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Direct Capture of Functional Proteins from Mammalian Plasma Membranes into Nanodiscs

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Supporting Information

ABSTRACT: Mammalian plasma membrane proteins make up the largest class of drug targets yet are difficult to study in a cell free system because of their intransigent nature. Herein, we perform direct encapsulation of plasma membrane proteins derived from mammalian cells into a functional nanodisc library. Peptide fingerprinting was used to analyze the proteome of the incorporated proteins in nanodiscs and to further demonstrate that the lipid composition of the nanodiscs directly affects the class of protein that is incorporated. Furthermore, the functionality of the incorporated membrane proteome was evaluated by measuring the activity of membrane proteins: Na^+/K^+-ATPase and receptor tyrosine kinases. This work is the first report of the successful establishment and characterization of a cell free functional library of mammalian membrane proteins into nanodiscs.

Plasma membranes (PMs) serve as a protective bilayer for cells by forming a permeability barrier for trafficking molecules into and out of the cell. These membranes are comprised of a diverse array of proteins and lipids that interact to form a complex superstructure with capacity to regulate various cellular processes. PM proteins are involved in cellular signaling, regulation, and homeostasis and serve as the largest class of druggable targets.[1] These proteins can be classified into various functional groups, including receptors, transporters, and enzymes.[2] Additionally, these membranes also contain various lipid components consisting primarily of different types of phospholipids, including POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine), and sphingomyelin, and other lipids, including cholesterol.[3−6] Furthermore, the PM contains microdomains such as lipid rafts that contain a percentage of cholesterol and sphingomyelin significantly higher than that of the regular plasma membrane that aid in protein signaling.[4,7] Taken together, the plasma membranes are highly ordered, and their complexity arises from the intricate interplay between membrane proteins and a variety of lipids.

The involvement of plasma membrane proteins in signaling processes and their importance in therapeutic development necessitate the discovery of innovative methods for interrogation of membrane proteins in a cell free medium.[1] Here we use nanodiscs to efficiently capture membrane proteins directly from the mammalian cellular membranes (Figure 1). Previous studies show that nanodiscs can be used to preserve the structural and functional integrity of membrane proteins, including cytochrome P450s, G protein-coupled receptors (GPCRs), and other receptors.[8−10] Additionally, they can be coupled to various downstream detection methodologies and assays such as SILAC and MALDI, nuclear magnetic resonance, and fluorescence microscopy.[11]

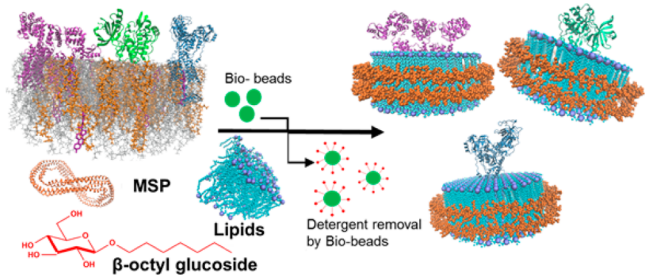

Figure 1. Schematic of nanodisc assembly. Membrane proteins in membranes were mixed with membrane scaffold protein (MSP), *β*-octyl glucoside, and lipids of choice. The detergent was removed by biobeads, leading to the onset of the assembly of the membrane protein nanodisc library (MPNL).

The application of nanodiscs has been predominantly limited to the incorporation of single purified proteins.[8−10,12−14] Recently, there have been studies to prepare membrane protein nanodisc libraries (MPNLs) by directly encapsulating proteins from the membranes of simpler organisms such as bacteria[15] and yeast.[16] This work is the first report of a mammalian membrane protein nanodisc library with detailed characterization of the incorporated proteome. Furthermore, we demonstrate that the incorporated proteins also retain functional activity. The capacity to stably incorporate membrane proteins as a library in nanodiscs provides a cell free platform suitable for facile downstream functional analysis and drug binding studies.

For the preparation of the MPNL, we utilized metastatic human osteosarcoma 143B cells[17] in the study presented here to serve as a model of mammalian plasma membranes for the preparation of the mammalian protein nanodisc library (MPNL). The immortalized 143B cell line originates from the malignant osteoblast lineage and is characterized by a short

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doubling time *in vitro* and a spontaneous metastatic potential *in vivo*.[18] The potential discovery of membrane biomarkers of osteosarcoma and drug discovery against identified receptor targets permit 143B cells to serve as a relevant and suitable mammalian cell model for this study. To capture the membrane proteins into nanodiscs, first, the membrane fraction from 143B cells was isolated using an aqueous two-phase partitioning system (ATPS).[19,20] This was followed by equilibration with mild, nonionic detergent, β-octyl glucoside, to extract the proteins from the membrane. Use of β-octyl glucoside has been previously shown to maintain the functional integrity of membrane proteins as compared to that of cholate or sodium dodecyl sulfate.[21,22] Detergent-solubilized lipids and membrane scaffold protein (MSP1E3D1) were added followed by removal of the detergents by Amberlite Biobeads to initiate nanodisc self-assembly (Figure 1). The ratio of these components was optimized and verified by using size exclusion chromatography (Figure S1). The following lipid compositions were chosen to make nanodiscs (NDs): 100% POPC (ND1), 20% POPS and 80% POPC (ND2), 20% cholesterol and 80% POPC (ND3), and 20% POPS, 72% POPC, and 8% cholesterol (ND4).

