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REVIEW

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Nanodiscs: A toolkit for membrane protein science

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Abstract

Membrane proteins are involved in numerous vital biological processes, including transport, signal transduction and the enzymes in a variety of metabolic pathways. Integral membrane proteins account for up to 30% of the human proteome and they make up more than half of all currently marketed therapeutic targets. Unfortunately, membrane proteins are inherently recalcitrant to study using the normal toolkit available to scientists, and one is most often left with the challenge of finding inhibitors, activators and specific antibodies using a denatured or detergent solubilized aggregate. The Nanodisc platform circumvents these challenges by providing a self-assembled system that renders typically insoluble, yet biologically and pharmacologically significant, targets such as receptors, transporters, enzymes, and viral antigens soluble in aqueous media in a native-like bilayer environment that maintain a target's functional activity. By providing a bilayer surface of defined composition and structure, Nanodiscs have found great utility in the study of cellular signaling complexes that assemble on a membrane surface. Nanodiscs provide a nanometer scale vehicle for the in vivo delivery of amphipathic drugs, therapeutic lipids, tethered nucleic acids, imaging agents and active protein complexes. This means for generating nanoscale lipid bilayers has spawned the successful use of numerous other polymer and peptide amphipathic systems. This review, in celebration of the Anfinsen Award, summarizes some recent results and provides an inroad into the current and historical literature.

KEYWORDS

lipid bilayer, membrane protein, Nanodisc, signaling complex

1 | INTRODUCTION

It is an honor to receive the Protein Science Anfinsen Award and provide this article for the journal *Protein Science*. Dr. Anfinsen[1] was a pioneer in efforts to understand the structure of proteins. Using ribonuclease to show that the folding of a protein was determined by its amino acid sequence,[2] he initiated a vast field of study as to how the three dimensional structure of a protein comes together from a random coil. Together with the assembly of multimeric protein complexes, Nature indeed has revealed the beauty of self-assembly. Further pioneering work by Fred Richards and others on the elucidation of the ribonuclease crystal structure and the re-assembly of the cleaved S-peptide fragment with the A-protein[3] revealed how complementarity drives the formation of higher order complex entities. As we will see, Nanodiscs, a complex of lipids and an amphipathic helical protein, are a wonderful example of self-assembly. As Anfinsen used protein modification to understand structure, so modifications of

Stephen Sligar is the winner of the 2020 Christian B. Anfinsen Award.

the helical proteins that self-assemble lipids into discoidal structures have enabled a plethora of Nanodisc applications in the study of membrane proteins and membrane protein/lipid complexes. Fittingly, Anfinsen was also active early in the structure of lipoproteins and their interconversion by enzymatic processes.[4]

Our term Nanodiscs refers to a discoidal self-assembled complex of lipids and a helical protein we have called the membrane scaffold protein (MSP). The current MSP based lipoprotein Nanodiscs are direct descendants of the human high-density lipoprotein (HDL) particles and the helical MSPs from the primary HDL protein component, apolipoprotein-A1 (Apo-AI). Their current wide-spread use by the biochemical and biophysical community has been aided by modifications to the original Apo-AI sequence, but the evolution was aided by a vast literature of lipoproteins and atherosclerosis as a human malady. Hence, a brief history is called for.

Human plasma lipoproteins were known to be complexes of lipids, proteins and cholesterol derivatives.[5] The "high density" fraction, defined via ultracentrifugation as a density of 1.063–1.21 g/ml, received study as the so-called "good cholesterol". Pioneering work by Brouillette and colleagues noted that the first 43 amino acids of the human Apo-AI sequence were not required for lipid binding.[6] Continuing with major contributions from Segrest, the subsequent 11- or 22-residue helices, punctuated by glycine or proline residues, were known to provide the major lipid binding domain and to also help control the size of the resulting particle.[6–8] The modularity of the alpha helix content of Apo-AI led to the hinge domain hypothesis[9] due to the existence of discrete steps in particle sizes. With the extensive work of Jonas and colleagues,[10–12] who honed the detergent dialysis method for forming "reconstituted" HDL particles, it was clear that a large degree of heterogeneity could exist in the size and composition of reconstituted HDLs.[13]

Our entry into the lipoprotein field came from two very different directions. The first was from a realization that much of the machinery of the living cell involved organized multi-component complexes of proteins, lipids and nucleic acids. Protein structure determination, via x-ray or NMR, could not easily capture these large entities. Hence we began adapting the then revolutionary idea of atomic force microscopy (AFM) to work under aqueous solutions.[14] In looking for something in the ~10–100 nm size range we were struck with the discoidal form of HDL that could be easily generated by the dialysis method developed by our Illinois colleague.[13] When we examined a population of HDL particles assembled from various Apo-AI to lipid stoichiometries, the heterogeneity was obvious.[15,16] While there seemed to be a preference for a certain ratios, the origin of the heterogeneity was not clear, even though fractionating by size exclusion chromatography could yield a much more narrow size distribution.[15] Nevertheless, the AFM experiments worked and part of the laboratory went on to use this tool to pattern and quantitate single bilayers on atomically flat surfaces.[15,17]

The second direction, that formed our branch point in the study of lipoproteins, came from a problem in need of a solution. Most of my laboratory was heavily involved in understanding the bioinorganic mechanisms of metalloprotein oxygenase, particularly the cytochromes P450.[18] Much of the functional understanding, and first crystal/NMR structure results came from the study of a soluble bacterial P450cam. However, a great relevance of P450s is their key role in human drug metabolism and hormone biosynthesis. Believing Alexander Pope's 1773 poem noting that "The proper study of Mankind is Man" we wanted to study the mammalian P450s. But, horror of horrors, these were all membrane bound! Frustrating were the human proteins isolated from their membranes via detergent. Often these were then studied in detergent, with sometimes a small amount of lipid added or as an aggregate following removal of the solubilizing agent. However, the mechanistic parameters derived from study of these "solubilized" P450s did not match those obtained from the bacterial versions, for example, Reference 20. Was there something fundamentally different, or did the isolation from the normal membrane bilayer environment render the protein either partially or wholly inactive? We needed to put these membrane proteins back into a bilayer environment. While reconstitution into liposomes was well known, these large heterogeneous entities were not stable to the techniques we had become used to with soluble proteins (e.g., stopped-flow spectrophotometry). Hence the wild idea: What about just adding the detergent solubilized membrane protein into the same mix of detergent solubilized helical Apo-AI and lipid that was used to assemble Nanodiscs? Would the membrane protein target magically assemble into the discoidal bilayer? Would this be stable and amiable to biophysical studies? The answer, of course, is that indeed, Nature's process of self-assembly allowed everyone to find their appropriate home.

