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Nanodiscs in the studies of membrane-bound cytochrome P450 enzymes

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SUMMARY

Cytochromes P450 from Eukaryotes and their native redox partners cytochrome P450 reductases both belong to the class of monotopic membrane proteins containing one transmembrane anchor. Incorporation into the lipid bilayer significantly affects their equilibrium and kinetic properties and plays an important role in their interactions. We describe here the detailed protocols developed in our group for the functional self-assembly of mammalian cytochromes P450 and cytochrome P450 reductases into Nanodiscs with controlled lipid composition. The resulting preparations are fully functional, homogeneous in size, composition and oligomerization state of the heme enzyme, and show an improved stability with respect to P420 formation. We provide a brief overview of applications of Nanodisc technology to the biophysical and biochemical mechanistic studies of cytochromes P450 involved in steroidogenesis, and of the most abundant xenobiotic metabolizing human cytochrome P450 CYP3A4.

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[20]Following is an example of how the reconstitution mixture is prepared, showing all the calculations involved. The example is based on 25 nmoles of P450.

Assume the following values for the concentration of the stock solutions that would be used in this mock disc assembly:

$[POPC]_{STOCK}$ = 75 mM

$[MSP1D1(-)]_{STOCK}$ = 150 μM

$[P450]_{STOCK}$ = 75 μM

POPC: MSP1D1(−): P450 = 1280:20:1

Note: The ratio between lipid and MSP would depend on the choice of lipid (POPC, POPS, DMPC, DPPC, etc.) and membrane-scaffold protein type (MSP1D1, MSP1E3D1, etc.) pair. The ratio between P450 and the number of Nanodiscs is kept high (typically 1:10) to ensure a high yield of incorporation.

Amount of P450 to be incorporated into discs = 25 nmoles

Amount of MSP1D1(−) needed = 25 × 20 = 500 nmoles

Amount of POPC needed = 500 × 64 = 32,000 nmoles

Volume of POPC-chloroform stock to be dried = 32000/75 = 426.67 μl, i.e., 427 μl

Amount of cholate needed to solubilize POPC = 32000/50 = 640 μl

For a final POPC concentration of 6 mM

Total volume of the solution should be 32000/6 = 5333 μl

To achieve a final cholate concentration of 15 mM

Volume of buffer with cholate to be added = ((5333 × 15) - (640 × 100))/100 = 160 μl

Volume of 150 μM MSP1D1(−) to be added = 500/150 = 3333 μl

Volume of 50 μM P450 to be added = 25/50 = 500 μl

Volume of buffer with cholate to be added = 5333 – (640 + 160 + 3333 + 500) = 700 μl

Keywords

Cytochrome P450; Nanodiscs; CYP3A4; CYP17; CYP19A1; Eukaryotic P450; Membrane mimetics

INTRODUCTION

Cytochromes P450 constitute a broad superfamily of heme enzymes with more than 20,000 isozymes identified in genomes of organisms from all biological kingdoms [1-2]. Prokaryotic cytochromes P450 are soluble. However, eukaryotic enzymes are monotopic integral membrane proteins with the N-terminal transmembrane fragment and the hydrophobic fragment between F and G helices inserted into the lipid bilayer with an estimated depth of insertion of 1 - 2 nm [3-9]. As with other membrane proteins, isolation, purification and biochemical studies of eukaryotic cytochromes P450 are mostly based on the use of detergents to improve solubilization of hydrophobic constituents of the membrane. However, in most cases, the resulting reconstitution systems of mixed detergents, lipid micelles and solubilized membrane proteins, do not contain a lipid bilayer and are a poor mimetics of biological membrane protein systems. Lipid vesicles represent a better approximation to the real *in vivo* arrangement of membrane protein assemblies, but working with small and large unilamellar vesicles is difficult because of limited stability and the inability to control the local environment of the membrane protein incorporated into the lipid bilayer. Therefore, there is a need for a new model system for the functional reconstitution of membrane proteins and their complexes [10].

The development of Nanodiscs, self-assembled nanoscale lipid bilayers solubilized by an amphipathic scaffold protein [11-14], has created a new list of methods and experimental approaches that can be used in studying the biophysical chemistry of membrane proteins [15-20]. Since its development, the Nanodisc technology has proved to be the method of choice for detailed mechanistic studies of G-protein-coupled receptor systems [21-26], cytochromes P450 [16, 20, 27-37], ion channels [38-41], transporters [42-47], chemotactic receptors [48-49], and other biological systems [50-53].

There are several important advantages for using Nanodiscs in biochemical and biophysical studies of membrane proteins. First is the precise control of oligomerization state of the target protein and subsequent separation of Nanodiscs containing populations of monomers and oligomers [15, 21, 27, 50-54]. This approach offers an unprecedented opportunity to study the functional properties of monomers and oligomers with no need to deal with the monomer - oligomer equilibria and simultaneous presence of multiple species with different properties. Nanodiscs allowed the first direct measurement of the binding and native photochemical properties of monomeric rhodopsin [21, 24-26] and green proteorhodopsin [55], activation of G-proteins by monomeric β2 receptors [22, 56], activation of unclustered integrins [53] and the identification and characterization of the functional unit of chemotactic receptors [49].

