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Biophysical characterization of membrane proteins in nanodiscs

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ABSTRACT

Nanodiscs are self-assembled discoidal phospholipid bilayers surrounded and stabilized by membrane scaffold proteins (MSPs), that have become a powerful and promising tool for the study of membrane proteins. Even though their reconstitution is highly regulated by the type of MSP and phospholipid input, a biophysical characterization leading to the determination of the stoichiometry of MSP, lipid and membrane protein is essential. This is important for biological studies, as the oligomeric state of membrane proteins often correlates with their functional activity. Typically combinations of several methods are applied using, for example, modified samples that incorporate fluorescent labels, along with procedures that result in nanodisc disassembly and lipid dissolution. To obtain a comprehensive understanding of the native properties of nanodiscs, modification-free analysis methods are required. In this work we provide a strategy, using a combination of dynamic light scattering and analytical ultracentrifugation, for the biophysical characterization of unmodified nanodiscs. In this manner we characterize the nanodisc preparation in terms of its overall polydispersity and characterize the hydrodynamically resolved nanodisc of interest in terms of its sedimentation coefficient, Stokes' radius and overall protein and lipid stoichiometry. Functional and biological applications are also discussed for the study of the membrane protein embedded in nanodiscs under defined experimental conditions.

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1. Introduction

In a typical cell, up to 30% of all open reading frames are predicted to encode membrane proteins [1]. They play key roles in many biological pathways [2] and represent important drug targets. In particular, G protein-coupled receptors (GPCRs) have intensively been targeted for therapeutic purposes [3,4]. Despite the tremendous progress in understanding the function of membrane proteins, limitations still exist, for example, due to the difficulties associated with obtaining sufficient quantities of the membrane protein of interest and incorporating them into a lipid environment in functional form. To fully understand the biology of a target membrane protein, biochemical information relating to the functional role of the membrane protein (e.g. ligand binding) needs to be complemented by structural studies (e.g. nuclear magnetic resonance or X-ray experiments).

Cell membranes are permeable barriers that maintain the internal environment of cells. The lipids do not only provide the physiological environment for membrane proteins but also control their function [5], in part through their composition that is known to change with organism [6], age [7] and disease [8]. A number of membrane proteins require unique lipids to modulate their mechanism of action [6,9,10], and for this reason, *in vitro* studies should preferably be carried out with membrane proteins embedded in a native lipid bilayer environment. Therefore, the following terms need to be satisfied to conduct a thorough biological characterization of the membrane protein: (i) precise control of the lipid environment surrounding the target membrane protein; (ii) control and definition of the membrane protein's oligomeric state; and (iii) access of the interacting partner protein or ligand to the membrane protein independent of the topology of insertion. Even though detergents, artificial lipid bilayers (e.g. liposomes) or bicelles have been used to provide native-like environments for

Abbreviations: 2xYT, double strength YT medium; AUC, analytical ultracentrifugation; CHAPS, 3-[(3-cholamidopyropyl)dimethylammonio]-1-propanesulfonate; CHS, cholesteryl hemisuccinate Tris salt; DDM, n-dodecyl-β-D-maltoside; DLS, dynamic light scattering; GPCR, G protein-coupled receptor; H10, decahistidine; H7-MSP1D1, MSP1D1 with an N-terminal heptahistidine tag; HPLC/MS/MS, HPLC combined with tandem quadrupole mass spectrometry; IMAC, immobilized metal affinity chromatography; MBP, maltose-binding protein; MSP, membrane scaffold protein; NT, neurotensin; NTS1, neurotensin receptor 1; $NTS1_f$, NTS1 expressed as a fusion protein; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; SE, sedimentation equilibrium; SV, sedimentation velocity; Tev, tobacco etch virus; T_m, phase transition temperature; TrxA, *E. coli* thioredoxin.

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membrane proteins [11,12], none of these satisfy all the criteria described above.

The nanodisc technology developed by Sligar and co-workers, on the other hand, does satisfy all of the above requirements for the study of membrane proteins *in vitro* [13,14]. A nanodisc is a discoidal phospholipid bilayer surrounded and stabilized by two molecules of a membrane scaffold protein (MSP). Unlike detergent micelles, bicelles, or liposomes, nanodisc reconstitution is highly regulated by the MSP to lipid ratio and the length of the MSP; at the proper lipid to MSP ratio this generates homogeneous and monodisperse entities. A membrane protein reconstituted within a nanodisc does not only gain the stability and functional activity provided by native environments, it can also be treated much like any soluble protein. To date, a number of membrane proteins have been successfully introduced into nanodiscs reflecting a variety of protein types, number of the transmembrane helices and oligomeric states. These include the rhodopsin monomer and dimer [15], the bacteriorhodopsin monomer and trimer [16], the SecYEG translocon complex [17], and cytochrome P450 [13] among others. In this manner various functional studies, such as protein–protein interaction studies in solution [18–20], ligand binding studies by immobilized surface plasmon resonance [21], and fusion pore assays [22] have been carried out demonstrating the power and versatility that the nanodisc technology provides for the study of membrane proteins.