Our first efforts were with a simple target that had only a short membrane anchor, with the main catalytic portion of the structure in a soluble "ecto" domain. The P450 reductase (a 68 kD flavoprotein) provided this initial test. Indeed it worked, could be imaged by the AFM, was shown to be active in the Apo-AI supported bilayer and even be structurally characterized by measuring to high precision the height above the bilayer surface.[16,19] Subsequent tries with a more complex membrane

anchoring topology, provide by a mammalian P450 that is "embedded" into the membrane (Figure 1b), also worked, and again AFM under physiological conditions could provide a model for how these two proteins could come together to effect electron transfer and catalysis.[21] Subsequent work over the past 20 years by many laboratories has shown that the same self-assembly process works for almost any membrane protein, including multi-span integral proteins and even complex multi-component assemblies such as the photosynthetic reaction center,[22] cytochrome oxidases[23] and transporters.[24,25] Multiple examples will be described in later sections.

1.1 | The question of size and functionality

In the historical context, we were still concerned with size heterogeneity as we ultimately would like to apply Nanodiscs to three dimensional structural determination. In addition, some uses, such as the reconstitution of large multi-protein complexes, may need discoidal bilayers larger than ~10 nm. Given the discussion in the HDL literature that one or more internal helices, in addition to the amino terminal "globular domain," may or may not be involved in lipid protein interactions, we thought to increase the length of the MSP by adding one, two or three additional 22-mer helices. This corresponds to what is now called MSP1E1, MSP1E2, and MSP1E3, made by adding another helix-4, helix-4 plus helix-5 or helix-4, helix-5 and helix-6 respectively.[26] But a long problem in the literature surfaced—what was the topology of the Apo-AI protein relative to the lipids? Two models were proposed. Much of the earlier experimental[9,27] and theoretical[8] work envisioned a "belt" configuration, yet infrared linear dichroism[28] and molecular dynamics simulations[29] proposed a "picket fence" configuration where the helical components were punctuated by turns and ran up-down. Ultimately, this was resolved by numerous experimental approaches including solid state NMR,[30] solution NMR,[31] chemical crosslinking and mass spectrometry[32] and molecular dynamics trajectories.[33] As will be discussed, recent high-resolution cryo-electron microscopy (cryo-EM) of Nanodisc embedded membrane proteins clearly show the belt configuration. More recent long-time molecular dynamics calculations have added motion to the picture of the encircling MSP, capturing the collective dynamical motion of the discoidal bilayer.[34]

An indirect quantitation of lipid count in the Nanodisc versus the length of the MSP could also give an indication as to which model was correct, since the width of a "picket fence" helix is clearly smaller than the length of a 22-mer "belt." Realizing that the most precise determination of lipid content was via radioactive counting, Denisov et al. measured the number of DPPC lipids in purified, homogeneous MSP1, MSP1E1, MSP1E2 and MSP1E3.[26,35] In a belt model, if the lipid count is plotted against the square root of the MSP length, then a straight line should form. This was observed, and clearly indicated the preference for the "belt" configuration. However, the fit to the data did not go through the origin, suggesting that the first part of the MSP sequence was

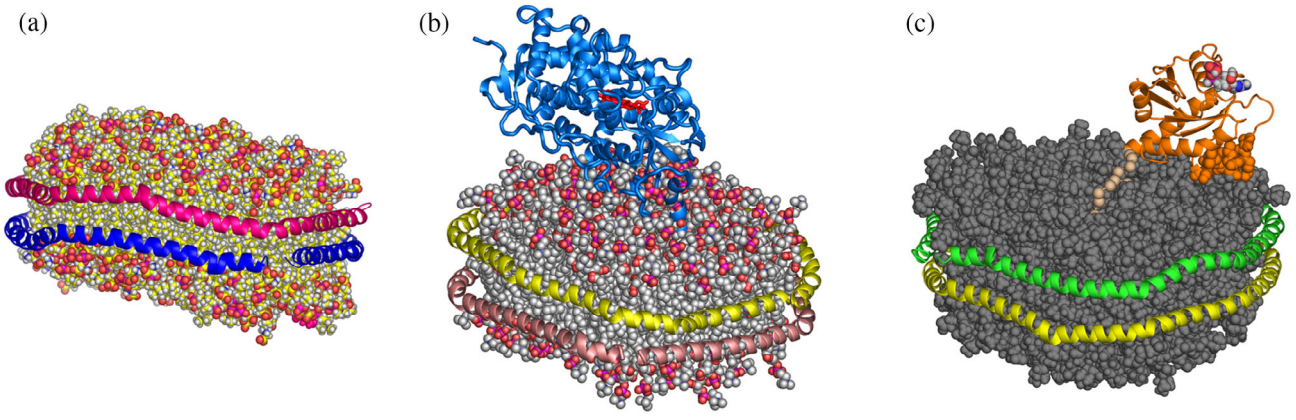

FIGURE 1 The Nanodisc System. All images are based on long-term molecular dynamics simulations and illustrate two main uses of Nanodiscs. (a) The self-assembled discoidal bilayer formed by DMPC lipids and two encircling MSP1D1 membrane scaffold proteins (MSPs; blue and magenta helices). (b) Human cytochrome P450 CYP3A4 as an example of an incorporated membrane protein target in its natural bilayer environment, rendered soluble in aqueous solution by the amphipathic MSP. (c) The use of Nanodiscs as a membrane surface of defined lipid composition. The fully processed oncoprotein KRas4b, with methylated carboxy terminus and covalently attached farnesyl group as it associates with the bilayer (grey surface). The polycationic hyper variable domain (wheat color, Cα atoms shown) and head group residues (helix 4 surface representation) both associate with the anionic bilayer surface. GDP substrate is shown in a Van der Waals atomic surface representation

not contributing to the formation of the phospholipid bilayer. Genetic engineering of the MSP synthetic gene that removed 11, 22 and 33 amino acids, size characterization[26] and molecular dynamics simulation[36] showed that deletion of the first 11 amino acids of the truncated Apo-AI sequence (already missing the residues 1–43 of the globular domain) gave the most stable and homogeneous Nanodiscs. These MSPs were then labeled with D1 (for deletion) as MSP1D1, MSP1E1D1, MSP1E2D1 and MSP1E3D1. While the MSP1D1 and MSP1E3D1, which generate ~9.8 nm and ~12.7 nm discs respectively, are used the most frequently, even larger constructs were made.[37] Also successful, and of important use in recent cryo-EM investigations, is MSP2N2.[38] This was an evolution from the earlier MSP2, which was simply a fusion of two MSP1 sequences, but did not form homogeneous or stable discs.[39] Removing the extra amino acids from the N-terminus and generating a fusion of two MSP1D1 sequences with a simple glycine-serine linker generates nice ~17 nm discoidal bilayers. How big can one go? At some point this will look like a mixture of lipids and amphipathic protein with the aspect ratio of a dinner plate, and fluctuations will result in collapse. Remember, Nature evolved the Apo-AI sequence to form the netting around the predominant spherical HDL particle.

