The development of Nanodisc technology as a model membrane system in membrane protein research.

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ABSTRACT

This literature research focuses on the recent development of a new artificial system in which membrane proteins can be reconstituted: Nanodiscs. It will introduce with a general overview of the model membrane systems used for membrane protein reconstitution in biochemical research. A short review of early research into the high density lipoprotein complex is then followed by in-depth reviews of the article describing the engineering of the original membrane scaffold protein that makes up the Nanodisc and two following studies that further developed the membrane scaffold protein. Another three in-depth reviews cover the first membrane protein to be reconstituted into Nanodiscs, the demonstration of the use for Nanodiscs in single-molecule studies and the development of oligomeric Nanodisc reconstitution, followed by recent examples of the Nanodisc technology applied to membrane protein research.

INTRODUCTION

Proteins associated or attached to the membrane of a cell or organelle are vital to all cellular life. Such membrane proteins are responsible for the transport of chemicals and information across the membrane, and as such they perform a variety of essential biological functions in cellular metabolism, communication and transport and the maintenance of membrane gradients and ion concentrations. Cellular structure, movement and enzymatic activity are also dependant on membrane proteins. The importance of membrane proteins to cellular life is reflected by several estimates. The protein content of membranes is generally 50%, ranging between 18% in myelinated neurons and 75% in mitochondrial membranes[1]. A genome-wide analysis of *Homo sapiens*, *Escherichia coli* and *Saccharomyces cerevisae* estimates that 30% of the proteins encoded by these organisms will be integral membrane proteins[2]. The importance of membrane proteins in pharmaceutics is demonstrated by the estimate that 70% of drug targets are membrane proteins[3].

Despite the increasing number and knowledge about membrane protein sequences, the three-dimensional structure and the behaviour of many membrane proteins within the membrane *in vivo* remains largely unsolved. The difficulties in determining these are caused by several characteristics that are inherent to most membrane proteins. Firstly, most membrane proteins are only expressed at very low levels *in vivo*, so that purification from their native environment will not yield sufficient amounts. Attempts at overexpression of the membrane protein often result in aggregation of the membrane protein in the cytoplasm, so purification of membrane proteins remains a challenge[4]. Secondly, the native environment of membrane proteins is a mosaic lipid bilayer. This means that many membrane proteins are insoluble in aqueous solution and need to be reconstituted in artificial systems that represent the native membrane. Not only is this reconstitution challenging in itself, but such artificial systems present problems of their own, since they limit the use of many biophysical techniques while the extent to which the artificial system represents the native environment is debatable. In this literature research, we discuss the development of the Nanodisc technology as a model membrane system over the past decade and to which extent these inherent problems have been dealt with.

MODEL MEMBRANE SYSTEMS

Detergent micelles

Detergents are amphiphilic molecules, structurally containing a hydrophilic head group attached to a hydrophobic tail group. In aqueous solution they self-associate to form micelles, which are spherical aggregates with the hydrophilic region of the molecules on the surface in contact with the solvent and the hydrophobic region of the molecules in the centre of the micelle. Membrane proteins are often soluble in detergent micelles, as the hydrophobic centre and hydrophilic circumference of the micelle provide similar conditions as those found in the native bilayer membrane environment. This has made detergents important tools in the study of membrane proteins and their use is often essential in the isolation, purification, solubilisation and manipulation of membrane proteins in preparation for further experiments, as well as in many methods for the reconstitution and recrystallization of membrane proteins. Specific detergents differ markedly from one another in properties such as the conditions under which micelles are formed, and the choice of which specific detergent to use in membrane protein research is based on the properties that different detergents have that make them suitable for different kinds of experimentation. While detergent micelles are very useful for solubilising and handling membrane proteins during many procedures necessary in membrane protein research, they also provide destabilizing conditions for membrane proteins and as a consequence, many membrane proteins are not functional in detergents, or are denatured over time. Additionally, detergent micelles are a poor model of the biological membranes that membrane proteins normally inhabit, so model membrane systems that more closely resemble the native lipid bilayer and has a smaller effect on the functionality of membrane proteins are required.