In this review, we focus on the biophysical characterization of membrane proteins inserted into nanodiscs. We provide a brief overview of the various techniques using modified membrane proteins and focus on novel methodological applications to the analysis of unmodified membrane protein targets using the class A GPCR neurotensin receptor 1 (NTS1) as a model system. We describe the purification of the protein components needed for generating nanodiscs, along with a detailed reconstitution procedure, followed by a description of their characterization by dynamic light scattering (DLS) and analytical ultracentrifugation (AUC). The last section includes examples of the functional and biological analyses of membrane proteins inserted into nanodiscs, emphasizing the versatility of this technology.

2. Reconstitution of the target membrane protein into nanodiscs

A variety of membrane proteins have been reconstituted into nanodiscs (summarized in Table 2.1). The basic protocol for reconstitution requires mixing of the purified membrane protein, detergent solubilized phospholipids and MSP in a previously determined stoichiometric ratio, followed by detergent removal to initiate the self-assembly and formation of the membrane protein-nanodisc. Typically this is followed by further purification on an affinity column or by size exclusion chromatography. In this section, we describe the reconstitution of NTS1 into nanodiscs and provide particular considerations for each step of the protocol.

Neurotensin (NT) is a 13 amino acid residue peptide [23,24] with diverse biological activities. It plays key roles in antinociception [25], hypothermia [26], modulation of dopamine neurotransmission [27], cancer growth [28], Parkinson's disease [29] and the pathogenesis of schizophrenia [30]. Most of the known effects of NT are mediated through NTS1, a class A GPCR transmembrane protein [31], and the targeting of NTS1 with synthetic agonists and antagonists for therapeutic purposes has been discussed [32]. NTS1 binds NT at its extracellular surface and couples preferentially to a Gq-type G protein at its intracellular surface. These properties distinguish NTS1 from the well-studied rhodopsin and beta-adrenergic receptors, which bind small ligands within their transmembrane cores and interact with Gi- and Gs-type proteins, respectively. The importance of the phospholipid environment for rhodopsin function has been well documented [9,33–39]; however, these observations may not be extrapolated to other GPCRs because of rhodopsin's specific properties and the unique lipid environment for visual signal transduction. We were therefore interested in studying the effect of lipid head group charges on the signaling properties of NTS1 and utilized the defined experimental conditions afforded by the nanodisc technology.

2.1. The target membrane protein

Nanodisc reconstitution requires relatively large amounts of the membrane protein both for the optimization of the nanodisc reconstitution procedure, as well as for the subsequent series of experiments we describe in this work. As approximately 0.5 mg of purified NTS1 is needed for the reconstitution of NTS1-nanodiscs at a 1 ml scale, direct purification from natural sources is not feasible, because NTS1, like many membrane proteins, is expressed *in vivo* at very low levels. Note that rhodopsin is among the very few membrane proteins, which are found in high quantities in natural tissue. For most targets under consideration, the establishment of a recombinant production system is essential and to date several heterologous overexpression systems have been developed and successfully utilized to express membrane proteins [40–45], such as *Escherichia coli* for the production of prokaryotic

Table 2.1
Biological studies with membrane proteins inserted into nanodiscs. The list is not exhaustive and the reader is referred to excellent recent reviews [14,49,103,104] for additional details. Representative references for each example are given.

Membrane proteins	Number of transmembrane domains in monomer	Inserted into nanodisc as	Measurements	Ref.
Bacterial chemoreceptor (Tar)	1	Dimer/trimer of dimers	Methylation and deamidation; activation of histidine kinase	[53]
Cytochrome P450 (3A4)	1	Monomer/co-incorporation with cytochrome P450 reductase	Redox potential upon ligand binding; cooperativity of ligand binding; role of lipids	[13,64]
Bacteriorhodopsin	7	Monomer/trimer	Spectroscopic properties	[14,68]
GPCRs	7			
μ-opioid receptor		Monomer	Agonist and antagonist binding; G protein activation; role of lipids	[18,19,59]
β_2-adrenergic receptor				
Neurotensin receptor 1				
Rhodopsin		Monomer/dimer	G protein activation; role of lipids	[15,61,69]
		Monomer	Phosphorylation by kinases; arrestin binding; role of lipids	[20,101]
Translocon complex SecYEG	15	Monomer	Interaction with SecA; role of lipids	[17]