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Non-micellar systems for solution NMR spectroscopy of membrane proteins

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Abstract

Integral membrane proteins play essential roles in many biological processes, such as energy transduction, transport of molecules, and signaling. The correct function of membrane proteins is likely to depend strongly on the chemical and physical properties of the membrane. However, membrane proteins are not accessible to many biophysical methods in their native cellular membrane. A major limitation for their functional and structural characterization is thus the requirement for an artificial environment that mimics the native membrane to preserve the integrity and stability of the membrane protein. Most commonly employed are detergent micelles, which can however be detrimental to membrane protein activity and stability. Here, we review recent developments for alternative, non-micellar solubilization techniques, with a particular focus on their application to solution NMR studies. We discuss the use of amphipols and lipid bilayer systems, such as bicelles and nanolipoprotein particles (NLPs). The latter show great promise for structural studies in near native membranes.

Introduction

Membranes represent hydrophobic barriers that separate intra- and extra-cellular spaces of cells, and allow the compartmentalization into organelles. They primarily consist of lipid bilayers, whose constitution varies largely between different membranes. In addition to forming hydrophobic barriers, membranes fulfill many other functions, such as energy transduction, import and export of nutrients and drugs, signal recognition, and cell-to-cell communication. These tasks are primarily mediated by membrane proteins in their specific membrane environments. Despite their importance, our knowledge on structural aspects of membrane proteins is still sparse [1]. Membrane proteins pose several challenges to their biophysical characterization, ranging from difficulties in expression (reviewed in [2]) to non-destructive extraction or refolding, and purification (reviewed in [3]). Beyond these difficulties, a major limiting factor is the requirement for a membrane mimicking environment that preserves the integrity and stability of a membrane protein outside its native cellular environment [4]. The most commonly employed method to solubilize membrane proteins in aqueous solution are micelle-forming detergent molecules [5], which are amphipathic molecules, consisting of a hydrophobic and a hydrophilic portion. At concentrations below their specific critical micellar concentration (cmc), detergent molecules exist as monomers in

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aqueous solution. Above the cmc, multimeric detergent micelles assemble that are in a highly dynamic equilibrium with detergent monomers. A large number of detergents with a wide range of chemico-physical properties are available.

However, detergents are often not optimal for the activity and stability of a membrane protein [3]. Micelles are highly dynamic assemblies and their intrinsic instability can result in protein unfolding and aggregation. This is particularly problematic if the membrane proteins contain larger moieties that reach out into the aqueous extra-membranous space as detergents may destabilize or unfold those portions. Additionally, the lateral forces acting on a membrane protein in the roughly spherical detergent micelle are significantly different from those in the native planar membrane [6,7]. Overall, a detergent micelle thus only poorly mimics the architecture and physical properties of a cellular membrane.

In many cases, the presence of detergent molecules interferes with the experimental conditions required for monitoring protein activity by enzymatic, ligand-binding or spectroscopic assays. Furthermore, the particular chemico-physical requirements of a given membrane protein is usually only met by few, if any, out of the numerous detergents available. There is currently no reliable method that could predict a compatible detergent for a given membrane protein. The search for a detergent that stabilizes a functional conformation of a membrane protein is thus a highly empirical and time-consuming process. As a consequence, suitable solubilization conditions have yet to be found for many membrane proteins.

For these reasons, alternative solubilization techniques are highly desirable for the functional and structural characterization of membrane proteins. Here, we discuss recent developments in the use of amphipols, bicelles and nanolipoprotein particles (NLPs) as alternative membrane mimics (Figure 1). These non-micellar systems are portrayed here with a particular focus on their applicability to solution NMR spectroscopy, which is a key technique for functional and structural studies of membrane proteins at atomic resolution [8,9]. Within the small number of available membrane proteins structures there are even much fewer structures of complexes of membrane proteins. In this respect, solution NMR shows great promises for the characterization of protein–protein and protein–ligand interactions. Moreover, it can also provide valuable insight into membrane protein dynamics.

In almost all cases, the non-micellar membrane-mimicking systems discussed here rely on detergents to extract the membrane protein of interest from its native membrane or precipitate. A noteworthy exception is the cell-free synthesis of membrane proteins in the presence of liposomes [10–13] or preassembled NLPs [14].