Interestingly, the nanodiscs prepared from different lipid compositions had similar sizes except for the cholesterol nanodiscs that show a later elution time, indicating a smaller size (Figure S1). The rigid structure of cholesterol possibly increases the fluidity of the nanodisc as it disrupts the phospholipid packing.[23] A similar reduction in size upon incorporation of cholesterol has been observed in molecular dynamics (MD) simulations of bilayers.[24]

For peptide fingerprinting analysis of the MPNL, the protein content of the nanodiscs was further characterized using peptide fingerprinting by mass spectrometry.[25,26] Prior to proteomic analysis, the MPNL nanodiscs were disassembled using sodium cholate followed by use of a Ni-NTA to remove His-tagged MSP1E3D1 as the presence of MSP1E3D1 overwhelms the ion signal from the other membrane proteins. The proteins identified in the nanodiscs using proteomics were classified into the following functional categories: structural, GPCRs, enzymes, transporters, chaperones, immune complexes, and other receptors (Figure 2 and Tables ST1–ST5 of the Supporting Information). Herein, we use two parameters for data analysis. Figure 2 and Table ST6 represent the percentage of a particular functional class of membrane protein in the protein mixture (eq SE-1). The second parameter is the incorporation of proteins into nanodiscs as compared to the initial membrane (Table ST7) indicating the percentage incorporation

Peptide fingerprinting of POPC nanodisc ND1 revealed that ~55% of membrane protein species in the initial membrane fraction were incorporated into nanodiscs (Table ST7). Although a majority of the protein categories were conserved in these nanodiscs, the percentage of structural proteins was only 19% in ND1 as compared to 56% in MP (Table ST6). An important class of structural proteins, annexins, was completely absent in ND1 (Table ST2-2), indicating that the protein–lipid electrostatic interaction that is important for annexin binding is lacking in the zwitterionic POPC lipid bilayer.[15,27]

Typically, the plasma membrane of cells consists of ~20% anionic lipids such as phosphatidylserine.[6] Various proteins, including phospholipases and annexins that contain positively charged residues, preferentially associate with anionic lipids to perform their biological activities.[28–30] Therefore, ND2 containing 20% POPS (Table ST3) was prepared. In these

