vary strongly.

In **Chapter VI**, a summarizing discussion is provided that puts the presented findings into perspective and gives an overview of the body of literature that has accumulated since the earliest studies. This chapter finishes with an outlook for the use of SMA in a variety of applications to study membranes and membrane proteins.

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Chapter II

Detergent-free Isolation, Characterization, and Functional Reconstitution of a Tetrameric K^+ Channel: The Power of Native Nanodiscs

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Abstract

A major obstacle in the study of membrane proteins is their solubilization in a stable and active conformation when using detergents. Here, we explored a detergent-free approach to isolating the tetrameric potassium channel KcsA directly from the membrane of *Escherichia coli*, using a styrene–maleic acid copolymer. This polymer self-inserts into membranes and is capable of extracting membrane patches in the form of nanosize discoidal proteolipid particles or “native nanodiscs”. Using circular dichroism and tryptophan fluorescence spectroscopy, we show that the conformation of KcsA in native nanodiscs is very similar to that in detergent micelles, but that the thermal stability of the protein is higher in the nanodiscs. Furthermore, as a promising new application, we show that quantitative analysis of the co-isolated lipids in purified KcsA-containing nanodiscs allows determination of preferential lipid–protein interactions. Thin-layer chromatography experiments revealed an enrichment of the anionic lipids cardiolipin and phosphatidylglycerol, indicating their close proximity to the channel in biological membranes and supporting their functional relevance. Finally, we demonstrate that KcsA can be reconstituted into planar lipid bilayers directly from native nanodiscs, which enables functional characterization of the channel by electrophysiology without first depriving the protein of its native environment. Together, these findings highlight the potential of the use of native nanodiscs as a tool in the study of ion channels, and of membrane proteins in general.

Introduction

Integral membrane proteins (MPs) are an abundant class of proteins that play key roles in a wide range of essential cellular processes [1]. To facilitate their study *in vitro*, detergent molecules are commonly used to extract MPs out of their native lipid bilayer environment [2]. However, the use of detergents has some inherent disadvantages. Most importantly, even though there are promising developments to improve their properties [3,4], the insufficient mimicking of a lipid bilayer by detergent micelles often leads to destabilization and rapid loss of function of the incorporated protein [5]. For many functional and structural studies, it is thus necessary to reconstitute the MP into a more stabilizing environment; for example, by replacing the detergent with amphipathic polymers (amphipols) [6] or incorporating the MP into lipid nanodiscs with a surrounding protein scaffold [7]. Both approaches have proven to be valuable tools for the study of structural and functional properties of MPs [8,9]; however, a limitation remains, as transfer of MPs into any of these systems requires initial solubilization by detergent.

Recently, a detergent-free approach has been described using amphipathic styrene–maleic acid copolymers (SMAs) as an alternative to solubilize MPs directly from biological membranes in the form of nanodiscs, referred to as “Lipodisq” or SMA–lipid particles [10–13] (Figure 1). The mechanism of action of SMA differs fundamentally from that of detergents: instead of disrupting the lipid bilayer completely, SMA spontaneously self-inserts and extracts intact membrane patches in the form of discoidal particles that are stabilized by a SMA annulus [14,15]. Because these nanodiscs conserve a spatially delimited native biomembrane including MPs, we term them “native nanodiscs.” One of the main advantages of this system is the straightforward extraction protocol without the need for detergent. It has been shown that the SMA polymer is capable of directly extracting native nanodiscs containing large functional protein complexes from yeast [12], bacterial proteins involved in cell division [16] and photosynthesis [17], and several members of the ABC transporter family [13]. The isolation of these proteins from a variety of different organisms suggests a general applicability of SMA solubilization for all MPs, irrespective of their expression host or native organism.

To further explore the potential of native nanodiscs, we used the SMA polymer to isolate an oligomeric bacterial membrane protein: the tetrameric potassium channel from *Streptomyces lividans* (KcsA) [18], expressed in *Escherichia coli*. KcsA is an ideal model protein for such

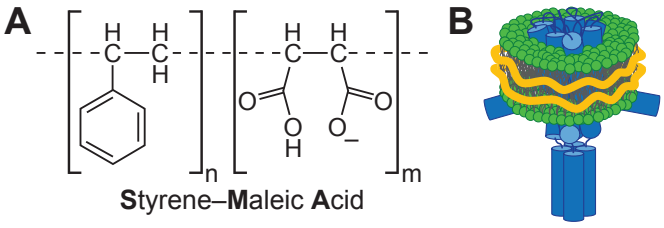

Figure 1: (A) Chemical structure of SMA polymers at neutral pH. For this study, a polymer with an average SMA ratio of n:m = 2:1 was used. (B) Schematic representation of a native nanodisc containing a KcsA tetramer (blue) and native lipids (green). The outer hydrophobic surface of the lipids is shielded by SMA (orange).

studies because it is well-characterized and because reconstitution studies have shown that both its function and stability are strongly affected by lipid composition [19–21]. In this work, we apply SMA to prepare and purify native nanodiscs with KcsA to compare the conformational properties and stability of the protein with those in detergent micelles. In addition, we use native nanodiscs to investigate preferential lipid–protein interactions by analyzing the composition of small patches of native membrane that are copurified with the protein. Finally, we study the functional properties of KcsA on reconstitution from native nanodiscs into a planar lipid bilayer system. Our results underscore the huge potential of SMA as a membrane-solubilizing agent, as well as the use of native nanodiscs as a membrane-mimetic system for biophysical studies on ion channels, and MPs in general.

Materials & Methods

Materials and SMA preparation

All chemicals and enzymes were purchased from Sigma-Aldrich unless otherwise indicated. *n*-docecyl-β-D-maltoside (DDM) was from Affymetrix, and all reference lipids used were from Avanti Polar Lipids. The used polymer was SMA2000, a styrene–maleic anhydride copolymer with a molar styrene-to-maleic anhydride ratio of 2:1, a weight average molecular weight of 7.5 kDa, and a molar mass dispersity (polydispersity index) of 2.5 (Cray Valley). Conversion into SMA was achieved by hydrolysis in 1 M KOH and reflux for 2 h while heating the suspension to 100 °C. Subsequently, the polymer was precipitated by the addition of HCl and washed 6 times with 100 mM HCl to remove K^+ ions. The sample was lyophilized, and hydrolysis was confirmed by Fourier transform infrared spectroscopy [17]. SMA stock solutions were prepared by dissolving 6 % (wt/vol) SMA powder in 50 mM unadjusted Tris buffer with gradual addition of NaOH solution until the pH reached the neutral range. The solution was then stored at –20 °C and adjusted to pH 8.0 and to the desired volume after thawing.

Gene expression and protein purification

KcsA was produced as described earlier [22]. Cell pellets were resuspended in 50 mM Tris buffer at pH 8.0 containing 600 mM NaCl and 30 mM KCl (10 mL per 2 g wet cell weight) and incubated with 10 µg/mL lysozyme and 5 µg/mL DNase for 45 min on ice. The suspension was aliquoted and mixed 1:1 with 6 % (wt/vol) SMA in 50 mM Tris at pH 8 for the preparation of native nanodiscs or 1:1 with 50 mM Tris buffer at pH 8 for detergent solubilization, respectively. The cell suspension with SMA was pushed through a disruptor at a pressure of 215 MPa and incubated overnight with gentle agitation at room temperature, followed by 45 min of centrifugation at 100,000 × *g*. SDS/PAGE analysis of supernatant and pellet after centrifugation revealed that 70–80 % of the total amount of tetrameric KcsA was solubilized by SMA. The supernatant was then incubated for at least 4 h at 4 °C with 2.5 mL HisPur Ni-NTA agarose beads (Thermo Scientific) per liter of culture medium. The beads were transferred to a gravity-flow column and washed three times each with buffers containing 10 and 50 mM imidazole. Protein in native nanodiscs was then eluted with buffer containing 300 mM imidazole, yielding 1–2 mg protein per liter of culture medium

on average, estimated by SDS/PAGE analysis and densitometric comparison of the protein bands after Coomassie staining with a gradient of BSA of known concentration, using the Quantity One software (Biorad). For biophysical studies, the pooled eluted fractions were loaded on a Superdex 200 10/300 GL size exclusion column (GE Healthcare) connected to an Äkta Prime Plus chromatography system (GE Healthcare) to separate the protein-containing nanodiscs from contaminating soluble proteins and free polymer. Thereby, the buffer was exchanged to 10 mM Tris at pH 8, 100 mM NaCl, and 5 mM KCl, which was used in all subsequent experiments.
Detergent purification was performed on aliquots of lysed cells, as described earlier [22], with the difference of using Tris buffer at pH 8.0 instead of Hepes. Eluted protein was extensively dialyzed against 1 mM DDM in the same buffer that was used for nanodiscs, using a dialysis membrane with a 50-kDa molecular weight cutoff. Protein concentration was determined by the absorption at 280 nm, using a calculated extinction coefficient of 34,950 $M^{-1} \cdot cm^{-1}$ [23]. For protein in native nanodiscs, this can only be considered an estimate, as the phenyl groups of SMA contribute to the absorption in this wavelength range.

Spectroscopy

Far-UV CD experiments were performed on a J-810 spectropolarimeter (Jasco) with a Peltier thermo control element (Jasco), using Teflon-sealed, polarimetrically checked quartz glass cuvettes with an optical pathlength of 1 mm and a volume of 350 µL (Hellma Analytics). Experimental parameters included a wavelength increment of 1 nm, a scan speed of 20 nm/min, a response time of 4 s, and a KcsA concentration of 0.06 mg/mL, as determined for DDM-solubilized protein. KcsA in native nanodiscs was diluted such that samples had the same signal intensity as protein in DDM, correcting for inaccuracies in concentration determination. Samples were allowed to equilibrate for 30 min at the desired temperature before measurement. All resulting spectra are buffer- and offset-corrected averages of 8–10 scans in the range of 200–250 nm.
Tryptophan fluorescence measurements were performed on a Cary Eclipse spectrofluorometer (Varian) equipped with a thermo control unit (Varian) in sealed quartz glass cuvettes with optical path lengths of 4 × 10 mm and an inner volume of 1.4 mL (Hellma). The excitation light beam had a wavelength of 295 nm at a slit size of 5 nm, and the fluorescence emission was recorded in the range of 300–400 nm, using a slit size of 5 nm, a wavelength increment of 1 nm, a scan speed of 60 nm/min, and an integration time of 1 s. The KcsA concentration was 0.01 mg/mL. All samples were allowed to equilibrate for 30 min at the desired temperature. Spectra were recorded in triplicate and corrected for buffer contribution before analysis by nonlinear least-squares fitting to a bimodal log-normal distribution, using the Excel add-in Solver (Frontline Systems) [24].

Lipid analysis

Before lipid isolation, KcsA-containing native nanodiscs that were eluted from Ni-NTA beads were washed with buffer on spin columns with a molecular weight cutoff of 30 kDa to remove imidazole. Lipids were then extracted according to a modified version of the method of Bligh and Dyer [25] and analyzed by quantitative TLC, as well as gas chromatography (for details, see Supporting Information).

Electrophysiology

Single-channel recordings of KcsA were performed on a Compact setup for planar lipid bilayer electrophysiology (Ionovation) connected to an EPC 10 amplifier (HEKA). Lipid bilayer formation was achieved by painting *E. coli* polar lipid extract dissolved in *n*-decane (50 mg/mL) over a 200-µm hole in a Teflon-septum separating two compartments. Both compartments contained 150 mM KCl solution that was buffered with 10 mM Hepes to pH 7.0 in the *cis* compartment and 10 mM succinic acid to pH 4.0 in the *trans* compartment, respectively. After channel insertion, the conductivity at a constant voltage of +100 mV was recorded for several minutes, using the PatchMaster software (HEKA). Data were sampled at 10 kHz and digitally filtered at 1 kHz. All measurements were performed at 22 °C.

For a more detailed description of the method used for incorporation of KcsA channels into planar bilayers as well as electron microscopy, see Supporting Information.

Results

KcsA can be solubilized and purified in native nanodiscs. Preparations of KcsA in native nanodiscs were obtained by the addition of SMA polymer directly to lysed *E. coli* whole cells with overproduced His-tagged KcsA, followed by isolation of the KcsA-containing nanodiscs by Ni-affinity chromatography. SDS/PAGE analysis of purified KcsA in native nanodiscs shows a pronounced band at a molecular weight of ~60 kDa (Figure 2A, lane 2), similar to that observed with tetrameric KcsA purified according to a standard protocol, using the nonionic detergent DDM (lane 4) [22]. Exposing the samples to 95 °C in the presence of SDS results in a virtually complete transition to the monomeric form of KcsA, as evident from the appearance of a band at ~17 kDa (Figure 2A, lanes 3 and 5). Thus, KcsA can be isolated in native nanodiscs as a stable tetramer that is resistant to SDS at room temperature, similar to what is described for KcsA in detergent [26]. In the isolations of KcsA in nanodiscs, we observed an additional pronounced double band of varying intensity at ~25 kDa that was heat-stable. Mass spectrometric analysis on a tryptic-digested sample of this band identified it as SlyD, a stress-upregulated, soluble 20.9-kDa protein endogenous to *E. coli* that contains multiple histidine residues in its C terminus [27]. This common contaminant of Ni-affinity purifications could be removed completely by size-exclusion chromatography (Figure 2B) [28]. The SMA polymer itself migrates at a low apparent molecular weight because of its high intrinsic negative charge and is observed as a blue-stained front on the depicted gels (Figure 2A, lanes 2 and 3).
The size and shape of purified KcsA-containing nanodiscs were further investigated, using negative-stain transmission electron microscopy (Figure 2C). The particles exhibit a round shape with a fairly homogeneous size distribution, with diameters of 10 ± 2 nm, which is in agreement with previous studies on native nanodiscs [10,12] and on protein-free nanodiscs obtained by SMA solubilization of synthetic liposomes [14,15].

Native nanodiscs surpass detergent micelles in conserving the structural stability of KcsA. The purity of KcsA nanodiscs after size-exclusion chromatography renders them directly suitable for studies with circular dichroism (CD) and tryptophan fluorescence spectroscopy. Far-UV CD spectra of KcsA in native nanodiscs and DDM micelles at different

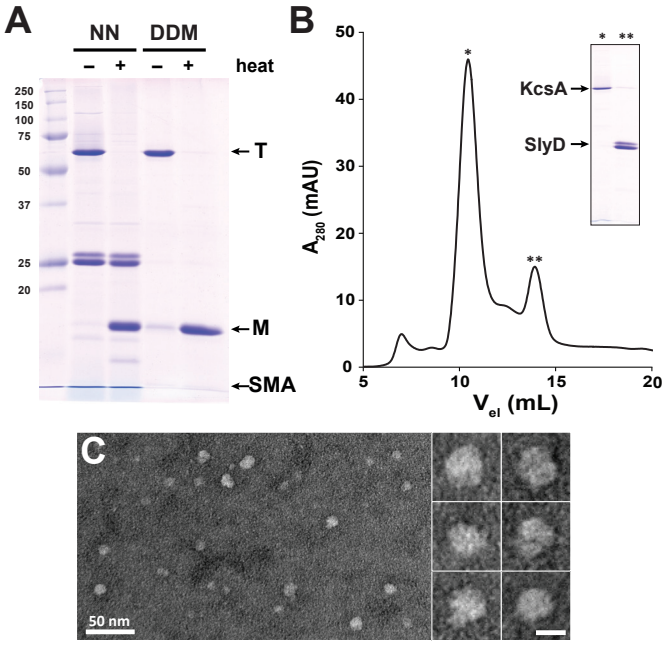

Figure 2: Purification of KcsA in native nanodiscs. (A) SDS/PAGE analysis of purified KcsA in native nanodiscs (NN) and DDM micelles at room temperature (–) and after incubation at 95 °C (+). Complete transitions from tetrameric (T) to monomeric (M) state are visible. (B) Size exclusion chromatogram showing effective separation of KcsA (*) from the soluble contaminant SlyD (**). (C) Negative stain transmission electron micrograph of native nanodiscs with KcsA after size exclusion chromatography. Round particles of an average size of 10 ± 2 nm are visible. (Scale bar in the enlarged images, 10 nm.)

temperatures are depicted in Figure 3A. The shapes of the spectra acquired at room temperature are virtually indistinguishable and show the features of a predominantly α-helical protein with minima at around 208 and 222 nm, in good agreement with previous studies [20,29] and available X-ray structures [30]. Upon heat exposure, differences between the spectra of KcsA in the different systems become visible. At 95 °C, KcsA in DDM shows a loss of ~50 % in secondary structure, with a well-defined transition at 72 °C, as measured by the ellipticity at 222 nm (Figure 3B). In contrast, KcsA in nanodiscs lacks a genuine transition as a function of temperature, and the amount of helicity decreases only slightly with increasing temperature, with a loss of ~20 % at 95 °C. In addition, the characteristic features of α-helical proteins are qualitatively conserved over the whole temperature range for KcsA in nanodiscs, whereas they are lost in DDM micelles above the transition.

Similar observations were made when the intrinsic fluorescence of KcsA in different environments was monitored. Increasing the temperature resulted in a strong decline in intensity of the recorded emission spectra of KcsA that was more pronounced in detergent than in nanodiscs (Figure 3C). At 20 °C, the maximum emission wavelengths on excitation at 295 nm were ~329 and ~332 nm for KcsA in nanodiscs and micelles, respectively. The blue-shifted emission maximum in nanodiscs can be explained by the decreased solvent

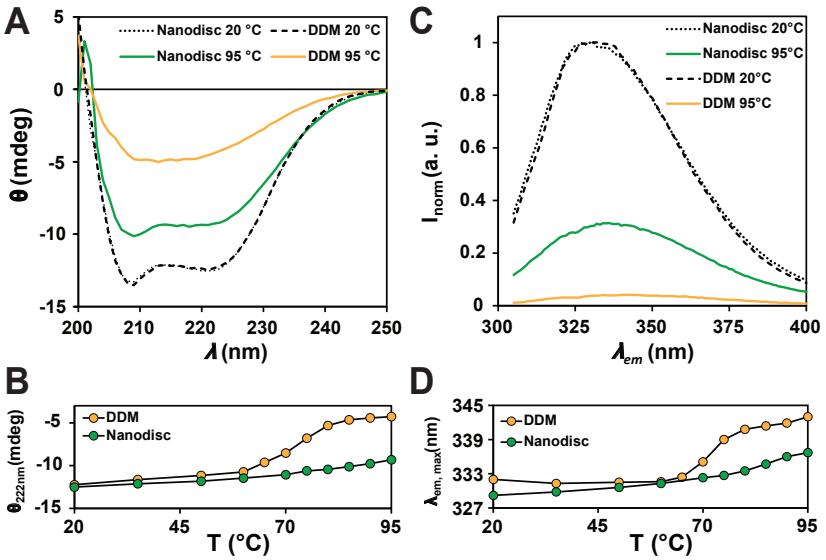

Figure 3: Comparison of the thermal stability of KcsA in different environments. (A) Circular dichroism spectra of KcsA in native nanodiscs and DDM micelles at 20 °C and 95 °C. Data are offset-corrected averages of 8–10 scans. (B) Corresponding thermal unfolding traces monitored by the ellipticity at 222 nm. Data are averages of two experiments with errors too small to be depicted. Solid lines are depicted to guide the eye. (C) Normalized fluorescence intensity of the intrinsic tryptophans of KcsA at 20 °C and 95 °C. Data are averages of three scans normalized to the intensity at 330 nm of the respective spectra at 20 °C. (D) Corresponding thermal unfolding traces monitored by the wavelength of maximum fluorescence emission. Solid lines are depicted to guide the eye.

exposure of some tryptophans resulting from better shielding by conserved lipid molecules than is seen in detergent micelles. The thermal stability of the tertiary structure of KcsA was investigated by following the shift in the emission maximum as a function of temperature. Consistent with the CD analysis, KcsA in detergent micelles shows a genuine transition at ~70 °C, whereas in nanodiscs, changes are less pronounced, without a clear transition for KcsA (Figure 3D). The emission maximum of micellar KcsA at 95 °C is ~343 nm, and is thus considerably more red-shifted than in nanodiscs (~337 nm). Virtually the same results were obtained when the intensity of the fluorescence at 330 nm was monitored as function of temperature. Thus, the tryptophan residues of detergent-solubilized KcsA show larger conformational changes than those in nanodiscs, where the tertiary structure is more stable even at high temperatures.

The higher stability of KcsA in nanodiscs compared with DDM micelles is further supported by the observation of a faint monomer band of KcsA purified in DDM on SDS/PAGE at room temperature that is absent for nanodiscs (Figure 2A, lanes 2 and 4). Upon storage at 4 °C, we observed an increase of this difference over time. Tetrameric KcsA in nanodiscs was stable over months, with a constant tetramer fraction above 95 %, whereas the tetramer fraction of DDM-solubilized protein decreased to ~60 % after 6 weeks. In line with this,

we found that neither freeze thawing nor lyophilization of KcsA in nanodiscs affects the integrity of the quaternary structure, whereas DDM-solubilized tetramers dissociate to some extent (Figure S1). Thus, using a complementary set of techniques, it is shown that native nanodiscs serve as a good tool to conserve the structural stability of the oligomeric KcsA. A possible explanation for the lower stability of detergent-solubilized KcsA is the dynamic equilibrium between monomeric and micellar DDM [2], which may promote the dissociation of the KcsA tetramer by segregation of monomers into separate micelles, thus favoring further unfolding. In contrast, native nanodiscs are stable particles, and no free SMA is needed in solution to maintain their stability. Thus, the more rigid structure of the particles could convey the higher stability of KcsA. However, it should also be noted that the mere presence of lipids can increase the stability of KcsA, as suggested by studies on reconstituted protein [20,22]. Conserving and stabilizing a lipid environment around MPs during their extraction by SMA polymers thus directly yields particles that maintain a higher stability of the trapped protein than detergent micelles.

Biochemical analysis of the local lipid environment of KcsA reveals enrichment of anionic lipids. The isolation of intact nanopatches of biological membranes in native nanodiscs provides a unique opportunity to investigate the immediate local lipid environment of KcsA, and thus characterize preferential lipid–protein interactions. Using TLC, the isolated lipids from native nanodiscs can be separated according to headgroup. Figure 4A shows a representative chromatogram with lipid samples from the total cell lysate, the complete SMA-solubilized fraction, and purified KcsA nanodiscs. In all samples, three pronounced bands are visible that can be identified by comparison with synthetic reference lipids as the zwitterionic phosphatidylethanolamine and the anionic lipids phosphatidylglycerol and cardiolipin. The lipid composition of independent bacterial cultures was found to be very sensitive to the growth conditions and time, resulting in a variation of absolute values in the range of 5–10 mol%. For comparison, data from each independent nanodisc preparation were therefore normalized to the corresponding total cell lysate. Quantitative analysis of the intensity of the chromatogram bands (Figure 4B) shows that the lipid compositions of the total cell lysate and the soluble fraction are very similar and in good agreement with other studies on lipids of *E. coli* K 12 wild-type strains [31,32]. Thus, SMA does not preferentially solubilize any specific lipids in the *E. coli* membrane. Strikingly, KcsA nanodiscs show higher amounts of anionic lipids, with increases of 36 % in phosphatidylglycerol and 61 % in cardiolipin content compared with the total extract. This isolation of enriched lipid species can hence be attributed to the preferential interaction of anionic lipids with KcsA.
Preferential lipid–protein interactions were further analyzed by investigating the composition of the acyl chains of all isolated lipids (Figure 4C). Lipids from all samples predominantly contained palmitic (16:0) and *cis*-vaccenic acid (18:1) chains with a combined weight percentage of ~70 %, in agreement with other studies [31]. In our analysis, no strong enrichment of specific acyl chains could be detected when comparing the lipids that were copurified with KcsA with the total extract or with the SMA-solubilized fraction. Only the amount of short lauric (12:0) and myristic acid (14:0) chains appeared to be slightly decreased in purified KcsA nanodiscs in favor of a slightly higher *cis*-vaccenic acid content. These results suggest a minor preference of KcsA for lipids with longer chains, possibly to promote hydrophobic matching [33,34].

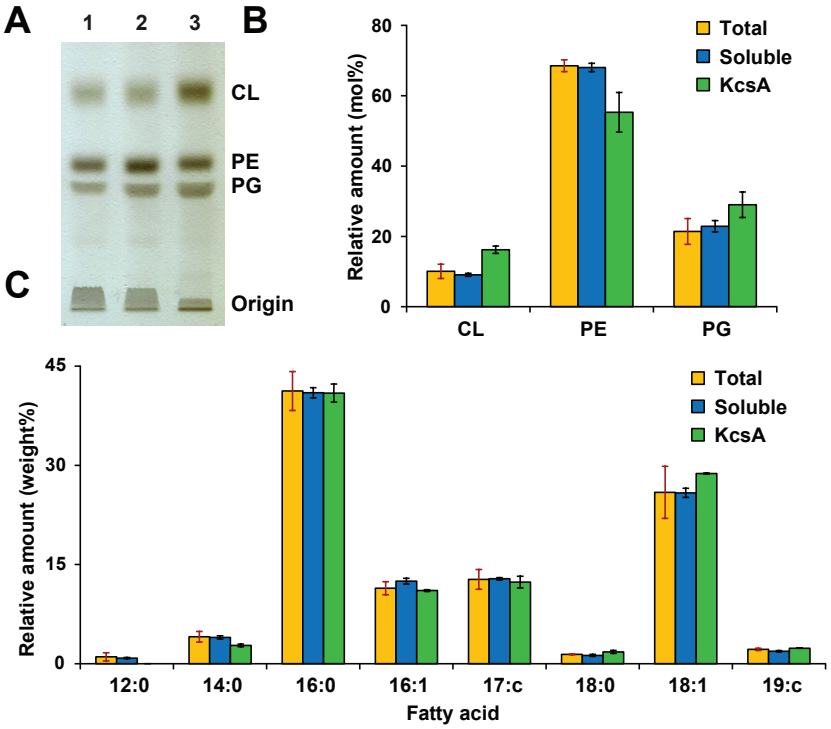

Figure 4: Analysis of coisolated lipids in native nanodiscs. (A) TLC on extracted lipids from lysed whole bacterial cells after addition of SMA (1), complete soluble fraction separated by ultracentrifugation (2), and native nanodiscs with KcsA purified by Ni-affinity chromatography (3). (B) Molar composition of the extracted lipid species according to headgroup of total lysate (orange), soluble fraction (blue), and KcsA nanodiscs (green). Data are shown as average mol percentages with standard deviations (red error bars) of three independent nanodisc isolations. Data for the soluble fraction and KcsA nanodiscs were normalized to the corresponding total lysate. Black error bars denote standard deviations of the change compared with total lysate. (C) Gas chromatography analysis of the fatty acid composition of all isolated lipids. Depicted data are averages of two independent experiments. Errors are given as in B.

Reconstitution of KcsA into planar lipid bilayers enables functional characterization. A final challenge remains in assessing the functionality of KcsA in native nanodiscs. Because analysis of ion transport is not possible in nanodiscs, we attempted to reconstitute the channel into a compartment-forming bilayer system that enables investigations of protein-facilitated potassium conductivity across membranes. To this end, we used a planar lipid bilayer setup that has been used successfully for functional studies on KcsA delivered in protein-stabilized nanodiscs [35]. Seconds to minutes after the addition of native nanodiscs with KcsA, single-channel conductivity was observed as transient discrete current changes with an amplitude of 10–15 pA (Figure 5), implying spontaneous fusion events of single nanodiscs with the bilayer. The recorded traces show both high and low open probability states that are characteristic for KcsA monitored under similar conditions by single-channel recordings of KcsA fused from liposomes [36] or incorporated via a cell-free expression

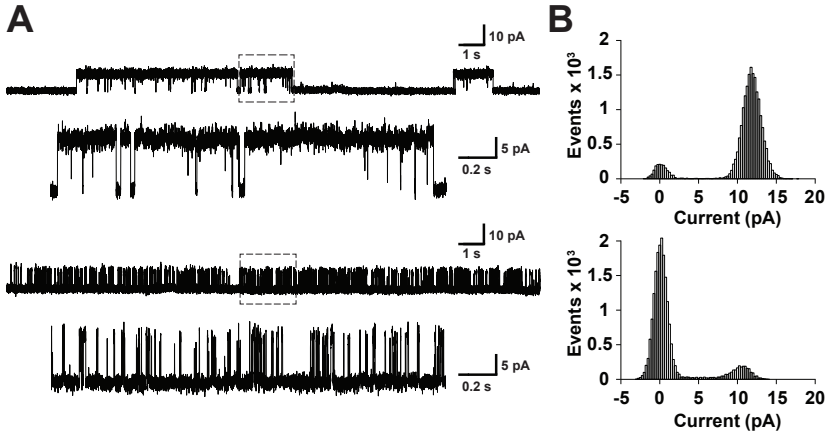

Figure 5: Functional characterization of KcsA reconstituted from native nanodiscs into a planar lipid bilayer of *E. coli* polar lipid extract. (A) Typical single-channel current traces on addition of KcsA nanodiscs to a setup with a symmetric 150-mM KCl solution at +100 mV. The top part represents a channel in the high open probability mode that is characterized by long opening dwell times, as shown in the enlarged section indicated by a box of dashed lines. Channels in the low open probability mode exhibit a distinctly different pattern, with short opening times resulting in sequences of bursts (bottom). (B) All-point histograms for open/closed distribution of channels with high (top) and low (bottom) open probability calculated from the enlarged 2-s single-channel current traces from A.

protocol [37]. The results are also comparable with those obtained by patch clamp studies of giant unilamellar vesicles with KcsA reconstituted from detergent [38]. Together, this suggests identical functional properties of KcsA reconstituted from native nanodiscs compared with standard reconstitution protocols. Upon addition of the K^+-channel blocker tetraethylammonium, we observed a reversible complete depletion of all opening events (Figure S2), further confirming the presence of reconstituted KcsA in the bilayer. Native nanodiscs thus may serve as a powerful alternative to conventional approaches for the functional reconstitution of ion channels.

Discussion

We have shown that native nanodiscs with embedded KcsA are formed spontaneously from bacterial cells upon addition of SMA polymer and that, using Ni-affinity and size-exclusion chromatography, sufficient protein can be purified in nanodiscs for extensive biophysical characterization. The isolation of KcsA in this manner is less cumbersome than standard detergent protocols and has the additional advantage of conserving the native conformation of the protein in a stabilizing environment comprising native lipids. Our data suggest that native nanodiscs in general convey a higher stability of the incorporated protein than detergent micelles, in agreement with previous findings on the thermostability of an ABC transporter [13] and photosynthetic reaction centers [17]. In addition, as shown in this study, the coisolation of MPs with a patch of biological membrane allows for the analysis of

preferential interactions and further facilitates structural and functional studies on purified protein in a (near) native state, as discussed next.

Insights into preferential lipid–protein interactions are important to understand structural and functional properties of MPs in a membrane environment. However, detailed information is not easily obtained with standard methods. Here, we exploited the extraction of intact nanopatches of biological membranes by the SMA polymer to directly identify preferential lipid–protein interactions that allow approximations of the situation *in vivo*. Analysis of the lipid composition revealed an enrichment of anionic lipids in close proximity to KcsA. This finding is unlikely to be an artifact of the extraction protocol because we found that none of the three main *E. coli* lipid species is preferentially incorporated into nanodiscs. This is intriguing, as the SMA polymer has a high negative charge density at pH 8.0 because of its many carboxylic acid moieties, which could be expected to lead to electrostatic repulsion of anionic lipids. The absence of a measurable effect of this repulsion hence emphasizes the promiscuity of the polymer with respect to lipid species in biological membranes. Similarly, SMA did not preferentially solubilize lipids containing specific fatty acids. This indicates that lipid solubilization by SMA is applicable to all phospholipids in any membrane, irrespective of headgroup or acyl chains, as further supported by the successful extraction of all major phospholipids of the mitochondrial membrane in yeast [12], as well as the membrane of the photosynthetic bacterium *Rhodobacter sphaeroides* [17].
The importance of anionic lipids for KcsA functionality and their close association with the channel has been well established by a wealth of studies using fluorescence quenching by brominated lipids [36], mass spectrometry [39], electrophysiology [36,40,41] and molecular dynamic simulations [42,43]. In addition, the diacylglycerol fragment found in crystal structures of KcsA was attributed to phosphatidylglycerol [19], and it has been shown that anionic lipids strongly stabilize the KcsA tetramer against dissociation by both heat [20,22] and small fluorinated alcohols [21]. In these studies, KcsA was purified in detergent, and preferential interactions were either deduced from the copurification of single tightly bound lipids or were observed indirectly after reconstitution into synthetic lipid bilayer systems. In contrast, the method described in this work facilitates direct biochemical analysis of preferential lipid–protein interactions that involve the native annular lipid environment. This paves the way for a new application to study specific lipid–protein interactions by using the SMA polymer to isolate nanodiscs with protein that has been conventionally reconstituted into bilayers of well-defined composition. By systematically varying the composition of these bilayers, one can then obtain very detailed information on the specificity of KcsA or other proteins for both lipid headgroups and acyl chains. Aside from assessing lipid–protein interactions, native nanodiscs offer the important additional advantage of copurifying proteins or other molecules that weakly interact with the target MP, providing unique insights into the composition of its immediate membrane environment. This feature has been used very recently for the isolation of a detergent-labile complex of penicillin-binding proteins in *Staphylococcus aureus* [16]. Native nanodiscs thus are a powerful tool to study preferential interactions of MPs both *in vitro* and *in vivo*.

In addition to purification of MPs and analysis of their preferential interactions, native nanodiscs also allow structural and functional characterization. Here, we used CD and fluorescence spectroscopy to study the thermostability of KcsA in a straightforward way.