In addition, highly homogeneous stable and reproducible preparations of solubilized membrane proteins allow for the application of a plethora of modern biophysical methods

and precise quantitative analysis of acquired experimental data. Examples include the application of global analysis to the deconvolution of binding constants and functional properties of binding intermediates during cooperative catalysis of testosterone hydroxylation by CYP3A4 [29], single-molecule experiments describing the allosteric effects in CYP3A4 substrate binding [37], stabilization of transient intermediates in CYP3A4 and CYP19 for spectroscopic studies at cryogenic temperatures [16, 34], and the functional immobilization of CYP3A4 on gold nanoparticles for sensing and screening using local surface plasmon resonance methods [57]..

In many cases Nanodiscs improve the stability of membrane proteins as compared with detergent-solubilized preparations. This was shown for CYP3A4 at room temperature (by the decreased rate of formation of inactive P420 form), for ATP synthase from *E. coli*, for human monoamine oxidase A, and for epidermal growth factor receptor [15, 58-60]. Although in most cases the stabilization mechanisms have not been identified, the general effect may be attributed to the native-like incorporation of membrane proteins into lipid bilayers and to the absence or reduced concentrations of detergents.

Control of the local lipid composition provides new opportunities for detailed microscopic studies of protein binding to membranes, influence of lipids on protein-protein interactions mediated by the membrane, and certain specific functional properties of membrane proteins [61-62]. For P450 systems this is especially important, because not only cytochrome P450 enzymes, but their redox partner proteins, hepatic cytochrome P450 reductase (CPR) and mitochondrial adrenodoxin reductase, can also be incorporated into the membrane. Therefore, mutual orientations and matching of protein–protein interfaces between interacting cytochrome P450 and reductase are defined by the depth of insertion and orientation of each protein with respect to the lipid bilayer, electrostatic interactions and metal ions at the bilayer interface, as well as the mobility and dynamics of all components. Equilibrium and kinetic parameters of hydrophobic substrates binding to membrane cytochromes P450 can be significantly modulated by presence of the lipid bilayer, since some substrates can access the active site of the heme enzyme directly from the membrane [7, 63-64]. In addition, the electrostatic field created by membrane lipids may significantly affect the redox potentials of cytochromes P450 [65] and of the flavoprotein redox partners [66]. Such effects demand a platform where one can perform mechanistic studies of cytochromes P450 reconstituted in the lipid bilayers, with an ability to control and vary lipid composition, oligomerization state of the proteins, and the stoichiometric ratio between protein components. Nanodiscs provide such a platform, making available a wide range of lipid-membrane protein systems that can be probed experimentally.

2. Materials

Prepare all solutions using ultrapure water with a sensitivity of 18MΩ at 25°C. Unless a specific vendor or product is listed, all reagents should be of the highest grade available.

2.1 Materials

Amberlite XAD-2 (Supelco)

Ni-NTA resin (Invitrogen) 2′-5′ ADP agarose resin

Size-exclusion column: Superdex 200 10/300 (GE Life Sciences)

Microcentrifuge filters, 0.22 μm (Millipore)

2.2 Buffers

Column buffer: 100 mM phosphate buffer, pH 7.4

Disc buffer: 100 mM phosphate buffer, pH 7.4, 50 mM NaCl

Buffer with cholate: 100 mM phosphate buffer, pH 7.4, 50 mM NaCl, 100 mM sodium cholate. 10 mM 2-mercaptoethanol

Buffer without cholate: 100 mM phosphate buffer, pH 7.4, 50 mM NaCl, 10 mM 2-mercaptoethanol

Buffer A: 100 mM phosphate buffer pH 7.4, 300 mM NaCl

Buffer W: Buffer A containing 15 mM imidazole

Buffer E: Buffer A containing 300 mM imidazole

2.3 Other Solutions

Lipid solubilization solution: 100 mM sodium cholate, 100 mM

NaCl

100 mM $NiSO_4$

Triton X-100 stock: 20% Triton X-100 (w/v) in ultrapure water

Palmitoyloleoylphosphatidylcholine (POPC) stock in chloroform

2.4 Protein stocks: P450 target in buffer of choice

His-tagged membrane scaffold protein (MSP) in disc buffer.

MSP1D1 for P450 Nanodiscs and MSP1E3D1 for P450-CPR

Nanodiscs.

3. Methods

3.1 Self-Assembly of P450 in Nanodiscs Using Cholate

1. Using a glass Hamilton syringe, dispense 427 μl of 75 mM chloroform-POPC stock into a disposable 18-ml glass culture tube (*see* Notes 1, [2], [3]).

[1]Starting with the desired amount of P450, calculate the required amount of lipid. We have used POPC as an example. Other lipids such as Dimyristoylphosphatidylcholine (DMPC) and Dipalmitoylphosphatidylcholine (DPPC) can also be used to make Nanodiscs [13, 68]. Other membrane-scaffolding proteins can be used (we have used MSP1D1 as an example), using the following ratios: P450: MSP = 1:10, and POPC: MSP1D1 = 1: 64.