Over the years, the utility of Nanodiscs has been expanded by including a variety of tags or altered amino acid residues into the MSP sequence. These have been summarized in the more recent reviews.[40,41] Briefly, histidine tags are available at both the N- and C- terminus of MSP and the N-terminal variants with Factor X or TEV protease sites. FLAG tags are available at both termini. Unique cysteine residues have been incorporated for attaching fluorescent or other labels. Most recently, "Dark" MSP with no tryptophans, "Ultra Dark" which also deletes the tyrosine residues, and "Ultra Bright" with five additional tryptophans per MSP have been generated.[42] These have found use in some forms of assay, such as analytical ultracentrifugation, fluorescence polarization or microscale thermophoresis.

1.2 | The current popularity of nanodiscs

The current use and overall utility of Nanodiscs can be summarized in two graphs. Following the discovery that Nanodiscs could be used to self-assemble essentially any membrane protein in a phospholipid bilayer with maintenance of function, it was initially utilized by only a few laboratories as evidenced from publications, quoted from PubMed by title or key word (Figure 2a).

Believing that new technologies should be disseminated to the widest academic audience, we provided the synthetic genes for MSPs, optimized for high expression in *Escherichia coli*, to the scientific community through AddGene. These constructs were widely popular, winning the "Blue Flame" designation from AddGene early on. As evidence of their continued popularity, Figure 2b illustrates that the number of samples provided to the academic community has doubled since 2016. For those not willing to express the MSP in a liter of bacteria, or for commercial entities, Millipore-Sigma offers a variety of purified and characterized proteins. With increasing use as a controlled membrane surface, in vaccines and in vivo delivery, we can expect the use of these nanometer scale lipid bilayers will only increase. Equally important is the explosion in the use other amphipathic molecules for bilayer stabilization, including full length Apo-AI, individual oligopeptides and a variety of complex organic polymers. As a general example of amphipathic protein-lipid assembly, it is amusing to note that even apo-myoglobin, with five helices, will associate with lipids to form discoidal particles.

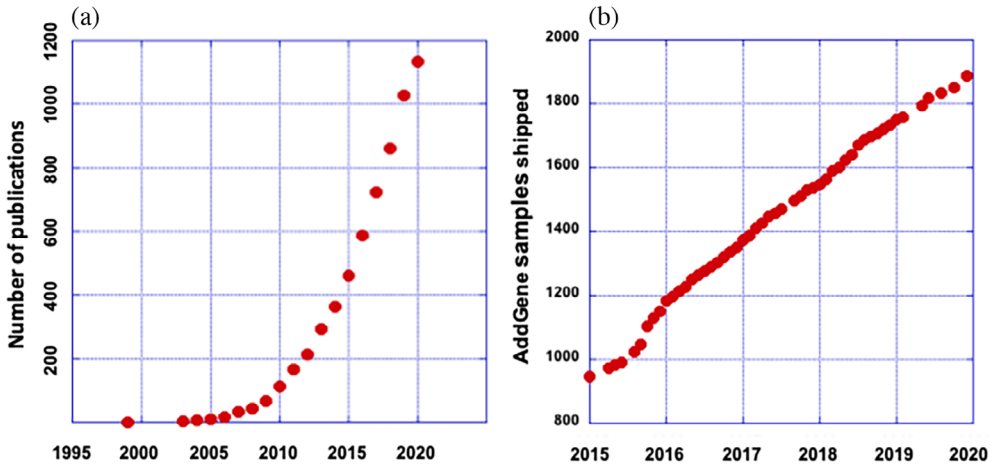

FIGURE 2 The popularity of Nanodiscs. (a) Indicates the cumulative number of publications that deal with Nanodiscs and their applications. (b) Shows the cumulative number of samples of the membrane scaffold protein (MSP) gene shipped by AddGene to laboratories worldwide. The abscissa indicates the value as of October 1, of the indicated year

1.3 | Recent reviews of nanodiscs

There have been numerous reviews of MSPs and the Nanodisc approach, both from our laboratory and others.[40,41,43–45] Other reviews also describe other amphipathic scaffolds, including zebrafish apolipoproteins,[46] the saposin family of lysosomal lipase activators,[47] and short amphipathic peptides.[48,49] Indeed, even α-synuclein[50] and apo-myoglobin, with its five helices, can solubilize lipids into 10-nm structures.[51] The use of amphipathic polymers, first those of styrene maleic acid pioneered by Dafforn and co-workers,[52] and extended to other block copolymers of defined charge and hydrophobicity[53–55] are also finding increasing use. An extensive comparison of these various means for rendering membrane proteins suitable for biophysical structure determination is beyond the scope of this article. Clearly, however, the choice of approach depends on the end point sought. We focus the remainder of this manuscript on summarizing the latest applications of MSP based Nanodiscs. We use a capital "N" to recognize the former copyright of Nanodisc and to distinguish the use of these MSP proteins, engineered from Apo-AI, that yield homogeneous and stable nanoscale soluble lipid bilayers.

1.4 | How do Nanodiscs assemble?

When the first presentation of the Nanodisc platform was published more than 20 years ago, George Whitesides noted that this was "an excellent example of self-assembly." How else to describe hundreds of lipids, long amphipathic MSPs and integral membrane protein targets all coming together into a final functional structure? These core process of lipids and Apo-AI rearranging, and interacting with enzymes such as LCAT and cholesterol exchange proteins, are at the heart of important transitions in the human lipoproteins that have been studied for many years,[56] aided by an in vitro procedure to form HDL particles with detergent removal, forming so-called "reconstituted" HDL.[57] The success of incorporating a wide variety of membrane proteins into the resultant discoidal bilayer in their correct configuration further encourages the question as to how the various components come together. How multi-subunit integral membrane proteins themselves can order correctly is amazing. In each case, the starting condition consists of a detergent solubilizing the lipid, encircling MSP and the individual subunits of the target. Dialysis, or more commonly, the addition of hydrophobic beads, provides a sink for the detergent, through a complex process involving desorption of the detergent from the surfaces of target, MSP and lipid. If there is also a solubilized target, a key question relates to the branch point between correct assembly and the aggregation of components. This leads to a variety of success rates for the incorporation of a membrane protein target into Nanodiscs. Some systems, with the correct stoichiometry of reactants, >90% of the target is functionally incorporated. Others (notably GPCRs) can result in <10% success. Careful optimization of conditions, including detergent choice, amount and exposure time to the hydrophobic beads, temperature and so forth can improve the overall yield. But what is clearly needed is a detailed understanding of the Nanodisc assembly process. Far from being just a philosophical issue, this is the single rate-limiting step in using the powerful Nanodisc technology to investigate membrane proteins where the quantities are limited or they are sensitive to conditions.