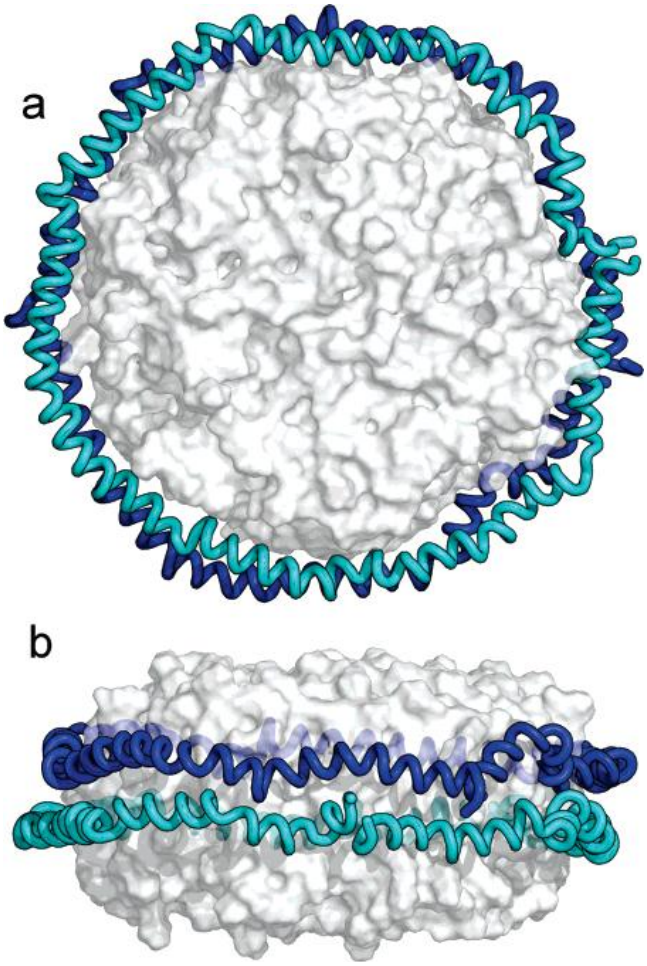

Fig. 1 Schematic model of Nanodisc, with two membrane scaffold proteins (blue and cyan) wrapped around the hydrophobic region of a phospholipid bilayer (white) to create a small discoidal phospholipid bilayer.[6]

Detergent-lipid micelles and bicelles

Lipids are a wide group of biological amphiphilic molecules that constitute the main structural components that make up biological membranes. The main difference between lipids and detergents is the multimolecular structures formed by aggregation, where detergents tend to form micellar aggregates while lipids are more likely to form bilayers. By mixing lipid and detergent solutions together, micelles containing both molecules can be formed. Membrane proteins can then be solubilised in these detergent-lipid micelles, where the hydrophobic tail groups of the lipids coat the hydrophobic regions of the membrane proteins. Such detergent-lipid micelles provide more stable conditions for membrane proteins, and have allowed the study of several membrane proteins that are not functional in detergent micelles. Under the correct conditions, mixing certain detergents with short chain lipids will lead to the formation of bicelles. Bicelles are discoidal structures that retain some bilayer characteristics and have low detergent concentrations, and are an even further improvement on solubilising membrane proteins in conditions that more closely resemble the native lipid bilayer. This allows for the functional reconstitution of a higher variety of membrane proteins as compared to

detergent-lipid micelles, although studies also indicate that membrane proteins show a lower activity when reconstituted in detergent-lipid bicelles.