Moreover, their small size makes native nanodiscs, in principle, suitable for characterization by the full range of biophysical techniques, including NMR spectroscopy, that have been successfully applied to MPs incorporated into similar lipid nanodiscs that are stabilized by scaffold proteins instead of SMA [9]. Native nanodiscs are also promising tools for studies on MPs in the rapidly developing field of single-particle cryo-electron microscopy. The use of amphipols to stabilize MPs has recently led to a high-resolution *de novo* structure determination of a cation channel at a resolution of 3.4 Å [44], breaking the side-chain resolution barrier. Together with promising studies using scaffold-protein nanodiscs [45,46], as well as initial studies with native nanodiscs [47], these new developments suggest that native nanodiscs may become a powerful alternative to enable high-resolution structural characterization of MPs in their native environment.

Several proteins have so far been functionally characterized in native nanodiscs by investigating their ligand-binding [13] and optical [10–12,17] properties, as well as their enzymatic activity [10,12]. Our data demonstrate that native nanodiscs can be also used to directly reconstitute KcsA into planar lipid bilayers, allowing electrophysiologic characterization of single channels in membranes with well-defined composition. To the best of our knowledge, this represents the first evidence of purification and transfer of an MP from the membrane of living cells to synthetic lipid bilayers without being deprived of its native lipid environment during any stage in the procedure. The ability of native nanodiscs to fuse with bilayers can be considered a promising first step toward enabling crystallization trials of MPs in a near-native environment. Other prospects of successful reconstitution include a plethora of assays available for MPs in liposomes and supported bilayers in applications that require a well-defined lipid environment. With the inherent advantage of conserving the native lipid environment of MPs, native nanodiscs constitute a highly promising and convenient alternative to detergent solubilization and may lead the way toward an *in situ* approach for structural and functional studies on MPs, including pharmacologic assays.

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Supporting Information

Storage potential of proteins in native nanodiscs

As shown in Figure 2 of the main article, KcsA in native nanodiscs is an SDS-stable tetramer that can be completely dissociated by incubation at 95 °C. Here, the storage potential of native nanodiscs was investigated by comparing the effect of freeze-thawing and lyophilization on the integrity of the tetramer. Figure S1 shows that the tetramer fraction in the nonheated nanodisc samples is close to 100 %, and neither freeze-thawing nor lyophilization leads to a decrease. By contrast, DDM-solubilized KcsA shows a minor fraction of monomer when stored at 4 °C. Here, freeze-thawing and lyophilization lead to an increased destabilization, as seen by a higher fraction of monomeric (and in the case of lyophilized protein also dimeric) KcsA. Thus, also the storage potential of native nanodiscs surpasses that of DDM micelles.

Channel blocking by tetraethylammonium

To further validate the functional reconstitution of KcsA, using the planar lipid bilayer setup, the effect of the selective K^+ channel blocker tetraethylammonium (TEA) on the observed conductivity was investigated. Figure S2 shows the baseline current of a stable bilayer (Figure S2 A) and KcsA-mediated K^+ currents after channel incorporation (Figure S2 B). To enhance

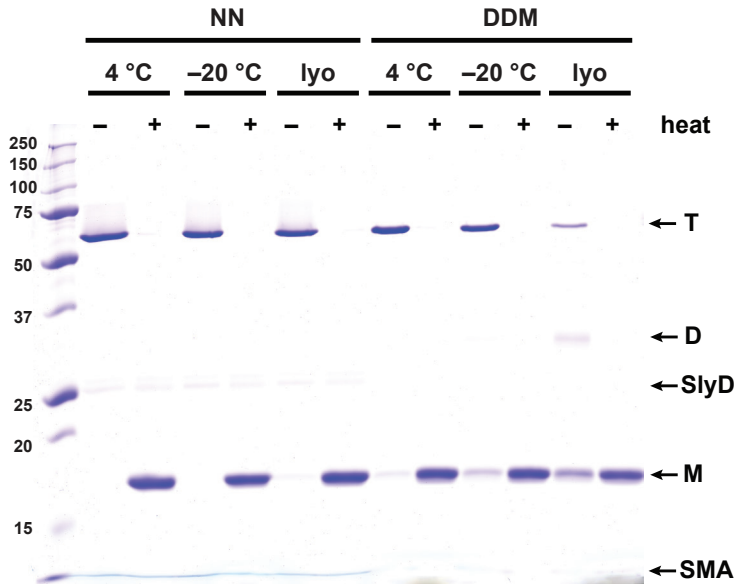

Figure S1: Storage potential of KcsA in native nanodiscs. Protein in native nanodiscs (NN) and DDM micelles was stored at 4 °C and freeze-thawed (–20 °C) or lyophilized (lyo) for 24 h. Before analysis by SDS/PAGE, samples were incubated for 15 min at room temperature (–) or 95 °C (+) in the presence of SDS. KcsA tetramer (T), dimer (D), and monomer (M) bands as well as SlyD and SMA polymer are marked with arrows.

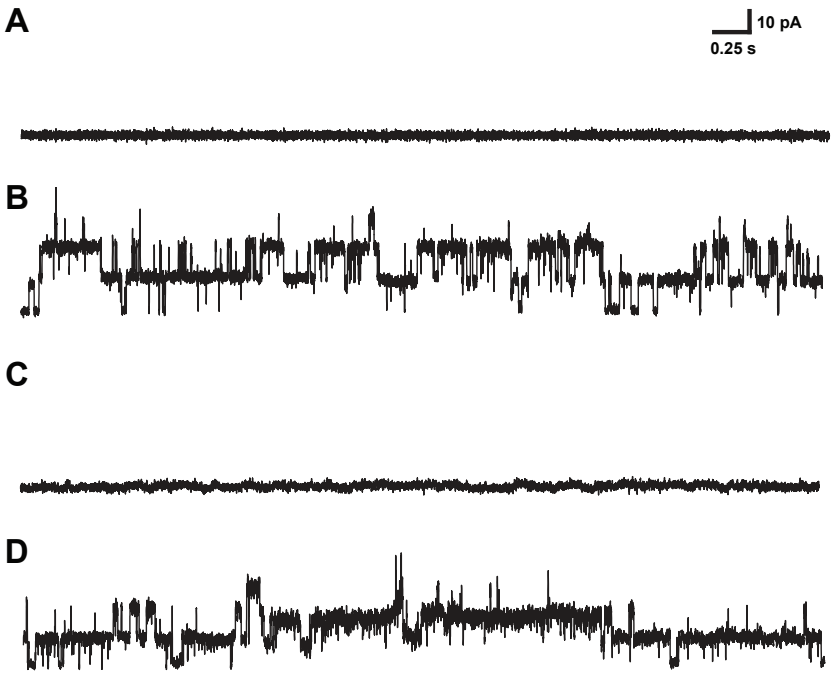

Figure S2: Effect of TEA on KcsA channel currents. (A) Background current of a stable bilayer before KcsA addition. (B) Discrete K^+ current changes mediated by at least three functional KcsA channels. (C) Fast channel blocking after addition of 20 mM TEA, as seen by a strong decrease in current amplitudes. (D) Recovery of KcsA-mediated currents after wash out of TEA. The slightly lower current amplitudes compared with (B) may be attributed to the presence of residual TEA in the vicinity of the channels.

clarity, a recording of a bilayer with several channels was selected for the experiment. Upon TEA addition and homogeneous distribution by gentle stirring for ~15 s, no more channel opening events could be detected, and only current fluctuations at baseline level were observed (Figure S2 C). Channel activity could be recovered to comparable channel open probabilities with close to original single-channel current amplitudes after a wash out of TEA (Figure S2 D), showing that the effect of TEA on KcsA is reversible.

SI Materials & Methods

Electron microscopy

KcsA-containing native nanodiscs purified by size-exclusion chromatography were concentrated to 1.5 mg/mL protein, using molecular weight cutoff spin columns (Merck), and 10 µL of sample was transferred to glow-discharged copper grids coated with a polymer film (Quantifoil) and adsorbed for 2 min, followed by two washing steps with water. Excess liquid was removed with filter paper in each step. Subsequently, grids were stained with 5 µL of 2 % uranyl acetate solution for 45 s, followed by air-drying. Electron micrographs

were recorded on a Tecnai12 electron microscope (Philips), operating at an acceleration voltage of 120 kV. For size determination, 20 individual particles where investigated using the software measureIT (Olympus).

Lipid extraction and analysis

Lipid-containing samples (0.5 mL) were added to 1.7 mL chloroform:methanol:1 M Tris at pH 8 [10:23:1 (vol/vol/vol)] and mixed extensively. To achieve phase separation, 1 mL of a 1:1 mixture of chloroform and 0.1 M Tris at pH 8 was added. The lipid-containing organic phase was then collected and washed with ion switch buffer (50 mM Tris, 100 mM NaCl, 100 mM EDTA at pH 8.2). For TLC, Nano-ADAMANT plates (Macherey-Nagel) were impregnated with 1.2 % (wt/vol) boric acid in ethanol:water (1:1) and dried for 1.5 h at 160 °C. Phospholipids were applied using a Linomat 5 setup (CAMAG), and plates were developed in an automated development chamber (ACD 2; CAMAG) at 50 % relative humidity, using a solvent mixture of chloroform:methanol:water:25 % ammonia [120:75:6:2 (vol/vol/vol/vol)]. Visualization of the lipids was achieved by immersing the plates in a solution of 10 % (wt/vol) $CuSO_4$, 8 % (wt/vol) H_2SO_4, and 8 % (wt/vol) H_3PO_4 in methanol, followed by uniform heating at 130 °C for 12 min. For quantification, a concentration gradient of total lipid extract of *E. coli* (Avanti) was applied to all plates, and band intensities were determined by densitometry, using the Quantity One software (Biorad). For fatty acid analysis by gas chromatography, isolated lipid samples were esterified in 2.5 % (vol/vol) H_2SO_4 in methanol for 2 h at 70 °C. The sample was mixed 1:1 with water, and fatty acid methyl esters were extracted by three washing steps with hexane. Separation was performed on a TraceGC ultra gas chromatograph (Interscience), using a Stabilwax column (Restek) and a temperature gradient from 180 °C to 220 °C. Peaks were identified by calibration with two standards: GLC 63b (Nu-Chek Prep) and Bacterial Acid Methyl Ester Mix (Sigma-Aldrich).

SDS/PAGE stability assay

KcsA in native nanodiscs or DDM micelles that was purified by Ni-affinity chromatography was diluted to a final concentration of 0.2 mg/mL with 10 mM Tris buffer at pH 8, supplemented with 100 mM NaCl, 5 mM KCl, and 300 mM imidazole. Aliquots of 100 µL were then stored for 24 h at 4 °C, –20 °C, or frozen in liquid nitrogen and subsequently lyophilized. The lyophilized sample was then resuspended in the same buffer. Before analysis by SDS/PAGE, samples were mixed with SDS-containing sample loading buffer, aliquoted, and subsequently incubated for 15 min at room temperature or at 95 °C.

Functional reconstitution

Aliquots of native nanodiscs with KcsA (1–5 µL, 0.3 mg/mL protein) were added to the cis compartment (V = 1.34 mL) of the planar bilayer setup, followed by gentle stirring to achieve incorporation of the channel. Using a “stir and wait” approach resulted in observations of channel conductivity in ~75 % of all cases, although lag times varied considerably from seconds to more than 30 min. However, once an initial fusion event was observed, breaking and reforming the bilayer in the presence of nanodiscs resulted in incorporation of channels after seconds. Bilayers with conductive KcsA channels were generally stable for several minutes but could be manipulated for up to 1 h in single cases. Using this approach, a total of ~20 independent fusion events were observed, resulting in a sum of recorded conductivity traces of more than 2 h, of which representative traces were selected. Channel

incorporation could also be achieved when the *trans* chamber instead of the *cis* chamber was supplemented with KcsA in native nanodiscs. Because the established pH gradient selectively activates channels with one orientation (pH-sensitive cytosolic domain in the *trans* compartment), it can thus be concluded that nanodiscs with KcsA fuse with the bilayer in both orientations. For experiments with channel blockers, tetraethylammonium was added to a final concentration of 20 mM to the *cis* compartment. Removal of the blocker was achieved by perfusion of the *cis* compartment with the pH 7 buffer for 30 s at a rate of 20 mL/min.

Chapter III

Solubilization of Lipids and Lipid Phases by the Styrene–Maleic Acid Copolymer

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Abstract

A promising tool in membrane research is the use of the styrene–maleic acid (SMA) copolymer to solubilize membranes in the form of nanodiscs. Since membranes are heterogeneous in composition, it is important to know whether SMA thereby has a preference for solubilization of either specific types of lipids or specific bilayer phases. Here, we investigated this by performing partial solubilization of model membranes and analyzing the lipid composition of the solubilized fraction. We found that SMA displays no significant lipid preference in homogeneous binary lipid mixtures in the fluid phase, even when using lipids that by themselves show very different solubilization kinetics. By contrast, in heterogeneous phase-separated bilayers, SMA was found to have a strong preference for solubilization of lipids in the fluid phase as compared to those in either a gel phase or a liquid-ordered phase. Together the results suggest that (1) SMA is a reliable tool to characterize native interactions between membrane constituents, (2) any solubilization preference of SMA is not due to properties of individual lipids but rather due to properties of the membrane or membrane domains in which these lipids reside and (3) exploiting SMA resistance rather than detergent resistance may be an attractive approach for the isolation of ordered domains from biological membranes.

Introduction

In recent years, the styrene–maleic acid (SMA) copolymer has evolved as an important tool for isolation and characterization of membrane proteins (for a review, see [1]). SMA has been shown to solubilize biological membranes in the form of nanodiscs allowing the isolation of membrane proteins directly from their native environment without the need for detergent [2–7]. The small size of these so-called "native nanodiscs" enables their characterization by a variety of biophysical approaches [1,4,5,8–11]. Furthermore, the presumed preservation of the annular lipid environment helps to maintain the stability of the embedded proteins and thereby allows the use of SMA as a convenient tool to study preferential lipid–protein interactions, simply by analyzing the lipid composition of purified protein-containing nanodiscs and comparing it with that of the native membrane [4,5,7].

For unambiguous analysis of preferential lipid–protein interactions using SMA, it is however of crucial importance to know whether or not SMA by itself exhibits any lipid preference during solubilization. This can be conveniently investigated by employing synthetic model membrane systems, which allow highly systematic variation of lipid composition. Solubilization of model membranes by SMA results in the formation of styrene–maleic acid lipid particles (SMALPs), which have similar sizes and properties as membrane protein-containing (native) nanodiscs [1]. Using such model systems, it has been shown that the interaction of SMA with membranes strongly depends on lipid composition, with the kinetics of solubilization being modulated by, e.g., surface charge, lipid packing and lipid chain length [12]. This would suggest that SMA might exhibit a lipid preference toward solubilization. However, experiments in which model membranes of an *Escherichia coli* total lipid extract were partially solubilized showed that the SMA-solubilized fraction exhibits no significant enrichment in specific lipid species [12]. Together these results suggest that SMA is promiscuous and that solubilization is determined by overall properties of the membrane rather than by properties of individual lipids. This was supported by a recent study using ^{31}P NMR [13].

So far, the lipid mixtures that have been used to study preferential solubilization by SMA represent only a few selected homogeneous lipid mixtures in the fluid phase, and no systematic studies have been reported yet on a possible lipid preference of SMA. Also, whether SMA exhibits any preference in heterogeneous membranes that exhibit domain formation and that arguably are biologically more relevant than homogeneous fluid bilayers has not been investigated.

To obtain insight into these matters, we here set out to investigate to what extent preferential solubilization of lipids by SMA occurs in simple binary lipid systems forming a single homogeneously mixed fluid phase and in heterogeneous phase-separated membranes exhibiting coexistence of a fluid phase with either gel or liquid-ordered phase. In order to maximize our "window" for monitoring any potential preferences of the polymer, the following strategy was employed. First, combinations of lipids were selected that on their own would have very different SMA solubilization kinetics. Second, to achieve partial solubilization short incubation times of 1 h were used thereby avoiding full equilibration of the system. This required adjustment of the concentration of SMA for each system individually in order to obtain sufficient material for reliable analysis. Third, multilamellar vesicles (MLVs) were chosen as the lipid system, which has the following advantages: (1) MLVs provide a large accessible surface area, which diminishes the chance of "all or nothing" effects that may occur for small vesicles, i.e., full solubilization of some membranes and no solubilization of

others, (2) by employing these larger structures any potential curvature effects are avoided, and (3) the use of these larger structures facilitates the separation of solubilized and non-solubilized material by centrifugation.
The results show that SMA is indeed highly promiscuous with respect to solubilization of lipid species in homogeneous fluid bilayers, but that there is a clear preference for solubilization of the fluid phase in phase-separated bilayers with either a gel phase or a fluid phase. We will discuss the implications of these findings regarding the general applicability of SMA as a tool to determine preferential lipid–protein interactions. We will also discuss the use of SMA for the isolation of SMA-resistant membranes (SRMs) as an alternative to conventionally studied detergent-resistant membranes (DRMs).

Materials & Methods

Materials

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). The used lipids were 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (di-18:1 PC); 1,2-dioleoyl-*sn*-glycero-3-phospho(1′-rac-glycerol) (di-18:1 PG); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (di-18:1 PE); 1,2-distearoyl-*sn*-glycero-3-phosphocholine (di-18:0 PC); 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (di-14:1 PC); 1,2-di-(9Z-hexadecenoyl)-*sn*-glycero-3-phosphocholine (di-16:1 PC); 1,2-di-(11Z-eicosenoyl)-*sn*-glycero-3-phosphocholine (di-20:1 PC); 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (di-22:1 PC); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0/18:1 PC); brain sphingomyelin (bSM); cholesterol; 23-(dipyrromethene boron difluoride)-24-norcholesterol (Top-Fluor-cholesterol); 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE).
Xiran 25010, a styrene–maleic anhydride copolymer with a molar ratio of styrene to maleic anhydride of 3:1 and an average molecular weight of 10 kDa, was a kind gift from Polyscope (Geleen, The Netherlands). Xiran 25010 (anhydride copolymer) was converted to the acid form by hydrolysis under base-catalytic conditions as detailed elsewhere [12]. All other chemicals used were from Sigma Aldrich (St. Louis, MO).

Preparation of multilamellar vesicles (MLVs)

Phospholipid stock solutions in chloroform/methanol (9:1 v/v) were mixed in predetermined ratios, and the solvent was removed under a stream of N_2. The resulting lipid film was dried in a desiccator under vacuum for at least 1 h. MLVs were obtained by hydrating the lipid films with solubilization buffer (50 mM Tris–HCl, 150 mM NaCl, pH 8.0). The samples were then subjected to ten freeze–thaw cycles, each consisting of 3 min of freezing in liquid N_2 (–196 °C) and 3 min of thawing in a water bath at 50 °C, well above the gel-to-fluid phase transition temperature (T_m) of the lipids. For vesicles containing di-18:0 PC (T_m = 56 °C [14,15]), the water bath was kept at 60 °C to ensure membrane fluidity.

Turbidimetry experiments

The solubilization of MLVs was monitored by turbidimetry, using a Lambda 18 spectrophotometer (PerkinElmer, Waltham, MA) as described previously [12]. Briefly, 700-µl aliquots of 0.5-mM dispersions of MLVs in solubilization buffer were transferred to a quartz cuvette and equilibrated at the desired temperature for 10 min. Next, different amounts of SMA were added as detailed in the legends of the corresponding figures, and the solubilization kinetics were followed at a fixed wavelength of 350 nm by monitoring the decrease of the apparent absorbance. Absorbance values were recorded every 0.4 s.

Analysis and quantification of the lipid composition of solubilized fractions

Partial solubilization of vesicles

To achieve partial solubilization while still obtaining enough lipid material for further analysis, the SMA concentration was tuned for each lipid mixture individually. Accordingly, 700-µl aliquots of 0.5-mM dispersions of MLVs in solubilization buffer were supplemented with an amount of SMA that led to a decrease in apparent absorbance to approximately 50–60 % after 1 h of incubation. Vesicles containing liquid-ordered domains required higher amounts of SMA for sufficient solubilization because of their higher solubilization resistance. Detailed incubation conditions for each sample are specified in the legends of the corresponding figures.

Lipid isolation

After 1 h of incubation with SMA, the samples were cooled down on ice and then transferred to a pre-chilled ultracentrifuge. The non-solubilized material was removed by centrifugation at 115,000 × *g* for 1 h at 4 °C, and the supernatant, containing the solubilized lipid material, was collected. The lipids from the supernatant and from an aliquot of non-treated MLVs were extracted according to the method of Bligh and Dyer [16] (see Supporting Information) prior to analysis.

Lipid analysis and quantification

The procedure for lipid analysis and quantification was selected depending on the lipid composition of the membrane. Lipids with different headgroups were separated by thin layer chromatography (TLC). Quantification was then achieved by densitometric analysis after copper staining [4,5]. Phospholipids with the same headgroup but with unsaturated acyl chains of different length were separated by reverse-phase TLC. After iodine staining, each spot was scraped off, and the amount of phosphate was determined by the method of Rouser [17]. For phospholipids with the same headgroup but with acyl chains differing in degree of unsaturation, reverse-phase TLC did not provide sufficient separation. These samples were therefore quantified by gas chromatography after esterification of the fatty acids [5,18]. For detailed experimental descriptions, see Supporting Information.

Transmission electron microscopy

Size characterization of the SMALPs present in the supernatant fractions resulting from turbidimetry experiments was performed by transmission electron microscopy. To this end,

copper grids were prepared following the carbon flotation technique. Briefly, samples were diluted with solubilization buffer to a lipid concentration of 0.5–1 mM, and small aliquots were adsorbed on carbon-coated mica. The mica was then transferred to a staining solution containing 2 % (w/v) sodium silico tungstate, causing detachment of the carbon film. Subsequently, a copper grid was placed on top of the detached carbon that was recovered and dried under air flow. Images were taken under low dose conditions at a nominal magnification of 49,000 with a T12 electron microscope (FEI, Hillsboro, OR) at an operating voltage of 120 kV using an ORIUS SC1000 camera (Gatan, Inc., Pleasanton, CA). The average size of the SMALPs was estimated manually from 16 well-defined individual particles randomly located through the image based on their maximum diameter using Adobe Illustrator software (San Jose, CA). This procedure was used to avoid potential artifacts such as stain-induced particle aggregation or inhomogeneous particle staining [12,19,20].

Fluorescence imaging

Fluorescence microscopy imaging was performed at room temperature using a Nikon A1 confocal microscope (Tokyo, Japan) equipped with a Perfect Focus system. Supported lipid bilayers (SLBs) were prepared in a custom-built chamber following the vesicle fusion procedure (see Supporting Information). Solubilization of SLBs by SMA was assessed under a continuous flow of solubilizing agent solution. Images were taken before addition of SMA and after 5 min of incubation using a 100× oil immersion 1.49-NA objective (Nikon) under identical conditions of laser power and gain for all samples. Top-Fluor cholesterol and rhodamine-PE were imaged sequentially using a 488- and 561-nm laser, respectively, to avoid spectral cross-talk. The images were acquired with a resolution of 512 × 512 pixels (pixel size 0.41 × 0.41 μm).
Fluorescence intensities were quantified from intensity histograms using NIS Elements software (Nikon). Intensity values are expressed as an average of the intensities calculated from five different snapshots randomly picked from the planar bilayer. A representative video of the solubilization process can be found in the Supporting Information (Video S1).

Supporting information

The supporting information includes turbidimetry data of all binary lipid mixtures shown in Figure 2 (Figure S1), a detailed size characterization of SMALPs by dynamic light scattering and EM (Figure S1, Figure S3 and Table S1), analysis of the lipid preference of SMA in di-14:1 PC-containing lipid mixtures (Figure S3), turbidimetry data on the SMA solubilization of membranes in an L_o phase (Figure S4), analysis of the lipid preference of SMA in a binary mixture of di-18:1 PC and bSM (Figure S5) and analysis of the lipid preference of SMA in 16:0/18:1-PC/bSM/cholesterol lipid mixtures (Figure S6).

Results

SMA does not display a preference for individual lipid species when solubilizing homogeneously mixed bilayers. It was previously demonstrated in model membrane systems that the lipid headgroup and acyl chain composition are important determinants

for the kinetics of SMA solubilization [12]. Here, we investigated whether two lipids that exhibit very different solubilization kinetics will be selectively solubilized by SMA when homogeneously mixed in a lipid bilayer. For this we selected mixtures of di-18:1 PC as "host" lipid with an equimolar amount of different "guest" lipids. The choice of these guest lipids was motivated by our previous observations [12] that (1) lipids with short chains are solubilized faster than lipids with longer acyl chains, most likely as a consequence of the lower number of van der Waals interactions between neighboring chains; (2) bilayers containing negatively charged lipids exhibit much slower solubilization kinetics than bilayers of zwitterionic lipids, presumably due to electrostatic repulsion by the negative charge of the polymer; (3) bilayers containing lipids in the gel phase, cone-shaped lipids or unsaturated lipids are solubilized more slowly than bilayers containing cylindrical lipids or saturated lipids in the fluid phase. These latter effects were ascribed to differences in the packing density of the acyl chains, with tighter packing hindering the insertion of the polymer and subsequent solubilization.
To obtain insights into a possible selectivity of SMA for certain lipids, an approach of partial solubilization of MLVs was used, as illustrated in Figure 1A for a mixture of di-18:1 PC with the anionic lipid di-18:1 PG, which is known to form homogeneously mixed bilayers [15,21]. The soluble fraction, after incubation with SMA for 1 h, was subjected to electron microscopy (EM) imaging (Figure 1B) and lipid composition analysis by TLC (Figure 1C). The EM data (Figure 1B) show a homogeneous distribution of particles of 6–8 nm size (Table 1), which is at the lower end of the range of commonly reported dimensions of

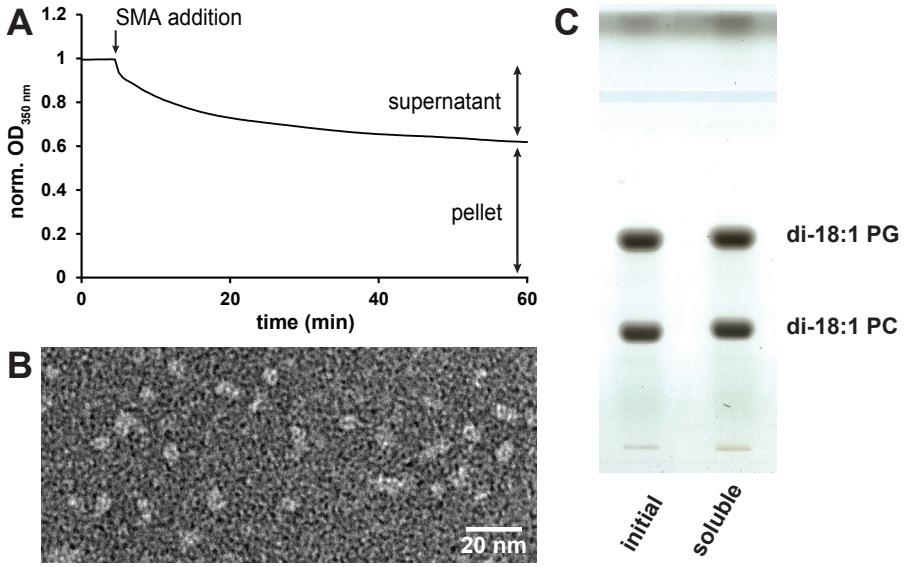

Figure 1: Partial solubilization of vesicles by SMA. (A) Kinetics of SMA solubilization of MLVs composed of an equimolar mixture of di-18:1 PC and di-18:1 PG (0.5 mM lipid, SMA-to-lipid mass ratio of 0.64) at 25 °C. Data are shown as normalized optical density at 350 nm. (B) Visualization of the SMALPs from the supernatant by negative-stain transmission electron microscopy. (C) Thin layer chromatography analysis of the lipid composition of lipids extracted from non-treated MLVs as well as the soluble fraction after partial solubilization by SMA.

Table 1: Nanodisc size characterization based on analysis of EM data

Lipid mixture (1:1, M)	Incubation temperature (°C)	Size (nm)
di-18:1 PC/di-18:1 PG	25	6–8
di-18:1 PC/di-18:1 PE	25	8–10
di-18:1 PC/di-14:1 PC	25	6–8
di-18:1 PC/di-18:0 PC	60	8–10
di-18:1 PC/di-18:0 PC	25	6–8

around 10 nm (see, e.g., [8,11,12]). Lipid composition analysis of the solubilized fraction after SMA incubation revealed that PC and PG are present in a similar molar ratio as in the initial vesicles (Figure 2A), indicating non-selective solubilization of both lipids. Considering the electrostatically unfavorable interaction of SMA with negatively charged lipids [12], this result is rather surprising and suggests that SMA does not perturb the bilayer homogeneity. Similar experiments were performed with other homogeneous lipid mixtures in the fluid phase. The results are summarized in Figure 2A and Table 1, while original solubilization traces and representative EM micrographs can be found in Figure S1. When di-18:1 PC

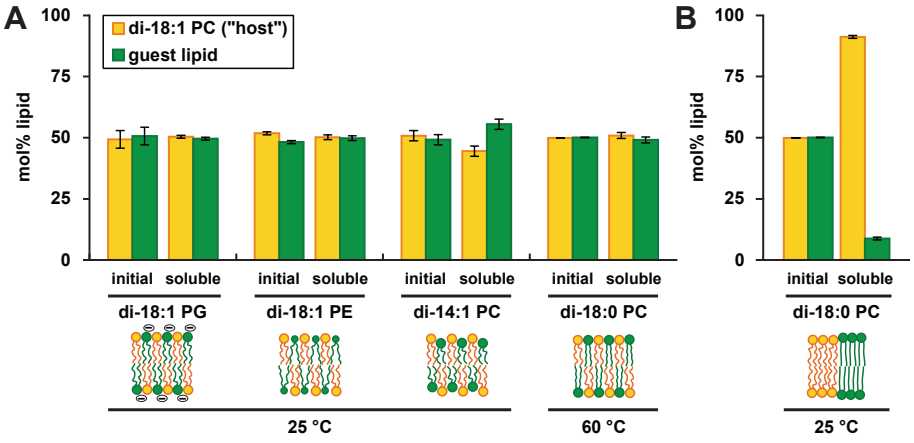

Figure 2: Solubilization preference of SMA in binary lipid systems with different properties assessed by lipid composition analysis after partial solubilization. (A) Equimolar mixtures of the zwitterionic unsaturated di-18:1 PC ("host", orange) with different guest lipids (green) under conditions of phase homogeneity. From left to right: anionic di-18:1 PG, cone-shaped di-18:1 PE, short chain di-14:1 PC and saturated di-18:0 PC. Respective SMA-to-lipid mass ratios at 0.5 mM lipid were 0.64, 1.31, 0.27 and 0.13. Phase homogeneity for di-18:1 PC/di-18:0 PC was achieved by elevating the temperature to 60 °C, above T_m of di-18:0 PC (T_m = 56 °C [14,15]). (B) Equimolar mixture of di-18:1 PC and di-18:0 PC under conditions of phase separation at 25 °C (SMA-to-lipid mass ratio 1.27). Cartoons show the schematic bilayer organization before addition of SMA. Error bars represent the standard deviation of three independent experiments.

was mixed with the cone-shaped lipid di-18:1 PE, again it was found that SMA does not show a lipid preference (Figure 2A), despite the slower kinetics of solubilization of PC/PE as compared to pure PC bilayers [12]. This non-selective solubilization is in line with results from a recent study of a very similar lipid system [13]. A different result was obtained for mixtures of lipids with varying acyl chain length, where a small preference was observed for solubilization of di-14:1 PC over di-18:1 PC (Figure 2A). To elucidate whether this preference might be related to hydrophobic mismatch effects, we also tested mixtures in which di-14:1 PC was kept as the shorter lipid, while the length difference between the lipid components was either increased or decreased (Figure S2A). In all cases, the results showed a similar small preference for di-14:1 PC, suggesting that this is an artifact related to a particular feature of di-14:1 PC, perhaps being more easily extracted from the membrane because of its short unsaturated acyl chains. This hypothesis is supported by the results obtained from partial solubilization of di-18:1 PC/di-22:1 PC membranes (Figure S2B), where the solubilized fraction has a similar lipid composition as the initial vesicles. Finally, a mixture of di-18:1 PC with di-18:0 PC was tested under conditions where both lipids were in the fluid phase. This was achieved by raising the incubation temperature to 60 °C, above the gel-to-liquid crystalline phase transition temperature of di-18:0 PC. Here again no preference for either lipid species was observed (Figure 2A).

For all the solubilized fractions corresponding to Figure 2, the formed SMALPs appeared to have a rather similar size in the range of 6–10 nm as visualized by EM imaging (Figure 1B, Figure S1) and as quantified in Table 1. Previously it was reported that the use of relatively low SMA concentrations might result in the formation of larger particles [10,22]. However, in our case the different populations of SMALPs were found to be fairly small with a relatively uniform size distribution, despite conditions of relatively low SMA concentrations (SMA-to-lipid mass ratio of 0.3–1.3). Importantly, similar particle sizes were found under conditions of using a higher SMA-to-lipid ratio, longer incubation times and higher lipid concentrations, which allowed characterization of the particles by both EM and dynamic light scattering (Figure S2, Table S1). Together these data support the validity of our partial solubilization approach.

Overall, the data show that SMA is highly promiscuous with respect to solubilization of lipid species when these are present as homogeneously mixed bilayers in the fluid phase. Whether preferences of SMA solubilization do occur in bilayers with a heterogeneous lipid distribution was investigated next.