Amphipols

One alternative to detergents are amphipathic polymers, short “amphipols”, which have been developed mainly by Jean-Luc Popot and colleagues [15]. Amphipols consist of polymeric backbones that are covalently modified with a stochastic distribution of hydrophobic and hydrophilic groups (Fig. 1d). The use of amphipols as detergent-free membrane substitutes that conserve the membrane-protein function, has been successfully shown in a number of cases, including the β -barrel proteins OmpA [16], FomA [16], and OmpX [17], as well as the α -helical protein diacylglycerol kinase [18], bacteriorhodopsin [16,19] and several G-protein coupled receptors [20].

The feasibility of studying amphipol-complexed membrane proteins by solution NMR spectroscopy has been demonstrated for the 171-residue transmembrane domain of the outer membrane protein A (tOmpA) from *Escherichia coli* [21] and the outer membrane protein X (OmpX) from *E. coli* [17]. Two-dimensional (2D) [^{15}N ; ^1H]-Transverse Relaxation-Optimized Spectroscopy (TROSY)-NMR spectra exhibited dispersed cross peak patterns similar to the

detergent-solubilized protein, indicating a native fold of the protein in complex with the amphipol. The solvent accessibility of amide protons was studied for OmpX by hydrogen/deuterium exchange NMR experiments [17]. In an attempt to apply amphipols for the investigation of α -helical integral membrane protein, we recently recorded 2D [^{15}N ; ^1H]-TROSY spectra of bacteriorhodopsin in complex with amphipols (Fig. 2f; unpublished data). A comparison with detergent-solubilized bacteriorhodopsin (Fig. 2d) showed an almost identical pattern of cross peaks, corroborating the utility of amphipols for the structural characterization of membrane proteins by solution NMR.

With respect to solution NMR studies, a limitation of the currently mostly used amphipol A8–35 is its low solubility at acidic pH. Therefore, NMR studies using this amphipol are limited to a pH above 7, which can lead to exchange broadening and loss of signals from solvent exposed amide protons. Thus, amphipols that are pH-insensitive might be more suitable for solution NMR studies [22].

Lipid bilayer systems

Amphipols, as well as detergent micelles, are able to stabilize membrane proteins in aqueous solution, but they do not closely resemble the native lipid bilayer. For many applications however, a membrane mimic comprising a lipid bilayer is preferable, as it may better maintain the structural integrity of a membrane protein. This requirement is met by three classes of membrane mimics, i.e. liposomes, bicelles and NLPs. In the case of liposomes, limited solubility, the formation of multilamellar vesicles and the inaccessibility of the vesicle interior may, however, interfere with functional investigations. Nevertheless, high resolution structural information of membrane proteins in liposomes may become accessible, predominantly by solid-state NMR techniques [23–26].

Bicelles

Bicelles are binary, water-soluble assemblies of lipids and detergents. An idealistic picture of a bicelle is that the lipids form the central part and the detergents form the edge of a disc-shaped assembly (Fig. 1b). In this way, bicelles can provide a roughly circular patch of a lipid bilayer in aqueous solution. In reality however, there is a large distribution of shape, size and composition [27].

Whereas several characteristics of bicelles are beneficial over micelles, a disadvantage for their wide use in solution NMR experiments is their larger molecular weight compared to micelles, leading to broad resonance lines. Recent reports have however indicated that bicelle studies with NMR are well feasible (e.g. [28–30]). The use of bicelles is favorable for the study of interactions that are not retained in micelles. An example is given by the $\alpha_{\text{IIb}}\beta_3$ integrin dimer, which consists of a lateral interaction of two transmembrane helices. This dimer is formed in bicelles, but not in micelles, and its structure could be determined by solution NMR in bicelles [31]. It was also shown that a β -barrel membrane protein in a bicelle is embedded in the central, lipidic phase and is probably not in contact with the detergent surrounding the lipidic phase [32]. Limiting factors for bicelles include the constricted number of lipid-detergent combinations amenable to bicelle formation, their instability, and their size heterogeneity.

Nanolipoprotein particles

The introduction of nanolipoprotein particles (NLPs), also referred to as nanodiscs or reconstituted high density lipoprotein particles (rHDLs), as membrane mimics has provided a novel tool for studying membrane proteins in a native-like membrane environment. NLPs consist of a non-covalent assembly of phospholipids arranged as a discoidal bilayer, surrounded by amphipathic apolipoproteins (Fig. 1e). In their native context, apolipoproteins assemble into

roughly spherical high density lipoprotein particles (HDLs), responsible for the reverse transport of cholesterol from the peripheral tissues to the liver [33]. The *in vitro* reconstitution of HDLs [34] has served as a basis for the development of disc-shaped rHDLs for membrane protein solubilization, a methodology pioneered by Stephen Sligar and colleagues [35,36]. The NLP technology has since been successfully explored to investigate a large number of membrane proteins by a multitude of biochemical and biophysical methods (reviewed in [37, 38] and representative examples shown Table 1).