Proteoliposomes

Liposomes are bilayer phospholipid vesicles, and are called proteoliposomes when they contain membrane proteins. These proteoliposomes resemble the native lipid bilayer even more closely than did detergent-lipid micelles and bicelles and are especially useful in transport activity studies. Several criteria need to be fulfilled before they can be used for functional studies, such as homogeneous liposome size, even distribution of protein over the liposomes, low membrane permeability and the stabilisation of membrane protein functionality. Although proteoliposomes closely resemble the native lipid bilayer and are very suitable for research that depends on the directionality and topology of the membrane proteins in the membrane, their large size and number of membrane proteins present per proteoliposome can be a restriction to structural and functional studies. For further reading on the properties of these three model membrane systems we direct towards a comprehensive review by Seddon et al[5].

Nanodiscs

This literature research will describe the development of the Nanodisc technology over the last decade. Nanodiscs are small bilayer discs of lipid, enclosed by membrane scaffold proteins covering the hydrophobic region of the bilayer **(Figure 1)**. Membrane proteins can be functionally reconstituted in such Nanodiscs through various methods. The lipid content of Nanodiscs is controllable and can closely resemble the native lipid bilayer, and coupled with the small and uniform size of the Nanodiscs this model membrane system is ideal for functional and structural research of membrane proteins on a single-molecule level[6].

DEVELOPMENT OF THE NANODISC TECHNOLOGY

High density lipoprotein complex

The transport of lipids within the lymphatic and circulatory systems is facilitated by lipoproteins. These lipoproteins are assemblies formed by the binding of apolipoproteins to the lipids to be transported. The smallest human lipoprotein is a lipoprotein complex called the high density lipoprotein (HDL)[7], and consists of human apolipoprotein A-I (apoA-I) binding to cholesterol[8]. Reconstitution of lipids other than cholesterol with this apolipoprotein resulted in the homogeneous formation of small discoidal bilayer lipoprotein complexes (rHDL) of rougly 5 nm in thickness and 10 nm in diameter[9,10,11]. Electron microscopy (EM)[9,10,13], small-angle X-ray scattering (SAXS)[11,12], neutron scattering (NM)[13], sequence analysis[14] and polarized internal reflection infrared spectroscopy (PIRIS)[14] examinations of rHDL lead to the development of a structural model where the apoA-I is wrapped around the edge of a bilayer disk of lipid with a series of amphipathic α-helix domains oriented parallel to the bilayer plane covering the hydrophobic section of the bilayer[15]. The complex also contains additional globular protein domains required for the interaction of HDL with cellular receptors and enzymes. The formation of rHDL complexes of homogeneous size, shape and stoichiometry was shown to be dependent on the initial lipid to protein ratio as well as on the physical state of the lipid[16].

The possible application of discoidal bilayer lipoprotein complexes based on Apo-I in membrane protein research was initially demonstrated by the reconstitution of functional cytochrome P450 oxidoreductase (POR) in rHDL[17]. In this study, rHDL formation from Apo-I and dipalmitoyl phosphatidylcholine (DPPC) was induced in the presence of solubilised POR. Analysis of the resultant lipoprotein complexes showed that rHDL complexes containing POR had formed without a decrease in the enzymatic activity of POR. These rHDL/POR particles were stably imaged over time with scanning force microscopy (SFM), revealing isolated POR enzymes bound to the bilayer of the rHDL complexes and defining the reconstitution of membrane proteins in rHDL complexes as a novel technique in structural and functional membrane protein research.

MSP	optimal lipid/MSP ratio	lipid/MSP	MSP/disk	diameter (nm)	bilayer area (nm²)
MSP1 DPPC	100	78 ± 3	2	9.3	42 ± 2
MSP1 POPC	75	62 ± 5	2	9.1	43 ± 3
MSP2 DPPC	200	171 ± 6	1	9.3	46 ± 2
MSP2 POPC	150	129 ± 9	1	9.0	45 ± 3

Tbl. 1 Properties and composition of Nanodiscs[18].