SMA preferentially solubilizes the fluid phase under conditions where gel phase and fluid phase coexist. A heterogeneous lipid bilayer can easily be obtained in mixtures of lipids with unsaturated (low T_m) and saturated (high T_m) acyl chains by lowering the temperature well below T_m of the saturated lipid. For instance, in the above-described equimolar mixture of di-18:1 PC and di-18:0, lowering the temperature to 25 °C promotes a situation where gel and fluid (liquid-crystalline) phases coexist [15]. Under these conditions, SMA shows a strong preference toward solubilizing the fluid phase, which is mainly constituted by di-18:1 PC (Figure 2B). This result is in accordance with the much faster solubilization kinetics of lipids in the fluid phase as compared to lipids in the gel phase [12,13]. For bilayers exhibiting phase coexistence, the lipid preferences of SMA under conditions of partial solubilization thus do appear to reflect the differences in solubilization kinetics between the lipids in their respective phases.

SMA preferentially solubilizes the fluid liquid-disordered matrix upon incubation with membranes containing liquid-ordered domains. The resistance of gel-phase lipids against solubilization by SMA raises the question whether this is a general phenomenon for phases in which the lipids exhibit a high degree of order. This was first tested by adding SMA to a binary mixture of brain sphingomyelin (bSM) and cholesterol that forms a liquid-ordered (L_o) phase [23,24]. Addition of an amount of SMA that is generally sufficient to rapidly solubilize homogeneous bilayers in the fluid phase (SMA-to-lipid mass ratio of 3.5) did not lead to any decrease in apparent absorbance after 1 h for this system (Figure S4), and neither did increasing the SMA concentration or prolonging incubation times (data not shown), indicating a very poor solubilization efficiency of SMA for lipids in the L_o phase. These results resemble those reported for the non-ionic detergent Triton X-100 (TX-100), for which the L_o phase shows a well-described detergent resistance (see, e.g., [25–30]).
The solubilization potential of SMA was further investigated in an equimolar ternary lipid mixture of di-18:1 PC, bSM and cholesterol. Over a wide temperature range, bilayers of such composition exhibit phase separation containing L_o domains enriched in sphingomyelin and cholesterol that coexist with a fluid liquid-disordered (L_d) matrix enriched in di-18:1 PC [15,24,31]. As shown from the TLC results in Figure 3A and as quantified in Figure 3B, the lipid material solubilized from these membranes after incubation with SMA at 25 °C is clearly enriched in di-18:1 PC, while it is depleted in bSM and cholesterol in approximately equimolar amounts. At 4 °C, the SMA-solubilized fraction has a rather similar lipid composition as at 25 °C, while at 37 °C the solubilized fraction resembles the non-treated case more closely (Figure 3B). These results are consistent with a preferential solubilization of the L_d phase over the L_o phase by SMA at lower temperatures, which may be ascribed to tight packing and preferential SM–cholesterol interactions that cause co-segregation from the fluid phase [23,26,28,31]. Indeed, in the absence of cholesterol, an equimolar mixture

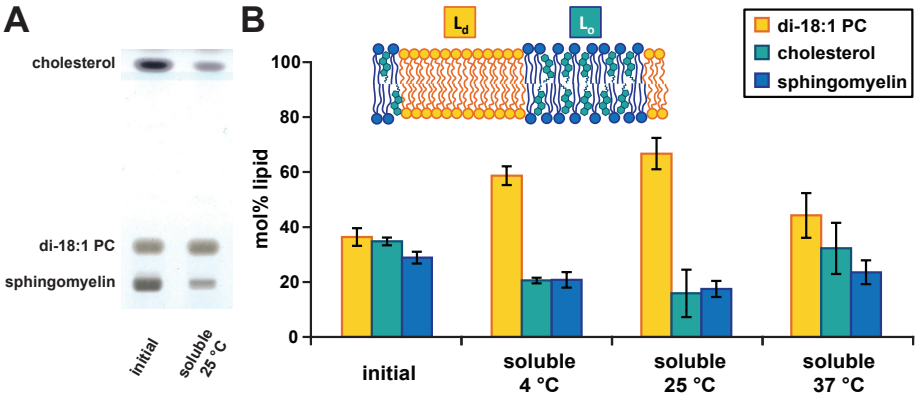

Figure 3: Lipid composition analysis after partial solubilization of MLVs composed of an equimolar ternary lipid mixture of di-18:1 PC, bSM and cholesterol by SMA. The inset shows a simplified schematic cartoon representation. (A) TLC plate with lipids extracted from non-treated vesicles and from the soluble fraction after incubation with SMA at 25 °C. (B) Quantification of lipid composition shown as mol% lipid (color coding consistent with cartoon) for non-treated vesicles as well as the solubilized fractions after the incubation with SMA (0.5 mM lipid, SMA-to-lipid mass ratio of 3.1) at different temperatures. Error bars represent the standard deviation of three independent experiments.

of bSM and di-18:1 PC was found to be solubilized in equimolar amounts of both lipids (Figure S5), demonstrating the large effect of cholesterol on lipid organization. At 37 °C, both cholesterol and bSM are more readily solubilized, which is likely to be related to the beginning of a gradual liquid ordered-to-fluid phase transition [32,33]. Based on our findings of non-preferential SMA solubilization in homogeneous bilayers (Figure 2A), it is likely that also this phase-separating ternary lipid mixture will be solubilized without a (strong) preference in case it exists in a homogeneous fluid phase. Similar results showing a high predisposition of the SMA copolymer to solubilize the L_d phase over the L_o phase were obtained for a phase-separating ternary lipid mixture of 16:0/18:1 PC, bSM and cholesterol (Figure S6).

To gain more insight into the mode of action of SMA in phase-separated bilayers, we performed additional experiments where the solubilization of di-18:1 PC/bSM/cholesterol membranes was monitored using fluorescence microscopy. For these experiments we used supported lipid bilayers (SLBs) that were supplemented with a small amount of lipid-derived fluorescent dyes that partition selectively into the L_o or L_d phase [34]. At room temperature, the SLBs showed a clear phase separation, with L_o domains varying in size from 0.1–2 µm (Figure 4A). After 5 min of incubation with 0.1 % (w/v) of SMA, the fluorescence intensity of the L_d probe dropped by more than 50 %, while the fluorescence emitted by the L_o probe decreased only marginally (Figure 4B). When the SMA concentration was increased to 0.5 % (w/v) only background levels of L_d fluorescence could be detected, while the L_o fluorescence was still at approximately 40 % of the initial intensity. Importantly, no further decrease in L_o fluorescence was observed when the SMA concentration was further increased to 1 % (w/v) (Figure 4B, see also Video S1) or when the sample was allowed to further incubate with the SMA solution for several hours (data not shown). Thus, the L_d phase enriched in di-18:1 PC is efficiently solubilized by SMA, while the L_o domains show a high resistance against solubilization, which is in agreement with the experiments performed with vesicles at the same temperature.

Discussion

The experiments described in this study reveal new insights into the process of membrane solubilization by SMA and how it depends on physicochemical properties of individual lipids and those of the membrane or membrane domains they reside in. Here, we will discuss our findings and the implications for the use of SMA as a tool to (1) study lipid–protein interactions and (2) isolate ordered domains from biological or model membranes.

SMA as a tool to study lipid–protein interactions

To obtain insight into whether SMA by itself has any preference for solubilization of specific lipids, we performed partial solubilization experiments on binary lipid mixtures. The results strongly suggest that under conditions of phase homogeneity there is no significant preference of SMA to solubilize any glycerophospholipid species. This is irrespective of differences in solubilization kinetics of the individual lipids upon changing properties such as headgroup charge, lipid shape, acyl chain saturation or acyl chain length. A potential exception is di-14:1 PC, which was found to be incorporated into SMALPs with a slight

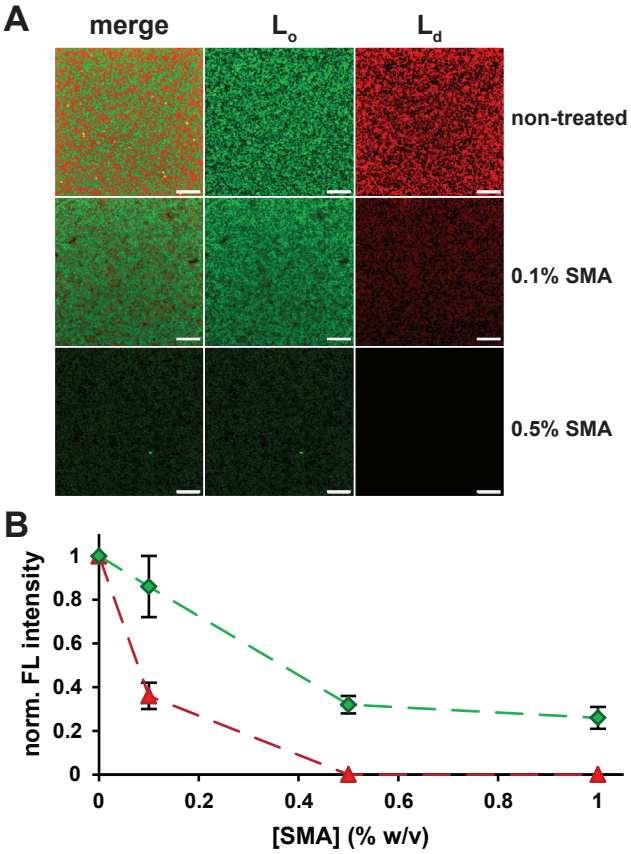

Figure 4: Preferential solubilization in supported lipid bilayers composed of an equimolar ternary lipid mixture of di-18:1 PC, bSM and cholesterol. (A) Fluorescence microscopy images are shown as merged (left column) and single channels (green: top-fluor-cholesterol, middle column; red: rhodamine-PE, right column) for non-treated samples and after incubation times of 5 min with different amounts of SMA in solubilization buffer. The scale bars correspond to 10 µm. (B) Quantification of the fluorescence intensity in the images from (A). Dashed lines are depicted to guide the eye. All experiments were performed at room temperature. Error bars represent the standard deviation of the fluorescence intensity of five snapshots randomly picked from the planar bilayer.

preference, probably due to its short and unsaturated acyl chains. Importantly, the observed full promiscuity of SMA in solubilizing homogeneous fluid bilayers suggests that SMA by itself does not perturb the bilayer homogeneity. Thus, our findings support the validity of the use of SMA to study preferential lipid–protein interactions of membrane proteins that are extracted from biological membranes [4,5,7]. Here, a snapshot view of the interplay of lipids and proteins in biological membranes can be obtained, provided that the proteins reside in a fluid lipid environment.

However, biological membranes in general are heterogeneous and may contain domains that are more ordered [35–37]. Our experiments with phase-separating lipid bilayers show that

distinct solubilization preferences of SMA do arise under conditions of phase coexistence of a fluid phase with either a gel phase or an L_o phase. In both cases, the soluble fractions consisted almost exclusively of lipids that were in the fluid phase. For gel phases it was previously postulated that it is the tight packing of the chains that is responsible for the poor solubilization yield, because it increases the energetic barrier for SMA molecules to penetrate into the bilayer core [12,13]. The same explanation would hold for the L_o phase, because it displays a similarly high degree of order of the acyl chains [38]. Together these results demonstrate that lipid packing plays a major role in the resistance against solubilization by SMA.

What are the implications of these results for the use of SMA for the investigation of preferential lipid interactions for proteins that reside in either gel phase domains or liquid-ordered domains? Obviously, such proteins will not easily be solubilized into SMALPs. However, they may be isolated as insoluble domains instead, as will be further discussed below. Although analysis of the lipid environment in such a case will not provide a snapshot of the immediate lipid environment, it may nevertheless provide relevant information on the lipid composition of the domains in which the protein resides.

SMA as a tool to isolate ordered domains

The clear preference of SMA to solubilize the fluid phase under conditions of phase coexistence holds promise for applications for the isolation of SMA-resistant membrane (SRM) domains. On the one hand, these could be applied to domains with a very high protein density, as was recently demonstrated by experiments in which SMA was used to prepare thylakoid membrane fractions that are enriched in specific photosystem complexes [39]. On the other hand, they could involve approaches similar to those exploiting detergent resistance of certain membrane domains.

Resistance against detergent solubilization is a well-known phenomenon in membrane research, which has been used extensively to prepare DRMs from biological samples [35,36,40,41]. In particular, TX-100 resistance at low temperatures has been exploited for the isolation of DRM fractions from mammalian plasma membranes. These DRM fractions have been associated to so-called “lipid rafts,” which are postulated to be specific membrane domains that are enriched in (glyco)sphingolipids, cholesterol and specific proteins and that have important roles in membrane function [36]. The basis of their detergent resistance is ascribed to the ordered nature of the lipid chains in these domains [36,37]. Our results with model membranes suggest that SMA may be used in a similar way as conventional detergent to isolate highly ordered membrane domains in the form of SRMs.

This raises the question of how the two approaches to isolate ordered domains from biological membranes would compare. DRMs are unlikely to have the same composition as postulated natively occurring lipid rafts in the plasma membrane at physiological temperature [33,42]. One reason for this is that conditions for DRM isolation usually include low temperatures, which will promote phase separation and thereby may cause further deviation from the composition of lipid rafts as they may occur at physiological temperature. Furthermore, by partitioning into the membrane, TX-100 shifts the thermodynamic equilibrium of phase separation [42] and thus likely affects the composition of lipid rafts that are isolated in DRMs. Alternatively, TX-100 has been postulated to increase the size of these domains [43].

It is not yet known to what extent this also holds for SMA. However, there is evidence that suggests that SMA may be less perturbing than detergent. It has been classified as an extraordinarily mild solubilizing agent [10,13] having a very low free energy cost for solubilization of lipids from membranes into SMALPs. This is reflected by the native-like bilayer organization of the solubilized lipids [11]. The results in the present study furthermore indicate that SMA is fully promiscuous in fluid bilayers, which suggests that SMA does not significantly perturb membrane homogeneity. Together, these results suggest that SMA could serve as an alternative for the isolation of highly ordered membrane domains that may have advantages over conventional methods using cold detergent solutions. However, whether indeed and to what extent SRMs isolated from biological membranes are superior to DRMs remains to be assessed.
Finally, an interesting novel possibility of this application of SMA may lie in the size of ordered domains existing in biological membranes. SMA may be capable of solubilizing very small membrane nanodomains in a conserved bilayer organization in case they are smaller than the average size of the SMALPs. This may for the first time make it possible to solubilize and characterize such small ordered domains directly from native membranes at physiological temperatures.

Conclusions

In this study, we show that in fluid membranes SMA does not exhibit a preference for solubilization of specific lipids, which supports the validity of studying preferential lipid–protein interactions in SMA-bounded nanodiscs derived from either model or biological membranes. In phase-separated membranes, SMA has a strong preference for solubilization of the fluid phase, with the potential application of isolating ordered domains from biological membranes by exploiting their SMA resistance. Our initial data suggest that the use of SMA for these approaches may be an alternative to cold detergent solutions, which are commonly used for this purpose.

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Supporting Information

SI Materials & Methods

Dynamic light scattering

1-mL aliquots of 15 mM dispersions of MLVs were incubated with SMA (SMA-to-lipid mass ratio of approximately 1.8) overnight at the specified temperature (see Table S1). The non-solubilized material was pelleted down by spinning at 115,000 × *g* for 1 h at 4 °C and the supernatant, containing the solubilized lipid material, was collected. Excess SMA was removed from the supernatant using Amicon Ultra 0.5-mL centrifugal filters with a molecular weight cut-off of 30 kDa (Millipore, Darmstadt, Germany). The filtered solution was diluted to 1 mL with solubilization buffer aiming for a final lipid concentration of approximately 10 mM. Dynamic light scattering (DLS) analysis was performed on the dialyzed samples using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples were measured at least 12 times, each measurement being an average of 20 sub-runs of 15 s. Size–intensity distributions were generated using Zetasizer software Ver. 6.20 and 7.03 and analyzed using the multiple narrow distribution. Hydrodynamic diameters were calculated from the intensity distributions with the assumption that nanodiscs have a spherical shape. All samples showed a polydispersity index (PDI) < 0.4.

Transmission electron microscopy

Size characterization of the SMALPs present in the supernatant fractions resulting from DLS experiments was performed by transmission electron microscopy (TEM). To this end, samples were diluted with solubilization buffer to a lipid concentration of 0.5–1 mM and small aliquots were adsorbed on EM grids. The further procedure was as described in the main text.

Lipid extraction

The lipids from solubilized fractions and from the initial vesicles were extracted according to the method by Bligh and Dyer [1]. Briefly, 700 µL of sample was mixed with 700 µL of $CHCl_3$ and 1.61 mL of MeOH in a glass tube and the mixture was vortexed vigorously. Next, 700 µL of $CHCl_3$ were added promoting phase separation and the organic phase was recollected. The aqueous phase was mixed once more with 700 µL of $CHCl_3$ and vortexed. The organic phase was again recollected and added to the previous organic fraction obtained. The organic fraction containing the lipids was washed with a buffer solution (Tris 50 mM, EDTA 50 mM, pH 8.0) and mixed with 100 µL of isopropanol. The solvent was evaporated under a stream of N_2 and the resulting lipid films were stored for further use.

Lipid analysis and quantification

For thin layer chromatography, lipids extracted from the solubilized fraction and from the non-treated vesicles were dissolved in 100 µL chloroform/methanol (9:1 v/v) of which 20 µL

and 10 µL, respectively, were applied to a high performance thin layer chromatography 10 x 10 cm Silica gel 60 plate (Macherey Nagel, Düren, Germany) using a Linomat 5 automatic TLC device (CAMAG, Muttenz, Germany). The lipids were separated in an ADC2 automatic development chamber (CAMAG), using chloroform/methanol/24 % (v/v) ammonia/H_20 (68:28:3:1 v/v/v/v) as the mobile phase. Next, the plate was dried for 5 min under a stream of N_2 and dipped in copper staining solution (10 % w/v $CuSO_4$, 8 % v/v H_2SO_4 98 % w/v and 8 % v/v H_3PO_4 85 % w/v). The spots were visualized by uniform heating at 130 °C for 15 min on a TLC plate heater (CAMAG) and subsequently quantified. The quantification was based on densitometry comparing the intensity of the lipid spots with a calibration curve on the same plate [2–4], using the Quantity One software (BioRad, Hercules, CA). In all cases, the amount of lipid in the samples was found to be in the linear range of the calibration curves. For reverse phase-TLC, the lipids extracted from the SMA-solubilized fraction and from the non-treated vesicles were dissolved in 100 µL chloroform/methanol (9:1 v/v) and 40 µL of each solution was applied manually to a silica gel C_{18} TLC plate (Millipore, Darmstadt, Germany). Next, the plate was developed in a TLC chamber, where the mobile phase consisted of methanol/dichloromethane/acetic acid (glacial) (80:20:1.5 v/v/v). After development, the plate was dried under a stream of N_2 and immediately placed in an iodine tank to visualize the lipid spots. The spots were scraped off and the total phosphate content in each spot was quantified according to the method of Rouser [5] as follows. Each sample was suspended in 300 µL of $HClO_4$ (70 % w/v) and heated for 1.5 h at 170 °C until organic samples were completely converted to inorganic phosphate. Next, the reaction was cooled down by adding 1 mL of H_2O followed by the addition of 0.4 mL of ascorbic acid (5 % w/v) solution and 0.4 mL of ammonium molybdate (VI) tetrahydrate (1.25 % w/v) solution The samples were agitated and heated in a boiling water bath for 6 min and cooled at room temperature for at least 10 min. Absorbance of the samples was recorded at 797 nm, and the total phosphate amount was quantified from a calibration curve.

For gas chromatography, analysis was conducted after subjecting the phospholipids to an acid-catalyzed esterification [6] as follows. The lipid films were suspended in 3 mL solution of 2.5 % w/v MeOH in H_2SO_4 (98 % w/v) and heated for 2 h at 70 °C. The reaction was stopped by adding 3 mL of H_2O and the methylated fatty acids were extracted with 3 mL of hexane. The extraction was repeated and the organic fractions were combined. The organic fraction was washed twice with H_2O after which 100 µL of isopropanol was added. The solvent was then removed under a stream of N_2 stream and the resulting methylated fatty acid films were redissolved in 90 µL of hexane and saved for further analysis. Subsequently, the samples were analyzed using a TRACE GC Ultra (Interscience, Breda, The Netherlands) equipped with a Stabilwax polar column (Thermo Fisher scientific, Waltham, MA) with an internal diameter of 0.31 mm and a film thickness of 0.25 µm. The retention times of different fatty acid methyl esters were assigned by comparison with two standards: GLC 63b (Nu-Check Prep, Elysian, MN), and Bacterial Acid Methyl Ester Mix (Sigma-Aldrich, St.Louis, MO).

Preparation of supported lipid bilayers

Substrate pretreatment
Glass slides were washed in 2 % (w/v) Hellmanex (VWR International, Chicago, IL) at 80 °C for 60 min, rinsed excessively with deionized water and then dried under a stream of N_2. Cleaned slides were then etched for 8 min in a solution of 98 % w/v H_2SO_4 and 30 % w/v H_2O_2 (3:1 v/v). The slides were kept in MilliQ water and used immediately.

Preparation of supported lipid bilayers
Multilamellar vesicles (MLVs) were prepared in solubilization buffer from a mixture of di-18:1 PC, bSM and cholesterol in an equimolar ratio, supplemented with 0.01 mol% rhodamine-PE and 0.05 mol% Top-Fluor cholesterol (see Preparation of MLVs). Large unilamellar vesicles (LUVs) were then obtained by extrusion of the MLV dispersion 21 times through 100-nm polycarbonate membranes at 50 °C. Next, supported lipid bilayers (SLBs) were prepared by vesicle fusion as follows: a custom-built chamber (V = 100 µL) was assembled on top of the pretreated, hydrophilic glass slide. The chamber was then completely filled with a LUV dispersion (250 µM lipid) and equilibrated for 30 min. at room temperature. Subsequently, the unfused vesicles were removed by buffer flow and 2 chamber volumes of solubilization buffer containing different amounts of SMA were flowed through the chamber at a flow speed of 50 µL/min using an oil-free pump.

Monitoring the process of solubilization in real time
The process of solubilization of supported bilayers (di-18:1 PC, bSM and cholesterol in an equimolar ratio, supplemented with 0.01 mol% rhodamine-PE and 0.05 mol% Top-Fluor cholesterol) was also monitored in real time for 5 minutes at a constant flow of 1 % w/v SMA-containing buffer solution at room temperature.

SI Results

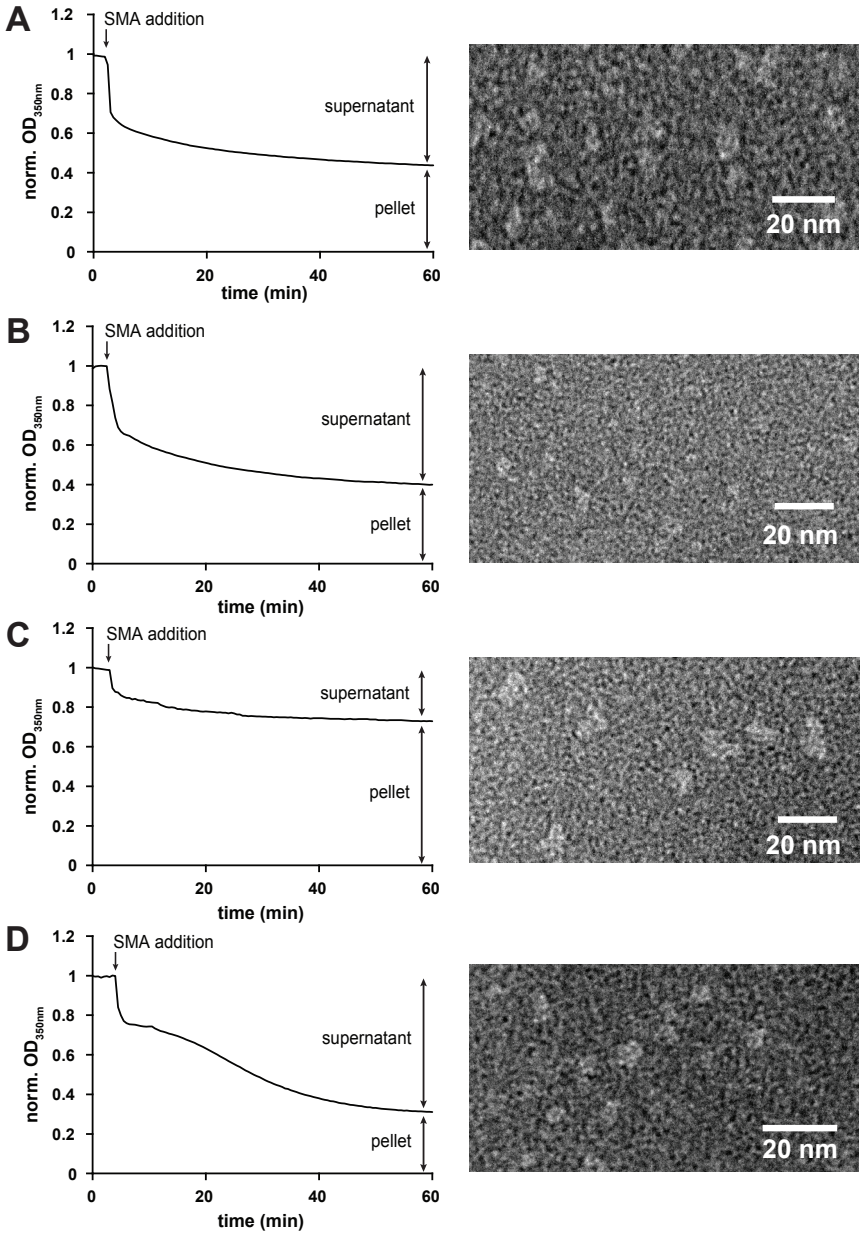

Figure S1: (Left) Kinetics of SMA solubilization of MLVs composed of equimolar lipid mixtures of 18:1-PC with di-18:1 PE (A), di-14:1 PC (B), di-18:0 PC (preparation at 60 °C) (C) and di-18:0 PC (D). Solubilization was assessed at 25 °C unless specified. Respective SMA-to-lipid mass ratios at 0.5 mM lipid were 1.31, 0.27, 0.13 and 1.27. Data are shown as normalized optical density (apparent absorbance) at 350 nm. (Right) Visualization of the nanodiscs from the corresponding supernatant fractions by negative-stain transmission electron microscopy.

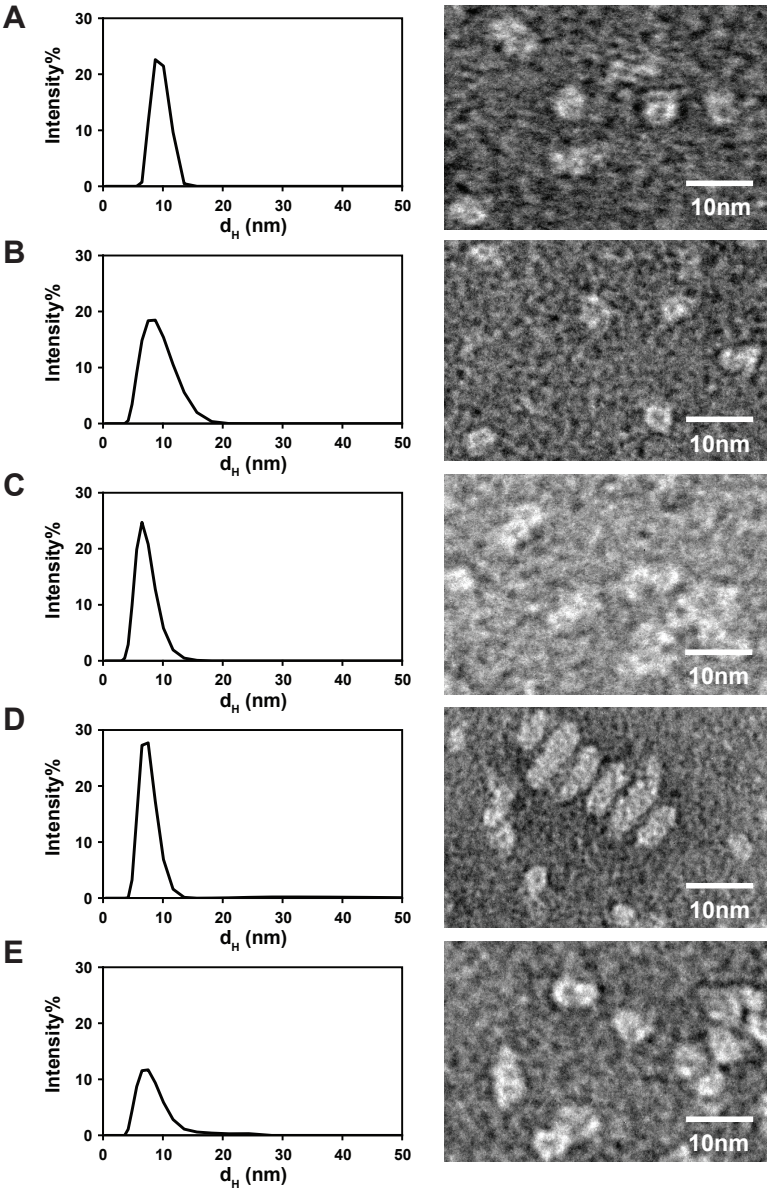

Figure S2: (Left) Size distribution of soluble nanodiscs after removal of non-solubilized material as quantified by dynamic light scattering at 25 °C for equimolar mixtures of di-18:1 PC with di-18:1 PG (A), di-18:1 PE (B), di-14:1 PC (C), di-18:0 PC (preparation at 60 °C) (D) and di-18:0 PC (E). The SMA-to-lipid mass ratio was kept constant at 1.8. Samples were prepared at 25 °C unless specified. (Right) Visualization of the SMALPs from the same sample by negative-stain transmission electron microscopy. In D, note the formation of stacks of discs in samples of di-18:1 PC and di-18:0 PC. Such "rouleaux" stacks have been observed before in nanodiscs bounded by amphipathic proteins [7,8] and were ascribed to an artifact resulting from specific interactions of the inorganic complexes of the staining solution with the positively charged choline headgroups.

Table S1: Nanodisc size characterization

Lipid mixture (1:1, molar)	Incubation temperature (°C)	Size EM (nm)	Size DLS (nm)*
di-18:1 PC/di-18:1 PG	25	6–8	8.2 ± 0.9
di-18:1 PC/di-18:1 PE	25	5–8	8.8 ± 0.7
di-18:1 PC/di-14:1 PC	25	5–7	8.3 ± 1.0
di-18:1 PC/di-18:0 PC	60	7–9	8.1 ± 0.3
di-18:1 PC/di-18:0 PC	25	6–9	7.2 ± 0.7

* Errors reflect the accuracy of the positioning of the peak maximum within 12 consecutive measurements. Note however that the actual size distribution as estimated from the DLS intensity plots in Figure S2 is several nm.

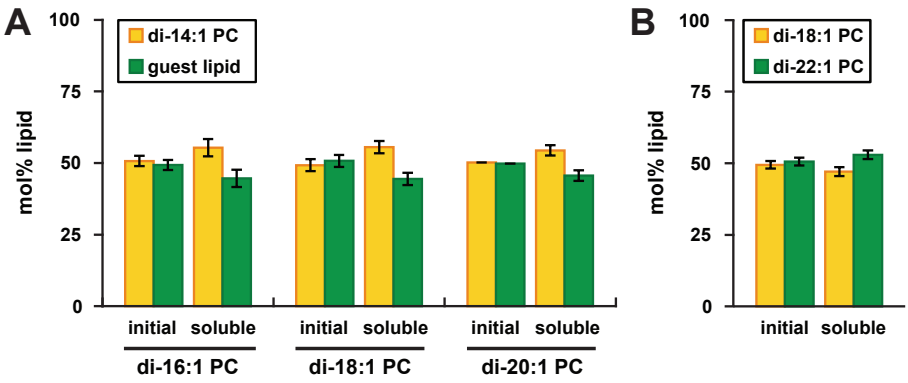

Figure S3: Solubilization preference of SMA in binary lipid systems with different properties assessed by lipid composition analysis after partial solubilization. Analysis was performed by reverse phase TLC (A) Equimolar mixtures of the zwitterionic unsaturated di-14:1 PC ("host", orange) with different guest lipids (green) under conditions of phase homogeneity. From left to right: di-16:1 PC, di-18:1 PC and di-20:1 PC. Respective SMA-to-lipid mass ratios at 0.5 mM lipid were 0.28, 0.27 and 0.26. (B) Solubilization preference of SMA in equimolar mixture of di-18:1 PC and di-22:1: PC at SMA-to-lipid mass ratio of 0.24. Cartoons show the schematic bilayer organization before addition of SMA. Error bars represent the standard deviation of 3 independent experiments.

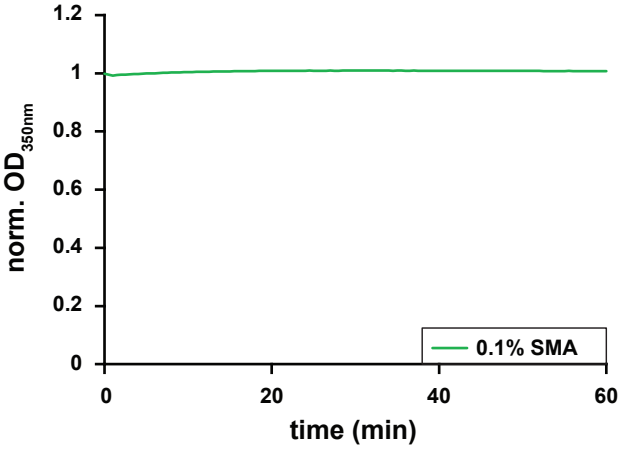

Figure S4: Inefficient SMA solubilization of membranes in the L_o phase. A turbidity trace is shown for MLVs composed of bSM and cholesterol (1:1, molar) that were incubated with SMA for 1 h (SMA-to-lipid mass ratio of 3.5).

