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Editorial

Structure and function of proteins in membranes and nanodiscs

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ABSTRACT

The field of membrane structural biology represents a fast-moving field with exciting developments including native nanodiscs that allow preparation of complexes of post-translationally modified proteins bound to biological lipids. This has led to conceptual advances including biological membrane:protein assemblies or "memteins" as the fundamental functional units of biological membranes. Tools including cryo-electron microscopy and X-ray crystallography are maturing such that it is becoming increasingly feasible to solve structures of large, multicomponent complexes, while complementary methods including nuclear magnetic resonance spectroscopy yield unique insights into interactions and dynamics. Challenges remain, including elucidating exactly how lipids and ligands are recognized at atomic resolution and transduce signals across asymmetric bilayers. In this special volume some of the latest thinking and methods are gathered through the analysis of a range of transmembrane targets. Ongoing work on areas including polymer design, protein labelling and microfluidic technologies will ensure continued progress on improving resolution and throughput, providing deeper understanding of this most important group of targets.

Isolation of membrane proteins from their native lipid environment typically requires the use of harsh detergents, however various non-detergents options also exist, including liposomes, bicelles, and nanodiscs, which can be formed using membrane scaffold proteins, amphipols styrene maleic acid (SMA)-related copolymers. This provides an expanding range of possible media solubilizing various membrane proteins for a range of techniques, as described in this issue. The Verhelst group provides a comprehensive review of these methods and furthermore focuses on their use with the intramembrane protease class of membrane proteins [1]. Application includes their use in kinetic assays, structural determination and lipid studies. The choice of extraction procedure and appropriate lipid mimetic must be tailored for the intended use of the protein being extracted.

Native nanodiscs have emerged as a revolutionary tool for preparing intact membrane:protein complexes. The efficiency of membrane solubilization into such nanodiscs under a wide range of solution conditions was compared by Killian's group [2]. Analysis of the tetrameric KcsA ion channel solubilized from *Escherichia coli* membrane with a short SMA(2:1) copolymer with a 2:1 ratio of S:MA subunits shows optimum copolymer concentrations of 0.5–1% under temperatures in the 25–37 °C range with incubation times of 1–2 h. Lower temperatures warrant longer incubations, while excessive polymer concentrations should be avoided to minimize aggregates. Optimal ionic strength (300–450 mM NaCl), divalent cations (< 2 mM) and pH (8–9) are evident for KcsA solubilization but can be protein and membrane dependent as they are subject to respective charge distributions. Comparisons with the performance of conventional detergents suggests that the development of neutral or basic copolymers related to SMA could offer advantages, providing avenues for solubilization and analysis of a broader array of biological membrane:protein assemblies (memteins).

The utility of SMA lipid particle (SMALP) technology for analysis of memteins by mass spectrometry (MS) and X-ray diffraction methods remains limited. This problem stems from the requirements of the respective gas phase or crystalline samples and the heterogeneity of currently used SMA copolymers. In order to address this, Muench et al. have developed a method to transfer membrane proteins from SMALPs into amphipols or detergents for downstream analysis [3]. The homotrimeric *E. coli* multidrug transporter AcrB was used as a test case. The vulnerability of SMA to precipitation in the presence of divalent cations was exploited by using stepwise addition of $MgCl_2$ from 0.5 to 2 mM to cause the solubilizing copolymer to precipitate in the presence of a rescue solution composed of amphipol A8–35 or 1% n-dodecyl-β-D-maltoside (DDM) detergent. The resulting amphipol and detergent complexes are more homogeneous, tolerate higher divalent cations levels, and yield observable signals by native mass spectrometry, including of phosphatidylglycerol in the amphipol-stabilized protein trimer.

New methods are being applied to analyze the process of forming nanodiscs from membranes in greater detail. In particular, a Microfluidic Diffusional Sizing (MSD) system which was recently developed to detect protein hydrodynamic radii between 0.3 and 20 nm has been adapted to probe nanodisc formation [4]. This microfluidics technology requires small sample volumes (< 10 μL) which are injected into a microfluidic chip. The complexes diffuse across from one side of the chamber to the other during a 15 min time course and are then sensed *via* primary amines of, for example, phosphatidylethanolamine. Several stages can be reliably seen from comparison of MDS and dynamic light scattering (DLS) data collected with increasing ratios of SMA(3:1) to lipid. First the polymer inserts into bilayers until it reaches saturation. This is followed by solubilization into particles with 30–10 nm diameters. As excess polymer is applied these become progressively smaller until reaching a limiting diameter of 6 nm. The lipid-

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specific association of an aggregation peptide derived from the Tau protein is also demonstrated, indicating utility for detecting how fibers are nucleated on membranes.