For the relatively simple assembly of "bare" Nanodiscs with the engineered MSPs, early work utilized molecular dynamics to examine the reverse process of detergent disassembly,[58] as well as the collapse of a prearranged MSP with lipids to form a discoidal particle.[59,60] This work was extended to examine the conversion of disc to sphere, as is the case for the maturation of circulating HDL particles.[61] However, there has been limited experimental work examining this complex self- assembly process. Preliminary solution X-ray scattering (SAXS) studies after rapid removal of detergent suggests that discs are formed on the minute time scale. One approach has been to examine the structure of the mixture of lipid and scaffold protein at varying concentrations of detergent. Our early work compared experimental SAXS data with calculations to provide a suggested path for assembly.[59] More recent work in our laboratory followed several biophysical parameters, including second derivative optical spectroscopy to monitor the polarity of tyrosine residues, Laurdan fluorescence to suggest when a bilayer-water interface was forming and the overall rotational correlation time of the particle.[62] By studying both addition and removal of detergent one came to the conclusion that the assembly process was reversible, with the bilayer beginning to form below the critical micellar concentration of the detergent and the association of MSP and lipids starting at even lower detergent levels. Other laboratories, notably that of Arleth,[63] are also beginning to address the details of Nanodiscs assembly. The more complicated process of how a membrane protein can find its correct home in the bilayer, as well as the sequential ordering necessary for the functional incorporation of multi-subunit proteins and entire assemblies, such as the photosynthetic reaction center or cytochrome oxidase, is only beginning to be addressed.

1.5 | Nanodiscs enable control of membrane protein aggregation state

Many complexes and assemblies of multi-component membrane proteins have been successfully incorporated into Nanodiscs, which are well suited to stabilize the critical protein–protein interactions in a native-like membrane. An impressive recent example is the multiprotein complex of the mitochondrial calcium uniporter assembled in Nanodiscs containing the cardiolipin necessary for function. Cryo-EM structures in the presence and absence of Ca^{2+} were resolved for the uniporter holocomplex, which was formed from MCU and EMRE subunits from the beetle *Tribolium castaneum* in complex with the human MICU1-MICU2 heterodimer.[64,65] An MCU-EMRE subcomplex was prepared by coexpressing TcMCU and TcEMRE in mammalian cells, purifying the TcMCU-EMRE assembly, and reconstituting it into lipid Nanodiscs with MSP1D1 and a lipid mixture (POPC: POPE:cardiolipin 2:2:1). This control over lipid composition of the membrane in Nanodiscs and purification of complex, with well-defined stoichiometry, enabled acquisition of high resolution data and to discern the mechanism of cation dependent gatekeeping.[64,66]

The stability of Nanodisc assembled membrane proteins, and lack of protein exchange between Nanodiscs, has provided a useful tool for addressing the question of oligomerization and functional relevance of oligomer formation. A first example was demonstrated early in our laboratory by monitoring the correct assembly of bacteriorhodopsin trimer in Nanodiscs,[67] and then by proof-of principle experiments comparing transducin activation[68] and arrestin binding to rhodopsin monomers and dimers.[69] As a result, the application of Nanodiscs provided direct evidence of fully active rhodopsin monomers with properties similar to those observed in proteoliposomes, where in this mimetic it is impossible to control the monomer-dimer equilibrium. In addition, the binding of arrestin was enhanced in the presence of POPG lipids, and conformational changes in visual arrestin upon binding to the phosphorylated rhodopsin monomer were similar to those observed in native membranes.[70]

A qualitative understanding of the process of multisubunit assembly was realized by the studies of bacterial chemotactic receptors in collaboration with the Hazelbauer laboratory.[71,72] The Tar receptor is a trimer of dimers[73,74] with separate assays for the formation of dimers and the final oligomeric configuration in the bilayer. This allowed the dissection of the specific roles of dimerization and their roles in activating of chemotaxis kinases and allosteric coupling of the trimer of dimers[75] to the histidine kinase signaling complex. While the extensive protein–protein interface between dimers drove their formation in the correct configuration, the trimer of dimers only forms a small interface at the apical position. Hence, during the assembly process, the sets of dimers are incorporated in multiple orientations relative to the bilayer. The subsequent cryo-EM structure of *E. coli* aspartate receptor dimers revealed the presence of two flexible hinges which allow for formation of higher order complexes without steric clash.[76] This example suggests that subunit components with high affinity will form correctly in the resultant Nanodisc, whereas weak or transient protein–protein complexes may incorporate into the bilayer in different overall configurations.

1.6 | Cell-free translation of membrane proteins into Nanodiscs

An alternative means of membrane protein incorporation into Nanodiscs is based on cell-free in vitro expression of membrane proteins in the presence of Nanodisc components or preformed Nanodiscs.[77] Binding to the preformed Nanodiscs prevents aggregation and precipitation of expressed protein.[78] Co-translational incorporation of membrane proteins into Nanodiscs significantly improves the yield of folded and fully functional membrane proteins.[79,80] This approach was optimized for several proteins expressed with various ^{13}C- and ^{15}N-labeling schemes for solution NMR studies.[81] Cell-free expression can be also used to bypass solubilization and purification step for unstable enzymes. For instance, Cook et al.[82] successfully used cell-free expression to assemble and purify to homogeneity the monomeric human UGT2B7 in Nanodiscs using the scaffold protein MSP1D1 with POPC lipids. The enzyme with intact C-terminal transmembrane helix is stable in Nanodiscs and functionally competent, similar to UGT2B7 in Supersomes®. Each Nanodisc is shown to harbor a fully active single UGT2B7 monomer, contrary to the previous suggestions that formation of dimers is needed for activity.

1.7 | Biophysical characterization of Nanodiscs

A number of reviews have summarized the plethora of biophysical tools that have been used to characterize Nanodiscs,[40,41] which can be only briefly summarized. First, is the case of "bare" Nanodiscs, composed of just lipid and the MSP. The overall shape is that of a circular disc as evidenced by numerous cryo-EM and TEM structures,[83–85] although depending on packing density of lipids and the models used for interpretation, solution

X-Ray scattering can indicate a more elliptical configuration.[63,86] As monitored by both Laurdan fluorescence and differential scanning calorimetry (DSC), the lipid bilayer contained by the encircling MSPs undergoes a thermal phase transition similar to that seen in liposomes, although broader.[87] This phase transition can also be monitored by SAXS where the mean area per head group and packing is quantitated.[88] More recent works have also monitored the phase transition and separated the contributions from the central lipid core and those juxtaposed to the MSP belt.[89] Here the core domain has properties similar to those measured for liposomes with 1–2 layers of more perturbed lipids due to their direct interactions with the scaffold protein. Recent DSC of the very small DMPC Nanodiscs[89] suggests a significantly perturbed bilayer. Long-time molecular dynamics studies contributed to a picture of fluctuations of the scaffold protein and internal motion of the Nanodisc.[34,90] The tumbling motion of the entire Nanodisc has been measured by time resolved fluorescence anisotropy,[91] yielding a rotational correlation time of ~50–70 ns for the MSP1D1 discs, similar to those reported from NMR studies.[92] Interestingly, the overall solution motion of the Nanodisc can be used to monitor the binding of molecules to the Nanodisc, thereby giving a label-free assay. One excellent example involved discovering a specificity for the association of the signaling protein KRas4b to surfaces of varying ionic lipid composition.[93]