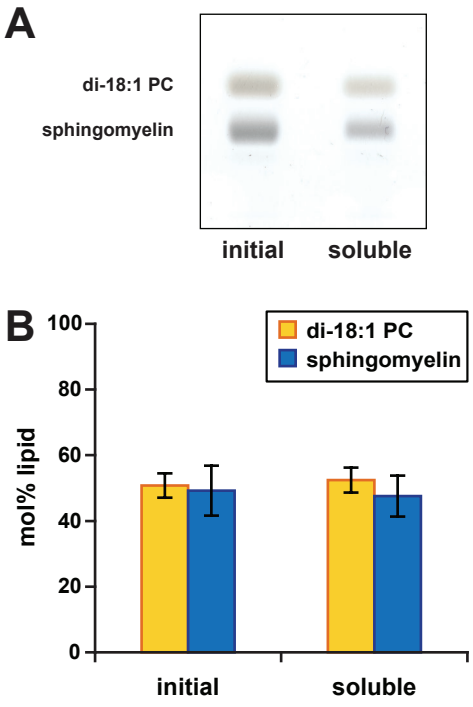

Figure S5: Lipid composition analysis after partial solubilization of MLVs composed of an equimolar binary lipid mixture of di-18:1 PC and brain sphingomyelin. (A) TLC plate with lipids extracted from initial vesicles and from the soluble fraction after incubation with SMA at 25 °C. (B) Quantification of lipid composition shown as mol% lipid for initial vesicles as well as the solubilized fractions after the incubation with SMA (0.5 mM lipid, SMA-to-lipid mass ratio of 2.6) at 25 °C. Error bars represent the standard deviation of 3 experiments.

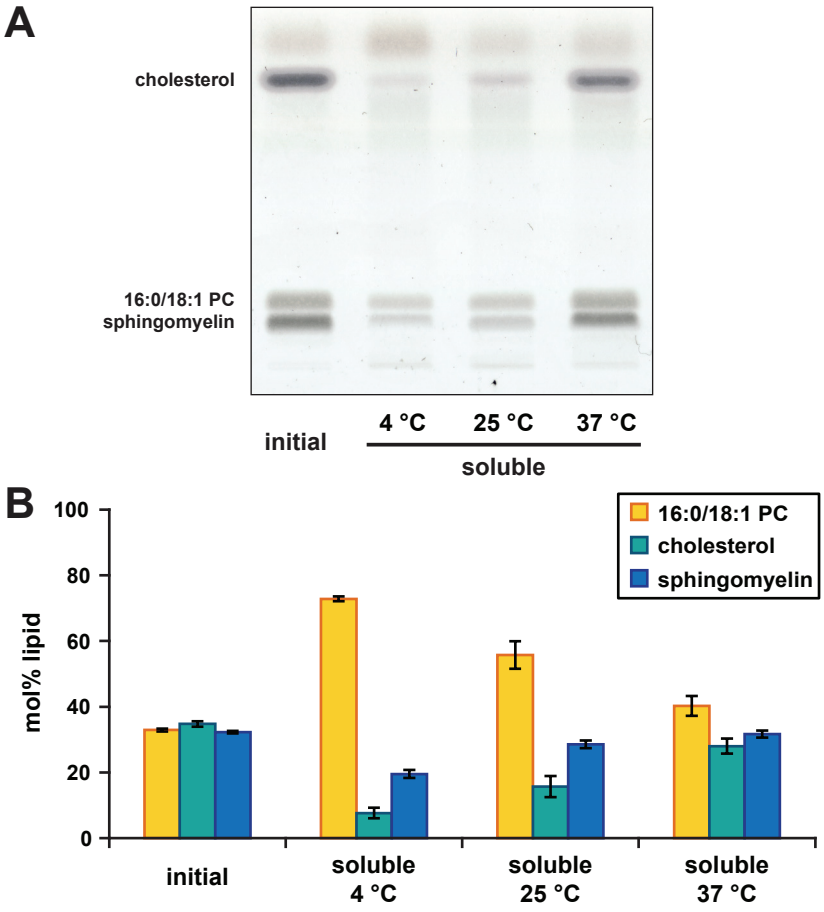

Figure S6: Lipid composition analysis after partial solubilization of MLVs composed of an equimolar ternary lipid mixture of 16:0/18:1 PC, brain sphingomyelin and cholesterol by SMA. (A) TLC plate with lipids extracted from initial vesicles and from the soluble fraction after incubation with SMA at 4 °C, 25 °C and 37 °C. (B) Quantification of lipid composition shown as mol% lipid for initial vesicles as well as the solubilized fractions after the incubation with SMA at different temperatures (0.5 mM lipid, SMA-to-lipid mass ratio of 3.2). Error bars represent the standard deviation of 3 experiments.

Video S1: Video showing the process of solubilization of a supported lipid bilayer of an equimolar mixture of di-18:1-PC/bSM/cholesterol, supplemented with the fluorescent lipids top-fluor-cholesterol (green, L_o domains) and rhodamine-PE (red, L_d domains) upon exposure to 1 % (w/v) SMA in buffer at room temperature. The SLB was flushed continuously with SMA-containing buffer solution from the start of the video (begin of SMA incubation) until its end after 5 min (real time). L_d domains (red) disappear completely in time, whereas L_o domains remain intact, only showing a decrease in fluorescence intensity (see Figure 4 of the main text). For better representation the play speed of the video was accelerated threefold.

SI References

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Chapter IV

Solubilization of Human Cells by the Styrene–Maleic Acid Copolymer: Insights from Fluorescence Microscopy

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Casper C. Hoogenraad
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Abstract

Extracting membrane proteins from biological membranes by styrene–maleic acid copolymers (SMAs) in the form of nanodiscs has developed into a powerful tool in membrane research. However, the mode of action of membrane (protein) solubilization in a cellular context is still poorly understood and potential specificity for cellular compartments has not been investigated. Here, we use fluorescence microscopy to visualize the process of SMA solubilization of human cells, exemplified by the immortalized human HeLa cell line. Using fluorescent protein fusion constructs that mark distinct subcellular compartments, we found that SMA solubilizes membranes in a concentration-dependent multi-stage process. While all major intracellular compartments were affected without a strong preference, plasma membrane solubilization was found to be generally slower than the solubilization of organelle membranes. Interestingly, some plasma membrane-localized proteins were more resistant against solubilization than others, which might be explained by their presence in specific membrane domains with differing properties. Our results underscore the general applicability of SMA for the isolation of membrane proteins from (sub)cellular membranes and suggest additional applications for isolation of proteins that are localized in ordered membrane domains.

Introduction

In recent years, styrene–maleic acid (SMA) copolymers have rapidly gained attention for applications in membrane research [1]. These include the detergent-free extraction and purification of integral membrane proteins from a variety of expression systems [2–4] and the study of lipid–protein interactions in the resulting native nanodiscs [4,5]. Importantly, these SMA-bounded nanodiscs generally mediate a higher protein stability than detergent micelles [2–4], which makes SMA extraction a promising alternative to established detergent-based methods.

Approaches involving lipid model membrane systems have contributed vastly to our understanding of the mode of action of membrane solubilization by SMA and the physico-chemical properties of the resulting SMA–lipid particles (SMALPs) [6–12]. However, systematic studies addressing the solubilization of cellular membranes are not available to date. These are important since the membranes of living cells are far more complex in composition and organization than are common lipid model membranes [13], with a substantial mass fraction being constituted by protein. These differing properties of biomembranes may have major consequences for their solubilization by SMA, especially if membrane proteins are isolated from more complex cells that contain subcellular compartments.

In this study, we employed HeLa cells to study the mechanism of action of SMA in solubilizing cellular membranes. HeLa cells were selected because (i) they are the most commonly used human cell line, (ii) they are relatively easy to culture and general protocols for transfection with various DNA constructs are available and (iii) they exhibit strong adhesion to surfaces, which is beneficial for in-plane microscopy in the presence of solubilizing agent. In particular, we investigated whether SMA solubilization of membrane proteins depends on their localization in different (sub)cellular membranes. To this end, we used a variety of fluorescent membrane protein fusion constructs targeted to the membranes of different organelles or to the plasma membrane and followed their solubilization by multi-channel fluorescence microscopy. In addition, water-soluble fluorescent proteins with differing cellular localizations were used to assess membrane perforation. The results provide new insights into the mode of action of SMA in standard human cell lines and into the susceptibility of different membranes and membrane domains for solubilization by SMA.

Materials & Methods

Materials

Xiran30010, a styrene–maleic anhydride polymer with a molar ratio of styrene to maleic anhydride monomer units of 2:1 and a weight-average molecular weight of 10 kDa, was a kind gift from Polyscope (Geleen, NL). Conversion to the acid form by hydrolysis and preparation of stock solutions at 5 % (w/v) was performed as described elsewhere [7]. Hoechst 33342 (NucBlue® Live ReadyProbes® Reagent solution) was from Thermo Fisher Scientific (Waltham, MA), polyethylenimine PEI MAX was from Polysciences (Warrington, PA), Triton X-100 and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

DNA constructs

All DNA constructs used have been described before. Constructs encoding for soluble proteins include tagRFP-ER (Molecular Probes, Eugene, OR) that contains an N-terminal calreticulin signal sequence and a C-terminal KDEL sequence resulting in localization to the ER lumen, MitoDsRed [14], encoding for the mitochondrial targeting sequence derived from the precursor of subunit VIII of human cytochrome C oxidase and a pEGFP N1 vector for marking the cytosol with GFP. Constructs encoding for membrane proteins were MARCKS-GFP and MARCKS-RPF (MARCKS–TagRFP-T) [15], both encoding for a truncated version of human myristolylated alanine-rich C-kinase substrate (MARCKS), comprising the 41 N-terminal amino acids in which Ala3 was mutated into Cys to introduce a palmitoylation site, TOM20-mCherry (TOM20-mCherry-LOVpep) [16], encoding for human mitochondrial import receptor TOM20 homolog, located in the outer mitochondrial membrane, CD8-ΔC-GFP [17], encoding for a truncated version of cluster of differentiation 8 that in T cells is plasma membrane localized but is not exported out of the ER in HeLa cells. Man II-GFP [18], encoding for the Golgi-resident human mannosidase 2 was a gift from John Presley, McGill University and NGL3-EGFP [19], encoding for rat Netrin-G ligand 3, a plasma membrane-localized protein was a gift from Eunjoon Kim, KAIST.

Sample preparation and fluorescence microscopy

HeLa cells were cultured in a 1:1 mixture of DMEM and Ham's F10 medium (Lonza, Basel Switzerland), containing 10 % Fetal Calf Serum and 1 % penicillin/streptomycin. Before splitting, cells were detached using trypsin/EDTA. For microscopy, cells were seeded on glass coverslips and transfection was performed using polyethylenimin. After transfection, cells were grown for additional 24–48 h prior to solubilization experiments. Hoechst nuclear dye was applied as specified by the manufacturer immediately before an experiment.
For life fluorescence microscopy, glass coverslips were mounted into an imaging chamber, which was filled with 150 µL of buffer solution (50 mM Tris, pH 8; 150 mM NaCl). Cells were imaged with a Nikon Ti inverted microscope (Tokyo, Japan) equipped with a Plan Fluor 40x/1.30 oil objective (Nikon). Imaging was controlled using the MicroManager software (Vale Lab, UCSF), which is an extension of ImageJ. Fluorescent images were taken using filters for blue (ET-EBFP2 (49021)), green (ET-GFP (49002)) or red (ET-mCherry (49008)) (Chroma Technology, Bellows Falls, VT). Additionally, cells were imaged in differential interference contrast mode. To improve reproducibility of the results, imaging areas were chosen in the center of the cover slips. Exposure times were optimized per channel and spacing between frames was set to ~3–6 s.
After acquisition of ~10 frames, 150 µL of SMA solution at twice the desired concentration in the same buffer were added to the sample chamber with a pipette and the focus was immediately readjusted manually. Imaging was then continued for 5–30 min depending on the experiment. To mimic general conditions for membrane protein solubilization by SMA, all experiments were performed at room temperature. Control experiments in buffer devoid of solubilizing agent showed no influence on cell morphology on the experimental time scale. In membrane perforation experiments, a SMA concentration of 0.1 % (w/v) was used, to be able to distinguish single steps in the process. Higher concentrations were found to accelerate the process up to the point that temporal resolution was insufficient to observe

single-cell effects or step-wise perforation, whereas lower concentrations were only partially effective in inducing leakage, as seen by the presence of intact single cells even after > 20 min of incubation (see Figure S1). For experiments with membrane protein solubilization, a higher SMA concentration of 0.33 % (w/v) was chosen since solubilization was found to be generally slower than membrane perforation. Higher concentrations of SMA were avoided because they promoted detaching of cells, which strongly impaired data acquisition.

Quantification of fluorescence intensity

For quantification, images were analyzed for mean intensity per cell with the multi measure plugin in the ImageJ software. The positions of individual cells were marked using the freehand selection tool and images were analyzed per single channel. Intensities were corrected for background effects by analyzing nearby regions of approximately the same area that did not show initial fluorescence. Background-corrected data were normalized for the fluorescence intensity at the point of addition of SMA and leakage or solubilization midtimes were determined as the midpoint of the quasi-sigmoidal decay of fluorescence intensity as a function of time. In cases where transitions were incomplete (MARCKS constructs), midpoints were approximated by the time at which fluorescence intensity had dropped to 50 % of the original value. For quantification of the effect of SMA on cell nuclei, the method had to be adapted since no decrease in fluorescence intensity of DNA-binding Hoechst dye was detected at the original location of the nucleus in the X/Y plane. Instead, fluorescence intensity increase in the direct vicinity of the nuclei was monitored to detect DNA release due to perforation of the nuclear membrane. Since quantification was mainly based on transition midpoints, photobleaching effects should only have marginal influence. Nevertheless, experimental settings were chosen such that photobleaching of the fluorophores generally did not cause a loss in fluorescence intensity of more than ~20 % over the total imaging time. For data representation, images were exported from MicroManager using identical contrast settings for all images recorded in the same channel.

Results

SMA solubilizes cells in a step-wise process. To address general features of the overall solubilization process of cells, we first studied SMA-induced membrane perforation by investigating leakage processes of soluble proteins present in different cellular compartments. Figure 1A shows fluorescence images of HeLa cells that were transfected to produce a cytosolic protein (GFP, top) together with a protein that is localized to the lumen of the endoplasmic reticulum (ER) (tagRFP-ER, middle). The addition of SMA, after an initial lag time, led to a fast leakage of cytosolic GFP, indicating plasma membrane perforation. This process affected single cells at a time at random positions in the observed area (see also Video S1). Perforation of the plasma membrane coincided with a morphological change of the cell, as seen by a higher contrast of the nucleus (see arrows in Figure 1A bottom). For every cell, the ER probe was released only after an additional lag time (see asterisks in Figure 1A). The differences between leakage of cytosol and ER lumen can be clearly seen from a quantitative analysis of the loss in fluorescence intensity per single cell (Figure 1B and C, for more details see Figure S2). Such a delayed leakage of the ER lumen is to

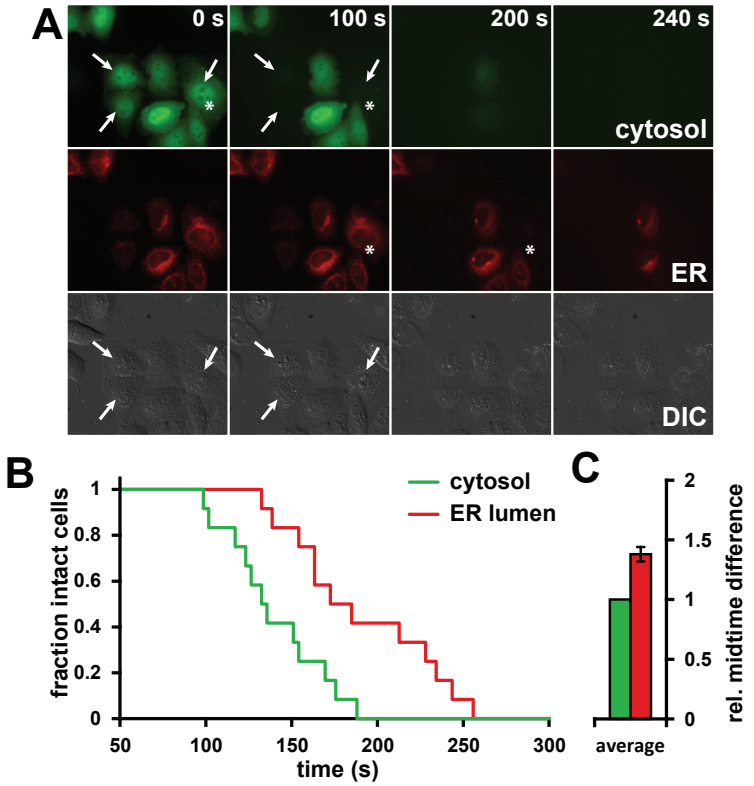

Figure 1: Perforation of HeLa cell membranes by SMA. (A) Top/middle: fluorescence changes of cells marked with cytosolic GFP (green) and ER luminal tagRFP-ER (red) as a function of time upon addition of 0.1 % (w/v) SMA. Asterisks indicate the step-wise membrane perforation for a single cell. Bottom: micrographs recorded in differential interference contrast (DIC) showing changes in overall cell morphology that correlate with GFP leakage (arrows). (B) Population analysis based on fluorescence decline of single cells. (C) Relative difference in leakage midtimes per cell. Data are given as averages of leakage midtimes of 12 individual cells with error bars indicating the standard deviation. Experiments were performed at room temperature. For details on quantification see Figure S2.

be expected since SMA first needs to cross the plasma membrane before it can attack intracellular compartments.

Intracellular membranes are solubilized faster than the plasma membrane. In a further set of experiments we assessed actual solubilization of membranes by SMA, using fluorescent membrane proteins. In order to achieve efficient membrane protein solubilization, the SMA concentration was increased to 0.33 % (w/v). To investigate possible preferences of SMA for specific (sub)cellular membranes, we used combinations of a lipid-anchored plasma membrane marker (MARCKS-RFP/GFP) with single-span integral membrane protein fusion constructs of complementary color that were targeted to different organelles. Upon addition of SMA, we observed a relatively fast and efficient solubilization of the markers for

ER (CD8-GFP, Figure 2A) and Golgi (ManII-GFP, Figure 2B). In both cases, plasma membrane solubilization was much slower and some residual fluorescence could still be detected after 5 min, indicating incomplete solubilization under these conditions.

A somewhat smaller time difference was observed between plasma membrane and the outer mitochondrial membrane (TOM20-mCherry, Figure 2C). This may be caused by an accumulation of MARCKS-GFP in the Golgi in these cells (see arrows). As a consequence, solubilization appears to be faster since the Golgi signal is also taken into account. An additional experiment with the markers for Golgi and mitochondria showed that both proteins indeed are solubilized similarly fast (Figure 2D). Together, these results suggest fast and non-preferential solubilization of organelles by SMA and a generally slower solubilization of the plasma membrane.

This finding is remarkable because one would expect that the plasma membrane, since it is exposed to SMA earlier and thus perforated first, should also be solubilized faster than intracellular membranes. An explanation for this contrasting behavior could be that MARCKs partitions into ordered domains (“lipid rafts”) that may exist in the plasma membrane due to its high cholesterol and sphingolipid content [13,20,21]. The MARCKS construct we used is palmitoylated at a cysteine close to its myristoylated amino terminus, which could promote its partitioning into ordered domains [22–25]. Such domains might be difficult to solubilize by SMA, as suggested by a recent model membrane study on phase-separating lipid mixtures [10] that showed a distinct preference of SMA to solubilize lipids in the fluid liquid-disordered phase.

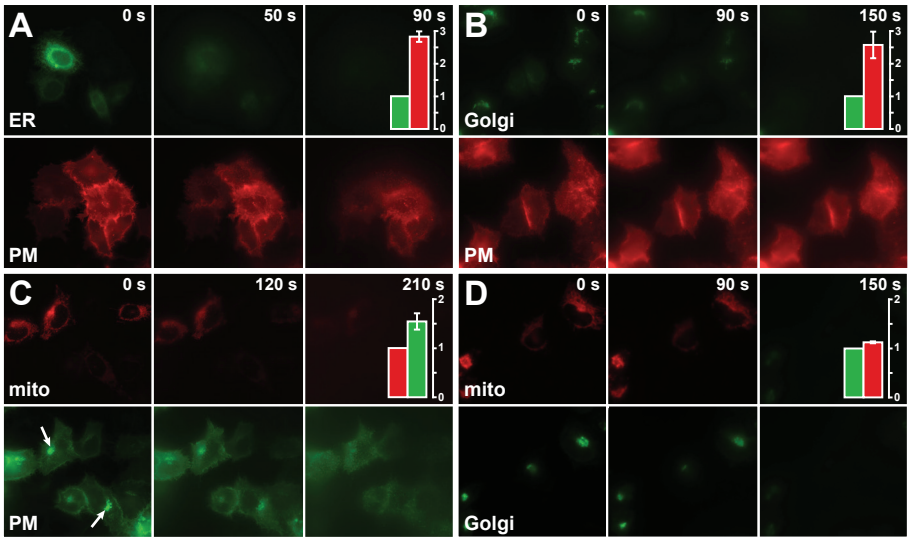

Figure 2: Solubilization of (sub)cellular membranes by SMA. (A–C) Single-channel representations of cells expressing markers for intracellular compartments (top, A: CD8-GFP, endoplasmic reticulum; B: ManII-GFP, Golgi apparatus; C: TOM20-mCherry, outer mitochondrial membrane) together with a plasma membrane marker (MARCKS-RFP/GFP, bottom). (D) Single-channel representation for cells co-expressing markers for mitochondria (top) and Golgi (bottom). Column diagrams represent relative differences in solubilization midtimes per channel and cell (see Figure 1C). SMA concentration was 0.33 % (w/v). Error bars are given as standard deviations of 4–10 individual cells.

Differential kinetics of plasma membrane solubilization suggest domain preference. To further investigate possible domain-associated solubilization preferences of SMA in the plasma membrane, we generated cells that contained the lipid-anchored MARCKS together with a plasma membrane-localized integral membrane protein (NGL3-GFP). Proteins of these two classes have been associated with different propensities for partitioning into membrane domains, with palmitoylated peripheral membrane proteins often showing a preference for partitioning into ordered domains [22–24], whereas integral membrane proteins generally favor more fluid domains [26,27].

The experiments revealed a localization of both proteins in the plasma membrane (Figure 3). Despite this, fluorescence intensities of the probes dropped at different rates upon addition of SMA, with NGL3 being solubilized 1.5 times faster than MARCKS (Figure 3A, Video S2) and the latter being incompletely solubilized after 5 min. We speculate that the differential solubilization kinetics are due to the presence of MARCKS and NGL3 in separate membrane domains. Such domains are likely very small with estimated diameters in the range of tens of nanometes [28], which makes it impossible to resolve them by standard diffraction-limited light microscopy. An influence of domain localization is further supported by the observation that using Triton X-100 instead of SMA leads to very similar differences in solubilization kinetics of MARCKS and NGL3. Also here NGL3 was solubilized faster (Figure 3B, Video S3). For

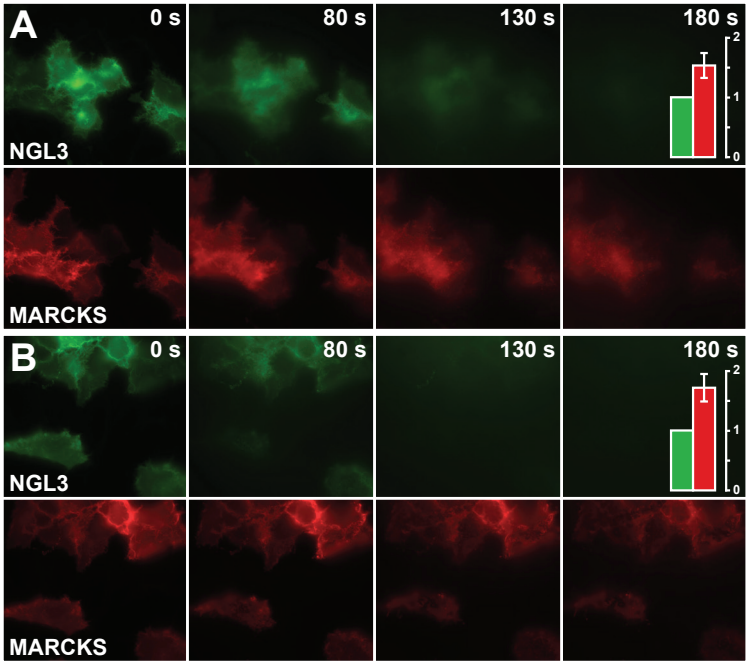

Figure 3: Differential solubilization of different plasma membrane markers by SMA (A) and Triton X-100 (B). Solubilizing agent was added to cells containing the integral membrane protein NGL3-GFP (top, green) together with lipid-anchored MARCKS-RFP (bottom, red). Images are shown in single-channel mode over time after addition of 0.33 % (w/v) solubilizing agent. Column diagram insets depict relative solubilization midtime differences analogous to Figure 1C.

Triton, a strong preference for solubilization of disordered membrane domains has long been established by studies with both model membranes [29] and cellular membranes [20]. Finally, we compared the kinetics of solubilization of proteins in fluid environments in the plasma membrane (NGL3) and an organelle membrane (TOM20) (Figure S5). The experiments showed that NGL3 was solubilized at a similar rate as the mitochondrial TOM20. In view of our observation of clear lag times between the perforation of these membranes by SMA (Figure S3), this suggests a general adverse influence of plasma membrane properties on solubilization by SMA. This may be connected to the fact that the plasma membrane is the natural barrier between a cell and its environment, which implies the necessity of a higher structural stability compared to organelle membranes.

Discussion

Our results suggest a hierarchical multi-step process of cell solubilization by SMA, which partially reflects a model for its mode of action based on model membrane studies [7]. Initially, polymer molecules bind to membranes until a threshold concentration is reached (lag time) and the polymer starts to insert into and subsequently perforates the plasma membrane. This causes cytosolic molecules to leak out and enables SMA to access intracellular membranes where this process is repeated. In a final step, membranes are fragmented into intermediates and eventually solubilized in the form of nanodiscs. Especially this final step seems to be strongly dependent on the properties of the target membrane as seen from our results with the plasma membrane. Here, membrane perforation occurs fast whereas solubilization of membrane proteins is relatively slow. This may in part be due to a higher stability conveyed by tight interactions of the plasma membrane with components of the cytoskeleton ("picket fence" model; see e.g. ref [30]). Another explanation is the higher cholesterol-to-phospholipid ratio in the plasma membrane compared to organelle membranes [13]. This strongly influences plasma membrane properties since cholesterol increases the order in lipid packing and it is essential for the formation of ordered domains in the plasma membrane, which would explain the observed differential solubilization kinetics for different plasma membrane-localized proteins (Figure 3). Our data suggest that SMA could potentially be used to isolate such ordered domains in the form of SMA-resistant membranes by selectively removing fluid membrane domains [10], as has been shown for SMA-resistant plant membranes [31]. We speculate that the mild solubilization of proteins by SMA may be advantageous over conventional protocols exploiting detergent resistance [20] but whether this approach is indeed beneficial remains to be addressed.

The data presented here suggest that SMA is capable of extracting integral membrane proteins from all cellular membranes including the plasma membrane, which would allow their purification and characterization with their native environment in a stable and soluble form. However, it is important to realize that there are a number of additional factors that may affect the efficiency of solubilization by SMA. These include protein type or protein-specific properties in general, such as surface charge or dimension of their membrane-embedded domain. Another important factor is the membrane environment, with protein-dense membranes with relatively low lipid content [31,32] and bilayers with a high degree of lipid order [7,8,10] being challenging for SMA. For such systems, solubilization efficiency could be increased by the addition of lipid before solubilization [33,34] or by elevating the temperature [7,10], respectively.

Acknowledgements

We thank John Preston, McGill Univsersity and Eunjoon Kim, KAIST for their gift of DNA constructs and we thank Cátia Frias and Max Adrian for helpful discussions. Financial support received from the European Union via the 7th Framework Program (Initial Training Network "ManiFold," grant 317371 to J.M.D.) and from the Netherlands Organization for Scientific Research (NWO-ALW-VICI to C.C.H.) is gratefully acknowledged. M.H.C.-H. was supported by the NutsOhra foundation (grant 1104-034), and by the Dutch Brain Foundation (Hersenstichting, grant 2012(1)-141).

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Supporting Information

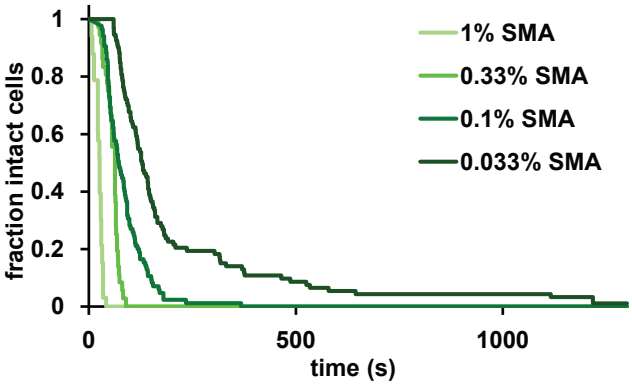

Figure S1: Effect of SMA concentration on leakage kinetics. Data are shown as cumulative population analyses of several data sets for different concentrations of SMA. The number of cells was 33 for 1 %, 36 for 0.33 %, 85 for 0.1 % and 93 for 0.033 % SMA.

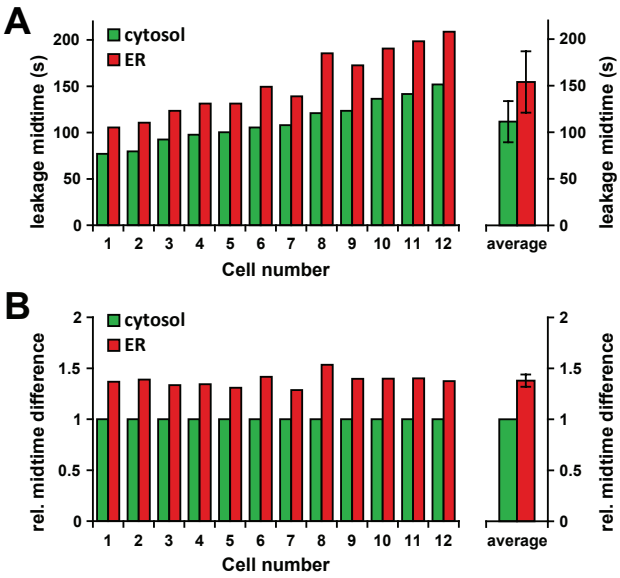

Figure S2: Quantification of SMA-induced membrane perforation corresponding to cells shown in Figure 1. (A) Leakage midtime raw data per protein marker. (B) Relative leakage midtime differences for the two proteins per individual cell obtained by normalization on cytosol leakage. Data are shown for individual cells (left) and as averages of all cells with error bars representing standard deviations (right).

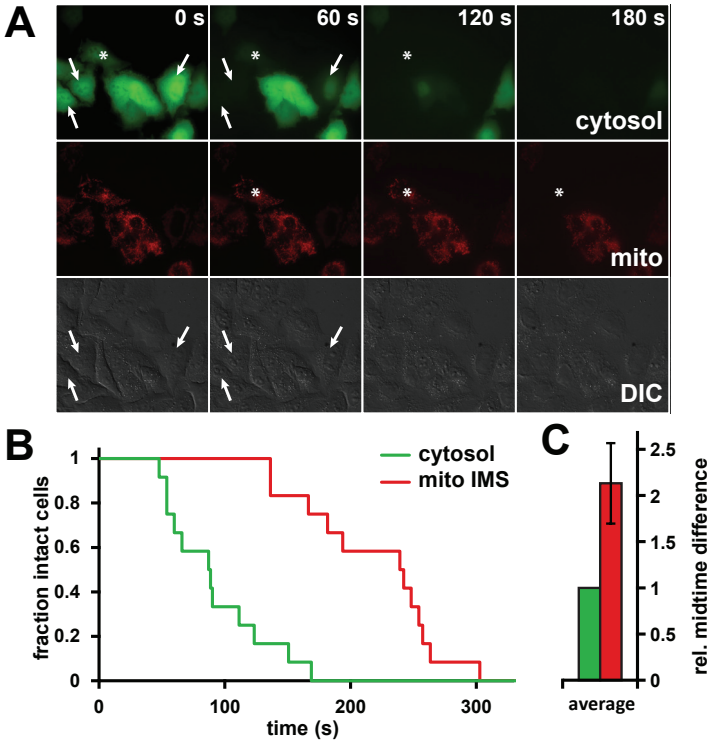

Figure S3: Perforation of HeLa cell membranes by SMA: mitochondria. (A) Top/middle: fluorescence changes of cells marked with cytosolic GFP (green) and MitoDsRed (red) that localizes to the mitochondrial intermembrane space (IMS). Images are shown as a function of time upon addition of 0.1 % (w/v) SMA. Bottom: micrographs recorded in differential interference contrast (DIC) showing changes in overall cell morphology. (B) Population analysis based on fluorophore leakage of single cells. (C) Relative difference in leakage midtimes per cell.