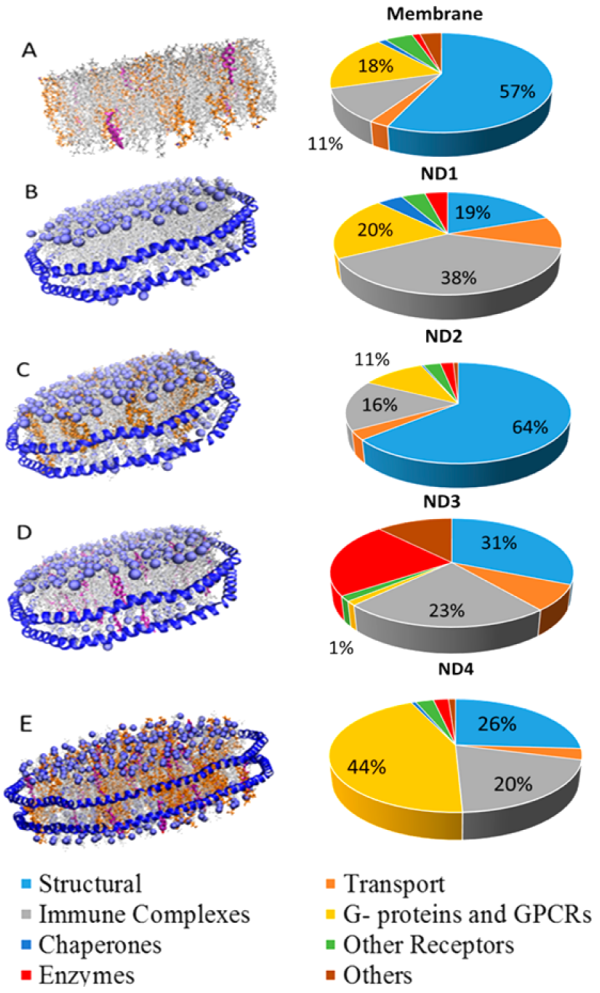

Figure 2. Schematics (left) of nanodiscs containing different lipid compositions. POPC lipids are colored white and POPS lipids orange, and cholesterol is colored pink. Phospholipid headgroups are colored purple, and membrane scaffold protein is colored blue: (A) MP, (B) ND1 (100% POPC), (C) ND2 (20% POPS and 80% POPC), (D) ND3 (20% cholesterol and 80% POPC), and (E) ND4 (20% POPS, 8% cholesterol, and 72% POPC). Functional classification (right) of the membrane proteome. The percentage represents the ratio of one particular class of membrane protein in the mixture (described in Supporting Information equation SE1 and ST6). The percentages of structural proteins (blue), immune complexes (gray), and GPCRs (yellow) are indicated. Further details are provided in the Supporting Information (ST1–ST6).

nanodiscs, the protein content recapitulated the initial membrane composition (Table ST6) and achieved an overall incorporation of 70% of the initial mixture of membrane proteins into nanodiscs (Table ST7). Unlike ND1, the structural proteins constituted the largest class of proteins in ND2 (Figure 2C and Table ST6) as seen for the original MP fraction (Figure 2A and Table ST6).

Next we incorporated cholesterol into nanodiscs. Cholesterol is the primary lipid component of lipid raft domains in the plasma membranes that have been implicated in clustering of GPCRs and other signaling proteins.[31,32] *In vivo*, these rafts contain a percentage of cholesterol (~30%) significantly higher than that of the plasma membrane (8–14%).[4] Because of the rigid nature of the sterol group, cholesterol partitions preferentially into the lipid rafts where acyl chains of the phospholipids tend to be more rigid and in a less fluid state.

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The proteomic analysis of 20% cholesterol-containing nanodiscs, ND3 (Table ST4), surprisingly showed an incorporation efficiency of 15% (Table ST6), and only a few protein classes were detected (Table ST7 and Figure 2D). Similar to ND1, cholesterol nanodiscs demonstrated a lack of proteins binding preferentially to anionic lipids. Interestingly, the percentage of enzymes incorporated into ND3 was higher than in ND1 and ND2 (Table ST6). However, the overall incorporation of membrane proteins in cholesterol ND3 was poor. MD simulation studies show that the volume of a bilayer decreases with an increase in cholesterol content.[24] The smaller size is also evident from our size exclusion chromatography, which showed that ND3 was smaller than other nanodiscs (Figure S1). The smaller volume of the nanodisc due to a high cholesterol concentration possibly results in a lower incorporation efficiency.

Finally, ND4 nanodiscs that closely mimicked the composition of the plasma membrane [POPC (72%), POPS (20%), and cholesterol (8%)] were prepared. Results from peptide fingerprinting show that ND4 achieved >75% incorporation of proteins, the highest incorporation efficiency among the different nanodisc classes (Table ST7). There was a dramatic enrichment of GPCRs and G proteins (Figure 2E and Tables ST5 and ST6). ND4 contains significantly less cholesterol than ND3 and therefore an overall greater nanodisc size as evidenced by size exclusion chromatography (SEC). Additionally, the presence of cholesterol has been linked to GPCR signaling either directly by binding to the GPCR[33] or by altering membrane physical properties,[34,35] or both. The significant enrichment of GPCRs in ND4 is probably due to favorable interactions of cholesterol with GPCRs.

For the enrichment of selective membrane proteins in the MPNL, all the nanodisc libraries show enrichment of the membrane protein population (Figures ST1–ST5). This led to identification of several low-abundance membrane proteins not originally detected in the membrane proteome. Therefore, nanodiscs preferentially capture and enrich membrane proteins and remove the soluble proteins and other cellular contaminants.

For Western blot validation of selected membrane proteins in the MPNL, we further validated the incorporation of two specific membrane protein targets into nanodiscs using Western blot analysis. It was determined that both GTPase KRAS, a peripheral membrane protein, and major histocompatibility complex (MHC) class I, an integral membrane protein, identified via peptide fingerprinting were present in Western blot analysis in all the different nanodisc preparations (ND1–ND4) (Figure 3A,B).