Two longstanding challenges are the characterization of endogenous heteromultimeric membrane proteins and the development of conformation-specific antibodies for memteins. A study by Mouro-Chanteloup and coworkers of red blood cell ghosts addresses these confounding issues [5]. The individual components of erythrocyte membranes including Rh proteins are well known, but their multimolecular protein lipid complexes are dissociated in detergent-based preparations and hence no longer recognizable or extractable using conformation-specific antibodies. In contrast using SMA(3:1) copolymer for solubilization preserves the native states of Rh complexes, and major membrane-associated cytoskeletal proteins can be gently removed by washing in very low ionic strength buffer. The biological relevant states of detergent-free Rh complexes can then be isolated using gel filtration and monoclonal antibodies. The native states are preserved based on detection by conformation-dependent antibodies, paving the way for antibody screening and structure-function analysis.

The extraction and purification of low abundance drug targets from membranes in stable, functional forms is a key goal for the pharmaceutical industry. The G-protein coupled receptor (GPCR) superfamily is critical, being targeted by many therapeutic agents. They respond to a diversity of signals and mediate cellular responses through interactions with heterotrimeric G proteins, GPCR kinases and arrestins. The complexity and conservation of the interactions of the phosphorylated and palmitoylated vasopressin receptor type 2, its peptide agonist vasopressin and arrestin is modeled by Fanelli and coworkers, revealing coupled motions between key GPCR sites [6]. Conformational changes that are induced by ligands including full agonists, partial agonist, antagonists and inverse agonists are membrane-dependent. The adenosine 2A (A2A) receptor is a well-characterized GPCR from a structural perspective, although the roles of dynamics and lipids remain unclear. After expression in *Pichia pastoris*, the wild-type A2A receptor and a pair of mutants with Trp to Tyr substitutions were solubilized into nanodiscs using SMA(2:1) copolymer by Wheatley et al. [7]. A fluorescent reporter (IAEDANS) was also attached to an introduced Cys residue by the sixth helix, providing an additional probe. Binding of specific ligands induces concentration-dependent changes in the fluorescence signals of Trp residues, which exhibit greater steric hindrance based on fluorescence anisotropy measurements. The association with phospholipids from the plasma membrane is apparent by MS/MS analysis of the SMALPs. Watts et al. show that the dopamine D1 receptor can be purified from human embryonic kidney (HEK) cells with a yield of 255 μg/L using SMA(3:1) copolymer [8]. As with the A2A receptor, the purification of this GPCR was carried out at 4 °C to preserve the native state. Binding studies of the protein in SMALPs provides estimated native-like affinities for a neurotensin peptide ligand and a specific antagonist.

The transport of materials including drug molecules out of cells is carried out by the ATP binding cassette (ABC) family of membrane transporters. The structures and functions of these proteins are reviewed by Kanelis and coworkers, with an emphasis on the intrinsically disordered regions that mediate key interactions in a phosphorylation-dependent fashion [9]. A set of ABCG2 protein constructs were designed with N-terminal GFP or SNAP and His_6 tags and solubilized from HEK cells using SMA(2:1) copolymer, as were CD28 and CD86 control proteins [10]. Kerr et al. then used fluorescence correlation spectroscopy (FCS) to study the diffusion of a fluorescent substrate molecule as it interacts with the ABCG2 protein, providing a useful alternative to radioactive ligand binding assays. Two binding components with differing diffusion coefficients are observed, with control nanodiscs and inhibitors demonstrating the specificity of substrate binding to the ABCG2 homodimer.

A diverse array of transmembrane proteins of agricultural interest remains structurally uncharacterized, yet is important targets for crop plants. A plant plasma membrane known as Salt Overlay Sensitive 1 (SOS1) is involved in sensing and responding to salt stress and is important for optimal plant growth. Constructs representing the 1146 residue *Arabidopsis thaliana* version of this Na^+/H^+ antiporter were designed with Enhanced Green Fluorescent Protein (eGFP) reporters by Fliegel's group. This allowed confirmation of relatively high levels of intracellular expression in *Pichia*. Following solubilization using SMA(2:1) copolymer and affinity purification of the His^8-tagged proteins, yields of 0.6–2 mg/L are obtained [11]. Comparisons of the expressed proteins indicate that inclusion of the large cytoplasmic domain, which binds cyclic nucleotides, reduces solubility, while a functional element is evident C-terminal to the last transmembrane helix. The proteins could be reconstituted from SMALPs into soy azolectin-based vesicles using $MgCl_2$, with the longest construct then showing native-like Na^+/H^+ exchanger activity upon addition of external NaCl. The cation/proton antiporters, which mediate the exchange monovalent metal ions for protons, are characterized by Stetefeld and coworkers [12]. The structure and interactions of the cytoplasmic domain of a K^+/H^+ antiporter from *Vibrio cholerae* known as NhaP2 are studied using NMR and small angle X-ray scattering. The 165 amino acid residue construct forms a monomeric state in solution that includes pair of structural domains joined by a flexible linker, with an ATP binding site in the N-terminal domain.