In general, the native-like bilayer architecture provided by NLPs is likely to support both protein stability and functionality of an incorporated membrane protein. The *in vitro* reconstitution of NLPs from purified lipids or membrane extracts offers the unique possibility to precisely mimic the native composition of a particular membrane and to probe the effect of selected lipids on membrane protein function. It is now well established that many membrane proteins require specific lipids for functional reconstitution, and specific lipid binding sites have been identified on several membrane proteins [39–41].

Many biophysical methods require monodisperse protein samples. Other than for bicelles, the confinement of the lipids in NLPs is achieved by an integral number of apolipoproteins, resulting in a discrete particle size distribution [42]. Currently, the process of NLP assembly can not be controlled entirely, thus resulting in a distribution of NLPs with discrete diameters due to different copy numbers of the integrated apolipoproteins [43].

NLPs render membrane proteins water-soluble in the complete absence of detergent molecules. The confinement of the lipid bilayer by the amphipathic apolipoprotein increases the stability of NLPs when compared to other membrane mimicking systems. Restricting the lateral motion of NLP-embedded membrane proteins by the proteinaceous edge further reduces potentially adverse protein–protein interactions, thus efficiently preventing protein aggregation. At the same time, NLPs are planar and of relatively small size, thus allowing the simultaneous access to both, the extracellular and cytoplasmic domain of an embedded membrane protein. These features make NLPs an ideal system for the application of functional enzyme assays that are often compromised by the presence of high concentrations of detergent. Moreover, NLPs permit the application of general biochemical purification methods.

NMR applications of NLPs

The application of solution NMR to membrane proteins in NLPs has been attempted only recently (Table 1). The membrane-active fungal peptide Antiamoebin-I (Aam-I), a member of the peptaibol family of peptides with antimicrobial activity, was incorporated into DOPG NLPs [44]. The 2D [^{15}N ; ^1H]-TROSY spectrum of Aam-I in DOPG NLPs exhibited similar chemical shift resonances when compared to DMPC/DHPC bicelles, however, showing significantly broader ^1H line-widths. The topology of membrane-associated Aam-I in lipid bilayers was further characterized with the spin-label 16-doxylstearate, revealing a peripheral membrane association of the single helix peptide. A comparison of the NMR spectra of Aam-I in various membrane systems suggested the structure and dynamics of Aam-I to be different in NLPs than in classical membrane mimics (DMPC/DHPC bicelles, LMPC and LMPG micelles) [45].

The feasibility to study membrane proteins by solution state NMR spectroscopy was further exemplified by incorporating the potassium channel KcsA from *Streptomyces lividans* into NLPs [45]. From a number of different tested lipids, KcsA reconstituted into DMPC NLPs yielded functional tetrameric assemblies, and 2D [^{15}N ; ^1H]-TROSY spectra revealed mobile domains of the potassium channel that are exposed to the aqueous solution.

The spectroscopic characterization of integral membrane proteins embedded in NLPs was further explored with the membrane spanning fragment of the human CD4 receptor [46]. Incorporation of an isotope-labeled CD4 fragment, comprising a single transmembrane helix, into POPC NLPs yielded 2D [^{13}C ; ^1H]-HSQC spectra of reasonable signal dispersion in both dimensions, resembling an equivalent spectrum recorded in DPC micelles. Based on the observed ^1H line-widths, additional local mobility is suggested for the observed residues, which might represent flexible regions of the protein in the solvent exposed termini.

Recently, we have shown with the human voltage-dependent anion channel (VDAC-1) that large polytopic integral membrane proteins reconstituted in NLPs can be studied by solution NMR [47]. 2D [^{15}N ; ^1H]-TROSY NMR spectra of VDAC-1 in DMPC NLPs exhibited well dispersed resonances, resembling the NMR spectra recorded in LDAO-micelles in terms of number of resonances and overall dispersion. The observation of distinct chemical shift changes upon addition of the native ligand NADH demonstrated functionality of VDAC-1 embedded in NLPs. Interestingly, the NLP/VDAC-1 particles could be imaged with negative stain electron microscopy, revealing pores with an identical diameter as determined structurally and observed in micrographs of native membranes [47]. Thus, by the use of NLPs, one has the possibility to study the same membrane protein preparations both by NMR and electron microscopy. Recently, we successfully recorded a 2D [^{15}N ; ^1H]-TROSY NMR spectrum of bacteriorhodopsin in DMPC NLPs, thus being able to compare spectral properties of bacteriorhodopsin in three different membrane systems, i.e. detergent micelles, amphipols, and NLPs (Fig. 1(d)–(f), unpublished data).