For the functional assay of proteins in the MPNL, from the proteomics data, we see that ND4, which most closely resembles the lipid composition of the native plasma membrane, is able to capture more than 75% of the initial membrane proteins (Table ST7). The primary advantage of incorporation of proteins into nanodiscs as compared to detergent-solubilized membrane preparations is the preservation of their function.[10,12] From the peptide fingerprinting analysis, we chose two representative classes of proteins to demonstrate that proteins are functionally active in nanodiscs: Na^+/K^+-ATPase and receptor tyrosine kinases (RTKs). The Na^+/K^+-ATPase transports sodium and potassium ions across their concentration gradient by hydrolyzing one molecule of ATP to ADP and inorganic phosphate (P_i). Ouabain is a specific inhibitor of Na^+/K^+-ATPase. The protein activity was

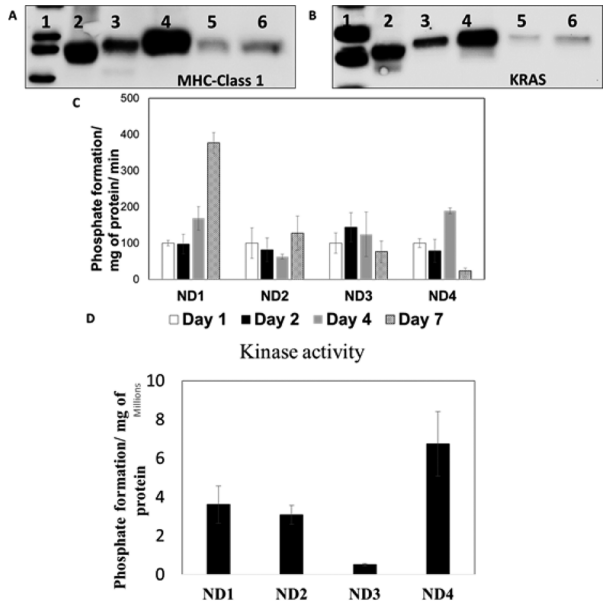

Figure 3. (A and B) Western blot showing incorporation of MHC-1 and KRAS protein into the different nanodiscs: (1) ladder, (2) 143B membrane, (3) 100% POPC ND1, (4) 20% POPS ND2, (5) 20% cholesterol ND3, and (6) 8% cholesterol and 20% POPS ND4. (C) Na^+/K^+-ATPase activity in nanodiscs monitored at 37 °C by measuring phosphate produced in the presence and absence of ouabain (Na^+/K^+-ATPase blocker) relative to day 1 (details in Table ST8). (D) Net kinase activity measured in the nanodiscs in the presence and absence of imatinib (RTK inhibitor) showing active RTK in nanodiscs is inhibited (Figure S3).

measured by phosphate liberated in the absence and presence of ouabain[36,37] (Figure 3C and Table ST8). It can be seen that Na^+/K^+-ATPase incorporated into the MPNL retains functionality over 7 days at 37 °C. Furthermore, the functional assay was conducted at a variety of temperatures [4 °C and room temperature (23 °C)] over different periods of time (days 1–7) and using freeze–thaw conditions (Figure S2). Under all tested conditions, Na^+/K^+-ATPase activity was retained in nanodiscs over a broad range of storage durations and temperatures.

Phosphorylation of proteins is a key process for signal transduction in enzymatic regulation. A major class of kinases is receptor tyrosine kinases (RTKs), which are involved in cancer, and there are various inhibitors that currently are available to target them.[38] We measured the activity of RTKs in nanodiscs by using malachite green-dependent detection of phosphate released from kinase activity[39] in the presence and absence of imatinib. This inhibitor inhibits multiple RTKs, including platelet-derived growth factor (PDGF) receptor, an overexpressed receptor in osteosarcoma.[40,41] All four nanodisc preparations retained RTK activity as evidenced by the decrease in activity in the presence of imatinib, indicating functional kinases that are inhibited by imatinib. Interestingly, the highest RTK activity was identified in POPC/POPS/cholesterol ND4. The activity of RTKs was lowest in the 20% cholesterol nanodisc, correlating with the absence of PDGF receptor and small amounts of RTKs detected by peptide fingerprinting. This demonstrates that the MPNL can be used for screening small molecules against a large variety of kinases in a model library.

In conclusion, this work establishes the formation of a functional mammalian nanodisc library from human osteosarcoma 143B cells and is the first to report a detailed analysis of

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the incorporated proteome in the nanodiscs. Furthermore, it is shown that the change in lipid composition strongly influences the subpopulation of proteins incorporated into the nanodisc. This study can be further extended to other lipid compositions, including sphingomyelin, cardiolipin, etc. In ND4 containing both anionic lipids and cholesterol, there was more than 75% incorporation of membrane protein, including enrichment of certain GPCRs and G proteins. These MPNLs are functionally active as shown by measuring Na^+/K^+-ATPase activity and RTK activity as proof-of-concept protein classes. This approach of using functional nanodisc libraries provides promise for stabilizing entire membrane proteomes in a cell free system that can be further utilized for evaluating protein interactions and drug binding studies. Notably, the methods used to prepare a functional MPNL from 143B cells can be broadly applied to other cell lines, including cancerous and noncancerous cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00954.

Experimental procedures, analysis tables, and proteomics data (PDF)

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Notes

The authors declare no competing financial interest.

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