Engineering of the membrane scaffold proteins

This lead to engineering of a synthetic gene expressing a protein derived from apoA-I, called membrane scaffold protein 1 (MSP1)[18]. MSP1 is a deletion 1-43 mutant of apoA-I, conserving the 200 amino acid sequence that forms the amphipathic α-helices of apohA-I while removing the N-terminal sequence that forms the globular protein domains of the HDL. MSP1 additionally contains an N-terminal 6-His tag followed by a Factor X protease cleavage site. The self-assembly of purified MSP1 with DPPC or 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) was analysed by size-exclusion chromatography (SEC) and resulted in the homogeneous formation of approximately 10 nm Nanodisc particles containing 2 MSP1 molecules and a quantized amount of lipid per nanodisk. A fusion protein of two MSP1 molecules with a GT-linker called MSP2 was engineered and also made to self-assemble into Nanodiscs containing DPPC and POPC, and several properties of MSP1 and MSP2 Nanodiscs containing DPPC and POPC were examined using gel filtration chromatograms (GFC), scanning probe microscopy (SPM) and circular dichroism spectroscopy (CDS). The optimal lipid to MSP ratio for formation of homogeneous Nanodiscs, the number of phospholipids per MSP, the number of MSPs per nanodisk, the diameter of the Nanodiscs and the bilayer area as calculated from the number of lipids per disk, are given in **Table 1**. The formation of MSP2 Nanodiscs was found to be less homogeneous and did not result in Nanodiscs of increased size. Instead, use of MSP2 seemed to abolish the requirement of bimolecular assembly. An important conclusion is that the optimal lipid to MSP ratio for homogeneous Nanodisc formation varies for different MSPs and lipids used, implying the importance of optimising the lipid to MSP ratio for Nanodisc self-assembly under different conditions using GFC.

A following study examined the effects of changing the length of the sequence coding for the amphipathic helices of MSP1[19]. One, two or three 22-mer amphipathic helices were added in a central part of MSP1 to produce a series of extended proteins, respectively MSP1E1, MSP1E2 and MSP1E3. A series of two truncated proteins was produced by removing the first 11 and 22 N-terminal amino acids of the amphipathic helices sequence, respectively MSP1D1 and MSP1D2. These MSPs were self-assembled into Nanodiscs containing DPPC or POPC, and the structure and composition of these Nanodiscs was examined by SEC and SAXS and compared. The results of this comparison are given in **Figure 2a**. While the Nanodiscs formed by MSP1E1, MSP1E2 and MSP1E3 show an exponential increase in size, the Nanodiscs formed by MSP1D1 and MSP1D2 do not show a similar decrease in size, indicating that the first 22 amino acids of the amphipathic helices sequence might not be necessary for the formation of the Nanodiscs. A comparison of Nanodiscs formed with the same MSP but different lipids showed that while there was no difference in the size of the Nanodiscs, the lipid to protein stoichiometry did change. From this was concluded that the MSP used is the determinant of the size of the Nanodiscs formed, while the lipid contained in the Nanodiscs is the determinant of the lipid to protein stoichiometry for a particular MSP. The optimal lipid to MSP ratio for homogeneous formation of Nanodiscs was again found to differ between the MSPs, further stressing the importance of optimising the lipid to MSP ratio for Nanodisc self-assembly under differing conditions.

A recent attempt to further increase the size of Nanodiscs was based on the hypothesis that the formation of 10 nm Nanodiscs by MSP2 was caused by a flexible domain composed of the first 22 N-terminal amino acids in the amphipathic helices sequence[20]. Three new MSPs were engineered, all based on the sequence of MSP1D1 fused together through a GT-linker to a second copy of the amphipatic helices sequence of MSP lacking the first 11, 22 or 17 N-terminal amino acids,