Antibiotics include lipid-specific peptides that self-associate into pores, which permeabilize bacterial membranes. However, the intact pores are difficult to resolve due to their dissociation in detergents. Palmer's lab studies daptomycin, a cyclic lipopeptide consisting of 13 amino acids and a decanoic acid group that is a critical line of defense against infections caused by Gram-positive bacteria. It works by binding phosphatidylglycerol-containing membranes in a calcium-dependent manner to form octameric pores. The design of alternating SMA copolymers with methylamine sidechains allows octamers and tetramers to be obtained by varying on the amount of added polymer, illuminating the assembly of membrane complexes [13]. This paves the way for structure-determination of the native pore state and design of improved antibiotics to combat multi-drug resistant bacteria. Aquaglyceroporin proteins allow the selective permeation of molecules across membranes. This family includes the *E. coli* glycerol facilitator, which mediates passive diffusion of glycerol across the inner membrane of the bacterium. Although prone to aggregation, careful optimization of its expression and solubilization in various media showed that this helical bundle protein is most monodispersed in lauryl maltose neopentyl glycol [14]. This detergent stabilizes the physiological tetramer, although a non-native octamer persists due to interactions between the disordered termini.

The dynamics of lipids in nanodiscs formed by SMA(3:1) and diisobutylene/maleic acid copolymer (DIBMA) copolymers, which differ in their hydrophobic groups, are compared by Steinhoff and coworkers [15]. The application of electron paramagnetic resonance (EPR) spectroscopy and a set of phosphatidylcholine lipids with nitroxide groups located at the 5th, 12th or 16th carbon atom positions reveal that the lipids are more constrained in the more stable SMALP discs. This can be explained by coarse-grain molecular dynamics simulations, which indicate that in the case of DIBMA a single belt of polymer contains lipids within the nanodisc with dynamics more closely resembling their mobility in a liposome.

In the Overduin lab, new amphipathic copolymers are being developed to broaden the utility of native nanodiscs. A series of alkylamine derivatives of SMA with alternating sidechains were designed to reduce polymer sequence heterogeneity and improve resolution [16]. Comparison of SMA(1:1) copolymers with methylamine, ethylamine and propylamine sidechains yields advantages including increased solubilization, particularly of protein multimers, and broader pH and divalent cation compatibility. The most generally useful appears to be the methylamine derivative, which solubilizes *E. coli* outer membranes containing the PagP protein into homogeneous nanodiscs with 14 nm

diameters. The SMA(1:1) derivatives with ethylamine and propylamine groups yield larger nanodiscs with diameters of approximately 25 and 32 nm, respectively, in line with their greater hydrophobicity.

In the major facilitator superfamily, various solute carrier (SLC) families exist. One of the challenges is to functionally characterize these transporters. In particular, a different transporter function may be present for the same transporter when it resides in different cell types. The Cordat lab determined that the SLC26A7 protein is a chloride/bicarbonate exchanger in kidney cells, which is in contrast to its role as a chloride channel in oocytes [17]. Furthermore, they show that the abundance of this protein at the cell surface is dependent on the osmolarity of the cell culture medium, and pH of the cells. This is important considering the SLC26A7 is expressed in outer medullary collecting duct cells near acid secreting cells that can render the local environment hyperosmotic compared to plasma.

In human cells, both concentrative and equilibrative nucleoside transporters (CNT and ENT) are found. With multiple transporters in this family having overlapping function it is important to characterize each individually. Radioactive HPLC was used by the Young lab to determine the metabolism of nucleosides in oocytes, for which little previous study has been conducted [18]. This study revealed little metabolism occurred in oocytes and helped develop this novel means to examine nucleoside levels. Next, in mice, the contribution of CNT and ENTs, which have overlapping specificities, were examined using radioactive HPLC. Nucleotide metabolites were assessed from plasma of mice with CNT or ENT knockouts, illuminating the specificities of these transporters *in vivo*.