The use of NLPs to screen detergent micelles or bicelles as membrane mimics was recently presented for the isolated voltage-sensing domain (VSD) of the potassium channel KvAP from the archaeobacteria *Aeropyrum pernix*. In the absence of a functional assay for the isolated VSD, a 2D [^{15}N ; ^1H]-TROSY spectrum recorded in NLPs served as a reference for the screening of a suitable membrane mimic [48].

These studies have demonstrated the feasibility of investigating NLP-embedded membrane proteins by solution NMR despite the relatively large size of the NLP–protein complex. The effective molecular weight of a protein–mimic complex has important implications for the applicability of solution NMR spectroscopy. An increase in size leads to larger rotational correlation times and thus to wide resonance line widths, which in turn are associated with low sensitivity and resonance overlap. The smallest reported NLP has a diameter of about 9.5 nm [42], corresponding to a theoretical rotational correlation time of 85 ns (τ_c) at 30°C and a molecular weight of about 200 kDa [44]. The experimental determination of the rotational correlation time for the polytopic integral VDAC-1 protein in NLPs yielded a rotational correlation time of 93 ± 15 ns [47]. These values suggest that the structure determination of integral membrane proteins in NLPs is in principle possible, provided that transverse relaxation optimized spectroscopy (TROSY) at high magnetic field strengths in combination with high deuteration levels of protein backbone and side chains is used.

Conclusions

The biophysical investigation of membrane proteins at atomic resolution is hampered by the difficulties in maintaining solubilized membrane protein in a native conformational state. Solubilization of membrane proteins by detergent micelles is well established, however, this artificial membrane-mimicking system often impairs protein integrity. In recent years, a number of non-micellar membrane systems have emerged, providing complementary means of membrane protein solubilization. These alternative membrane systems have properties distinct from detergent micelles, facilitating novel approaches towards a functional and structural characterization of membrane proteins. Another approach, not covered in the current

review, employs reverse micelles to solubilize membrane proteins. The use of reverse micelles for solution NMR studies has been developed mainly by the lab of Joshua Wand [49]. Recently, their application to integral membrane proteins has become possible as demonstrated for the potassium channel KcsA [50,51]. Whereas the general applicability has yet to be verified, this approach may open intriguing new possibilities for structural and functional studies of membrane proteins. Here, we have reviewed the use of amphipols, bicelles and NLPs with a particular focus on their applicability to solution NMR spectroscopy.

Whereas bicelles represent a well-established membrane environment for solution NMR studies, amphipols and NLPs represent relatively new membrane mimics. Noteworthy to say, that solution NMR spectroscopy is currently the method of choice to pursue an investigation of membrane proteins at atomic resolution in either amphipols or NLPs, as membrane proteins in these membrane mimics have so far not been accessible to crystallization. As evident from the progress made in recent years, both amphipols and NLPs represent promising alternatives to the prevalent use of detergent micelles for the characterization of integral membrane proteins by solution NMR spectroscopy. The NMR spectra of the 32 kDa β -barrel protein VDAC-1 and the heptahelical protein bacteriorhodopsin exhibit comparable spectral properties in the different membrane mimicking environments (Figure 2), thus encouraging more detailed studies. The observed spectroscopic sensitivity and resolution of the non-detergent systems is expected to enable a detailed structural characterization and their use should be strongly considered if protein stability needs to be increased and/or native structure is not retained in a micelle environment. The feasibility to record 2D [^{15}N ; ^1H]-TROSY spectra of integral membrane of sufficient quality lays the foundation for more sophisticated experiments aiming at a high-resolution structure determination. In particular, the highly favorable properties of NLPs are anticipated to facilitate the NMR characterization of integral membrane proteins in an environment that contains the constituents of the native membrane. This approach has the promise to provide relevant novel biological insights.