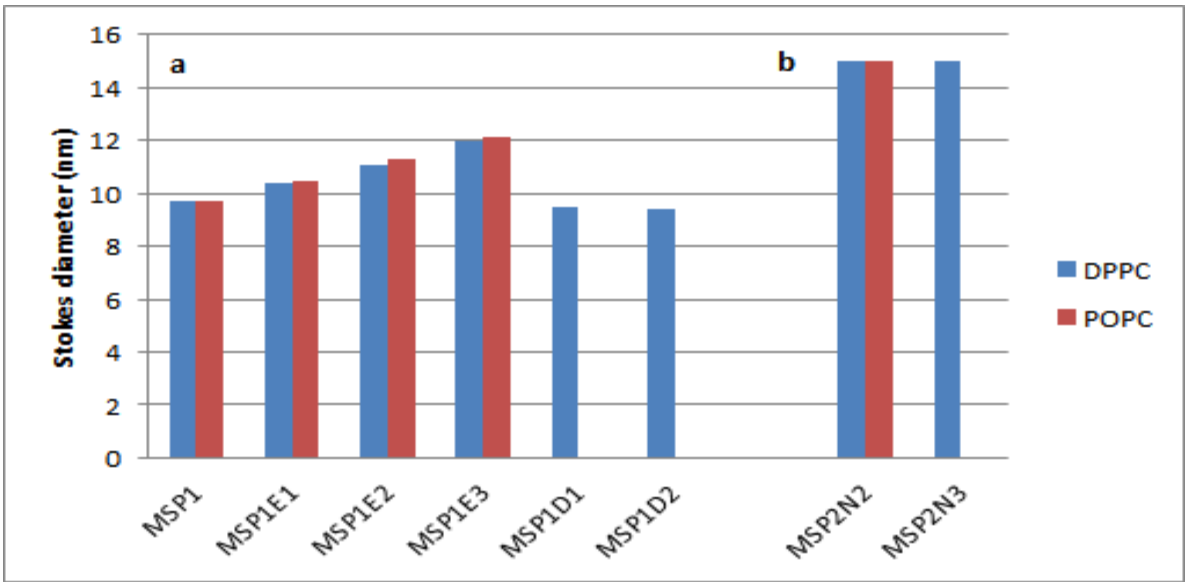

Fig. 2 Stokes diameter for several MSPs experimentally determined by SEC. **a** Results from Denisov et al., 2004[19]. **b** Results from Grinkova et al., 2010[20].

respectively MSP2N1, MSP2N2 and MSP2N3. Reconstitution of these MSPs into Nanodiscs containing DPPC and analysis by SEC and SAXS resulted in a significant increase in Nanodisc size, although homogeneous formation of Nanodiscs was not achieved for MSP2N1. MSP2N2 was also reconstituted with POPC to test assembly with different lipids, and the results can be seen in **Figure 2b**. These results support the hypothesis of a flexible N-terminal fragment and that the MSP used is the determinant of the size of the Nanodiscs. A simple formula (**Equation 1**) has been described where the size of the Nanodiscs, as given by the product of the number of lipids per Nanodisc (N_L) and the mean surface area per lipid in the Nanodisc (S), is a function of the number of amino acids in the MSP that interact with the bilayer disk (M). This formula can be used to approximate the optimal lipid to MSP ratio for homogeneous formation of empty Nanodiscs.

$$N_L S = (0.423M - 9.75)^2 \qquad \textbf{(Eq. 1)}$$

Reconstitution of membrane proteins in Nanodiscs

The cytochrome P450 superfamily played a large role in the early development of the Nanodisc technology. Not only was cytochrome P450 oxidoreductase the first membrane protein to be reconstituted into rHDL[17], its family member cytochrome P450 monooxygenase was similarly the first membrane protein to be reconstituted into Nanodiscs[21]. For this first reconstitution of a membrane protein in Nanodiscs, *Spodoptera frugiperda* was infected by a baculovirus to heterologously overexpress the N-terminally anchored cytochrome P450 monooxygenase CYP6B1. The cell membranes were collected and solubilised in detergent and added to a solution containing MSP1. Removal of detergent by BioBeads induced formation of Nanodiscs, which were isolated by affinity chromatography on the His-tag of the Nanodiscs. SEC showed that the Nanodiscs formed were of similar size and shape as empty Nanodiscs while an SDS-PAGE gel confirmed the presence of CYP6B1 as well as endogynous membrane proteins, with a stoichiometry of one CYP6B1 per 10 Nanodiscs. Examination of the phospholipids present in the Nanodiscs by thin-layer chromatography (TLC) showed that the lipid content of the cell membranes had been preserved in the Nanodiscs, demonstrating the ability of Nanodiscs to mimic the native membrane bilayer conditions. A microtiter assay for the catalytic activity of CYP6B1 indicated that the membrane protein function had been preserved over the Nanodisc self-assembly process. The successful reconstitution of CYP6B1 directly from cell membranes into Nanodiscs while preserving the lipid composition of the native cell membrane and the catalytic activity of CYP6B1 demonstrated the potential of the Nanodisc technology as a model membrane system in membrane protein research.