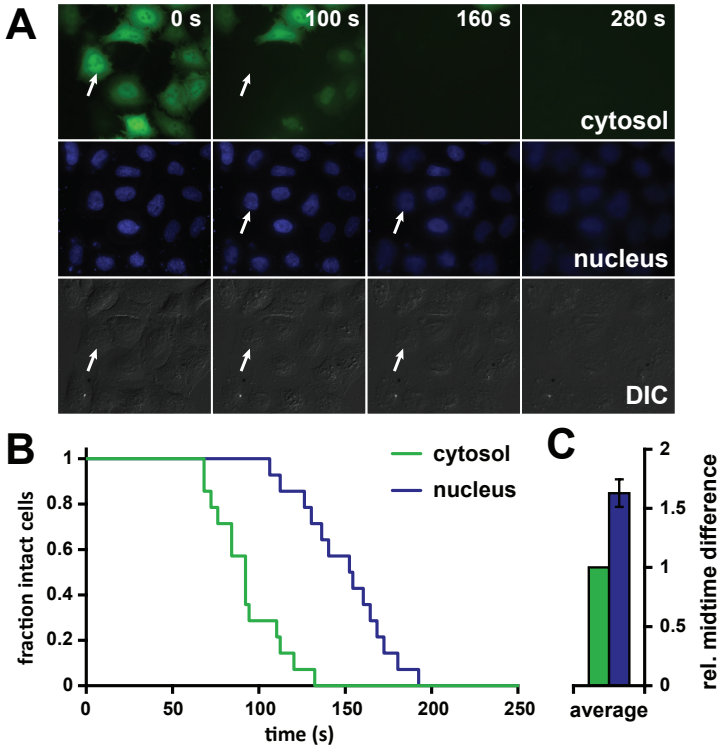

Figure S4: Perforation of HeLa cell membranes by SMA: nucleus. (A) Top/middle: fluorescence changes of cells marked with cytosolic GFP (green) and a DNA-binding Hoechst dye (blue) indicating the nucleus. Images are shown as a function of time upon addition of 0.1 % (w/v) SMA. Bottom: micrographs recorded in differential interference contrast (DIC) showing changes in overall cell morphology. (B) Population analysis based on fluorophore leakage of single cells. (C) Relative difference in leakage midtimes per cell.

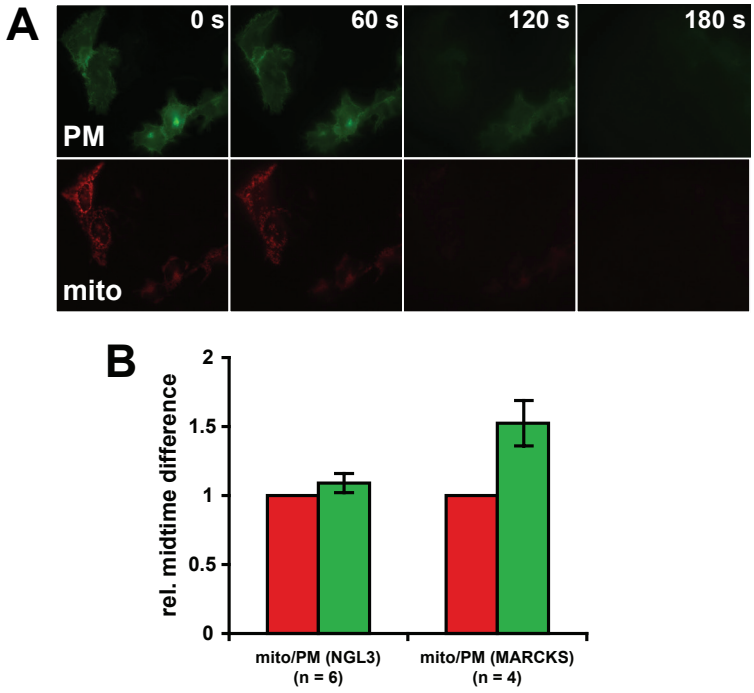

Figure S5: Solubilization of plasma membrane-localized proteins relative to mitochondrial proteins. (A) Single-channel representations of cells containing NGL3-GFP (plasma membrane, top) and TOM20-mCherry (mitochondria, bottom). SMA concentration was 0.33 % (w/v) (B) Relative solubilization midtimes for NGL3 and MARCKS compared to TOM20. Data for MARCKS correspond to images in Figure 2C.

Video S1: Membrane perforation of HeLa cells containing cytosolic GFP together with tagRFP-ER (ER lumen) by 0.1 % (w/v) of SMA corresponding to Figure 1. Images were recorded every 3 s and video speed is accelerated 20-fold.

Video S2: Solubilization of the plasma membrane of HeLa cells by SMA. Cells contain the integral membrane protein NGL3-GFP together with palmitoylated MARCKS-RFP corresponding to Figure 3A. Images were recorded every 3 s and video speed is accelerated 20-fold. SMA concentration was 0.33 % (w/v).

Video S3: Solubilization of the plasma membrane of HeLa cells by Triton X-100. Cells contain the integral membrane protein NGL3-GFP together with palmitoylated MARCKS-RFP corresponding to Figure 3B. Images were recorded every 3 s and video speed is accelerated 20-fold. Triton concentration was 0.33 % (w/v).

Chapter V

Factors Influencing the Solubilization of Membrane Proteins from *Escherichia coli* Membranes by the Styrene–Maleic Acid Copolymer

Jonas M. Dörr
Martijn C. Koorengevel
J. Antoinette Killian

Abstract

The isolation of membrane proteins in stable nanodiscs surrounded by styrene–maleic acid (SMA) copolymers holds great promise as an alternative to detergent isolation for downstream applications. However, the demand for sample amount and purity may remain a challenge. In order to rationalize membrane protein solubilization by SMA from biological membranes we here employ *Escherichia coli* membranes containing KcsA as a model to investigate the influence of experimental conditions on solubilization efficiency. We show that several parameters influence the amount of protein that is extracted by SMA. pH was found to be a particularly dominant determinant of solubilization efficiency, with increasing pH strongly increasing solubilization yield. This is in striking contrast to recent observations in lipid-only model systems that show decreasing solubilization efficiency with increasing pH. This discrepancy is likely due to the differing composition and complexity of biological membranes, highlighting the necessity to expand the regime of model systems currently employed for the study of membrane solubilization by SMA.

Introduction

In the past years, styrene–maleic acid (SMA) copolymers have been used to isolate native nanodiscs with a wide range of membrane proteins differing in size and properties (e.g. [1–4]). Importantly, these nanodiscs provide a stable environment for membrane proteins, which facilitates their study. While the available data suggest a general applicability of the isolation method, it is largely unknown whether there are general factors influencing solubilization from biomembranes.

Many of the studies so far reporting the use of SMA to isolate membrane proteins can be considered case studies where protocols have been adapted, in part crudely, from existing detergent-based protocols, often using relatively high concentrations of SMA in the range of 2.5–3 % (w/v) to ensure efficient solubilization. However, high concentrations of SMA in the solution may have a negative effect on the efficiency of affinity purification (Dörr and Killian, unpublished observations), which in turn can hamper downstream analyses that require significant amounts of protein. Since the preparation of relatively large amounts of proteins still is a major bottleneck in membrane protein research [5], optimization of solubilization conditions for SMA may thus result in important benefits.

A general goal in optimizing solubilization conditions would be to derive a set of parameters that ensures efficient protein extraction at the lowest possible amounts of SMA, thus minimizing unfavorable effects on purification. These parameters may be very different from those important for detergent isolation since the molecular properties of SMA vary strongly from those of most head-and-tail detergents. In view of protein degradation due to proteases and/or intrinsic instability, experimental conditions for protein isolation preferably should also avoid prolonged incubation times and exposure to elevated temperatures. In addition, other environmental conditions may be important, such as ionic strength or pH.

Several of these factors have been recently shown to affect SMA solubilization in systematic model membrane studies [6–10] and based on such studies a model was proposed for the mode of action of SMA [6]. Especially pH was shown to be a strong determinant of solubilization efficiency [9], which was mainly attributed to pH-dependent conformational changes of the polymer. However, it is not clear to what extent experiments with pure liposomes translate to the solubilization of proteins from biological membranes. Systematic studies on SMA solubilization of biological membranes are still scarce, with the available reports focusing mainly on comparison of the solubilization efficiency of different polymer variants [11,12].

Here, we employ *Escherichia coli* (*E. coli*) membranes containing KcsA as a model system that allows straight-forward analysis of the effect of varying experimental conditions, such as pH, ionic strength, temperature and SMA concentration. Because of its favorable properties in the solubilization of both artificial liposomes [9] and biological membranes [12], we used a SMA variant with a ratio of styrene to maleic acid units of 2:1. KcsA was chosen as a model protein because of several reasons: (i) it can be obtained in membranes at high copy numbers due to its spontaneous membrane insertion and oligomerization, constituting one of the most abundant proteins after initiated gene expression [4,13,14], (ii) the robustness of the protein allows studies at a wide range of conditions with small impact on structural integrity of the protein, and (iii) the stability of the KcsA tetramer on sodium dodecylsulfate polyacrylamide gel-electrophoresis (SDS–PAGE) [13,15] allows for a simple means of identification of the target protein band that does not require Western

blot analysis, resulting in a higher throughput. Our results demonstrate drastic differences in solubilization yield depending on some of the tested parameters and suggest the necessity to expand currently used model systems to better approximate bacterial membranes.

Materials & Methods

Materials

n-dodecyl-beta-D-maltopyranoside (DDM) was from Anatrace (Maumee, OH). All other chemicals were from Sigma Aldrich (St. Louis, MO). Styrene–maleic anhydride copolymer Xiran30010 (weight-average molecular weight of 10 kDa, styrene-to-maleic anhydride ratio of 2) was a kind gift from Polyscope Polymers (Geleen, NL). Conversion into the acid form was achieved by hydrolysis under base-catalyzed conditions as described earlier [4]. Lyophilization was generally omitted since it was not found to have a significant effect on sample quality. SMA stock solutions were prepared at final concentrations of 5 % or 1 % in Tris base solution (pH unadjusted) at 10 mM per 1 % of SMA. The desired pH of the solution was adjusted by adding NaOH. All concentrations of solubilization agent-containing solutions are given in percent weight per volume (% w/v).

Preparation of *E. coli* membranes

Total membrane fractions of *E. coli* cells producing KcsA were obtained as described previously [4]. Briefly, cells were transformed with the plasmid containing the *kcsa* gene and were grown after induction of gene expression until cultures reached an OD_{600} of ~0.8. Cells were then harvested by centrifugation and stored as pellets at –80 °C. Membrane preparations were then obtained by differential centrifugation after cell wall lysis and mechanical disruption. To remove any remaining soluble proteins, the obtained membrane pellet was resuspended in physiological buffer and sedimented again by ultracentrifugation at 100,000 x *g*.
Membrane pellets corresponding to 800 mL of cell culture were then resuspended in 2–4 mL Tris buffer (5 mM, pH 8.0, 10 mM KCl) to an OD_{600} of ~4. The total phosphate content was determined to be 5.5 mM using the method of Rouser. Membrane suspensions were aliquoted and stored at –20 °C until further use.

Membrane solubilization experiments

Membrane pellets were thawed on ice and diluted 4x into premixed solutions of Tris buffer to a final volume of 100 µL, with the membrane material corresponding to a phosphate content of 1.4 mM and to an OD_{600} of 1. Standard solubilization was performed at a final concentration of 10 mM Tris, pH 8.0, 300 mM NaCl and 15 mM KCl and different final concentrations of SMA or DDM. For standard experiments, samples were incubated for 2 h in an Eppendorf sample mixer that was set to shaking at 600 rpm at a temperature of 25 °C. To assess how strongly different conditions affect solubilization yield, variations were made in one parameter at a time, while keeping other conditions constant.
After incubation at the desired conditions, 60 µL of the solution were subjected to centrifugation at 100,000 x *g* for 45 min at 4 °C to separate solubilized from non-solubilized

material. The supernatant (solubilized material) was then transferred to a separate tube and the pellet (non-solubilized material) was resuspended in 60 µL of buffer containing 1 % SDS. Since the presence of SMA was found to influence densitometric analysis, buffers for resuspending pellets of SMA-containing samples were supplemented with the corresponding amount of polymer. Both supernatant and pellet samples were then mixed with 4x concentrated SDS–PAGE sample buffer and pairs of 8-µL aliquots were loaded on 13-% acrylamide gels. After ~1 h of electrophoresis at 175 V, gels were fixed and stained in a 0.1-% solution of Coomassie Blue R-250 in methanol:water:acetic acid (50:40:10 by volume) for 1 h. Destaining was achieved by gentle agitation overnight in a solution of water:methanol:acetic acid (70:20:10 by volume) devoid of Coomassie dye. Trays were supplemented with tissue paper to accelerate the destaining process by providing a "sink" for the dye in solution.

For the evaluation of the effect of different parameters on solubilization efficiency the standard solubilization conditions were adjusted in the following ways:
To ensure better comparison in experiments with incubation time differences, sample volumes of 300 µL were used, of which aliquots were centrifuged after different periods of incubation.
For assessing salt concentration dependence, a constant ratio of NaCl and KCl of 20:1 was used. The sample devoid of NaCl contained a final concentration of 5 mM KCl to ensure protein stability.
For experiments at different pH values, SMA stock solutions at 1 % in 10 mM Tris were prepared at every pH value used. In addition, 100-mM Tris stock solutions were prepared at the same pH with a slightly larger pH difference from pH 8. This was done in order to compensate for the final amount of Tris, pH 8, in solution that originated form the membrane suspension aliquots. Samples were then mixed in a ratio that yielded a final Tris concentration of 20 mM at the desired pH, which corresponds to a 16-fold excess of the original amount of Tris at pH 8. Control experiments were performed with 0.25 % DDM instead of SMA at otherwise identical conditions.

Densitometric analysis

Destained gels were scanned using an Epson Perfection V750 Pro scanner (Long Beach, CA) at 300 dots per inch (dpi) for general validation. The area of the gel containing KcsA bands was additionally scanned at 1200 dpi for densitometry. The band intensity was determined using the Quantity One software package (Biorad, Hercules, CA) using the volume contour tool. Values were corrected for background intensity that was determined by analyzing an area of approximately 4 times that of the band in its immediate surroundings.
For densitometric analysis of the efficiency of solubilization of KcsA, we compared the intensity of the tetramer band in the solubilized fraction relative to the sum of the intensities of the solubilized and non-solubilized fractions. This procedure was chosen because, for reasons that are not understood at this point, a more straightforward comparison of the KcsA bands from the solubilized and initial samples before centrifugation often resulted in apparent solubilization yields far above 100 % (up to 150 %) for SMA-containing samples. For all data shown, KcsA bands from every independent sample were analyzed in duplicate pairs of solubilized and non-solubilized fractions, with each pair being loaded on a different gel.

For every tested set of conditions, control experiments were performed with samples that were not supplemented with solubilization agent. Solubilization yields of KcsA never exceeded background levels of 5 % in these experiments. Such apparent solubilization of KcsA in the absence of solubilizing agent is likely an artifact owing to partial resuspension of the sedimented material when removing the supernatant.

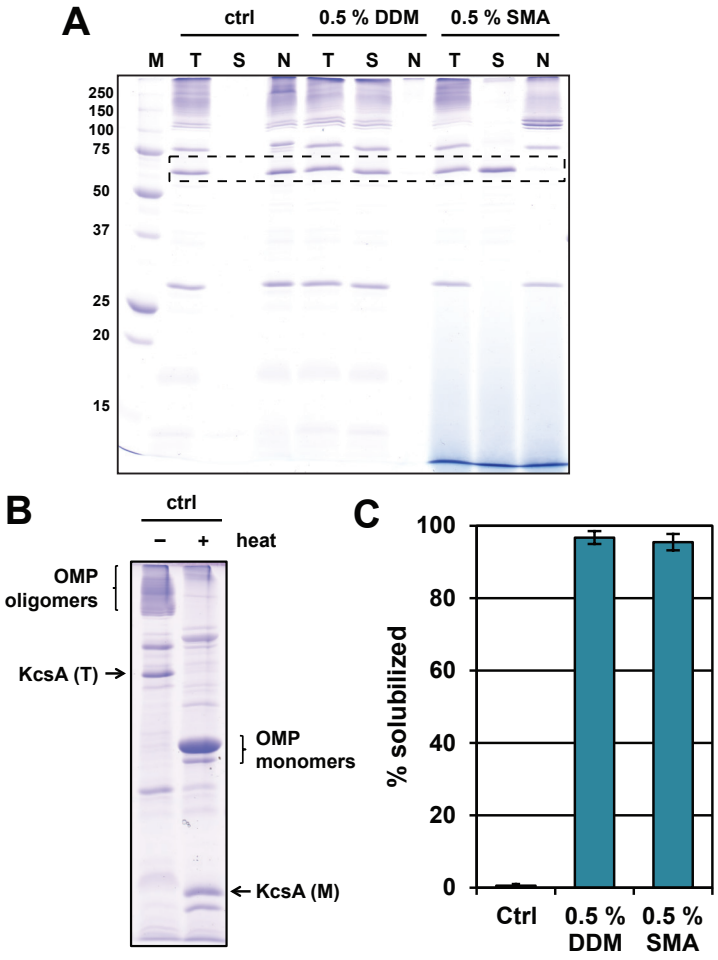

Figure 1: (A) Representative coomassie-stained acrylamide gel showing solubilization of *E. coli* membranes by DDM and SMA. Total samples are shown next to the supernatant after ultracentrifugation (solubilized fraction, S) and the pellet that was resuspended in SDS-containing buffer (non-solubilized fraction, N) The KcsA tetramer band is highlighted by a dashed box. (B) Control experiment showing the conversion of the KcsA tetramer band (T) into a monomer band (M) as well as the conversion of oligomeric complexes of outer membrane proteins (OMPs) into monomers upon heating for 15 min at 95 °C in the presence of SDS. (C) Densitometric analysis of the KcsA tetramer band. Solubilization yields are shown for a relative comparison of the solubilized fraction vs. the sum of solubilized and non-solubilized fractions (S + N). Error bars indicate differences in analyzing intensities for one sample on two different gels.

Results

To determine the amount of KcsA that was solubilized from *E. coli* membranes under a given set of parameters we employed densitometric analysis of the tetramer band after separation via SDS–PAGE. Representative results are shown in Figure 1A for the addition of 0.5 % (w/v) of either SMA or DDM, a commonly-used head-and-tail detergent. In both cases, > 90 % of the total amount of KcsA tetramer was solubilized in 2 h at 25 °C, at the same ionic strength and pH that had been used for KcsA isolation from whole cells by SMA [4]. SMA solubilization was effective for KcsA, but interestingly some other abundant proteins seemed to be completely resistant against solubilization. This can be seen from the broad band pattern in the molecular weight range above 100 kDa as well as a band at ~30 kDa that are not visible in the solubilized fraction but are present in the non-solubilized fraction after SMA incubation in similar intensities as in the total sample before centrifugation. The pattern in the high molecular weight range likely consists of oligomers of porin-forming outer membrane proteins (OMPs), as is suggested by their conversion into an intense band at ~37 kDa upon heating in the presence of SDS (Figure 1B). This band likely corresponds to the highly abundant OmpF of OmpC which is indicated by detection with a porin-specific antibody after Western blotting (data not shown). For DDM, no such selectivity was observed and all proteins were solubilized efficiently, which indicates differences in mode of action of both solubilizing agents.

In order to assess the influence of the ratio of SMA to membrane material on solubilization efficiency, in a first set of experiments the SMA concentrations was varied (Figure 2). The results indicate that the solubilization yield increases upon increasing SMA concentration up until a concentration of 0.5 %, at which an apparent maximum solubilization capacity of SMA of 80–90 % was reached.

For variations of other experimental conditions, a SMA concentration of 0.25 % was chosen, since SMA concentrations in this range lead to significant solubilization while still allowing a convenient window to detect deviations in solubilization yield in both directions.

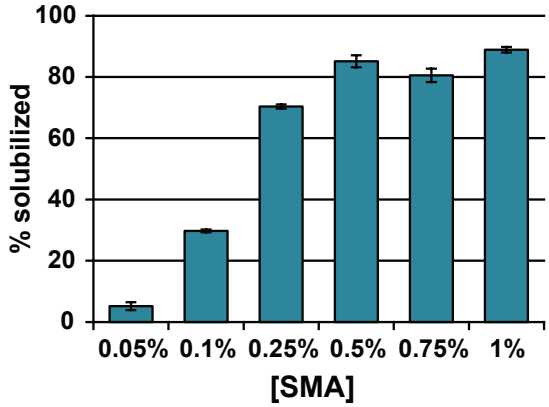

Figure 2: Influence of SMA concentration on solubilization of KcsA at 25 °C. Constant amounts of membrane material were incubated with SMA at different final concentration in the range of 0.05–1 %. All samples contained 300 mM NaCl, 15 mM KCl and 10 mM Tris at pH 8. Data are averages of 2 independent samples, with the error margin indicating the difference between both samples.

In a next set of experiments, the incubation time and temperature were varied at a constant ionic strength at pH 8 (Figure 3). Performing the solubilization experiment at 4 °C instead of 25 °C resulted in a strong decrease of the solubilization yield of KcsA whereas a solubilization temperature of 37 °C led to a moderate increase. Temperature differences were more pronounced at short incubation times and decreased over time. Increasing the solubilization time had a favorable effect at all temperatures. This was particularly strong at 4 °C, where longer incubation times improved the solubilization yield by more than 2-fold. For incubation temperatures of 25 °C and 37 °C, the effect was less pronounced with most of the total KcsA content being solubilized already after 1 h of incubation. Thus, prolonging incubation time did not lead to a drastic improvement in case of these already favorable solubilization conditions.

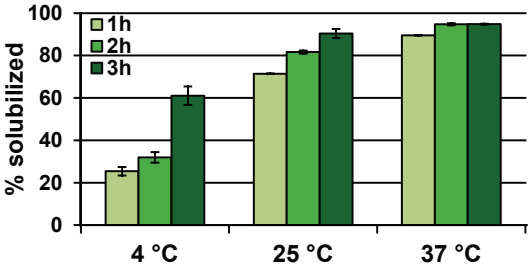

Figure 3: Influence of incubation time and temperature on KcsA solubilization by SMA. Solublization yield of KcsA is shown as a function of incubation time at different temperatures. All samples contained 0.25 % SMA, 300 mM NaCl, 15 mM KCl and 10 mM Tris at pH 8. Data are shown as averages of 2 independent samples, with the error margin indicating the difference between both samples.

The effect of the ionic strength of the solution was investigated by varying the salt concentration (Figure 4). The presence of salt was found to strongly improve KcsA solubilization by SMA, with hardly any solubilization being detected in the absence of NaCl, when only 5 mM KCl was present to ensure KcsA stability. Addition of increasing amounts of NaCl resulted in higher solubilization yields with a broad optimum range between 300–450 mM, where SMA solubilization was most efficient. Concentrations of NaCl exceeding this range, however, led to a decrease in solubilization yield.

Since pH is a major determinant of SMA properties [9,16], in a final set of experiments it was also investigated in what way pH influences the solubilization of KcsA. The results show that the solubilization yield is drastically lower when decreasing the pH from 8 to 7.5 or 7 (Figure 5). Increasing the pH to 9, however, leads to an increase in solubilization yield, with only small differences between pH 8.5 and 9, indicating that the maximum solubilization efficiency is reached. A pronounced KcsA-specific pH dependence is unlikely to be the cause for this effect since control experiments with detergent solubilization show no appreciable changes in the solubilization of KcsA in the same pH range. Additional experiments at a lower concentration of 0.1 % of SMA show a very similar pH dependence (Figure S 2A), supporting the generality of the observed effect.

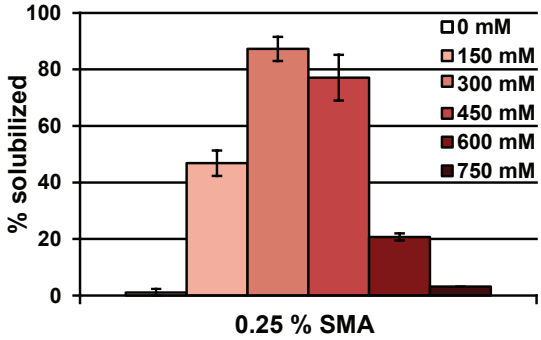

Figure 4: Influence of salt concentration on solubilization efficiency of KcsA by SMA. All samples contained 0.25 % SMA and 10 mM Tris at pH 8. Different amounts of NaCl were added at a constant ratio of NaCl/KCl of 20. The sample devoid of NaCl contained 5 mM KCl to ensure the structural stability of the KcsA tetramer. Data are shown as averages of two independent experiments with the error margin indicating the difference between both samples.

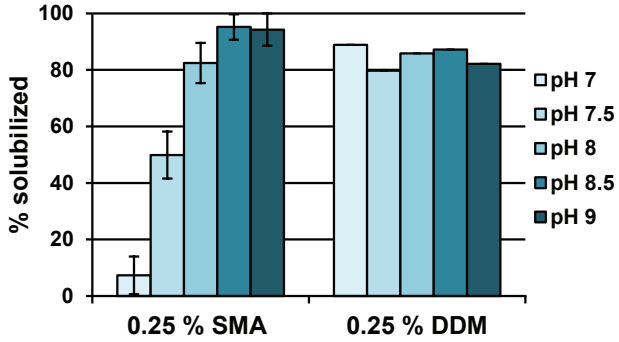

Figure 5: pH effect on the solubilization of KcsA by SMA. Solublization yields of KcsA after the addition of SMA or DDM at different pH values. All samples contained 0.25 % solubilizing agent, 20 mM Tris, adjusted to the desired pH, 300 mM NaCl and 15 mM KCl. Data for SMA are averages of two independent experiments and data for DDM are from a single experiment.

Generally, the intensity of bands corresponding to other solubilized proteins showed a similar dependence as that of KcsA upon changing experimental conditions. Although quantitative assessment of the solubilization yield of those proteins was hampered by their very low intensity, the observed behavior indicates a general validity of the effects derived from KcsA for the solubilization of other proteins from the (inner) *E. coli* membrane. OMPs, however, were generally not solubilized by SMA under any of the conditions tested, suggesting a high resistance of these proteins.

Discussion

The results obtained for testing the efficiency of SMA solubilization of *E. coli* membranes upon variation of different parameters suggest a high susceptibility towards changes in environmental conditions. One should note however, that the results presented here are obtained by a semi-quantitative method and thus should not be considered accurate in absolute terms. Nonetheless, the observed trends therein, since they were derived from relative comparison in data sets obtained on the same day, represent robust qualitative indications for how strongly the tested parameters influence SMA solubilization of proteins from *E. coli* membranes. In this section we will first discuss some general observations on SMA solubilization of *E. coli* membrane proteins, and then will zoom in on the importance of the different parameters that were varied, i.e. SMA concentration, time of incubation, temperature, salt concentration and pH.

Selective solubilization of subsets of membrane proteins by SMA

SMA, unlike DDM, was incapable of solubilizing some proteins including oligomers of OMPs. These molecules adopt a fold, known as β-barrel, that is fundamentally different from inner membrane proteins that generally form α-helices. However, since a β-barrel protein has been among the earliest examples of proteins that were incorporated into SMA-bounded nanodiscs [17] this is unlikely to be the cause for the solubilization resistance of *E. coli* OMPs. A possible explanation for this may lie in the specific properties of the outer *E. coli* membrane that they reside in. In contrast to the inner membrane that harbors KcsA, the outer membrane is highly asymmetric, with its outer leaflet containing high amounts of lipopolysaccharide (LPS). LPS molecules contain a complex network of branched sugars forming a very bulky molecule, that is tightly anchored in the membrane by six attached acyl chains [18]. This results in specific properties that are markedly different from typical phospholipids [19] and may thus hamper SMA solubilization of these membranes. In addition, the fact that OMP porins are some of the most abundant proteins in *E. coli* [20] may also lead to a high (local) protein content that impairs SMA solubilization in similar ways as in photosynthetic membranes [21,22]. Thus, the outer membrane of Gram-negative bacteria, as a whole or in part, may also represent an example for a SMA-resistant membrane [10].

SMA concentration and SMA-to-lipid ratio

The perhaps most straight forward of our findings is that increasing SMA concentration increases yield and efficiency of KcsA solubilization, which is line with observations in model membrane studies [6,7]. To put our findings into perspective, the SMA-to-lipid mass ratio used here can be approximated by using the mass of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) of 718 Da as a representative lipid to calculate the total phospholipid mass based on the measured phosphate content of the membrane suspension. While this approximation likely overestimates the phospholipid content since phosphate can also originate from other sources such as phosphorylated proteins or LPS it may still be useful as a qualitative measure. A SMA concentration of 0.25 % that leads to partial solubilization of KcsA would then correspond to a SMA-to-phospholipid mass ratio of ~2.5. This ratio is at the higher end of the range that is generally used in solubilization

studies of synthetic lipid-only model membranes, where such ratios lead to very fast and complete solubilization [6,7,23].
A major reason for the requirement of higher amounts of SMA may lie the complexity of bacterial membranes that, unlike artificial phospholipid vesicles, also have a considerable content of proteins and LPS that contains bulky sugar moieties [24]. This results in a higher total membrane mass that far exceeds that of the phospholipid fraction, which should be taken into account when comparing the solubilization of artificial vesicles and biomembranes. In addition, *E. coli* membranes differ in lipid composition, with the most abundant lipid being PE at 70–80 mol% [24,25]. Despite the non-selective solubilization of equimolar PC/PE mixtures [8,10], the presence of PE in PC bilayers has been shown to decrease solubilization rates [6,10] and to increase the thermodynamic boundary for complete solubilization [8], which was attributed to differences in packing of the cylindrical PC and the cone-shaped PE. Thus, also the lipid composition of *E. coli* explains the necessity of higher amounts of SMA for efficient solubilization, which indeed was demonstrated in model membrane experiments with vesicles composed of *E. coli* polar lipid extract [6].
In view of our findings with *E. coli* membranes, the very high concentrations of SMA of 2.5–3 % that are typically used for membrane protein extraction [1–4] may be excessive and therefore it may prove useful to reevaluate these concentrations in order to avoid negative effects on purification or other downstream processes.

Solubilization temperature and incubation time

The more efficient solubilization of KcsA at higher temperature is likely caused by the changes in membrane fluidity that occur in the temperature range tested. Lipid extracts of the bacterial inner membrane generally show a very broad gel-to-liquid crystalline phase transition, with midpoints between 20 and 30 °C for *E. coli* cells grown at 37 °C [26,27]. At 25 and 37 °C, the membrane is thus fluid which facilitates efficient solubilization, making long incubation unnecessary. With a more gel-like membrane at 4 °C, solubilization would then be slower due to impaired insertion of SMA into the hydrophobic core of the bilayer [6] and longer incubation times are required to reach higher solubilization yields. In addition, the solubilization process may have been further accelerated at higher temperatures simply due to the increase in thermal energy, with faster molecular motion facilitating polymer insertion into the membrane.
These findings indicate that temperature and incubation time can be adjusted in order to best match specific properties of a target protein. Conditions can then be chosen such that favorable solubilization kinetics balance unfavorable protein degradation.

Ionic strength

We found moderate salt concentrations in the range of 300–450 mM NaCl to lead to most efficient solubilization, with both lower and higher salt concentrations causing a decrease in solubilization yields. This may be explained by the interplay of two different processes. At low ionic strength, we suggest that the dominant effect is electrostatic repulsion of SMA by the anionic membrane surface, which hampers SMA binding to the membrane and thus slows down solubilization [6]. Increasing the ion concentration in the solution leads to a screening of these repulsive electrostatic interactions between anionic membrane lipids and

SMA, which aids polymer association with membranes, thus accelerating solubilization. In addition, a low ionic strength could affect the conformation of the polymer and promote a more long-stretched shape that is incapable of membrane solubilization. The presence of salt ions might then make this conformation energetically less favorable, thus making SMA competent for membrane interaction and solubilization.
A high ionic strength, however, may lead to the adoption of a collapsed globular conformation of SMA since the exposure of its hydrophobic styrene units becomes more and more unfavorable in an increasingly polar environment. The high ionic strength might then prevent efficient solubilization by acting as a "barrier" for the unburying of styrene units, which is essential for membrane binding and insertion. This is supported by the observation that an increase in salt concentration also raises the pH at which SMA molecules start to aggregate and precipitate [9]. Similar aggregation of SMA was also observed upon addition of divalent cations that coordinate with the carboxylates of the maleic acid units, thus efficiently neutralizing their charge and thereby strongly increasing the effective hydrophobicity of SMA [2,12].

pH dependence

Our most remarkable finding with *E. coli* membranes is the increase in solubilization efficiency with increasing pH in the range from pH 7 to 9, which is in striking contrast with recent findings for PC bilayers where solubilization kinetics slowed with increasing pH [9]. There, the effect was attributed to the charge density on single polymer chains that is higher at higher pH leading to a long-stretched conformation due to a higher overall hydrophilicity that impairs membrane insertion. At lower pH, however, polymer molecules are more hydrophobic making them more prone to interact with and insert into membranes. This increased hydrophobicity at lower pH also leads to the precipitation of SMA below an aggregation point that is dependent on polymer composition and salt concentration [9]. If this was the cause for the lower solubilization yield at lower pH under the experimental conditions used here, decreasing the salt concentration should help since it decreases the pH of aggregation of SMA. However, a reduction of the ionic strength did not alleviate the negative effect of decreasing pH but led to a further decrease in solubilization yield (Figure S 2B). This indicates that a different effect might be at play in the solubilization of *E. coli* membranes that cannot be explained based on polymer properties alone but instead is dominated by the properties of the targeted membrane.
One major difference in the *E. coli* membranes studied here is the presence of protein. This may drastically change bilayer properties as compared to protein-free liposomes and thus modulate SMA–membrane interactions. Particularly, a high local protein density might hamper efficient solubilization as is suggested for photosynthetic membranes [21,22]. This could also be important for KcsA which has a tendency to form supramolecular assemblies (channel clusters) at neutral pH in both artificial lipid systems [28–30] and *in vivo* in hyphae of *S. lividans* [31]. However, such cluster formation has not been systematically studied as a function of pH under conditions that are similar to those used in the present study. In addition, a recent study with a different SMA copolymer found a similar pH dependence of protein solubilization from *E. coli* membranes [32], which suggest a general effect that is not specific to KcsA.