The SecA protein works to facilitate the transport of proteins destined for secretion through the SecYEG translocon. Despite structural information that reveals helical nucleotide binding domains, how SecA interacts with diverse secretory proteins remain a question in the field. To address this, the Bondar group conducted a sequence analysis of bacterial SecA proteins (425), which was guided by sequence alignment, structural analysis and phylogeny [19]. They identified that SecA proteins have varied length as a result of insertions and deletions, with clustering revealing three main groups. The first 220 residues housing the nucleotide binding region 1 (NBD1) is highly conserved in the family, with preferred residues at the N-terminus, which include a conserved Phe at position 10, followed by hydrophobic sequences, and an Arg/Lys, are important for lipid anchoring. In addition they highlight the Tyr residue located at the end of a helix finger structural motif, and other charged residues that may play a role in recognition of the positively charged pre-sequence. They further suggest diversity in sequence length contributes to increased conformational dynamics that may influence target interactions.

The development of new tools to produce and analyze of integral membrane proteins is covered in a timely review by Danmaliki and Hwang [20]. These targets remain challenging to characterize at atomic resolution by any method. Their analysis by nuclear magnetic resonance (NMR) spectroscopy is complicated by the requirement for many milligrams of pure protein containing ^{2}H, ^{13}C and ^{15}N isotope labels to ensure high sensitivity and resolution, as well as the complex dynamics within the bilayer that broaden signals from transmembrane regions. Advanced isotope labelling protocols allow chemical groups such as methyl and methylene groups and ^{19}F nuclei on specific residue types to be resolved, yielding structural information from such moieties. Successes include an array of β barrel structures, which have been solved in various detergent micelles, as well as α-helical structures that are inherently more challenging as they exhibit greater chemical shift degeneracy and fewer observable distances between secondary structure elements. Hence NMR is well-positioned to provide unique insights into dynamics and interactions, as well as structures of proteins that are small or difficult to crystallize.

Single pass transmembrane proteins pose challenges for structural biology, being particularly dynamic, prone to aggregation and dependent on lipid microenvironments. Consequently, very few have been characterized at high resolution. Ramamoorthy et al. solubilized the full length form of the microsomal protein cytb5, which includes a heme-containing soluble domain, transmembrane helix and flexible linker, into nanodiscs using an 18-residue amphipathic helical peptide and various synthetic lipids [21]. Through expression in *E. coli* the four tryptophan residues could be labelled with fluorine-19 for detection by ^{19}F NMR spectroscopy. Due to the rapid tumbling of the ~8 nm diameter nanodiscs, the ^{19}F signals are resolvable by solution state NMR. The resonance from the single Trp in the soluble domain is particularly sharp, while the other three from within the transmembrane domain are broadened, becoming resolved only at higher temperatures such as 48 °C. Chemical shift perturbations observed in presence of phosphatidylserine and line broadening induced by addition of full-length cytochrome P450 2B4 protein indicate functional relevancy to the endoplasmic reticulum-resident form.

Multi-domain proteins that reversibly bind membrane surfaces provide technical challenges for unravelling biological pathways. The Phafin2 protein plays a critical role in inducing autophagy, but its membrane recognition mechanism is unclear. This modular protein contains a pleckstrin homology (PH) domain and a (Fab1, YOTB, Vac1, and EEA1) FYVE domain, both of which bind phosphoinositol 3-phosphate (PI3P), a key signaling lipid that is found in endocytic membranes. The Capelluto group showed that its FYVE domain is indispensable for constitutive and specific PI3P recognition [16]. In contrast, its PH domain binds acidic lipids such as phosphatidylserine or PI3P when they are present in bilayers (but not as soluble lipids). The PH domain is autoinhibited by interactions with a conserved acidic C-terminal motif. Thus the multivalent and tightly regulated binding of proteins to membrane surfaces can deciphered *via* the individual interactions. In the future soluble and homogeneous nanodiscs may offer opportunities to reveal the native binding mechanisms *via* concerted recognition of multiple lipids and protein elements that are heavily phosphorylated.

Together, these studies illustrate the challenges and potential of the growing field of membrane structural biology. Membranes have traditionally been the least well understood components of the cellular ecosystem, and new tools have been sorely needed. The coming years promise further gains as the interfaces between proteins and lipids become better understood, and are certain to yield many more secrets into memtein formation and function. Our ability to probe such mechanisms in native states and recombinant forms will in turn lead to improved understanding and exploitation of membrane targets for applications including synthetic biology and drug discovery.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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