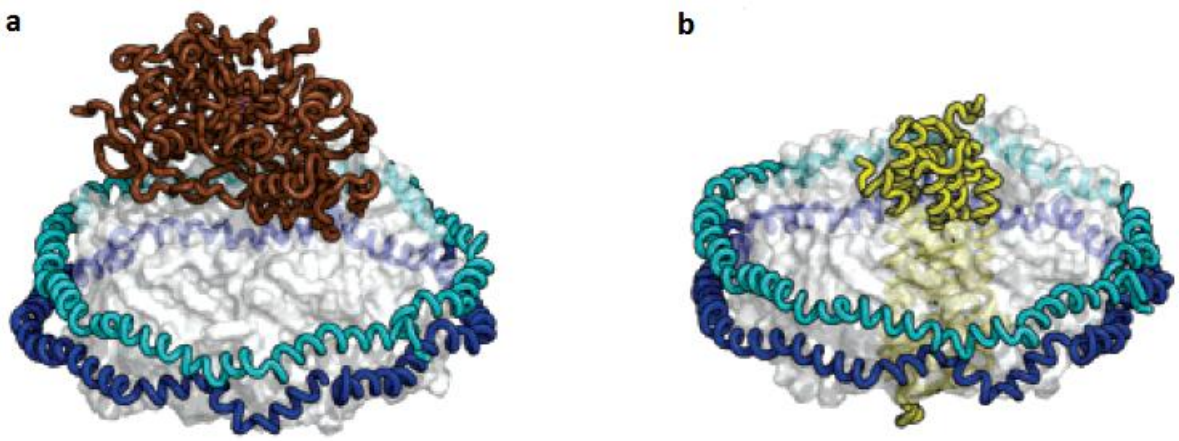

Fig. 3 Schematic models of membrane proteins reconstituted in Nanodiscs consisting of two MSP molecules (blue and cyan) wrapped around a phospholipid bilayer (white). **a** Reconstituted CYP6B1 (brown)[21]. **b** Reconstituted monomeric bacteriorhodopsin (yellow)[22].

The monodispersity of the oligomeric state of membrane proteins reconstituted in Nanodiscs was first examined by the reconstitution of *Halobacterium salinarum* bacteriorhodopsin (bR) into MSP1 Nanodiscs[22]. bR is a well-studied G-protein–coupled receptor of the seven-transmembrane class of integral membrane proteins notable for the formation of a purple trimeric state *in vivo*[23] that can be easily distinguished from its monomeric state by spectroscopic analysis, making it ideal as a model protein in studies on oligomeric state. Reconstitution into Nanodiscs of an excess of MSP1 and dimyristoylphosphatidycholine (DMPC) relative to solubilised bR was reconstituted into Nanodiscs. Analysis using EM, SFM, SEC, CDS, linear dichroism spectroscopy (LDS) and bilayer probe orientation measurements (BPOM) showed the inclusion of functional monomeric bR into monodisperse soluble Nanodiscs of similar size, shape, and stoichiometry as found for empty MSP1 Nanodiscs as shown in **Figure 3b**. This demonstrated the use of Nanodiscs as a tool for solubilisation, purification and biophysical analysis of monodisperse single-molecule membrane proteins.