Another possible explanation for the apparent paradox is related to differences in the physicochemical properties of PC and PE lipids. Overall both lipids are zwitterionic molecules that are similar in chemical structure, with the only difference being the positively charged terminal amino group of PE that is three times methylated in PC. This causes a difference in effective headgroup size due to changes in hydration properties which leads to differences in packing properties of cylindrical PC and cone-shaped PE. As a result, bilayers containing PE are characterized by tight acyl chain packing, which has been suggested to slow down SMA solubilization (see above). Aside from the influence on the effective shape of the molecule, the triple methylation in the PC headgroup also strongly affects the formation of intermolecular interactions, since the high hydrogen-bond donor capacity of PE headgroups is absent in PC [33]. As a consequence, PE unlike PC also shows a pH dependence of its ionization state in the basic pH range, with a beginning deprotonation above pH 8.5, which leads to a drastic decrease in the gel-to-fluid phase transition temperature at high salt concentration [34]. A beginning deprotonation of PE would lead to a more anionic character, which intuitively suggests an adverse effect on SMA solubilization due to electrostatic repulsion of the anionic carboxylates of SMA. However, deprotonation also would disturb the PE H-bond network and thus alleviate curvature stress, leading to less tightly packed acyl chains of anionic PE that then is expected to resemble the behavior of the cylindrical PC. An acceleration of the solubilization kinetics upon increasing pH in PE-rich membranes may thus be due to changes in lipid packing that promote faster insertion of SMA into the bilayer core and thus accelerate solubilization.

On the basis of the available data, we speculate that two opposing pH-dependent effects may be at play in SMA solubilization of membranes at pH values of 7 or higher. One effect that is mostly dependent on polymer properties leads to faster solubilization of (protein-free) PC-rich bilayers with decreasing pH [9], whereas another lipid property-driven effect dominates in (protein-rich) PE-rich membranes and there causes faster solubilization with increasing pH. Evidently, further experimental effort is needed to test these hypotheses, with possible insightful experiments including both biophysical studies on lipid-only model systems of varying composition and extending the range of the biological systems studied by protein solubilization experiments, e.g. by using yeast membranes that contain higher amounts of PC.

Conclusion and Outlook

In summary, we show that the majority of parameters derived from model membrane studies are transferable to the extraction of membrane proteins from bacterial membranes to a high degree. However, some environmental conditions, particularly pH, show very different effects on solubilization yield. Thus, using a physiological pH of 7.4 for SMA solubilization may be far from the optimal to achieve efficient protein extraction. Based on the data underlying this study, a tentative recommendation for the solubilization of proteins from *E. coli* membranes can be made. This includes using a moderate ionic strength (salt concentrations in the range of 200–500 mM), at a slightly basic pH between 8 and 9 and short incubations below 2 h at room temperature. To take into account differences in bacterial strains and culture conditions, initially trial experiments should be performed with a range of different SMA concentrations to determine the minimum SMA-to-membrane ratio for

efficient solubilization. However, one should be careful in extrapolating these findings to other biological membranes, where differences in membrane lipid and protein composition may change parameters such as the pH dependence and the optimal SMA concentration. Clearly, further work is needed to address the origin of some of the observed effects, but nonetheless we present compelling evidence that for some of the factors determining the efficiency of solubilization of proteins from (bacterial) membranes pure PC liposomes may not be the best indicators, calling for an extension of the employed model systems. It should also be noted that the study presented here solely focused on solubilization yield and that the properties of the obtained nanodisc particles have not been further investigated at this point. Although, SMA-bounded nanodiscs generally exhibit a high degree of similarity irrespective of membrane composition, one cannot exclude an influence of altered solubilization conditions on the properties of the resulting particles.

Acknowledgements

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Supporting Information

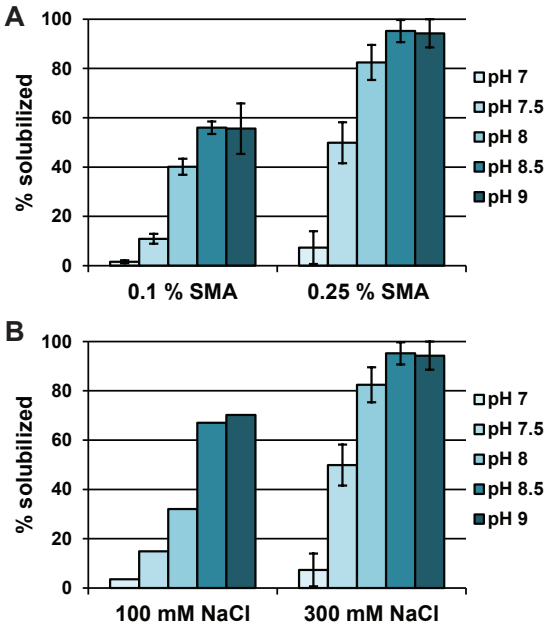

Figure S1: Extended data on pH dependence of the solubilization of KcsA from *E. coli* membranes. (A) pH dependence at different SMA concentrations (n = 2). (B) Influence of salt concentration on pH dependence ($n_{100\ mM\ NaCl}$ = 1). Data on the right-hand side in each panel correspond to the data shown in Figure 5.

Chapter VI

Summarizing Discussion

This chapter is partly based on:
Jonas M. Dörr, Stefan Scheidelaar, Martijn C. Koorengevel, Juan J. Dominguez Pardo, Marre Schäfer, Cornelis A. van Walree, and J. Antoinette Killian. **2016**. The styrene–maleic acid copolymer: a versatile tool in membrane research. *Eur. Biophys. J.* 45:3–21.

Abstract

In this final chapter the experimental work that has been presented so far will be put into context of other studies reporting the use of SMA in membrane protein research. First, the preparation of native nanodiscs from biological membranes will be described and some challenges that may be encountered are highlighted. This is followed by a description of the properties of native nanodiscs and an overview over the analytical methods that can be used to study membrane proteins that are isolated in this way. A focus is then laid on approaches to use SMA to investigate native interactions of membrane components, both in solubilized nanodiscs and non-solubilized SMA-resistant membranes. Furthermore, some current limitations of the use of SMA are discussed and finally an outlook is given on future applications and some remaining challenges of the method.

Extraction of Membrane Proteins from Cellular Membranes

Solubilization

As has been shown in Chapters II and IV of this thesis, SMA polymers can be used to extract membrane proteins directly from intact membranes of cells and organelles without addition of conventional detergent. This now has also been demonstrated in a number of other recent studies with biological sources including bacteria and yeast as well as cultures of insect and human cells, which together account for all major biosystems that are used for heterologous membrane protein production (for a review see [1]). In addition, a very recent paper reported the isolation of enzyme complexes from plant microsomens [2], complementing the set of available sources. The proteins that have thus been incorporated into native nanodiscs span a wide variety of sizes ranging from those with a single membrane spanning α-helix [3] to large oligomeric complexes comprising up to 36 transmembrane helices [4]. Also the types of extracted membrane proteins encompass a diverse set of molecules including enzymes, receptors and different kinds of transporters [1]. Membrane proteins can be directly extracted in these stable particles from whole cells (Chapters II and IV) or purified membrane fractions (Chapter V) and the reported solubilization yields using SMA are generally comparable with those of established detergent-based protocols with deviations in both directions.
The extraction of membrane proteins in native nanodiscs hence seems to be independent from the host organism and to be generically applicable to all members of this class of proteins. However, it is also evident from Chapters IV and V that some membrane proteins are relatively difficult to solubilize from native membranes. In addition, we show in Chapter III that the fluidity of the membrane plays a major role in determining solubilization efficiency. Thus, resistance of membrane proteins against SMA solubilization is likely connected to the properties of the membrane they reside in, as will be discussed below. Furthermore we demonstrate in Chapter V that the solubilization efficiency can strongly depend on different experimental conditions such as salt concentration and pH, which should be taken into account if protocols are adapted to other proteins.

Purification

Once solubilized in the form of native nanodiscs, the protein of interest can be purified using standard methods. So far, Ni-affinity chromatography has been most commonly used to purify His-tagged proteins (see [1]), which was also demonstrated for the purification of KcsA in Chapter II. In addition, purification strategies based on immunoaffinity [5] or cofactor binding to enzymes [2] have been employed successfully. All these approaches render protein yields and purities that are similar to those obtained by detergent-based protocols. However, the properties of native nanodiscs may also represent challenges for purification protocols. For instance in Ni-affinity purifications, based on the physico-chemical properties of SMA several interactions are possible that could impair His-tag binding in the presence of SMA in solution and/or associated to nanodiscs. These include electrostatic interactions between His tags and SMA due to the anionic character of the polymer, binding of the phenyl groups of SMA to His tags, and occupation of the free coordination sites of immobilized Ni by the carboxylates in SMA. Possibilities to improve purification yields in

this case include removal of excess SMA by prior filtration, decreasing the amount of SMA used for solubilization (Chapter V), increasing the amount of available Ni, increasing the number of histidines in the tag sequence to increase binding affinity or simply diluting the solution before addition of Ni-containing material to decrease the effective concentration of excess SMA. The use of other standard purification strategies (Strep tag, FLAG tag, etc.) has not been reported so far for proteins after SMA solubilization, but also in those systems inhibitory effects on binding might occur, with similar possibilities to improve the yields. The body of available literature suggests that native nanodiscs with any membrane protein can be purified without major difficulties by using established protocols, provided that fusion tags are sterically accessible to the chromatography resin.

Characteristics of Native Nanodiscs

Stability

One of the most important properties of native nanodiscs is that they provide an environment that stabilizes proteins better than detergent micelles. This has been demonstrated for KcsA (Chapter II) and many other proteins (see e.g. [5–7]). A particularly interesting example for such proteins are members of the family of G-protein coupled receptors that are notoriously difficult to study since they have a high intrinsic flexibility and are thus unstable in detergent micelles [8]. Yet, also members of this family have been successfully isolated using SMA polymers [9,10]. Other advantages of nanodiscs over detergent micelles are an improved storage potential [9,11] and the fact that native nanodiscs constitute a more stable environment in the sense that there is no rapid exchange of polymer molecules and hence there is no requirement for a pool of soluble polymer molecules to maintain particle stability. Proteins in native nanodiscs thus essentially behave like soluble proteins and are amenable to standard biochemical and biophysical analysis.

Size and composition

The size of native nanodiscs incorporating MPs is fairly uniform for each individually studied protein, but diameters vary between 10 and 24 nm for different proteins. Although many reports suggest that the presence of incorporated proteins increases the particle size [6,12,13], a comparison of different studies does not show a clear correlation of the disc size and the dimensions of the membrane-spanning domain of the protein [1]. For instance, nanodiscs containing a KcsA tetramer with 8 transmembrane (TM) helices have an average size of 10 nm, whereas the smaller bacteriorhodopsin (7 TM helices) yields slightly bigger particles with diameters of 12 nm.

For some studies, the ratio of lipid to protein in native nanodiscs was determined and also here a comparison of different proteins reveals considerable variations. While a photosynthetic reaction center was isolated and purified together with 150 lipid molecules from bacterial membranes [7] whereas only 11 lipid molecules were coextracted with PagP [6] and 40 with AcrB [4], respectively. Note that neither of the latter low values would be in accordance with a full annular ring of lipids around the incorporated protein and thus some of the hydrophobic surface of the protein would be in direct contact with the polymer.

However, given the huge variations in experimentally determined lipid-to-protein ratios and in view of the considerable differences in nanodisc size such estimations may perhaps not be very reliable. There are several other potential explanations for the large variation in diameter and lipid-to-protein ratios in native nanodiscs. For example, low amounts of lipids could originate from the formation of oligomeric complexes of the incorporated proteins. Furthermore, variations in experimental conditions, such as the SMA-to-lipid ratio used for initial solubilization could affect the composition and size of native nanodiscs [14]. Systematic studies on this with biological membranes have not been reported yet, but the heterogeneity of SMA in terms of length and composition, the use of different polymer variants (SMA 2:1, SMA 3:1), the presence of structurally different membrane proteins and differences in membrane lipid composition might all result in further deviations between samples. Thus, many different parameters will probably influence whether or not a full annular ring of lipids is coextracted with the protein or whether there are alternating polymer and lipid contacts with the hydrophobic surface of the protein.

In all studies where the lipid content of native nanodiscs has been investigated, it was demonstrated that native lipid molecules are coisolated [7,11,12,15]. Similar to the situation in protein-free SMALPs, these lipid molecules are likely to be organized in a limited version of a bilayer [16,17], which makes native nanodiscs the only system that is capable of extracting membrane proteins out of the membrane while conserving their native environment. This lipid environment—whether organized in a genuine full bilayer or not—is likely an important contributor to the high stability of MPs in native nanodiscs.

Structural and functional investigations of membrane proteins

Native nanodiscs are small soluble particles that readily allow structural characterization of the incorporated proteins by solution-based techniques. The small size of the nanodiscs is highly advantageous because of the low degree of light scattering, facilitating the use of optical techniques like circular dichroism and fluorescence spectroscopy as well as absorption measurements in the UV and visible light range (see [1]). The strong structural resemblance of nanodiscs bounded by SMA and MSP should in principle make native nanodiscs (and also MPs reconstituted into synthetic SMALPs) suitable for the complete range of methods that has been established for MSP nanodiscs [18,19].

While electron paramagnetic resonance spectroscopy has been successfully used to characterize membrane proteins in SMALPs [13,20,21], studies on protein structure involving the methodologically-related NMR spectroscopy are still scarce. This could be related to the particle size of the nanodiscs that is rather close to the limitations of solution-state NMR. However, both solution- and solid-state NMR approaches have been established for MSP nanodiscs [22] and therefore, in principle, they should also be applicable to native nanodiscs. Indeed, it has been recently shown, that solid-state NMR methods enable structural characterization of a protein in native nanodiscs [23], which is promising for future applications to assess MP structure in a native environment. Alternative solid-state NMR approaches using aligned systems, as reported for relatively large bicelles [24], unfortunately do not appear to be feasible, since neither standard MSP-nanodiscs nor SMALPs tend to align in magnetic fields: in both cases isotropic peaks in 31P NMR are observed that suggest fast reorientation of the particles in all directions [14,25].

The single-particle character of nanodiscs bounded by SMA also makes them promising targets for structural investigation by electron microscopy (EM). The EM field has recently undergone a drastic transition due to substantial improvements in both experimental equipment and data analysis software [26]. This now results in electron density maps of a quality that allows the *de novo*-determination of structures even of relatively small proteins (MW < 200 kDa) at (near) atomic resolution [27]. The fact that neither crystals nor large amounts of purified proteins are required in single-particle EM methods makes this approach particularly interesting for MPs and their complexes. While high-resolution structures for proteins in native nanodiscs have not been reported to date, EM characterization has recently resulted in the determination of the structure of the TRPV1 channel embedded in similar MSP nanodiscs [28], which suggests similar possibilities for native nanodiscs. This approach may be particularly useful for membrane protein complexes that are relatively unstable in detergent. In combination with complementary techniques like solid-state NMR spectroscopy that allow the study of protein dynamics, native nanodiscs could thus become a powerful platform for *ex vivo* structural biology of MPs.

Biophysical characterization of MP function in nanodiscs largely benefits from the fact that the soluble domains of the protein on either side of the membrane are accessible to the solvent. Thus, addition of solutes can be studied while a native-like environment is conserved. Indeed, binding studies with small molecules have been effortlessly performed with proteins in native nanodiscs using radioactive ligands [5,9] or detection of binding by fluorescence methods [5,10]. In general, the activity of the proteins investigated in native nanodiscs is either similar to that in detergent micelles or even improved, again indicating the superiority of the conserved (native) lipid environment over detergent. Together with the generally unperturbed protein structure it can thus be assumed that both the native structure and function of MPs are conserved in native nanodiscs.

Native Interactions of Membrane Components

Lipid–protein interactions

An important implication of the conservation of a native environment around a membrane protein in native nanodiscs is the possibility of direct biochemical analysis of native interactions of the protein with surrounding lipids or with other membrane components. This was originally established for preferential interaction of lipids with the potassium channel KcsA (Chapter II) and later also for the bacterial translocon SecYEG [15]. In both cases it was found that the composition of the lipids in the purified native nanodiscs was significantly different from that of the bulk membrane or the total solubilized fraction. Native nanodiscs containing either protein (complex) were enriched in anionic lipids, consistent with the functional relevance of these lipids for the proteins as deduced by model membrane studies.

In order to obtain reliable information on preferential lipid–protein interactions from native nanodiscs it is important to exclude preferential solubilization of certain lipid species by SMA. While this was validated for the *E. coli* inner membrane by analysis of the total solubilized fraction in the above mentioned studies, in Chapter III we provide a more general systematic comparison. By testing a variety of model membranes containing lipids with

different properties we found that SMA does not exhibit a preference for lipid species, provided that the formed bilayers are fluid and homogeneously mixed. Our observation that a strong preference for solubilization does exist for phase-separated membranes has important consequences for certain kinds of biomembranes, as will be discussed below. It should be noted here that the copurification of a larger number of (annular) lipids in a stable complex in native nanodiscs is distinctly different from coextraction of lipids with MPs in detergent-based methods. In nanodiscs part of the native lipid environment is retained, while detergent extraction generally results in a selective copurification only of those lipids that are tightly bound to the target protein [29,30]. Examples of the copurification of more extensive lipid material also exist, but this process is strongly dependent on the detergent used [31]. Moreover, due to the dynamics in the micellar organization, such complexes of lipids with the protein in detergent will likely not be stable in time, thus leading to a biased picture of the situation in biomembranes.

Protein–protein interactions

A further application of the extraction of membrane proteins in native nanodiscs is the study of interactions between different proteins, since proteins whose membrane-embedded parts are closely interacting can be captured together in the same nanodisc. This readily allows the isolation of stable homooligomers of membrane proteins [4,5,11] and large functional protein complexes [7,12], but can also be exploited for investigations of more dynamic interactions. For example, the detergent-labile binding of two penicillin binding proteins could be confirmed by an approach using coimmunoprecipitation after solubilizing bacterial cells with SMA [3]. More recently, the purification of the SecYEG channel as functional complex with an interacting peripheral membrane protein in native nanodiscs was reported. This provided information on the interactome of the channel, which could not be obtained in detergent-based approaches due to the detergent sensitivity of the complexes [15]. Similar results were obtained for the isolation of a multicomponent enzyme complex from plants, where a detailed lipidomics and proteomics analysis was carried out [2]. Native nanodiscs thus serve as an elegant tool to study preferential interactions of a membrane protein of interest with both lipids and other proteins.

Influence of Membrane Organization on SMA Solubilization

As is illustrated in Chapter III, SMA is promiscuous with respect to solubilization of phospholipid species in fluid homogeneously mixed bilayers. Similar results were also obtained in other model membrane studies [32,33] and for the solubilization of membrane proteins from biological membranes [7,11,12,15]. Our observations with HeLa cells furthermore indicate that there is little, if any, influence of the subcellular localization (Chapter IV).

However, SMA does solubilize certain types of membranes more easily than others. For example, membranes with lipids in a fluid phase are more efficiently solubilized than membranes with gel-phase lipids [32]. In phase-separated bilayers this leads to a distinct preference for solubilizing the fluid phase over a gel or liquid-ordered phase (Chapter III). It is thus tempting to also associate the observed differences in solubilization kinetics of plasma membrane-localized proteins (Chapter IV) in the context of domain formation.

However, such differences may also be caused by other reasons such as interactions with the actin cytoskeleton.
A high resistance against SMA solubilization has also been reported for photosynthetic membranes that exhibit a very low lipid-to-protein ratio [34,35] and it was hypothesized that this may be caused by the high protein density. The resulting low lipid content would then be insufficient to facilitate efficient SMA insertion into the membrane and thus impair solubilization. A similar mechanism could also explain the high SMA resistance of outer membrane proteins in *E. coli* (Chapter V), although the influence of other properties of the outer bacterial membrane cannot be excluded.
In summary, fluid membranes generally allow for efficient protein solubilization by SMA whereas membranes or membrane domains that are characterized by tight lipid packing or high protein density are resistant against SMA solubilization (Figure 1). Such SMA-resistant membranes (SRMs) have been isolated as the non-solubilized fraction after addition of SMA and have been found to be enriched in certain protein complexes [34]. Such approaches may provide valuable information on the interplay of membrane components in the native membrane as will be discussed below.

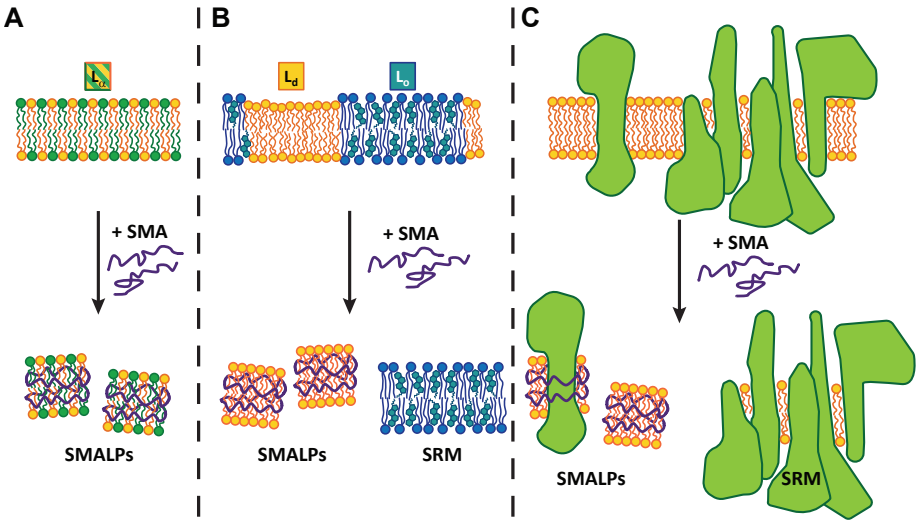

Figure 1: Influence of membrane properties on SMA solubilization. Model membranes are solubilized without a preference, in case they form fluid homogenously-mixed bilayers (A), whereas phase-separated bilayers exhibit pronounced solubilization preferences (B). Here, domains in the fluid phase are solubilized efficiently but ordered domains form SMA-resistant membranes (SRMs). (C) Membranes with a high protein density represent another kind of SRM. While membrane proteins that reside in lipid rich regions are incorporated into nanodiscs with ease, clustering of membrane proteins may prevent solubilization.

A possibility to improve nanodisc incorporation of proteins residing in SRMs may lie in the addition of synthetic DMPC lipid before solubilization. This lipid is very efficiently solubilized by SMA [32] and it has been used in early studies for assisted solubilization of proteins

from native membranes [13]. Addition of DMPC might then increase the overall fluidity of the membrane and shift the equilibrium towards a more homogeneously-mixed bilayer, thus destabilizing ordered domains. In protein-rich membranes, DMPC addition would lead to a dilution of the proteins in the membrane which might improve the solubilization capacity of SMA [35].

Limitations of the Method

As described in the previous section, SMA cannot be used for the solubilization of any biomembrane, irrespective of its properties. While supplementing such SRMs with lipid material such as DMPC may allow for solublilization by SMA, this will be at the expense of the "nativeness" of the resulting nanodiscs, which will hamper investigations on the native interactome of the isolated proteins.

One should also note that the use of SMA has further potential limitations. For instance, the higher order of lipids in SMALPs as determined for particles derived from model membranes [17] could cause a higher rigidity of the lipid environment. Apart from a favorable increase in protein stability this could also hinder conformational transitions or helical movement and thus interfere with protein function in native nanodiscs.

Some specific properties of SMA may render it problematic under certain conditions. For instance, measurements at low pH or assays that require high amounts of Mg^{2+}-stabilized nucleotides may be a problem due to destabilization of the nanodiscs by protonation or chelated divalent cations, respectively. Furthermore, the presence of SMA could impair binding studies involving the use of (multiple) positively charged molecules, because the high negative charge density of SMA can interfere with binding of these molecules to their target proteins. The high stability of SMA-bounded nanodiscs may also present challenges since it will impede activity assays that require the addition of membrane-embedded substrates for enzymes [36]. Also, the fact that proteins in nanodiscs are accessible to the same solvent on both sides of the membrane is not necessarily always an advantage. It is for example incompatible with functional studies on vectorial transport of small molecules by ion channels or transport proteins, since such assays usually require compartment-forming systems. One way to resolve this is the reconstitution of the protein directly from native nanodiscs into such a system. In Chapter II, we present promising results with KcsA, indicating that this is indeed feasible on a single channel level. While this proof-of-principle example opens the door to a wide range of potential applications, clearly further effort is required to generalize the approach for quantitative reconstitution to study proteins that require larger than single molecule amounts.

Outlook

The studies described in this thesis together with a number of recent reports on the use of SMA polymers highlight various applications of these versatile molecules to study membranes and membrane proteins. This shows that the system is rapidly gaining acceptance in the field. However, current use of the technique is still far from exploiting its full potential. In this last section, some possibilities offered by current methods of SMA extraction will be reviewed and a discussion is provided of some new applications of SMA that may become available in future membrane research.

Applications of native nanodiscs and new possibilities offered by transfer to other environments

Figure 2 illustrates established and potential future applications of membrane solubilization by SMA. The various applications are depicted as numbered arrows in the figure that will be referred to in the text. In the following paragraphs the possibilities associated with each of the different arrows will be discussed in detail.

Arrow 1. As discussed extensively already, the isolation of membrane proteins in native nanodiscs offers exciting possibilities for their structural and functional characterization in an *ex vivo* approach, i.e. membrane proteins are extracted directly from intact cells while conserving a native environment. Native nanodiscs are readily amenable to solution-based techniques right after purification and can thus be used for structural and functional characterization of membrane proteins. Given a sufficient sensitivity of any technique employed, it would even be possible to study isolated MPs and their complexes at endogenous levels of expression. These properties make native nanodiscs a promising tool that will likely develop into a powerful platform in membrane protein research.

Arrow 2 involves the copurification of lipids and other proteins with MPs in the same nanodisc. The results reported to date support the assumption that SMA-based extraction renders a snapshot view of a membrane protein in its natural context, allowing determination of preferential lipid–protein and protein–protein interactions [3,11,15]. Combined approaches involving different pull-down assays and separation techniques on native nanodiscs may thus provide a convenient tool to obtain detailed information on the interaction profile of membrane proteins, as was recently demonstrated for the interactome of a plant enzyme complex [2]. In this context, the solubilization of several proteins in one native nanodisc could be considered a form of noncovalent “mild crosslinking”.

Arrows 3–5. SMA can also be applied to model membranes in which proteins are reconstituted in the conventional way by using detergent. This allows the preparation of SMALPs containing a membrane protein embedded in a defined lipid environment. The big advantage would be that the lipid composition can be systematically varied in a similar way as has been established for MSP-bounded nanodiscs [19].

Arrow 6. SMALPs derived from synthetic bilayers of specific lipid and/or protein composition can also be used as a tool to analyze preferential interactions of membrane proteins with lipids or other proteins. The underlying principle is similar to that already presented for native nanodiscs, but the advantage is that the interactions can be studied in a more controlled way. For example, hydrophobic matching preferences could be assayed by reconstituting proteins into bilayers composed of two lipids with acyl chains of different lengths and by subsequently monitoring whether one of the lipids is enriched in isolated and purified protein-containing nanodiscs. It is also possible to reconstitute the proteins into bilayers at different lipid/protein ratios and then analyze the protein content of the purified nanodiscs. This would allow straightforward studies on protein–protein interactions and oligomerization processes and enable investigations on how they depend on, for instance, the lipid environment.

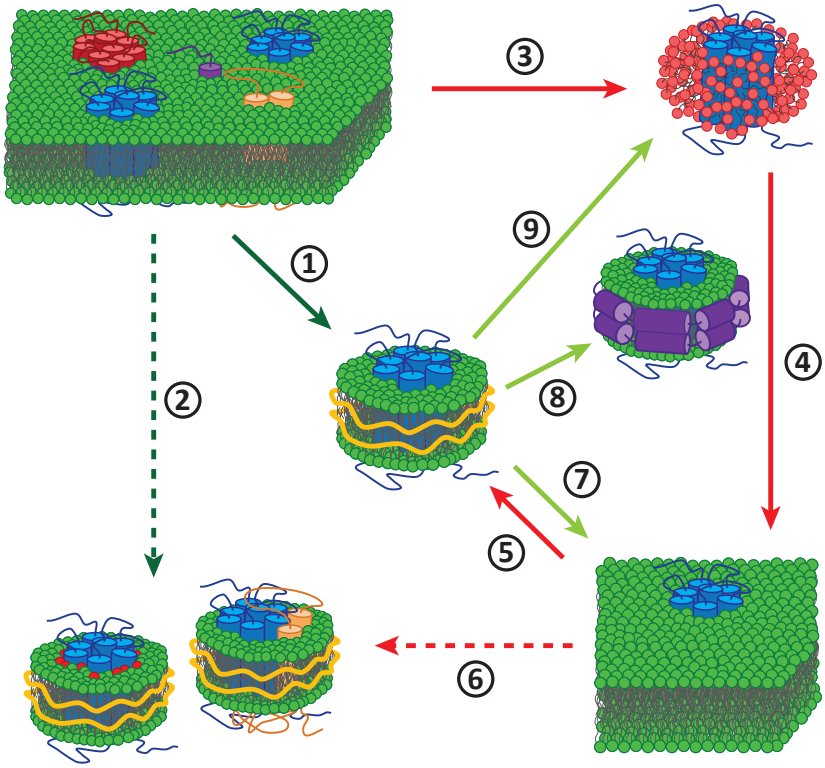

Figure 2: Different applications of native nanodiscs. Dark green arrows indicate conservation of the native environment, light green arrows display possibilities for the transfer to controlled environments. Red arrows display approaches that generally use synthetic environments. The dashed arrows represent approaches in which complexes of MPs with specific lipids (red) or other proteins (yellow) can be isolated from native (2) or synthetic (6) environments.

Arrow 7. A limitation of native nanodiscs is that they may be incompatible with intended downstream applications. Examples are techniques that require the presence of separate compartments such as transport assays and electrophysiology. In those cases it would be convenient to reconstitute the protein from the native nanodisc environment directly into synthetic bilayers, as is demonstrated in Chapter II for the channel protein KcsA. Membrane proteins treated this way may thus be studied in a controlled and compartmentalized environment without ever being destabilized in detergent. It should be noted however that in this example of KcsA the successful reconstitution of a single channel would be sufficient for functional measurements. A big challenge still remains in the quantitative reconstitution of the entire native protein material including surrounding lipids. Very promising results in this respect have been recently reported for the transfer of an MP into a lipidic cubic phase, which enabled the determination of the protein structure at high resolution by X-ray crystallography [37]. Extending this approach to protein reconstitution from native nanodiscs into other lipid systems may however be hampered by the high affinity of SMA for lipids.

This could complicate the removal of the polymer and may impair the formation of extended bilayers. It has been suggested that lowering the pH would solve this problem [38]. However, in our hands this approach was not successful (Dörr, Koorengevel, Scheidelaar and Killian, unpublished observations). Reconstitution via a decrease in pH is likely problematic because the increased hydrophobicity of a protonated polymer causes precipitation of the nanodiscs together with enclosed lipids and proteins. Nevertheless, it is an intriguing idea to exploit the pH dependence of SMA to destabilize nanodiscs, and for instance the presence of excess lipid may help to facilitate reconstitution into bilayers, as is indicated by recent findings with model membranes [33].

Arrows 8 & 9. As discussed above, there are several reasons why native nanodiscs may be incompatible with the assays or methods to be used. In such cases purification and stabilization of an MP in native nanodiscs and further transfer into other membrane mimics may be a convenient approach. Depending on the applications, this environment may be compartment-forming systems, as discussed above, or it could be MSP nanodiscs or detergent micelles. In particular, transfer to MSP nanodiscs could be convenient because many applications already have been developed and tested for these particles. However, reconstitution directly from native nanodiscs into MSP nanodiscs has not been reported yet. Similarly, it could be advantageous to purify proteins in the form of native nanodiscs and replace the nanodiscs for detergent prior to functional or structural studies. This is true for example when a protein is not stable enough in detergent for purification, but the analysis method is incompatible with nanodiscs bounded by SMA.

Isolation of SMA-resistant membrane fractions

Preferential solubilization of membrane domains by SMA may become a useful application in the future. By exploiting selective solubilization of fluid membranes, SMA-resistant membranes (SRMs) can be obtained, as has been demonstrated in plants [34]. The same approach could be applied to isolate clusters of membrane proteins or ordered domains in the plasma membrane of mammalian cells. In view of their high local protein density or their tight lipid packing, respectively, such domains are likely to be more resistant against SMA solubilization than are more lipid rich or more fluid membrane domains, as is suggested by our findings with biological membranes (Chapter IV) and model membranes (Chapter III). Thus, SMA could be used as an alternative to conventional detergent for their isolation as SRMs. This is important since detergent-based approaches can affect the phase equilibrium in membranes and might thus introduce artifacts [39,40]. Since the solubilization by SMA conserves basic bilayer properties [16,17] it can be considered a rather mild solubilizing agent. Thus, using SMA instead of detergent may better conserve native interactions of membrane components in SRMs. However, whether indeed SRMs have beneficial properties over conventional protocols for obtaining resistant membrane fractions remains to be addressed by future research.