As will be described, many investigations use the Nanodisc to provide a surface containing mixed charged lipids. Since one can imagine the difficulty in packing over a 100 charged head groups in ~7–10 nm area, a key question becomes understanding the actual charge on the Nanodisc. To this end, in collaboration Professor Laue, membrane confined electrophoresis was used to determine the actual Debye–Huckel–Henry charge for different lipid mixtures.[94] Not surprisingly, Nanodiscs behave as polyelectrolytes. When the pK of the head group can be altered by the local electrostatic field, such as with POPS, Nanodiscs can be formed with very high percentage of the charged lipid. When quantitating binding events, the true thermodynamic characterization requires analysis of actual charge rather than the number of incorporated lipids.[93]

Recent progress in native mass-spectrometry, in collaboration with Professor M. Gross, enabled the direct counting of lipids in Nanodiscs.[95,96] The next step applied this method to studies of membrane proteins and peptides in Nanodiscs, as described in several original papers and reviews.[97–99] An elegant extension of Nanodisc technology for mass-spectrometry applications via generation of the mutant MSP was designed in order to change total Nanodisc mass and to improve resolution of overlapping species in a single spectrum.[100] An advantage of Nanodiscs is the precise control of lipid composition, which was used in order to probe the effect of specific lipids on dynamic properties of two membrane transporters XylE and LacY.[101] Systematic analysis of HDX data in Nanodiscs of various lipid compositions, combined with molecular dynamics simulations, indicated significant stabilization of the inward-open conformational state in the presence of PE, as compared to PC headgroups.

Hydrogen-deuterium exchange experiments performed with MSP1D1 Nanodiscs revealed a picture of scaffold protein dynamics, as compared to the lipid-free MSP1D1.[102] As expected, MSP1D1 in Nanodiscs had much higher level of protection than lipid free protein on a short time scale, but reached the same level of exchange at 2 hr or longer. The largest dynamic range was found to be at the C-terminal helices, while the central helices were significantly more stable. This observation is in a good agreement with multiple MD simulations of Nanodiscs, starting from our first publication with only 6 ns long trajectories.[36] Subsequently, a variety of Nanodiscs of different sizes were explored both with all-atom and coarse-grain modeling, providing a consistent picture of the dynamic bilayer surrounded by two helical belts having distorted circular or elliptical structures.[34,89,103,104] Direct contact with the scaffold protein usually resulted in more disordered and dynamically constrained lipids at the lipid-protein interface. The same effect was observed in several NMR studies of Nanodiscs, in both solid-state[105] and solution.[31]

1.8 | Nanodiscs as imaging agents

The large surface area of Nanodiscs prompted the idea that they could be used as a vehicle for in vivo imaging. One challenge in magnetic resonance imaging with heavy metal contrast reagents, such as gadolinium (Gd^{3+}), is the relatively short plasma lifetime and overall sensitivity. Nanodiscs are descendent from human HDL particles, which are immunologically neutral and have significant plasma lifetime until cleared by the hepatic scavenger receptors. Additionally, the possibility that multiple sensitizers could be loaded into a single 10 nm particle could offer dramatic increase in sensitivity. Our first efforts with commercially available lipid anchored chelates resulted in precipitation and formation of rouleaux due to crosslinking, whether or not the Gd^{3+} was incorporated before or after the lipids were self-assembled into the Nanodisc. Although HDL particles have been suggested for use as a contrast agent platform,[106] Nanodiscs offer the advantages of improved stability

and the potential for multi-mode imaging. Improved Gd^{3+} chelating agents and lipid anchors developed by Meade and co-workers[107] were self-assembled into Nanodiscs together with a fluorescent dye covalently attached to the MSP, producing particles that label cells with high efficiency and provide a dramatic sensitivity increase for magnetic resonance imaging.[108] Further advancing the multi-factorial aspects of Nanodiscs was realized in collaboration with Shively and co-workers. Here [64] Cu-DOTA-azide click linked to PEGylated lipids were incorporated into Nanodiscs together with an antibody targeting carcinoembryonic antigen (CEA) positive tumors.[109] In addition, the same chemical derivation approach was shown to load therapeutic drugs such as doxorubicin, thus realizing the goal of a three pronged platform of targeting, imaging and treatment. Further examples of Nanodisc utility in positron emission tomography diagnostics were recently reported.[110]

1.9 | Membrane proteins in nanodiscs

Structural studies of membrane proteins in the phospholipid bilayer of Nanodiscs have made major advances using cryo-EM and NMR spectroscopy, taking advantage of sample size and lipid composition that mimic the natural membrane environment. These enabled improved resolution through extensive statistics collected with class-averaged single-particle images revealing the correct lipid-protein interactions of the incorporated target. Many such examples were published during recent years, when cryo-EM reached a new level of resolution with new sophisticated methods of analysis.[111] Because of size limitations of this review we selected only a minor sampling that illustrates various aspects of Nanodisc utility in this field (Figure 3).

In a recent work, the Lefkowitz group incorporated the M2 muscarinic and β2-adrenergic receptors in Nanodiscs to study structural and functional properties of their complexes with β-arrestin. They found that MSP1E3D1 Nanodiscs provided optimal size for interaction of the β-arrestin C-domain with the lipid bilayer, as seen by cryo-EM.[115] Allosteric activation of proto-oncogene kinase Src was shown to require interaction with the complex of β-arrestin and phosphorylated at C-terminal tail GPCR receptors in Nanodiscs, while chimeric M2 receptor with non-phosphorylated C-terminus did not activate Src.[116]

A spectacular high resolution example is provided by the cryo-EM structure of the phospholipid ABC transporter, MlaFEDB, in Nanodics formed with MSP1D1 and an *E. coli* polar lipid extract (PDB 6XBD) that explicitly resolves the encircling MSP (Figure 3a).[112] This transporter was found with two lipid molecules bound as substrates, which may indicate transport of two lipids in each catalytic cycle, or involvement of this transporter in the transport of cardiolipin, which has four acyl chains.