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Figure 1. Schematic representation of different solubilization methods for integral membrane proteins. Micelle (a), bicelle (b), reverse micelle (c), amphipol (d), and nanolipoprotein particles (NLP; e). Shown are cartoons depicting each solubilization system. The membrane protein is depicted in gray, with its hydrophilic and hydrophobic regions in light and dark gray, respectively. Detergents are colored in magenta and lipids in green. The amphipol polymer chain is shown as black lines; the negative charges are indicated by minus signs. The apolipoprotein is represented as a blue ring. With the exception of the reverse micelle assembly (c) all systems are in aqueous solution. The reverse micelles (c) are dissolved in organic solvents; areas containing aqueous solution are indicated.

Figure 2.

Characterization of human VDAC-1 and bacteriorhodopsin in different membrane mimicking media. 2D [^{15}N ; ^1H]-TROSY spectrum of VDAC-1 incorporated in LDAO micelles (a) and in DMPC-nanolipoprotein particles (NLPs, b). Electron micrograph of negatively stained VDAC-1 in DMPC-NLPs (c). 2D [^{15}N ; ^1H]-TROSY spectra of bacteriorhodopsin in DDM micelles (d), DMPC-NLPs (e) and A8-35 amphipols (f). Note that the experimental NMR time and protein concentration vary for these samples. In particular, the protein concentrations in the micelle samples were substantially higher than those in the respective non-micellar preparations, resulting in increased quality of TROSY cross peaks in (a) and (d). (panels (a)–(c) reproduced with permission from [47], (d)–(f) unpublished data).

Table 1

Selected studies of membrane proteins and membrane associated peptides in complex with nanoporoprotein particles (NLPs).

Membrane associated peptide	
Antiamoebin-I	Solution NMR spectroscopy [44]
Channels and transporters	
Potassium-channel (KcsA)	Solution NMR spectroscopy [45]
Voltage-dependent anion channel (VDAC-1)	Solution NMR spectroscopy, TEM [47]
Voltage-sensing domain (VSD) of the potassium channel (KvAP)	Solution NMR spectroscopy [48]
Translocon complex (SecYEG)	Protein interaction studies, crosslinking assays, steady-state FRET [52]
Multidrug transporter (EmrE)	Cell-free protein expression, ligand binding assays [14]
Anthrax toxin pore	TEM [53]
Cytochrome P450 enzymes	
Cytochrome P450 (CYP)	AFM [54], Solution SAXS and enzymology [55], spectropotentiometric titrations [56], ligand screening by localized SPR (LSPR) [57], solid state NMR spectroscopy [58], ligand binding assays [59], scanning stop-flow kinetics and time-resolved fluorescence spectroscopy [60], Co-incorporation of cytochrome P450 and P450 reductase, enzymology [61], UV/Vis spectroscopy, X-band EPR spectroscopy [62]
Cytochrome P450 reductase (CPR)	AFM [35], spectropotentiometry [63], fluorescence spectroscopy and spectroelectrochemical titrations [63]
7-TM?receptor proteins	
β 2-adrenergic receptor (β 2AR)	Ligand binding assays [64,65], Co-incorporation with heterotrimeric G-protein, TEM, single-molecule fluorescence imaging, FRET analysis [64]
Bacteriorhodopsin	AFM [66–68], UV/Vis spectroscopy, TEM and fluorescence-detected linear dichroism (LD) [66], solution SAXS and visible circular dichroism spectroscopy [69], Cell-free protein expression [14,67], time-resolved FTIR spectroscopy [67]
CD4 peptide	Solution NMR spectroscopy [46]
Chemoreceptor Tar	Enzyme assays [70]
Epidermal growth factor receptor (EGFR)	Enzyme assays [71]
Glycolipid receptor G_{M1}	Immobilization and kinetic analysis of cholera toxin binding by SPR [72]
Integrin	Analytical ultracentrifugation, TEM, protein binding studies [73]
Rhodopsin	UV/Vis spectroscopy, SAXS, protein interaction studies and fluorescence spectroscopy [74], SAMDI-TOF mass spectroscopy and SPR [75]
Miscellaneous proteins	
Tissue factor (factor III, CD142)	Protein interaction studies with plasma serine protease (factor VIIa), enzyme assays, SPR [76]
Annexin	Lipid interaction studies using microfluidic channels [77]
Membrane-bound hydrogenase	Enzyme assays [78]

Abbreviations: AFM, atomic force microscopy; TEM, transmission electron microscopy; SAXS, small angle X-ray scattering; SPR, surface plasmon resonance; FRET, Förster resonance energy transfer; NMR, nuclear magnetic resonance.