Other applications

Other potential applications of SMA-bounded nanodiscs include the use of SMALPs as membrane mimics for interactions with water-soluble proteins or as acceptor system for

cell-free protein production. A particular advantage of SMALPs in the latter case would be that the particles might be able to expand upon incorporation of a protein due to the flexibility of SMA that is less restricted to a specific arrangement than is MSP, for which the use as acceptors has been demonstrated successfully [41].
As discussed above, the special properties of SMA could cause potential problems, for instance due to the strong pH dependence or sensitivity to divalent cations. However, design of new types of polymers with modified functional groups may solve these problems. This is nicely illustrated by the recent discovery that a structurally-related diisobutyl–maleic acid copolymer solubilizes membranes with a similar efficiency [42]. The resulting nanodiscs are less sensitive to divalent cations and due to the absence of phenyl groups they exhibit beneficial optical properties. Generally, the chemical structure of both these polymers provides plenty of opportunities for modification to tune their properties in order to avoid undesired interactions. Also, the incorporation of fluorescent or radioactive labels or affinity tags is possible and thus an extensive tool box of different SMAs and their derivatives may be generated for various different applications.

Conclusion

In conclusion, SMA copolymers have been successfully employed in the solubilization of a diverse set of membrane proteins, generally showing the superiority of the obtained native nanodisc environment over detergent micelles. The nanodiscs have been subject to complementary studies proving their suitability for a plethora of biophysical and biochemical techniques that allowed insights into protein structure and function as well as native interactions of membrane proteins with both lipids and other proteins. In addition, many other potential applications of the use of SMA copolymers and chemically-modified or tailor-made variants thereof remain to be explored and discovered. These include approaches were solubilization preferences are exploited to prepare SMA-resistant membranes. A major future challenge will be the elucidation of the details of the mode of action of SMA-mediated membrane solubilization, as this will be invaluable for adaptions of the method to more difficult systems or conditions. Altogether, it can be concluded that SMA copolymers are highly promising versatile tools for the study of diverse membrane-related processes and that they are likely to make a large contribution to the field of membrane research in the future.

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Appendix

Layman's Summary
Deutsche Zusammenfassung
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Curriculum vitae
List of Publications
Acknowledgements – Danksagung

Layman's Summary

This chapter is addressed to a layman readership and to everyone who doesn't want to bother with all the details but still would like to know about the essentials of the work I did during my PhD. To you, I hope to be able to give some insight into the world of biophysics and into the sometimes weird concepts of basic research.

The research project that I have been keeping myself busy with primarily deals with **biomembranes**. Such membranes are a fundamental component of every single cell in our body and they are essential for life. Their main task is the structural organization of the cell and they delimit a cell from its environment and thus define it as a unit. In addition, they organize the cell into smaller compartments that execute many diverse functions. Since membranes are essentially impermeable to many water-soluble molecules, they ensure that each molecule stays in the right place to be able to fulfill its designated role. One can therefore think of membranes as gatekeepers that bring some order into the chaos of cellular molecules. This impermeability of membranes also enables the generation of concentration gradients of molecules across the membrane. Such concentration gradients are the basis for innumerable biochemical processes ranging from energy generation to signal transduction in neurons. Hence, biomembranes are of great importance for cellular function.

In order to better understand the function of biomembranes, one needs to zoom in to the molecular level. And since membranes on average are only about four nanometers (that is four millionths of a millimeter) in thickness, one has to zoom in quite a bit. From close up, biomembranes are complex dynamic structures whose main components can be divided into two classes of molecules: lipids and proteins.

Lipids are relatively small molecules consisting of a hydrophilic (water-loving) head group and hydrophobic (water-repellent or fat-loving) fatty acid chains. Since these hydrophobic fatty acid chains are very poorly soluble in water—think oil droplets in water—lipids spontaneously form a bilayer structure in aqueous solution (see Figure 2 in Chapter I). In such bilayer membranes, the fatty acid chains are oriented towards the interior where they are shielded from the aqueous environment, while the head groups are facing the water. Such lipid bilayers form the scaffold for a biomembrane and they are the cause of its impermeability for many water-soluble substances.

The second main component of biomembranes is **membrane proteins**. These are polymeric molecules consisting of chains of amino acids and they are generally much bigger than lipids. The large number of possible combinations of different amino acids results in an enormous variety of (membrane) proteins with different forms and properties which fulfill diverse tasks. Some of these membrane proteins accelerate biochemical reactions, for example in the respiratory chain or in photosynthesis, which occur mainly in membranes. Others ensure that the structure of a cell or of tissue is maintained. Membrane proteins are also responsible for selective transport processes of molecules through the membrane and they mediate the communication between different cells, e.g. as receptors that recognize messenger molecules or that activate cells of the immune system. This multitude of different functions also leads to a great importance of membrane proteins in medical research, which is nicely illustrated by the fact that about half of all currently-available drugs target membrane proteins.

If one wants to look at individual membrane proteins more closely to better understand their function one easily faces a rather big problem. It is important to realize that membrane proteins in order to function properly depend on their natural environment, i.e., on the company of lipids. Ideally, one would thus like to study proteins in a living cell. However, there is hardly any analytical method that would be sensitive enough for this. In order to study a particular protein, one thus generally needs to isolate it from the cell and separate it from other molecules. For water-soluble proteins, such as hemoglobin in our red blood cells, this is relatively straight forward. Membrane proteins, however, are firmly anchored in the membrane due to their hydrophobic surfaces. While this is a necessity for them to function properly, it implies that the membrane must be destroyed in order to separate different membrane proteins. The standard method for achieving this is the **solubilization** of the membrane by means of detergents. In everyday life, detergents are, of course, used to dissolve dirt or fat. In a similar process, the detergents used in the laboratory render membrane components water-soluble and thus enable their separation. Unfortunately, this method comes with the disadvantage that the protein is often destabilized and thereby possibly inactivated and prone to aggregation. Such protein aggregation is comparable to the processes that cause a chicken egg to solidify during cooking and in such an aggregated state, proteins obviously give very little information about their functioning in the cell. The investigation of membrane proteins is therefore considerably more problematic than that of soluble proteins and the characterization of a single membrane protein far exceeds the average duration of a PhD project. As a consequence, our understanding of the function of many membrane proteins is of yet not very solid.

With that, after a mere one and a half pages, I have arrived at the point where my research project starts. But bear with me, this is where it's actually getting interesting! My project is about developing a method to efficiently remove membrane proteins from the membrane, but at the same time to conserve their native state and protect them from aggregation. To achieve this, we make use of a copolymer of **styrene and maleic acid (SMA)**. This compound has originally been used as an additive in the chemical industry. However, SMA has also been characterized as a potential carrier system for the administration of drugs in medical research. In this context, British scientists discovered more or less by chance that SMA also efficiently solubilizes biomembranes. The special feature here is that SMA does not completely dissolve the membrane like detergents do, but rather cuts out intact disc-like fragments, in a way similar to a cookie cutter. These membrane discs have a diameter of about ten nanometers and are therefore referred to as **nanodiscs**. In 2009, it was also shown that membrane proteins can be transferred into such nanodiscs and that these represent a stable environment for the protein.

At the time when I started my research project, this method had only been adopted by very few scientists and it was not yet clear to what extent SMA is compatible with established methods in membrane protein research. Thus, we aimed at characterizing SMA nanodiscs in order to assess their potential for the investigation of membrane proteins. The insights we have gained along the way are described in this dissertation and they are summarized below.

Chapter I is a general introduction containing a detailed description of biomembranes and their components. Furthermore, various methods are presented that allow for the

solubilization of membrane proteins for subsequent study. The focus herein lies on SMA copolymers and on their application as membrane-solubilizing agents, which is the main topic of my dissertation.

Chapter II describes a first research project in which we investigated how well the polymer method works for the solubilization and purification of a membrane protein directly from a biomembrane. As a model protein, we chose a bacterial potassium ion channel that is structurally very similar to many human potassium channels.
Based on this model protein, we were able to show that the solubilization by SMA is a very good alternative to established detergent methods and that it readily enables purification of the protein. In addition, we found that the potassium channel was more stable in the SMA nanodiscs than in detergent, which is a great advantage with regard to downstream investigation. We also exploited the fact that the direct lipid environment of the channel is co-purified in the nanodiscs to investigate its molecular composition. In this way, we established a new analytical method, which allows conclusions on preferred lipid partners of the membrane protein. A nanodisc may thus be regarded as a snapshot of a protein in its natural membrane environment. Finally, we succeeded in transferring the channel into an artificial lipid membrane and could show that it still conducts potassium ions, thus proving that its functionality had not been affected by the isolation method. Importantly, this experiment represents the first example of the transfer of a membrane protein into an artificial environment, without ever having been in contact with potentially damaging detergent. This last result in particular holds great potential for studies on other membrane proteins, especially if they are very detergent sensitive.

In another study, described in **Chapter III**, we looked at how different lipid compositions of the membrane affect its solubilization by SMA. To this end, we used model membranes without proteins to check whether there are lipids that are solubilized by SMA with a preference. Information on such a preferential solubilization is important because this might potentially affect conclusions on preferred lipid partners of membrane proteins (see Chapter II).
By systematically varying the lipid composition of the model membranes, we were able to demonstrate that there is no preferential solubilization for membranes in a fluid state in which the fatty acid chains of the lipids are relatively mobile. Such membranes were very efficiently solubilized by SMA and transferred to nanodiscs. Membranes in a gel state with rigid, tightly packed fatty acid chains, however, are very inefficiently solubilized. The solubilization efficiency thus strongly depends on the state of the membrane lipids. As a result, a preferred solubilization can be observed in specific membranes which exhibit a so-called phase separation. Such phase-separated membranes include, at the same time, domains (regions) in the fluid state and domains in the gel state, which differ in their respective lipid composition. Here, fluid domains are solubilized much faster by SMA.
Such a preferential lipid solubilization could possibly find applications for the study of so-called “lipid rafts”. Such rafts are highly ordered membrane domains that may be present in the membrane of living cells (e.g., in the immune system) and they are believed to fulfill a specific function. However, for the last twenty years, membrane researchers have been debating the importance of such lipid rafts very controversially. In fact, the mere existence of such domains has neither been confirmed nor ruled out to date and there is still active research in this area.

Chapter IV describes a study in which we investigated the effect of SMA on human cancer cells in order to obtain further information on how the solubilization process works. Using a special microscopy technique, we could record videos that allowed us to follow live how living cells are solubilized by SMA.
For this purpose, we supplemented cell cultures with artificial DNA, which is then taken up by the cells and used as a blueprint to make various proteins. These blueprints are designed such, that the proteins produced by the cells are fluorescent. This means that when they are irradiated with light of a specific wavelength, that is, of a specific color, they send out light of a higher wavelength, that is, of a different color. This fluorescence light can then be detected with a special microscope which allows the visualization of individual cellular compartments.
With this method we investigated whether there are differences in the speed at which the membranes of the different cellular compartments are solubilized by SMA. Surprisingly, we found evidence that the plasma membrane (the outer envelope of the cell) is solubilized most slowly, even though it is the first that is in contact with SMA. Furthermore, we observed that different proteins in the plasma membrane are solubilized at different rates. These results also suggest a possible influence of lipid rafts on membrane solubilization (see Chapter III).

For the research project described in **Chapter V**, we returned to the above-mentioned bacterial membranes with the potassium channel (see Chapter II). We used these as an example system to optimize the experimental conditions for the solubilization of membrane proteins by SMA.
With this approach we could show that different factors influence the solubilization efficiency to varying degrees, and we derived conditions that favor the solubilization of proteins. We found that the influence of salt concentration and pH are particularly drastic. Very low or very high salt concentrations prevent the solubilization of membrane proteins almost completely, which can be explained primarily by the properties of the SMA polymer itself. Only salt concentrations that are similar to those in our body promote solubilization. For the pH, we found a reverse effect for the bacterial membranes compared to what had previously been observed for model membranes without protein. This seems paradoxical, but there are several possible explanations. The most probable of these is that the pH dependence of membrane solubilization is strongly related to the lipid composition of the membrane. However, further studies are needed to clarify this. This example shows very nicely that there is still a lot of work to be done in order to completely understand how membrane solubilization by SMA works. However, a better understanding of this process is indispensable to be able to exploit the full potential of the method.

In **Chapter VI**, our findings are put into the context of scientific studies by other authors within the framework of a general discussion. In addition, an outlook for future applications of solubilization of membranes by SMA is presented and possible limitations of the method are discussed. As a conclusion, it can be stated that the SMA studies carried out to date have yielded very promising results which suggest versatile applications in membrane research. It is therefore very likely that SMA polymers will make a big contribution to a better understanding of biomembranes.

Finally, I would like to briefly try to answer the frequently asked question "**And what is this good for?**". First of all, this really is a very good question. Unfortunately, the answer is not exactly straight forward and a complete answer would definitely go beyond the scope of this thesis. The shortest answer I can think of is: "Everything and nothing". Everything because the knowledge we have generated might be used to develop a plethora of applications. And nothing, because we do not really pursue any of these applications ourselves and they are not the focus of our work either. Our research much rather has the general goal of better understanding biomembranes and membrane proteins, and for this purpose we have investigated a new method.

This type of knowledge-oriented science is called **basic research**. According to the expression "knowledge is power", insights gained in this way can then serve as a basis for further applied research. As basic researchers, we are thus less concerned with a specific application, e.g. to develop a treatment for a specific disease. One could however argue that the findings of our research may help to cure all kinds of diseases. This is because diseases are often associated with membrane processes in one way or another and an understanding of their basic functional principles is crucial for drug development. In this context, I would also like to point out that many groundbreaking scientific achievements have been made possible by basic research. For example, the discovery of penicillin, the first known antibiotic, was a rather fortunate accident (a mold had infected a petri dish with bacteria and had killed them). And it is only thanks to the curiosity of the scientist that made this observation that this became the foundation for the development of antibiotics. Also the SMA copolymer entered the field of membrane research by mere chance. It is however very difficult today to estimate what will have become of our work with SMA and membrane proteins in ten or fifty years from now. It probably won't trigger a revolution in medicine comparable to that of penicillin, and it may even end up being forgotten altogether. But perhaps our findings will contribute to another breakthrough, who knows? Only one thing is certain: it is for sure worth the effort!

Deutsche Zusammenfassung

Diese Zusammenfassung richtet sich hauptsächlich an eine Laienleserschaft und an alle, die mich über die vergangenen Jahre (wiederholt) gefragt haben, was ich denn da jetzt eigentlich überhaupt so mache in Utrecht. Ich hoffe, mit diesem kurzen Abriss ein wenig Abhilfe schaffen zu können und ein paar (verspätete) Erklärungen zu liefern, die ein bisschen Licht ins Dunkel bringen und vielleicht sogar bei dem ein oder anderen ein gewisses Interesse am Thema wecken.

In meiner Forschungsarbeit habe ich mich in erster Linie mit **Biomembranen** beschäftigt. Diese Membranen sind ein fundamentaler Bestandteil jeder einzelnen Zelle unseres Körpers und sie sind absolut lebensnotwendig. Ihre Hauptaufgabe besteht in der strukturellen Organisation der Zelle. Membranen grenzen eine Zelle von ihrer Umwelt ab und definieren sie so als Einheit. Darüber hinaus gliedern sie die Zelle in verschiedene Untereinheiten mit unterschiedlichen Aufgaben. Da Membranen für viele wasserlösliche Moleküle undurchlässig sind, sorgen sie für deren örtliche Trennung und ermöglichen es so, dass jedes Molekül seine Aufgabe am richtigen Ort erfüllt. Man kann sich Membranen also in etwa als molekulare Ordnungshüter vorstellen, die das Chaos aus allen möglichen unterschiedlichen Biomolekülen in geordnete Bahnen lenken. Die Undurchlässigkeit von Membranen hat außerdem zur Folge, dass sich ein Konzentrationsgefälle von Molekülen über die Membran ausbilden kann. Ein solches Konzentrationsgefälle ist die Grundlage für unzählige biochemische Prozesse, die von der Energiegewinnung durch Zellatmung bis hin zur Reizweiterleitung in Nervenzellen reichen. Biomembranen sind also aktiv an vielen Vorgängen in der Zelle beteiligt und es kommt ihnen so eine große Bedeutung zu.

Um nun die Funktionsweise von Biomembranen genauer verstehen zu können, muss man sie auf molekularer Ebene betrachten. Und da Membranen im Schnitt nur ungefähr vier Nanometer (also vier Millionstel eines Millimeters) dick sind, muss man dazu schon ziemlich weit hineinzoomen. Aus der Nähe betrachtet sind Biomembranen komplexe dynamische Strukturen, deren Hauptbestandteile sich in zwei Klassen von Molekülen aufteilen lassen: Lipide und Proteine.

Lipide sind vergleichsweise kleine Moleküle, die aus einer hydrophilen (wasserliebenden) Kopfgruppe und aus hydrophoben (wasserabweisenden bzw. fettliebenden) Fettsäureketten bestehen. Da diese hydrophoben Fettsäureketten sehr schlecht in Wasser löslich sind — man denke an Öltropfen im Kochwasser —, ordnen sich Lipide in wässriger Lösung spontan in einer Doppelschicht an (siehe Abbildung 2 in Kapitel I). In solchen Doppelschichtmembranen richten sich die Fettsäureketten nach innen aus, wo sie von der wässrigen Umgebung abgeschirmt sind, während die Kopfgruppen dem Wasser zugewandt sind. Eine solche Lipiddoppelschicht bildet das Grundgerüst jeder Biomembran und sie ist die Ursache für deren Undurchlässigkeit für viele wasserlösliche Stoffe.

Der zweite Bestandteil von Biomembranen sind **Membranproteine**. Diese sind Eiweißmoleküle, bestehend aus Ketten von Aminosäuren, die im Allgemeinen deutlich größer sind als Lipide. Aufgrund der Vielzahl an möglichen Kombinationen verschiedener Aminosäuren ergibt sich so eine enorme Vielfalt an (Membran-) Proteinen mit verschiedenen

Formen und Eigenschaften, die die unterschiedlichsten Aufgaben erfüllen. Manche dieser Membranproteine beschleunigen biochemische Reaktionen, wie z. B. in der Zellatmung oder der Photosynthese, die beide hauptsächlich in Membranen stattfinden. Andere sorgen dafür, dass die Struktur einer Zelle oder eines Gewebes aufrechterhalten wird. Membranproteine sind außerdem für selektive Transportprozesse von Molekülen durch die Membran verantwortlich und sie vermitteln die Kommunikation zwischen verschiedenen Zellen, z. B. indem sie als Rezeptoren bestimmte Botenstoffe erkennen oder Zellen des Immunsystems aktivieren. Diese Vielzahl an verschiedenen Funktionen führt auch zu einer großen Bedeutung von Membranproteinen aus medizinischer Sicht. Dies kann man leicht dadurch veranschaulichen, dass etwa die Hälfte aller auf dem Markt befindlichen Medikamente an einem Membranprotein wirkt.

Will man sich nun einzelne Membranproteine genauer anschauen und ihre Funktionsweise untersuchen, gibt es da allerdings einen ziemlich großen Haken. Es ist nämlich wichtig zu wissen, dass Membranproteine auf ihre natürliche Umgebung, also auf die Gesellschaft von Lipiden, angewiesen sind, um richtig zu funktionieren. Im Idealfall möchte man Proteine daher in einer lebenden Zelle untersuchen. Weil Zellen aber vollgepackt sind mit allen möglichen Molekülen, gibt es leider kaum eine Analysemethode, die empfindlich genug wäre, um sich einzelne Proteine anzusehen. Um ein bestimmtes Protein zu untersuchen, muss man es deshalb im Allgemeinen aus der Zelle isolieren und von anderen Molekülen trennen um es so in Reinform zu erhalten. Für wasserlösliche Proteine wie z. B. das Hämoglobin in unseren roten Blutkörperchen ist das vergleichsweise einfach. Membranproteine sind hingegen durch ihre hydrophoben Oberflächen fest in der Membran verankert. Das ist zwar notwendig, damit sie ihre Funktion richtig erfüllen können, hat aber zur Konsequenz, dass man die Membran zerstören muss, um Membranproteine voneinander zu trennen. Die Standardmethode, um dies zu erreichen, ist die **Solubilisierung** (Auflösung) der Membran mit Hilfe von Detergentien. Im täglichen Leben findet man solche Detergentien zum Beispiel in Seife oder in Spülmittel, wo sie die Aufgabe haben, Fette zu lösen. In einem ähnlichen Prozess machen die im Labor verwendeten Detergentien Membranbestandteile wasserlöslich und ermöglichen so deren Auftrennung. Leider hat dieses Herauslösen von Membranproteinen oft den nachteiligen Nebeneffekt, dass das Protein destabilisiert wird und dadurch womöglich inaktiviert wird oder sogar verklumpt. Dieses Verklumpen ist vergleichbar mit den Prozessen, die dazu führen, dass ein Hühnerei beim Kochen fest wird, und in einem solchen Zustand geben Proteine natürlich nur wenig Auskunft über ihre Funktionsweise in der Zelle. Die Erforschung von Membranproteinen ist daher deutlich aufwendiger als die von wasserlöslichen Proteinen, und die Charakterisierung eines einzelnen Membranproteins übersteigt die durchschnittliche Dauer eines Promotionsprojekts um ein Vielfaches. In der Konsequenz ist unser Verständnis der Funktionsweise vieler Membranproteine noch nicht sehr ausgeprägt.

Und damit bin ich nach „nur“ knapp zwei Seiten schon an dem Punkt angekommen, an dem mein Forschungsprojekt ansetzt. Und zwar geht es darum, eine Methode zu entwickeln, um Membranproteine effizient aus der Membran herauszulösen, aber gleichzeitig ihren natürlichen Zustand zu konservieren, um sie so vor dem Verklumpen zu schützen. Um das zu erreichen, benutzen wir ein Mischpolymer aus Styrol und Maleinsäure (engl. **styrene maleic acid**, kurz **SMA**). Diese Verbindung findet vielseitige Anwendungen als Zusatzstoff

in der chemischen Industrie. Aber auch in der medizinischen Forschung wurde SMA als potentielles Trägersystem zur Verabreichung von Medikamenten untersucht. In diesem Zusammenhang entdeckten britische Wissenschaftler mehr oder weniger durch Zufall, dass SMA auch effizient Biomembranen auflösen kann. Die Besonderheit hierbei ist, dass SMA die Membran nicht wie Detergentien komplett zersetzt, sondern scheibenweise intakte Stückchen daraus herauslöst. Diese Membranscheibchen haben einen Durchmesser von etwa zehn Nanometern und werden daher **Nanodiscs** (von *Nano*meter und „disc", dem englischen Wort für Scheibe) genannt. Im Jahr 2009 wurde außerdem gezeigt, dass man Membranproteine in solche Nanodiscs überführen kann und dass diese eine stabile Umgebung für das Protein sind.

Zu dem Zeitpunkt, als ich mit meinem Forschungsprojekt begann, war diese Methode allerdings erst von sehr wenigen Wissenschaftlern aufgegriffen worden und es war noch nicht klar, inwieweit SMA mit etablierten Methoden in der Membranproteinforschung kompatibel ist. Somit setzten wir uns zum Ziel, SMA-Nanodiscs zu charakterisieren, um ihr Potential zur Erforschung von Membranproteinen zu beurteilen. Die Erkenntnisse, die wir seither gewonnen haben, sind in dieser Dissertation beschrieben und sind im Folgenden zusammengefasst.

Kapitel I ist eine allgemeine Einleitung, die eine detaillierte Beschreibung von Biomembranen und ihren Bestandteilen enthält. Des Weiteren werden verschiedene Möglichkeiten vorgestellt, wie Membranproteine für anschließende Untersuchungen isoliert werden können. Ein Schwerpunkt liegt hierbei auf SMA-Polymeren, und ihrer Anwendung als Lösungsmittel für Membranen, was das Kernthema meiner Doktorarbeit ist.

In **Kapitel II** ist ein erstes Forschungsprojekt beschrieben, in dem wir untersucht haben, wie gut die Polymermethode für das Herauslösen und Aufreinigen eines Membranproteins direkt aus einer Biomembran funktioniert. Als Modellprotein wählten wir hierfür einen bakteriellen Kaliumionenkanal, ein Protein, das selektiv Kaliumionen durch die Membran durchlässt. Dieses Kanalprotein kommt zwar in einem Bakterium vor, aber es hat große strukturelle Ähnlichkeit mit vielen menschlichen Kaliumkanälen, die z. B. in Nervenzellen wichtig sind. Anhand dieses Modellproteins konnten wir zeigen, dass die Solubilisierung mit SMA eine sehr gute Alternative zu etablierten Detergenzmethoden darstellt und dass sie ohne Weiteres ermöglicht, das Protein aufzureinigen, also es von anderen Molekülen zu trennen. Zusätzlich zeigte sich, dass der Kaliumkanal in den SMA-Nanodiscs stabiler ist als in Detergenz, was ein großer Vorteil im Hinblick auf nachfolgende Untersuchungen ist. Außerdem nutzten wir aus, dass die direkte Lipidumgebung des Kanals in den Nanodiscs mit aufgereinigt wurde, und konnten so ihre molekulare Zusammensetzung untersuchen. Damit etablierten wir eine neue Analysemethode, die es ermöglicht, Rückschlüsse auf bevorzugte Lipid-Partner eines Membranproteins zu ziehen. Eine Nanodisc kann somit als eine Art Schnappschuss eines Proteins in seiner natürlichen Membranumgebung aufgefasst werden. Schließlich gelang es uns noch, den Kanal in eine künstliche Lipidmembran zu überführen und so nachweisen, dass der Kanal immer noch leitfähig für Kaliumionen ist. Seine Funktionalität war also durch die Isolationsmethode nicht in Mitleidenschaft gezogen worden. Zusätzlich konnten wir mit diesem Experiment zum ersten Mal zeigen, dass ein Transfer eines Membranproteins in eine künstliche Umgebung möglich ist, ohne dass es jemals in Kontakt mit potentiell schädlichem

Detergenz gekommen ist. Dieses letzte Ergebnis birgt ein großes Potential für Studien an anderen Membranproteinen, besonders an solchen, die empfindlich gegen Detergenz sind.

In einer weiteren Studie, die in **Kapitel III** beschrieben ist, haben wir uns näher angesehen, wie unterschiedliche Lipidzusammensetzungen der Membran die Solubilisierung durch SMA beeinflussen. Hierzu haben wir Modellmembranen ohne Proteine verwendet, um zu überprüfen, ob es Lipide gibt, die bevorzugt von SMA solubilisiert werden. Informationen über eine solche bevorzugte Solubilisierung sind wichtig, weil diese z. B. Rückschlüsse auf bevorzugte Lipid-Partner von Membranproteinen (s. Kapitel II) potentiell verfälschen könnte. Indem wir die Zusammensetzung der Modellmembranen gezielt variierten, konnten wir zeigen, dass es für Membranen in einem fluiden (flüssigen) Zustand, in dem die Fettsäurereste der Lipide relativ frei beweglich sind, keine bevorzugte Solubilisierung gibt. Solche Membranen werden von SMA sehr effizient aufgelöst und in Nanodiscs überführt. Membranen, die sich in einem Gelzustand mit starren dicht gepackten Fettsäureketten befinden, werden hingegen nur sehr ineffizient solubilisiert. Die Solubilisierungseffizienz hängt also stark vom Zustand der Membranlipide ab. Dies hat zur Folge, dass in speziellen Membranen, die eine sogenannte Phasentrennung aufweisen, sehr wohl eine bevorzugte Solubilisierung beobachtet werden kann. Solche phasengetrennte Membranen beinhalten gleichzeitig Domänen (Bereiche) im fluiden Zustand und Domänen im Gelzustand, die sich in ihrer jeweiligen Lipidzusammensetzung unterscheiden. Die fluiden Domänen wurden hier deutlich schneller von SMA herausgelöst.
Eine solche bevorzugte Solubilisierung fluider Domänen könnte möglicherweise eine Anwendung in der Forschung an sogenannten „Lipid Rafts“ (wörtlich Lipidflöße) finden. Lipid Rafts bezeichnen stark geordnete Membrandomänen, die möglicherweise in der Membran lebender Zellen, z. B. im Immunsystem, vorkommen und dort spezifische Aufgaben erfüllen. Allerdings führen Membranforscher seit mittlerweile zwanzig Jahren eine sehr kontroverse Debatte über die Bedeutung von Lipid Rafts. Tatsächlich konnte deren bloße Existenz bis heute weder bewiesen noch widerlegt werden, und es wird weiterhin aktive Forschung in diesem Bereich betrieben.

In **Kapitel IV** haben wir den Effekt von SMA auf menschliche Krebszellen untersucht, um weitere Informationen über den Solubilisierungsprozess zu erhalten. Mit einer speziellen Mikroskopiemethode konnten wir hiervon Videos aufnehmen und so live beobachten, wie lebende Zellen von SMA solubilisiert werden.
Hierzu versetzten wir Zellkulturen mit künstlicher DNS, die dann von den Zellen aufgenommen wird und als Bauplan für verschiedene Proteine benutzt wird. Diese Baupläne sind so konstruiert, dass die von den Zellen hergestellten Proteine von selbst fluoreszieren, d. h. wenn sie mit Licht einer bestimmten Wellenlänge — also einer bestimmten Farbe — bestrahlt werden, strahlen sie Licht einer höheren Wellenlänge — also einer anderen Farbe — aus. Dieses Fluoreszenzlicht kann man dann mit einem speziellen Mikroskop sichtbar machen und so bestimmte Zellbestandteile separat beobachten.
Mit dieser Methode untersuchten wir, ob es Unterschiede in der Schnelligkeit gibt, in der die Membranen von verschiedenen Zellbestandteilen von SMA aufgelöst werden. Überraschenderweise fanden wir so Hinweise, dass die Plasmamembran—die äußere Hülle der Zelle—am langsamsten solubilisiert wird, obwohl sie am längsten mit SMA in Kontakt ist. Außerdem konnten wir zeigen, dass verschiedene Proteine unterschiedlich

schnell aus der Plasmamembran herausgelöst werden. Auch diese Ergebnisse deuten auf einen möglichen Einfluss von Lipid Rafts auf die Membransolubilisierung hin (s. Kapitel III).

Für das in **Kapitel V** beschriebene Forschungsprojekt griffen wir wieder auf die oben erwähnten Bakterienmembranen mit dem Kaliumkanal zurück (s. Kapitel II). Diese verwendeten wir als Beispielsystem, um die experimentellen Bedingungen für die Solubilisierung von Membranproteinen durch SMA zu optimieren.
So konnten wir zeigen, dass verschiedene Faktoren die Solubilisierungseffizienz unterschiedlich stark beeinflussen, und wir konnten Bedingungen ableiten, die die Solubilisierung von Membranproteinen begünstigen. Besonders drastisch ist hierbei der Einfluss von Salzkonzentration und pH-Wert. Sehr geringe oder sehr hohe Salzkonzentrationen verhindern das Herauslösen von Membranprotein fast völlig, was hauptsächlich durch Eigenschaften des SMA-Polymers selbst erklärt werden kann. Nur Salzkonzentrationen im Bereich, in dem sie auch in unserem Körper vorkommen, sind vorteilhaft für die Solubilisierung.
Für den pH-Wert fanden wir interessanterweise für die Bakterienmembranen einen entgegengesetzten Effekt zu dem, der vorher schon für Modellmembranen ohne Protein beobachtet wurde. Das erscheint zwar paradox, aber es gibt hierfür mehrere Erklärungsmöglichkeiten. Die wahrscheinlichste davon ist, dass die pH-Abhängigkeit der Membransolubilisierung stark mit der Lipidzusammensetzung der Membran zusammenhängt. Es bedarf allerdings weiterer Studien, um dies abschließend zu klären. Dieses Beispiel zeigt sehr gut, dass es noch immer viel zu tun gibt, um genau zu verstehen, wie die Membransolubilisierung mit Hilfe von SMA funktioniert. Ein besseres Verständnis davon ist aber unumgänglich, um das komplette Potential der Methode ausschöpfen zu können.

In **Kapitel VI** werden die von uns gefundenen Ergebnisse im Rahmen einer allgemeinen Diskussion in den Kontext von wissenschaftlichen Studien anderer Autoren gesetzt. Zusätzlich wird ein Ausblick für zukünftige Anwendungen der Solubilisierung von Membranen durch SMA präsentiert und es wird auf mögliche Beschränkungen der Methode eingegangen. Als Fazit lässt sich festhalten, dass die bisherigen Studien mit SMA sehr vielversprechende Ergebnisse lieferten, die vielseitige Anwendungen in der Membranforschung in Aussicht stellen. Es ist also sehr wahrscheinlich, dass SMA-Polymere einen großen Beitrag zum besseren Verständnis von Biomembranen leisten werden.