A subsequent study further examined the effect of several variable properties of Nanodisks on the oligomeric state of bR captured in the Nanodisc[24]. bR was reconstituted at a 3:2 bR-MSP ratio in Nanodiscs formed by MSP1, MSP1E1, MSP1E2 or MSP1E3 with DMPC to provide an average of three bR molecules per Nanodisc. The optimal DMPC to MSP ratios for Nanodisc self-assembly containing bR were approached for each MSP using SEC. The optimal ratios were found to be consistent with the displacement of about 135 DMPC molecules per Nanodisc containing three bR molecules as compared to the ratios for empty Nanodiscs. The displaced lipid surface area is in agreement with earlier studies on the amount of lipid displaced by bR trimers in archaeal membrane[25]. These results indicate that subtraction of lipid to account for the surface area of a membrane protein of known structure is a valid approximation for the optimal lipid to MSP ratio for homogeneous Nanodisc self-assembly. CDS was then used to determine the presence of trimeric bR in the Nanodiscs. Although multiple bR molecules were present in all samples, only MSP1E2 and MSP1E3 Nanodiscs were shown to contain trimeric bR. MSP1E3 Nanodisc self-assembly with 3:2 bR-MSP ratio was then induced under varying bR-DMPC ratios, and analysis using CDS showed that an an optimal ratio was reached for 4 bR molecules per MSP1E3 Nanodisc. These results imply that capturing a membrane protein in its desired oligomeric state in Nanodiscs can be accomplished using MSPs forming Nanodiscs of optimised size at optimised initial membrane protein to Nanodisc ratios during the self-assembly process.

Nanodisc technology has since then been utilised in a diversity of functional and structural studies on a variety of membrane proteins. Monomeric CYP3A4 displayed clean monophasic reduction kinetics when reconstituted in Nanodiscs[26] and similarly allowed redox potential measurements[27], presenting the Nanodisc technology as a useful tool to monitor the molecular function of monomeric membrane proteins. Reconstitution of CYP3A4 into Nanodiscs enabled the use of Magic-angle solid state NMR[28] as well as single-molecule fluorescence[29] spectroscopy on respectively the structure and function of CYP3A4. The model of interaction of the peptide translocon

complex SecYEG with the motor protein SecA has been examined using Nanodisc-reconstituted SecYEG in known oligomeric state[30] while the effect of the local phospholipid environment on blood coagulation protein tissue factor has been investigated by reconstitution in Nanodiscs of variable lipid content[31]. The reconstitution of various receptors in Nanodiscs successfully solubilised the receptors while preversing their functionality, allowing for structural and functional studies of G-protein coupled receptors[32,33,34], bacterial chemoreceptors[35] and epidermal growth factor receptor[36]. These examples demonstrate how the use of Nanodiscs can provide new insights on the structure and function of a range of membrane proteins through a variety of biochemical and biophysical methods.

CONCLUSION

Over the last decade there has been a steady increase in the understanding of and control over the Nanodisc self-assembly process. Nanodiscs of diameter sizes ranging from 9 nm to 17 nm can be obtained commercially and a simple formula can be used to approximate the initial conditions necessary to obtain monodisperse Nanodiscs containing the desired membrane protein at the desired oligomeric state in the desired lipid environment. Nanodiscs have also been shown to enable a diversity of biophysical and biochemical methods that are not compatible with any other model membrane system. This facilitates the novel reconstitution of a broad range of membrane proteins and gives researchers the ability to tightly control the conditions under which they carry out their experiments and analyse their results on a single-molecule level. This makes Nanodiscs ideal for biochemical research and it is likely that the use of Nanodisc technology in membrane protein research is going to increase in coming years to become an important tool for the structural and functional characterization of membrane proteins. Although some theoretical papers have been published on the molecular mechanism of the self-assembly process[37], little empirical evidence has been obtained yet. A proper understanding of the mechanism of Nanodisc self-assembly is going to be increasingly important to further improve the quality of experiments utilising Nanodisc technology.

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