Nanodiscs are used in SARSCoV2 studies, with the cryo-EM structures of both dimer and tetramer of the 3a ion channel in MSP1E3D1 Nanodiscs were obtained with a novel fold of 3a channel resolved.[117] A co- and post-translational insertase, a large nine-subunit membrane protein complex from the endoplasmic reticulum (EMC), was incorporated in MSP2N2 Nanodiscs, and the cryo-

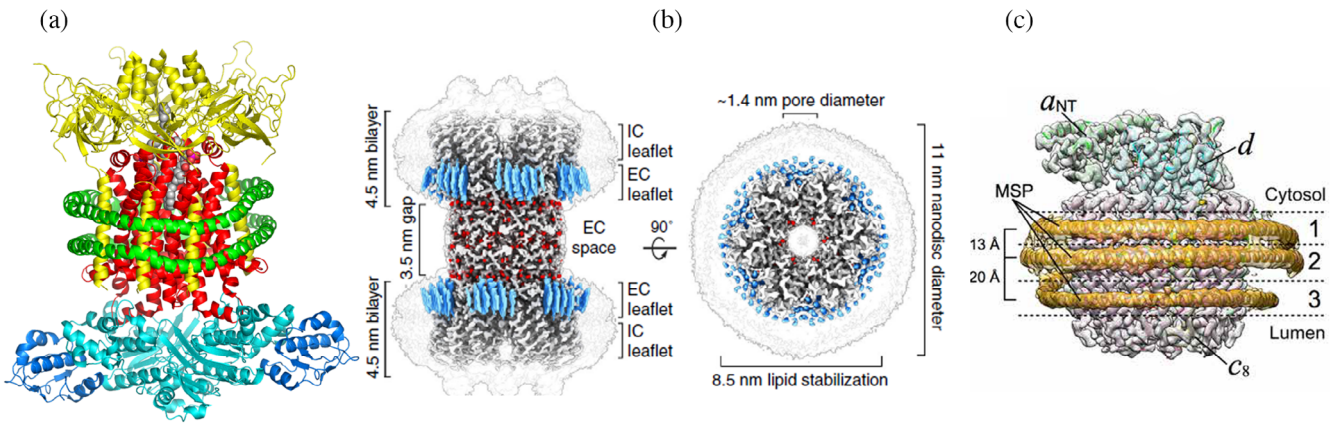

FIGURE 3 (a) Cryo-electron microscopy (cryo-EM) structure of the ABC transporter complex MlaFEDB. Hexamer MLAD (yellow), dimeric transport permease MLAE (red), ATP binding protein (cyan) and transporter-binding protein MLAB (blue) are enclosed into MSP1D1 Nanodisc (two parallel helices shown in green).[112] (b) Cryo-EM structure of connexin-46/50 incorporated in two Nanodiscs (light gray contours). Well resolved lipids are shown in blue, pore with diameter 1.4 nm is shown as the top view on the right. Reproduced with permission from Flores et al.[113] (c) Cryo-EM structure of the Vo proton channel of yeast vacuolar ATPAse. In this structure three molecules of MSP1E3D1 scaffold stabilize the protein complex by forming rings of different diameter around middle transmembrane part and around c-ring. Reproduced with permission from Roh et al.[114] Open access article published under the CC BY license

EM structure resolved to 3.4 Å in order to decipher the mechanism of substrate insertion.[118] Use of the larger Nanodisc was required to provide sufficient area for interactions with the surface-oriented helices in addition to the 12 trans-membrane helices of the seven subunits of the complex. Local thinning of the membrane on one side of EMC was suggested to lower the barrier for the substrate insertion into a hydrophilic conduit formed by three subunits of the complex, with the help of other parts on both sides of the membrane.

Another example of cryo-EM resolving lipid-protein interactions is the structure of Cx46/50, a complex of the connexins Cx46 and Cx50, each in Nanodiscs (MSP1E1 scaffold, DMPC lipids) forming a pore between two membranes, mimicking cell-to-cell junction with 3.5 nm gap.[113] Connexins form intercellular channels for communication of ions and small molecules between cells, often assembling into arrays, termed gap junctions, which are critical for synapse responses, and signaling. Interactions of connexins with membranes and their assembly strongly depend on the lipid environment, hence molecular mechanisms of these junctions should be studied in direct interactions with membranes. Nanodiscs provide an optimal tool for the single-particle cry-EM structural analysis of these interactions, revealing highly ordered lipids shown in blue in Figure 3b.

Very recently a collaboration from several laboratories demonstrated that long chain lipids and expanded hydrophobic surface of Vo-channel of vacuolar ATPase could assemble with three MSP1E3D1 scaffold proteins and revealed the structure of the resulting nanodisc by Cryo-EM (Figure 3c).[114] This work provided the membrane environment necessary to reveal the detailed mechanism of proton translocation by the vacuolar ATPase and a high resolution structure of both the proton channel and scaffold protein.

Nanodiscs have also been used for structural studies of membrane proteins in a detergent-free lipid environment by NMR.[45,119,120] Although the large sizes of Nanodiscs often put limitations on high resolution NMR spectroscopy in solution, the advantages of this method of membrane protein solubilization can be used by applying new experimental tools to solve this problem.[121] As previously mentioned, ^{13}C- and ^{15}N-enriched proteins for solution NMR studies can be made in cell-free expression systems[81] or in *E. coli*[122] with subsequent incorporation into Nanodiscs. Interactions of cytochrome P450 and its redox partners cytochrome b5 and NADPH dependent P450 reductase in Nanodiscs have been documented using NMR.[123–127] NMR was also used for screening of the small molecule binding to the membrane enzyme DsbB.[128]

Essentially the complete tool-kit of spectroscopic methods has been applied to reveal the structure and function of membrane proteins self-assembled into Nanodiscs. In addition to providing a close fit to the native membrane environment, allowing sampling of functionally important conformations, the Nanodisc platform provides an optically transparent medium, is robust to rapid mixing and variable temperature and offers a low viscosity aqueous solution. Uses of resonance spectroscopies, including all forms of paramagnetic resonance, fluorescence energy transfer (FRET), circular dichroism, hydrogen exchange, calorimetry, electrochemistry and high pressure have been applied. An extensive list of citations is provided in our recent reviews.[40,41]

1.10 | Human cytochromes P450: An example of Nanodisc utility

Eukaryotic cytochromes P450 are membrane proteins with a single N-terminal trans-membrane helix and a hydrophobic patch embedded in the lipid head groups of the membrane bilayer. This large superfamily has over 300,000 members and is found across all life forms.[116] Fifty-seven members perform critical roles in human biochemistry, but tend to form aggregates in aqueous solution. In most cases they require significant concentrations of detergent in order to solubilize the oligomers and aggregates to gain even partial activity. As a result, their catalytic properties, measured under various conditions in different solubilizing mixtures, have often been poorly reproducible with a large degree of variability in catalytic parameters such as substrate binding affinity, induced spectral change and product forming rates.[129–131] Another complication arises because of possible heterogeneity of the P450 sample preparation and substrate partitioning into the membrane, which is hard to control.[131] This represents a well-known problem in all biophysical and biochemical studies of membrane proteins, which carry out their normal function while inserted in the lipid bilayer, or interacting with the membrane surface. For the cytochromes P450 these interactions are even more important, because their redox partners, cytochrome P450 reductase (CPR), adrenodoxin reductase (Adr) or cytochrome b5, are also membrane bound proteins, and their functional interactions with P450 enzymes is dictated by the membrane surface to control height and orientation.[19]

As with other membrane proteins, the historic choice for gaining in vitro activity utilized proteoliposomes and mixed micelles consisting of detergent and lipids to solubilize the protein and ascertain function.[43] Both approaches have advantages and disadvantages.