Abschließend möchte ich noch kurz (diesmal wirklich) auf die oft gestellte Frage „**Und was macht man jetzt damit?**“ eingehen. Zunächst einmal sei bemerkt, dass das wirklich eine sehr gute Frage ist. Allerdings ist die Antwort darauf nicht ganz einfach und eine vollständige Antwort würde definitiv den Rahmen dieser Arbeit sprengen. Hier die kürzeste Antwort, die mir einfällt: „Alles und nichts“. Alles, weil sich aus den von uns erworbenen Erkenntnissen potentiell eine Vielzahl von Anwendungen ergeben, und nichts, weil wir eben keine dieser Anwendungen wirklich selbst entwickeln und diese auch nicht im Zentrum stehen. Unsere Forschungsarbeit hat vielmehr das allgemeine Ziel, Biomembranen und Membranproteine besser zu verstehen, und hierfür haben wir eine neue Methode untersucht.
Diese Art der erkenntnisorientierten Wissenschaft wird als **Grundlagenforschung** bezeichnet. Frei nach dem Ausdruck „Wissen ist Macht“ kann auf diese Weise gewonnenes Wissen dann wiederum als Grundlage für weitergehende angewandte Forschung dienen. Es geht

uns Grundlagenforschern also weniger um einen konkreten Zweck, wie z. B. ein Heilmittel für eine bestimmte Krankheit zu entwickeln. Man könnte im Gegenteil aber sagen, dass die Erkenntnisse aus unserer Forschung einen Beitrag dazu leisten, alle möglichen Krankheiten zu heilen. Denn diese hängen oft auf die ein oder andere Weise mit Membranprozessen zusammen. In diesem Zusammenhang möchte ich auch darauf hinweisen, dass viele bahnbrechende wissenschaftliche Errungenschaften durch Grundlagenforschung ermöglicht wurden. So wurde beispielsweise das Penicillin, das erste bekannte Antibiotikum, mehr oder weniger zufällig entdeckt (ein Schimmelpilz hatte eine Petrischale mit Bakterien infiziert und tötete diese ab). Und es ist nur der Neugier des Wissenschaftlers zu verdanken, dass diese Beobachtung den Grundstein für die Entwicklung von Antibiotika legte.
Auch das SMA-Polymer fand seinen Weg in die Membranforschung eher durch Zufall. Allerdings ist es heute noch sehr schwer abzuschätzen, was in zehn oder fünfzig Jahren aus unserer Arbeit mit SMA und Membranproteinen geworden sein wird. Eine Revolution in der Medizin, die mit Penicillin vergleichbar wäre, wird sie wohl nicht auslösen, und möglicherweise landet sie sogar im Papierkorb der Geschichte. Aber vielleicht trägt unsere Forschung ja auch zu einem weiteren Durchbruch bei, wer weiß? Eines ist jedenfalls sicher: die Anstrengung ist es wert!

Nederlandse Samenvatting

Dit hoofdstuk is gericht aan een leken lezerspubliek en voor iedereen die ik niet wil lastig vallen met alle details, maar die toch graag zou willen weten over de essentie van het werk dat ik gedaan heb tijdens mijn promotie. Ik hoop dat ik met dit korte stukje in staat ben om enig inzicht te geven in de wereld van de biofysica en in de soms bizarre concepten van het fundamenteel onderzoek.

Tijdens mijn onderzoek heb ik mezelf voornamelijk bezig gehouden met **biomembranen**. Dergelijke membranen zijn een fundamenteel onderdeel van elke cel in ons lichaam en zijn essentieel voor het leven. Hun belangrijkste taak is de structurele organisatie van de cel, deze te scheiden van zijn omgeving en daarmee te definiëren als een eenheid. Bovendien organiseren zij de cel in kleinere compartimenten die verschillende functies vervullen. Membranen zijn ondoordringbaar voor de meeste in water oplosbare moleculen en daardoor zorgen zij ervoor dat elk molecuul op de juiste plek in de cel blijft waar het zijn functie vervult. Men kan membranen dus voorstellen als poortwachters die wat orde brengen in de chaos van cellulaire moleculen. Doordat de membranen een barrière vormen voor in water oplosbare moleculen creëren zij concentratiegradiënten van deze moleculen. Een dergelijke concentratiegradiënt is de drijvende kracht voor talloze biochemische processen, variërend van energieproductie tot signaaltransductie in neuronen. Vandaar dat biomembranen van groot belang zijn voor het functioneren van cellen.

Om beter te kunnen begrijpen hoe membranen functioneren moeten we inzoomen naar het moleculair niveau. En aangezien membranen gemiddeld slechts ongeveer vier nanometer (dat wil zeggen vier miljoenste van een millimeter) dik zijn, moet men nogal een beetje ver inzoomen. Van dichtbij zijn biomembranen complexe dynamische structuren waarvan de belangrijkste componenten kunnen worden onderverdeeld in twee klassen van moleculen: lipiden en eiwitten.

Lipiden zijn relatief kleine moleculen, bestaande uit een hydrofiele (water-minnende) kopgroep en hydrofobe (waterafstotende of vet-minnende) vetzuurketens. Omdat deze hydrofobe vetzuurketens zeer slecht oplosbaar zijn in water—denk aan oliedruppels in water—vormen lipiden dus spontaan een dubbele laag in waterige oplossingen (zie figuur 2 in Hoofdstuk I). In dergelijke dubbellaagse membranen worden de vetzuurketens gericht naar de binnenzijde waar zij worden afgeschermd van de waterige omgeving, terwijl de kopgroepen contact maken met het water. Deze lipide dubbellaag vormt de basis van een biologische membraan en is verantwoordelijk voor de ondoordringbaarheid voor wateroplosbare stoffen.

De tweede component van biomembranen zijn de **membraaneiwitten**. Dit zijn moleculen die bestaan uit ketens van aminozuren en ze zijn over het algemeen veel groter dan lipiden. Het grote aantal mogelijke combinaties van aminozuren resulteert in een enorme verscheidenheid aan (membraan-) eiwitten met verschillende vormen en eigenschappen die verantwoordelijk zijn voor diverse taken. Sommige van deze membraaneiwitten versnellen biochemische reacties, bijvoorbeeld in de ademhalingsketen of tijdens de fotosynthese, processen die vooral plaatsvinden in membranen. Andere eiwitten zorgen dat de structuur

van een cel of weefsel wordt gehandhaafd. Membraaneiwitten zijn ook verantwoordelijk voor selectieve transportprocessen van moleculen door de membraan en ze vormen een belangrijke schakel in de communicatie tussen verschillende cellen. Een voorbeeld hiervan zijn receptoren die boodschappermoleculen kunnen herkennen of betrokken zijn bij het activeren van cellen van het immuunsysteem. Deze complexiteit aan functies draagt bij tot het grote belang van membraaneiwitten in medisch onderzoek, iets wat goed geïllustreerd wordt door het feit dat ongeveer de helft van alle momenteel beschikbare geneesmiddelen werken op membraaneiwitten.

Als men een individueel membraaneiwit beter wil bestuderen om meer inzicht te verkrijgen in hun functie loopt men snel tegen een vrij groot probleem aan. Het is belangrijk te beseffen dat membraaneiwitten afhankelijk zijn van hun natuurlijke omgeving om goed te functioneren, dat wil zeggen, van het samenleven met lipiden. Idealiter zou men dus graag eiwitten bestuderen in een levende cel. Er zijn echter weinig analysemethoden beschikbaar die daar gevoelig genoeg voor zijn. Dus om een bepaald eiwit te kunnen bestuderen is het nodig het eiwit te isoleren uit de cel en deze te scheiden van alle andere moleculen.
Voor in water oplosbare eiwitten, zoals het hemoglobine in onze rode bloedcellen, is dit relatief eenvoudig. Membraaneiwitten zijn echter stevig verankerd in de membraan door hun hydrofobe oppervlakken. Hoewel dit noodzakelijk is voor hun functioneren, impliceert dit dat de membraan moet worden afgebroken om de verschillende membraaneiwitten te scheiden. De standaardmethode voor het afbreken van deze membranen is deze te **solubiliseren** (oplossen) met behulp van detergenten. In het dagelijks leven zijn deze aanwezig in zeep en ze worden gebruikt om vuil en vet op te lossen. Op soortgelijke manier, lossen de in het laboratorium gebruikte detergenten membraancomponenten op in water om hun scheiding mogelijk te maken. Een nadeel van deze methode is dat tijdens dit proces het eiwit vaak gedestabiliseerd raakt, waarbij er een risico is op inactivatie en aggregatie. Eiwitaggregatie is vergelijkbaar met de processen die plaatsvinden tijdens het stollen van een kippenei tijdens het koken, en in een dergelijke toestand geven eiwitten natuurlijk erg weinig informatie over hun functioneren in de cel. Het onderzoek naar membraaneiwitten is dus aanzienlijk problematischer dan onderzoek naar oplosbare eiwitten en de karakterisering van een enkel membraaneiwit overschrijdt dan ook veruit de gemiddelde duur van een promotietraject. Het gevolg daarvan is dat ons begrip van de functie van vele membraaneiwitten nog niet heel goed is.

Hiermee, na “slechts” twee pagina’s, ben ik aangekomen op het punt waar mijn onderzoeksproject begint. Maar geloof me, dit is waar het eigenlijk pas interessant wordt! Mijn project gaat over het ontwikkelen van een methode om membraaneiwitten efficiënt te verwijderen uit de membraan en daarbij hun natuurlijke omgeving te conserveren en ze te beschermen tegen aggregatie. Om dat te doen maken we gebruik van een copolymeer bestaande uit styreen en maleïnezuur (Engels: **styrene maleic acid**, kort **SMA)**. Deze polymeer werd oorspronkelijk gebruikt als additief in de chemische industrie. Echter, SMA is ook onderzocht als een mogelijk transportsysteem voor het toedienen van medicijnen. In deze context hebben Britse wetenschappers min of meer bij toeval ontdekt dat SMA ook efficiënt werkt voor het oplossen van biomembranen. Het bijzondere is dat SMA de membraan niet volledig oplost zoals detergentia doen, maar het snijdt intacte ronde fragmenten uit de membraan, vergelijkbaar met een “cookie cutter”. Deze membraan schijven hebben een diameter van

ongeveer tien nanometer en worden daarom aangeduid als **nanodiscs** (van *nano*meter en *disc*, het Engelse woord voor schijf). In 2009 werd al aangetoond dat membraaneiwitten kunnen worden toegevoegd aan zulke nanodiscs die dan vervolgens een stabiele omgeving voor het eiwit vormen.

Op het moment dat ik mijn onderzoek startte, werd deze methode pas door zeer weinig wetenschappers gebruikt en was het nog niet duidelijk in welke mate de SMA verenigbaar is met bestaande methoden in het onderzoek aan membraaneiwitten. Daarom hebben we ons gericht op het karakteriseren van SMA-nanodiscs om de mogelijkheden te kunnen beoordelen voor de studie van membraaneiwitten. De inzichten die we gaandeweg hebben opgedaan zijn beschreven in dit proefschrift en zijn hieronder samengevat.

Hoofdstuk I is een algemene inleiding met een gedetailleerde beschrijving van biomembranen en hoe ze zijn opgebouwd. Daarna worden verschillende werkwijzen besproken die de solubilisatie van membraaneiwitten voor vervolgonderzoek mogelijk maken. De focus ligt hier op SMA-copolymeren en de toepassing daarvan als oplosmiddel voor membranen, wat het belangrijkste onderwerp van mijn proefschrift is.

Hoofdstuk II beschrijft een eerste onderzoeksproject waarin werd onderzocht hoe goed het polymeer werkt voor het oplossen en de zuivering van een membraaneiwit direct uit een biologische membraan. Als modeleiwit, kozen we een bacterieel kalium-ionkanaal dat structureel zeer vergelijkbaar is met veel humane kaliumkanalen.
Op basis van dit modeleiwit konden we aantonen dat de solubilisatie met behulp van SMA een goed alternatief is voor gevestigde detergentmethoden waarmee gemakkelijke zuivering van het eiwit mogelijk is. Bovendien werd er vastgesteld dat het kaliumkanaal stabieler is in de SMA-nanodiscs dan in detergentia, wat een groot voordeel bleek tijdens latere experimenten. Daarnaast gebruikten we het gegeven dat de directe lipide omgeving van het kanaal werd meegezuiverd in de nanodiscs om zijn moleculaire samenstelling te onderzoeken. We hebben dus nu een nieuwe analytische methode die ons informatie kan geven over de voorkeur van het membraaneiwit voor zijn lipiden partners. Een nanodisc kan dus worden gezien als een momentopname van een eiwit in zijn natuurlijke membraanomgeving. Ten slotte zijn we erin geslaagd om het kanaal te transformeren in een kunstmatige lipide membraan, waarbij bleek dat de functionaliteit van het eiwit niet was aangetast door de isolatiemethode. Bovendien was dit experiment het eerste voorbeeld van de overdracht van een membraaneiwit in een kunstmatige membraan zonder dat het ooit in contact is geweest met potentiële schadelijke detergentia. Dit laatste in het bijzonder heeft een groot potentieel voor het onderzoek naar andere membraaneiwitten, vooral als deze erg gevoelig zijn voor detergentia.

In een verdere studie, beschreven in **Hoofdstuk III**, hebben we gekeken naar hoe verschillende lipide samenstellingen van de membraan van invloed zijn op de oplosbaarheid door SMA. Hiervoor gebruikten we modelmembranen zonder eiwitten om na te gaan of er een voorkeur was in het oplossen van verschillende lipiden door SMA. Informatie over een dergelijke voorkeur is belangrijk omdat dit potentieel van invloed kan zijn op conclusies over de geprefereerde lipide partners van membraaneiwitten (zie Hoofdstuk II).

Door het systematisch variëren van de lipide samenstelling van modelmembranen, waren we in staat om aan te tonen dat er geen voorkeursbehandeling is van SMA tijdens het oplossen van membranen in een vloeibare toestand waarin de vetzuurketens van de lipiden relatief mobiel zijn. Dergelijke membranen worden zeer efficiënt opgelost door SMA en omgezet naar nanodiscs. Membranen in een geltoestand, met rigide opeengepakte vetzuurketens, worden echter zeer inefficiënt opgelost. De efficiëntie van het oplossen is dus sterk afhankelijk van de toestand waarin de membraanlipiden zijn. Een gevolg hiervan is dat er wel een voorkeur in de oplosbaarheid van membranen wordt waargenomen als deze zogenaamde fasescheiding vertonen. Membranen die fasescheiding vertonen bevatten tegelijkertijd domeinen (gebieden) in de vloeibare toestand en domeinen in de geltoestand, en beide domeinen verschillen in lipide samenstelling. In deze situatie worden de vloeibare domeinen veel sneller opgelost door SMA.
Dergelijke voorkeur in de oplosbaarheid van vloeibare domeinen zou mogelijk een toepassing kunnen hebben voor het bestuderen zogenaamde “lipid rafts” (letterlijk lipide vlotten). Dergelijke rafts zijn sterk geordende membraandomeinen die mogelijk aanwezig zijn in de membraan van levende cellen, bijvoorbeeld in het immuunsysteem, en er wordt aangenomen dat deze domeinen een specifieke functie vervullen. Echter, in de afgelopen twintig jaar is er een zeer controversieel debat gaande onder membraanonderzoekers over het belang van lipid rafts. En inderdaad is tot op de dag van vandaag het bestaan van deze rafts noch bevestigd noch ontkracht en wordt er nog steeds actief onderzoek gedaan op dit gebied.

Hoofdstuk IV beschrijft een studie waarin het effect van SMA op menselijke kankercellen onderzocht wordt om verdere informatie te verkrijgen over de oplosbaarheid. Met behulp van een speciale microscopietechniek konden we video’s maken waardoor we live konden volgen hoe levende cellen worden opgelost door de SMA.
Hiervoor werden de celkweken gesupplementeerd met kunstmatig DNA, dat vervolgens door de cellen werd opgenomen en gebruikt als bouwplan voor het maken van verschillende eiwitten. Deze bouwplannen zijn zodanig ontworpen dat de eiwitten fluorescent zijn. Dit betekent dat wanneer de cellen worden blootgesteld aan licht met een specifieke golflengte, dat wil zeggen licht met een specifieke kleur, ze licht uitstralen met een hogere golflengte, dat wil zeggen, een andere kleur. Het fluorescente licht kan vervolgens worden gedetecteerd met een speciale microscoop waardoor de visualisatie van individuele cellulaire compartimenten mogelijk is.
Met deze methode hebben we onderzocht of er verschillen zijn in de snelheid waarmee de membranen van de verschillende cellulaire compartimenten worden opgelost door SMA. Het was verrassend dat we aantoonden dat de plasmamembraan (de buitenlaag van de cel) het langzaamst oplost, ook al is deze laag de eerste die in contact komt met SMA. Verder zagen we dat verschillende eiwitten in het plasmamembraan met verschillende snelheden werden opgelost. Ook deze resultaten suggereren dat lipid rafts mogelijk invloed hebben op het oplossen van de membraan (zie Hoofdstuk III).

Voor het onderzoek beschreven in **Hoofdstuk V**, keerden we terug naar de hierboven genoemde bacteriële membranen met het kaliumkanaal (zie Hoofdstuk II). We gebruikten deze als modelsysteem om de experimentele condities voor de solubilisatie van membraaneiwitten door SMA te optimaliseren.

Met deze aanpak konden wij aantonen dat verschillende factoren bestaan die in verschillende mate invloed hebben op de efficiëntie van de solubilisatie, en hebben we condities geoptimaliseerd die de solubilisatie van eiwitten bevorderen. We vonden dat de invloed van de zoutconcentratie en pH bijzonder groot is. Zeer lage of zeer hoge zoutconcentraties voorkomen het oplossen van de membraan bijna volledig, dit kan vooral worden verklaard door de eigenschappen van de SMA polymeer zelf. Alleen zoutconcentraties die lijken op de fysiologische zoutconcentratie in ons lichaam bevorderen het oplosbaar vermogen van de SMA.
Voor de pH vonden we het omgekeerde effect voor de bacteriële membranen vergeleken met wat eerder is waargenomen voor modelmembranen zonder eiwit. Dit lijkt paradoxaal, maar er zijn hiervoor verschillende mogelijke verklaringen aan te dragen. De meest waarschijnlijke daarvan is dat de pH-afhankelijkheid van de membraanoplosbaarheid sterk gerelateerd is aan de lipide samenstelling van de membraan. Er zijn echter nog verdere studies nodig om hier een duidelijker beeld van te krijgen. Dit voorbeeld toont dan ook heel mooi aan dat er nog veel werk gedaan moet worden om volledig te begrijpen hoe het oplossen van een membraan met behulp van SMA werkt. Echter, een beter begrip van dit proces is noodzakelijk om het volledige potentieel van deze methode te kunnen begrijpen.

In **Hoofdstuk VI** worden onze resultaten in de context geplaatst van wetenschappelijke studies van andere auteurs in het kader van een algemene discussie. Verder wordt er een vooruitzicht gegeven op toepassingen van solubilisatie van membranen door SMA en worden eventuele beperkingen van de methode besproken. Als conclusie kan worden gegeven dat de studies die tot nu toe uitgevoerd zijn met SMA veelbelovende resultaten laten zien die veelzijdige toepassingen in het membraanonderzoek kunnen hebben. Het is daarom zeer waarschijnlijk dat SMA-polymeren een grote bijdrage gaan leveren aan een beter begrip van biomembranen.

Tot slot wil ik graag kort (dit keer echt) proberen om een veel gestelde vraag te beantwoorden: "**En waar is dit goed voor?**". Allereerst is dit een hele goede vraag. Helaas is het antwoord niet heel eenvoudig en een volledig antwoord valt echt buiten de strekking van dit proefschrift. Het kortste antwoord dat ik kan bedenken is: "Alles en niets". Alles, omdat de kennis die we hebben gegenereerd kan worden gebruikt in een groot aantal toepassingen. En niets omdat we zelf niet echt als doelstelling hebben om deze applicaties zelf te ontwikkelen en dit is ook niet de focus van ons werk. Ons onderzoek heeft als algemene doelstelling het verkrijgen van een beter begrip over biomembranen en membraaneiwitten, en daarvoor hebben we de mogelijkheden van een nieuwe methode onderzocht.
Dit soort op kennis gefocuste wetenschap heet daarom **fundamenteel onderzoek**. Volgens de uitdrukking "kennis is macht", kunnen inzichten die worden verkregen op deze manier dan vervolgens dienen als de basis voor verder toegepast onderzoek. Als fundamentele onderzoekers zijn we dus minder bezig met een specifieke toepassing, bijvoorbeeld om een remedie voor een specifieke ziekte te ontwikkelen. Men kan echter stellen dat de resultaten verkregen met ons onderzoek kunnen helpen om nieuwe remedies voor allerlei ziekten te vinden. Dit omdat ziekten vaak op een één of andere manier geassocieerd zijn met membraanprocessen en inzicht in hun fundamentele principes is cruciaal voor de ontwikkeling van geneesmiddelen. In dit verband wil ik er ook graag op wijzen dat vele baanbrekende wetenschappelijke prestaties mogelijk zijn gemaakt door fundamenteel

onderzoek. Bijvoorbeeld, de ontdekking van het allereerste antibioticum, penicilline, was een nogal gelukkig toeval (een schimmel had een petrischaal met bacteriën geïnfecteerd en bleek ze te doden). En het is alleen te danken aan de nieuwsgierigheid van de wetenschapper die deze waarneming maakte dat de basis werd gelegd voor de ontwikkeling van antibiotica. De SMA-copolymeer is ook slechts bij toeval ontdekt voor zijn toepassing op het gebied van het membraanonderzoek. Het is echter zeer moeilijk om nu al in te schatten wat er over tien of vijftig jaar geworden zal zijn van ons SMA-onderzoek naar membraaneiwitten. Het zal waarschijnlijk niet leiden tot een revolutie in de geneeskunde vergelijkbaar met penicilline, en wellicht is het uiteindelijk totaal vergeten. Maar misschien zal ons onderzoek een bijdrage leveren aan een andere doorbraak, wie weet? Enkel één ding is zeker: het is de moeite waard!

Curriculum vitae

I was born on April 2nd, 1987 in Saarbrücken, Germany and grew up on my parents' farm in the nearby village of Wiesbach, where I also obtained my primary education. My secondary education was provided by Illtal Gymnasium Illingen from which I graduated in 2006 as best of year. After a year of Civilian Service that I spent working with disabled people, in 2007 I enrolled in an interdisciplinary programme in biophysics (Biophysik Diplom) at the University of Kaiserslautern, Germany. In 2011, I spent a very enjoyable exchange semester at Uppsala University, Sweden. On my return to Kaiserlautern, I started my final research project in the group of Sandro Keller. I graduated in 2012 with a thesis on the "characterization of novel denaturants for membrane protein unfolding".

Later in 2012, after a brief extension of my stay with Sandro as a research assistant, I moved to the Netherlands, to work on a PhD project with Antoinette Killian in the Membrane Biochemistry and Biophysics group at Utrecht University, where I started in early 2013. The project was part of the ManiFold network on protein folding and assembly at the Bijvoet Center for Biomolecular Research and its results are described in this thesis. At many conferences and workshops both in the Netherlands and abroad, I presented our work on SMA copolymers to study membrane proteins, which was honored by several poster awards. During my time in Utrecht I was also actively involved in the organization of doctoral education both inside the Bijvoet Center and as a member of the PhD council of the Graduate School of Life Sciences at Utrecht University.

Currently, I am working on a project on interactions of the complement system with membranes together with Piet Gros at the Bijvoet Center.

List of Publications

Jonas M. Dörr, Martijn C. Koorengevel, Marre Schäfer, Alexander V. Prokofyev, Stefan Scheidelaar, Elwin A. W. van der Cruijsen, Tim R. Dafforn, Marc Baldus, and J. Antoinette Killian. **2014**. Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K^+ channel: the power of native nanodiscs. *Proc. Natl. Acad. Sci. U. S. A.* 111:18607–18612.

Jonas M. Dörr, Stefan Scheidelaar, Martijn C. Koorengevel, Juan J. Dominguez Pardo, Marre Schäfer, Cornelis A. van Walree, and J. Antoinette Killian. **2016**. The styrene–maleic acid copolymer: a versatile tool in membrane research. *Eur. Biophys. J.* 45:3–21.

Stefan Scheidelaar, Martijn C. Koorengevel, Cornelis A. van Walree, Juan J. Dominguez Pardo, **Jonas M. Dörr**, and J. A. Killian. **2016**. Effect of polymer composition and pH on the solubilization of lipid membranes by styrene–maleic acid copolymers. *Biophys. J.* 111:1974–1986.

Juan J. Dominguez Pardo, **Jonas M. Dörr**, Aditya Iyer, Vinod Subramaniam, and J. Antoinette Killian. **2017**. Solubilization of lipids and lipid phases by the styrene–maleic acid copolymer. *Eur. Biophys. J.* 46:91–101.

Beate Bersch, **Jonas M. Dörr**, Audrey Hessel, J. Antoinette Killian, and Paul Schanda. **2017**. Proton-detected solid-state NMR spectroscopy of a zinc diffusion facilitator protein in native nanodiscs. *Angew. Chem. Int. Ed. Engl.* In press, DOI: 10.1002/anie.201610441.

Jonas M. Dörr, Marleen H. van Coevorden-Hameete, Casper C. Hoogenraad, and J. Antoinette Killian. Solubilization of human cells by the styrene–maleic acid copolymer: insights from fluorescence microscopy.
Manuscript submitted.

Jonas M. Dörr, Martijn C. Koorengevel, and J. Antoinette Killian. Factors influencing the solubilization of membrane proteins from Escherichia coli membranes by the styrene–maleic acid copolymer.
Manuscript in preparation.

Juan J. Dominguez Pardo, **Jonas M. Dörr**, Mike F. Renne, Tarik Ould-Braham, Martijn C. Koorengevel, Mies J. van Steenbergen and J. Antoinette Killian. Thermotropic properties of phosphatidylcholine nanodiscs bounded by styrene–maleic acid copolymers.
Manuscript in preparation.

Yao Liu, Elisabete G. M. M. Moura, **Jonas M. Dörr**, Stefan Scheidelaar, Michal Heger, Maarten R. Egmond, J. Antoinette Killian, Tamimount Mohammadi, and Eefjan Breukink. Purification and characterization of Bacillus subtilis MraY in detergent or detergent-free styrene–maleic acid copolymer systems.
Manuscript in preparation.

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Then I almost had a third paranimf. Sort of. **Camilla**, it's a great pity you won't be able to make it to my defense. Thanks for powering up the now for me and for your shared passion for tree hugging. We need to have whiskys again more often and we're totally gonna finish that Almodóvar movie one day! Also, I clearly need a lesson from you on "How to date on festivals". Thanks a bunch also to your significant other Tim—although I secretly hate him a little because *GuessWho* is responsible that you won't be around for my defense? And Camilla, I will never quite eat tofu(e) the same way again…
Thanks to you all for everything! How about a summer in the woods Captain Fantastic style with the four of us? I mean, now that we'll have time…

None of those people I would have met if it hadn't been for a certain ManiFold project. And there were of course a couple more people involved. Martina thanks so much for your big heart (btw, it's clearly bigger than… you know…). You always managed to cheer me up, no matter what, and usually you didn't even need laser tag for that. Tania, I hardly ever met anyone with such a positive attitude towards, well, just about everything. I guess I even got used to your kisses in the end. Thanks for all the good vibes. Daniel, I much enjoyed our science discussions, particularly those over beers at Sterk. A belated sorry for being such a pedantic dork in the beginning sometimes. I might actually even have adjusted to a somewhat more Southern European attitude towards punctuality by now. Also, let me know if you need new clothes again at any point. If our shopping is gonna be as successful as our last attempt we'll end up with a perfect outfit for a certain bike ride of yours... ;) Saran, the most important lesson I might have learned from you: don't EVER eat the chilis in Indian food!!! Yang, the cutest, most harmless, adorable Chinese there is. I would even

say my favorite one but I don't do rankings. I'm gonna miss your friendly face around. All the best for Switzerland. Can't wait for a picture of Suri on her pony! Also to the rest of the pack, Anna, Ivan and Klemen, thanks for your company. I'm gonna miss our dinners, everyone. Best of luck for finishing up your theses! And a final ManiFold thank you goes to Stefan for putting together the grant that lured such delightful people to the Netherlands.

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MBB of course is not just a SMA lab although some Friday mornings may have given that impression. Thanks also to everyone else for your help in the lab and for interesting lunch discussions on diverse topics ranging from banana berries to…, well, let's just keep it at *very* diverse topics, shall we? I much enjoyed all the cakes and cookies and the borrels we had. Lisette, cheers for sharing much and caring more. Keep up the good spirits, crazy bird lady! Hope you find fulfilment in your teaching. I'm gonna miss you around, especially when the beat says Sean da Paul! Sabine, from an incredibly successful first language tandem dinner to some later help with certain summaries: your language input was much appreciated as were our exchanges on vegetable gardening. And if Heiko one day is up for a rematch in Catan, do let me know. Thanks also for all the events you hosted at your lovely place! Inge, thanks for bringing a pleasant calmness to the lab and for all the fun stuff you organized. Borrels aren't quite the same without you and Peter. Yao, multitasking is exactly your thing, right? Thanks for many a nice chat and for triggering speculations on the existence and identity of your *companion*. Michal, even after all the years I'm still not sure whether you actually did buy your PhD. Thanks for remaining enigmatic. Dr. Furse, please allow me to offer thee my gratitude for our office cohabitation for two years in length. Thanks for all that talk on politics and language, even if the latter sometimes rather contributed to confusion than clarification. Hope you found some more challenging bike trails up there north! Remko, or should I say Dr. Zig-a-zig-ah?, my gels look so much prettier since you showed me a couple of tricks. Pity you left the angels already so early on. Xue, your soundtrack choices were always a welcome alternative to the sometimes bizarre compilations by our colleagues and it was always nice to come to the lab and

see a friendly face, even on the weekend. Margriet, I much enjoyed having you here and cracking codes together. Super nice that you got this fungus job! Mike, thanks for being a partner in the fight against the chaos in the lab. Further good luck with this struggle! I'm afraid I smell cherries nowadays when it comes to that… And btw, I'm really jealous that you won that ticket to Corsica. Well, yes there was Corsica! Eefjan, thanks so much for organizing and taking me along. This was one of the best experiences I made in quite a while. Mónica, thanks for your shared appreciation of the creative use of toilet brushes in the creation of sound effects and good luck with finishing your projects. Many thanks also to everyone else: Tine (really miss your bread), Toon, Joost, Ruud, Paulien, Greg, Patricia (those chocolates…), Amrah, Tami, Xiaoqi, Yang, Thomas (thanks for reminding me of one of my better quotes), Beth and everyone else who was here for a while. Thanks also to all the secretaries that made sure that the chaos stayed in the lab. Cécile, you made it a super smooth start for me. Caroline, I much enjoyed your company. Next time we'll do weizenbier *with* the alcohol! And Noortje, I would have been lost without you. Thanks for everything! Most of all for providing a stern voice when needed. ;)

I was fortunate enough to be a member of not only one but two amazing labs. So cheers to all you structural biologists! Much enjoyed my time up there with you. Deniz, treasure hunts, pub quizzes,…: we need to team up organizing stuff again soon! But I heard you're busy enough with planning a wedding. Also thanks for sorting out everything with the printing deal. And btw, I'm not gonna leave this country before we have our McFlurry! Remco, it's a pity to see you leave but maybe it's the right decision to tell those membrane proteins to go screw themselves. But how are we gonna implement Deep Fryday now? Dimphna, *getver*, what was it with all this name confusion? Was it Heddi or Matwich or both? Keep mixing them up… Well, thanks for that! Great to have you, though. I'm a huge fan of your humor. Hedwich, right? Thanks for an amazing wadlopen experience and for many a (critical) conversation over and on AiO evening food. And Robbert, I'll be sure to drop by to fresh up my French before my next trip down there. Matti, always fun to be around a trumpeter (less so a trumpeteer…). I'll miss your wits but I guess they're in rather good company in the Valhalla of structural biology. Double-u-out, Wout, glad you took over all the Bijvoet AiO stuff. It's gonna be a fantastic experience. It's the best. Really. Knock on wood! And thanks for the *rose* and for teaming up with Dimphna to sort out pronunciation issues once and for all. Ramon, I still don't get why such a famous singer would do a PhD on the side… Don't forget to switch off that light in Brabant next time! And I really hope to meet your girlfriend one day. Revina, I absolutely admire your tenaciousness in going after this Wnt stuff while still keeping up high spirits. Guess such an attitude is required in that field. Why else would they have they named that thing Dickkopf? Vivi, much appreciate your efforts to make sure everyone stays healthy and thus providing a platform for many a nice chat. Tim, your sarcasm always came in quite refreshingly. Pity though that you prefer Jan Primus over Hugo Kruyt. Joke and XG, I much appreciate your help with all the complement stuff! Also thanks to the Frenchies, Matthieu et Lucas, Louris, Bert, Eric, Arie, Martin, Loes, Nick, Thomas and everyone else, as well as Laura and Robert and the ever-growing EM crowd. You're all making up a very fine group and it's always been nice to drop by for a chat.

Well, actually there was even a third lab that sort of adopted me from time to time. Particularly for activities that rather happened after regular working hours. Thanks dear experts of

molecular weighing for bringing that much friendliness and fun to the sixth floor. Be it an Oktoberfest, public viewing or karaoke: Ain't no party like an NPC party! Michiel, much appreciate your second opinion, both on language and on religious issues such as the ManiCult. Clément, pub crawls will never be the same without you. The word insistence comes to mind… Esther, thanks to you *Daar gaat ze* made it to the top of my Winter 2015 playlist! Clochard next Friday? Alice (that was your name, right?), thanks for keeping me (Alex, that is) company, I miss you here already. Cheers also to Arjan, Domenico, Kylie, Fan, Andrey, Martin, Celine and everyone else. Thanks for pulling the most amazing group costume ever. Down that rabbit hole with all of us!
Andrea, thanks for many a dip in the Kromme Rijn and for an amazing trip to Andermatt. It's a pity that the times of coffee on campus are over but it's always nice to grab a beer at Sterk (provided fingers are still strong enough) and discuss the *wulps*ness of it all.

I much enjoyed my time here at the Bijvoet Center and the great environment it offers. Thanks, Reinout for doing such an amazing job in organizing everything and for your contribution in attracting such an impressive amount of outstanding speakers.
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And talking about Dutch lessons: Yassine I'm really glad we managed to stay in touch. Always fun to discuss politics with you. And man: Sufjan!

Ook alle Vlerken bedankt voor de mooie tijd op het speelveld en bij de borrels na het volleyballen!

And for the last bit I'm gonna switch to German.

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