Proteoliposomes are good mimics for biological membranes, but hard to use with various spectroscopic methods because of turbidity, and impose diffusional limitations for substrate access due to phase heterogeneity. Since proteins are mobile in the liposome bilayer, control of the stoichiometry of interacting proteins is very difficult. Micelles provide more homogeneous and transparent media, but often destabilize membrane proteins because of the lack of native membrane bilayer environment. Nanodiscs emerged to combine the essential advantages of both systems, making stable, soluble and homogeneous samples of membrane proteins and their functional complexes in bilayers with precisely controlled composition. For P450 enzymes this was initially reported in 2002–2004, when methods were developed to incorporate P450 CYP2B4,[21] CYP6B1,[132] CYP3A4,[133] and CYP73A5[134] in Nanodiscs using self-assembly from a mixture of target protein, MSP and lipids. From these and further studies,[135–138] it became clear that P450 enzymes in Nanodiscs are fully functional and significantly more stable with respect to conversion to the inactive P420 form.

Substrate binding and the association of small ligands by P450 enzymes in Nanodiscs can be described by single or double exponential kinetics.[135,139–141] The same is true for the autoxidation reaction in the presence or absence of substrates,[135,138,139] Because of homogeneity of P450 monomers in Nanodiscs, these results can be analyzed in terms of molecular properties with no complications arising from limited access to the active site due to oligomerization or to the competitive binding of detergent molecules at the active site.[129,130] In addition, incorporation of cytochromes P450 in Nanodiscs opened the door for various nanoscale sensing techniques, such as localized surface plasmon resonance,[142,143] nanoplasmonic cup arrays,[144] and microring resonator arrays[145] yielding highly sensitive detection of interactions with substrates and inhibitors.

Stable and homogeneous monomeric preparations of human CYP3A4 in Nanodiscs allowed for detailed analysis of the homotropic and heterotropic cooperativity of this important drug metabolizing enzyme.[136,146–151] An important result from these investigations is that all signature features, such as non-Michaelis steady-state kinetics and allosteric effects, are observed to a full extent in a CYP3A4 monomer incorporated in the native-like lipid bilayer. Thus, this approach helped to identify the remote effector site at the P450-membrane interface, making possible more detailed understanding of allosteric mechanism in CYP3A4 responsible for drug–drug interactions.[147,148,150] A similar approach was recently used for analysis of heterotropic interactions of multiple substrates with P450 CYP2J2 in Nanodiscs,[152,153] suggesting a generalizable means to understand the mechanisms of cooperativity and drug–drug interactions.

To address a long-standing question of redox potential coupling to substrate binding in the human P450 enzymes, we used CYP3A4 incorporated into POPC lipid bilayer in Nanodiscs.[137] Careful anaerobic spectroelectrochemical titrations of CYP3A4, saturated with several different substrates, demonstrated a linear correlation between the fraction of high-spin heme iron induced by the presence of substrate, and the measured mid-potential value, validating a universal feature of regulation first reported for the soluble bacterial P450cam[154,155] and CYP102.[156] Nanodiscs also provided a convenient tool to measure the effect of electrostatic field from the charged bilayer lipids on the redox potential of CPR.[157] An effect of lipid composition on the activity and redox coupling of CYP3A4 and CPR in Nanodiscs was also demonstrated by the Scrutton group,[158] where they found that specific liver microsomal lipids improved functional properties of cytochrome P450. The importance of the lipid composition for the cytochrome P450 activity was also shown for CYP5A1 in Nanodiscs.[159]

Nanodisc incorporation of the human steroidogenic P450 enzymes CYP17A1 and CYP19A1 enabled application of cryoradiolytic reduction and the trapping of unstable intermediates for spectroscopic characterization and mechanistic studies. With CYP19A1, the ferrous dioxygen complex was prepared and its autoxidation to the ferric state documented.[138,160] Cryoreduction yielded the peroxo states resulting in the visualization by EPR spectroscopy.[161,162] With CYP17A1, we used a combination of biochemical and biophysical studies in order to decipher the mechanism of carbon–carbon bond scission in the formation of androgens, which had remained a subject of debate. Multiple studies using CYP17A1 incorporated in Nanodiscs, together with its redox partners CPR and cytochrome b5, demonstrated that the steady-state kinetics of the C—C cleavage (lyase) reaction catalyzed by CYP17A1 with 17-hydroxypregnenolone (17OH-PREG) and 17-hydroxyprogesterone {17OH-PROG) is much more consistent with a peroxo-ferric intermediate as the main catalytically active intermediate. There is very little involvement of the classic high valent Compound I intermediate that is the main pathway in most P450 oxygenation events.[163] Kinetic solvent isotope effects proved to be a robust probe for the proton transfer events that control the population of iron-bound oxidants.[164,165] Cryotrapping and annealing at increasing temperatures monitors the protonation events and led to the identification of a new hemiacetal intermediate, characterized by resonance Raman spectroscopy, in human P450 CYP17A1.[163,166,167]

The first proof-of-principle report on Nanodiscs potential for NMR studies of membrane bound cytochromes P450 was published in 2007,[168] where we expressed and purified ^{13}C,^{15}N-uniformly enriched CYP3A4 and incorporated in Nanodiscs. Solid state NMR (SS-NMR) monitored the ^{13}C-^{13}C 2D chemical shift correlation spectra with 0.5 ppm line width, and the secondary structure distribution within seven amino acids was consistent with the substrate-free X-ray structure. CYP3A4 remained fully functional after precipitation. SS-NMR and solution NMR was applied by Ramamoorthy and collaborators to study interactions of CYP2B4 with redox partners CPR and cytochrome b5 in Nanodiscs formed with amphipathic peptides.[123,126,169–172] These efforts demonstrated the importance of generating well-characterized complexes of P450 with redox partners with known stoichiometry for structural studies.

Another advantage of this platform is that membrane proteins in Nanodiscs can be affixed to as surface without denaturation, enabling a variety of sensing modalities. For example, adsorption of Nanodisc on mica surface,[19,21] specific binding of chemically modified lipids (i.e., biotinylated) to the high affinity sites on a streptavidin surface,[173] or interaction via a histidine tag or genetically engineered or chemically modified site on the MSP belt, be can be used to keep a membrane protein in its native bilayer environment but within close proximity to an electrode or sensor surface without altering activity.[143,174,175] These features are useful for applications of surface methods, such as surface plasmon resonance,[175,176] localized plasmon approaches[142,143] and single molecule studies.[177–180]

The dynamics of membrane proteins incorporated in Nanodiscs have been studied using hydrogen-deuterium exchange (HDX). This method provides detailed information on the protein mobility, making possible detection of dynamic changes as a result of substrate or effector binding.[181] In the case of the cytochrome P450s, a substantial difference in the dynamics of the highly specific steroidogenic P450 aromatase CYP19A1[156] and the promiscuous drug metabolizing CYP3A4[182] was noted upon substrate binding. The backbone dynamics of substrate-specific CYP19A1 is significantly more sensitive to the substrate binding, possibly indicating the hidden dynamic contribution to the high specificity, a hypothesis to be explored further.

Many other enzymes have been incorporated into Nanodiscs for functional studies. Examples include the dimeric heme cyclooxygenase (COX)[183,184] the flavoprotein human monoamine oxidase A[185] and methane monooxygenase.[186] DHHC acyltransferase (containing four trans-membrane helices) was incorporated into the larger MSPE3D1-POPC Nanodiscs for functional studies to show that significant membrane deformation is enabling hydration of catalytic cysteine at the active site inside the membrane.[187]

1.11 | Nanodiscs as a controlled surface of defined composition

Another major use of Nanodiscs has been as a vehicle to investigate the role of a membrane surface in the assembly and control of multiprotein complexes. Many cellular reactions act at a membrane surface, including systems where integral membrane proteins carry out catalysis, transport and signal transduction. The membrane surface itself can also play a critical role in bringing multiple protein players to a surface to interact with embedded membrane proteins. Specific phospholipids have been implicated in contributing to overall affinity and the resulting detailed topology necessary for function. This utility of Nanodiscs has been recently reviewed,[188] but an overall summary of the work from our laboratory is included here. Important is the ability to provide a surface of defined size and composition. While lipid specificities and interactions with soluble proteins have been measured using liposomes,[189] a challenge is the mobility of the lipids over the large area of the bilayer that makes determination of stoichiometric interactions difficult. Here the precise homogenous size of the Nanodisc membrane surface and ability to control the lipid, fatty acid, cholesterol and so forth composition is an advantage.

One of the first applications was in the quantitation of the lipid specificity for the recruitment of blood coagulation factors and their interaction with the integral membrane protein tissue factor. Using the Nanodisc system it was shown that the local phospholipid environment modulates the activity of blood clotting.[176,190,191] Furthermore, the utility of Nanodiscs for solid state NMR studies, and tethering to a sensor surface, enabled a structural understanding of how the carboxyglutamate (GLA) resides on the coagulation factors formed specific a complex with anionic lipids.[192,193]

A major application of Nanodiscs as a controlled surface has been in the area of signaling. For example, integrins play a central role in the dynamics of cell adhesion and cell–cell interaction and communication, including the control of embryonic development, cell migration, and the immune response.[194] Integrins are membrane-bound, heterodimeric receptors that are involve interaction with many signaling partners that result in both inside-out and outside-in communication between the extracellular matrix and the cytoskeleton.[195,196] Talin is a key player in the activation of integrins. Nanodiscs enabled the first documentation of

inside-out signaling by talin via cryo-EM[195] as well as development of FRET measures that allowed documentation of the lipid specificity of talin binding.[197,198]

More recently, our laboratory has used Nanodiscs to examine the recruitment of oncoproteins, such as KRas4b, with a membrane surface. Here again, the ability to precisely control the lipid composition enabled the determination of protein affinity with membranes of various lipid compositions. Ras proteins are small GTPases playing a key role in the signaling pathways responsible for cell growth and proliferation.[199] These proteins bind guanine nucleotide and are "off" when GDP is bound and "on" when GTP is at the active site. The signal transduction pathway connects receptor tyrosine kinase integral membrane through Ras activation by guanosine exchange proteins. Nucleotide binding induces a conformational switch that permits the binding and activation of downstream kinases that control cellular function. Nanodiscs have been critical in identifying the lipid specificity in the association of the KRas4b isozyme using a variety of biophysical techniques including FRET and single molecule measurements using AFM.[93,200] A structural picture of KRas4b interacting with the Nanodisc membrane has been recently realized.[201]

There are several other examples where Nanodiscs have been used to investigate protein binding to membrane surfaces, such as the documentation the association of the 69 kD cytotoxic protein BteA with characterization by combination of NMR, SAXS, X-ray and fluorescent quenching[202] and the binding of antimicrobial peptides as determined by mass spectrometry.[98,203] A particularly interesting use of Nanodiscs was to show the membrane involvement in the activation of the cell adhesion receptor CEACAM1 and the stepwise association of the cytosolic domain to calcium-calmodulin and then actin.[204]

1.12 | Nanodiscs in high throughput screening

The robustness of Nanodiscs to hard physical handling has allowed them to be used in a variety of high throughput screening applications. Their stability when on surfaces and, as described earlier, the variety of tags that can be added, has enabled the development of robotic high throughput approaches to drug discovery. One example is the use of Nanodiscs to identify the membrane protein components responsible for the association of the peptide aggregates involved in the initiation of neurodegenerative maladies, such as a-beta oligomers (ABO) and Alzheimer's disease.[205,206] In the ABO hypothesis, the causative process is the binding of ABO to a membrane protein in the post-synaptic junction that would initiate a damaging signaling event. How to identify the culprit? In collaboration with the Klein laboratory at Northwestern, we solubilized brain membranes and generated a "soluble membrane protein library" where each membrane protein is assembled into a separate Nanodisc.[207] ABO were added and a double pull down using a magnetic bead on the Nanodisc and an ABO antibody.[208] Six potential targets were identified. Subsequent work has focused on the a3-subunit of the Na/K ATPase.[209] This approach provides an important application of Nanodisc technology for screening not only other toxic oligomers, but a variety of other pharmaceutical targets that are membrane proteins.[210]

2 | SUMMARY

In this article we have attempted to provide a brief description of the many Nanodisc applications. The Nanodisc platform, consisting of engineered MSPs derived from the human Apo-AI lipoprotein, has clearly provided an enabling platform for a plethora of investigations focused on understanding membranes and membrane protein structure and function. Perhaps more relevant, has been their use in understanding self-assembly and molecular recognition events that build on the pioneering work of Christian Anfinsen. Nanodisc successes have encouraged further advances through development of other amphipathic molecules, such as peptides and polymers, that also efficiently self-assemble lipids and membrane proteins into robust soluble nanoscale entities. Future expansion into larger and more complex interacting components will provide a new look at cellular dynamics and function.

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expansion into new arenas of scientific inquiry. All are gratefully acknowledged.

AUTHOR CONTRIBUTIONS

Stephen Sligar: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing-original draft; writing-review and editing. **Ilia Denisov:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing-original draft; writing-review and editing.

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