[2]The size of the membrane-scaffolding protein variant (MSP1D1, MSPE3D1, etc.) and the lipid molecule determine the optimal ratio of lipids to MSP to be used for a Nanodisc preparation [13-14, 18, 68].

2. In a fume hood, dry the chloroform using a gentle stream of nitrogen gas. A thin, uniform film of lipid on the lower walls eases lipid solubilization in an aqueous medium later, and can be obtained by rotating the tube while holding it at an angle.
3. Place the tube in a vacuum desiccator for at least 4 hours to remove any residual solvent (*see* Note 4).
4. Add 640 μl of solution containing 100 mM sodium cholate and 100 mM NaCl to the tube (*see* Note 5).
5. Solubilize lipids by immersing the tube in warm water (50-70 °C) for 30-60 seconds and then vortexing. Repeat this until no lipid remains on the walls of the tube.
6. Sonicate the tubes in an ultrasonic bath for 5-10 minutes (*see* Note 6). *Steps 7-13 are done at 4 °C, or on ice unless otherwise mentioned*
7. Add 640 μl of buffer with cholate to the tube (*see* Note 7).
8. Add 160 μl of buffer without cholate to the tube (*see* Note 7).
9. Add 3333 μl of 150 μM MSP1D1(−) to the tube (*see* Notes 8, [9]).
10. Add 500 μl of 50 μM P450 to the tube (*see* Note 10)
11. Let the mixture incubate on ice for 20-30 minutes.
12. Add 6 ml equivalent of wet Amberlite XAD-2 beads of the disc reconstitution mixture (*see* Note 11).

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[3]Lipid stocks are maintained in chloroform, usually in concentrations between 25 and 100 mM. Lipids stocks are stored in 4-ml glass vials with Teflon-lined screw caps, to minimize changes in concentration between usages. The stock concentration is determined by analyzing the total phosphorus, by a colorimetric assay [69-70].

[4]For best results, leave the tube in the vacuum desiccator overnight.

[5]A final cholate concentration of 50 mM ensures quick and efficient solubilization.

[6]The lipid solution should be clear at this point. If not, repeat steps 5 and 6 until the solution becomes transparent. For hard-to-solubilize lipid films sonicate in short cycles of 2-3 minutes, vortexing the tube between cycles.

[7]Given the amount of lipids, calculate the total volume of the disc reconstitution mixture that would result in a final lipid concentration of 5-8 mM. Using the calculated final volume and the amount of sodium cholate used to solubilize the lipids calculate the amount of buffer with cholate that would need to be added to the tube to achieve a final cholate concentration of 14-20 mM. Add the required amount of MSP and P450 and adjust the volume of the mixture to equal that calculated earlier using Buffer without cholate.

[8]MSP1D1(−) denotes MSP1D1 without the histidine tag. The histidine tag on membrane-scaffold proteins can be cleaved with tobacco etch virus (TEV) protease. It is best to prepare His-tag-cleaved MSP beforehand and have it ready in a concentrated stock of 150-250 μM in disc buffer. Use of a His-tag-cleaved variant of MSP and a His-tagged target protein allows a simple isolation of Nanodiscs with embedded P450 from bare discs, using Ni-NTA chromatography.

[9]Measure the concentration of MSP every time a frozen stock solution is thawed. The extinction coefficients of more-routinely used MSP variants are [18]:

	Σ_{280} (in M^{-1} cm^{-1})	
MSP variant	With His-tag	Without His-tag
MSP1D1	21000	18200
MSP1E3D1	29400	26600

[10]A stock solution of concentration 25-50 μM of the protein works best.

[11]For best results, use 0.8-1 ml equivalent of wet BioBeads per ml of sample. Remove all excess water from the beads before use. This can be done by spreading the beads on a stack of KimWipes on a glass plate.

13. Place the tubes in an ice bucket secured on a shaker, to gently agitate the mixture. Make sure all the beads are suspended in solution. Alternatively, one may put the tube on a shaker platform placed inside a cold cabinet (*see* Note 12).

14. After at least 6 hours of incubation with Amberlite XAD-2, filter the solution to remove the hydrophobic beads with the help of plastic disposable filter columns (*see* Note 13).

15. Load the solution on to a 1-ml Ni-NTA column (*see* Note 14), pre-equilibrated with Buffer A.

16. Wash the column with 5 ml of Buffer W and elute with Buffer E (*see* Note 15).

17. Concentrate the eluate from the Ni-NTA column and filter it using disposable microcentrifugal filters (0.22 μm).

18. Load the filtrate onto a calibrated Superdex-200 10/300column (GE Life Sciences), pre-equilibrated with the column buffer.

19. Collect fractions and pool those containing P450 Nanodiscs and having the desired level of enzymatic activity, substrate-binding characteristics, or with the anticipated size to obtain a homogenous, monodisperse, stable membrane-P450 preparation (*see* Note 16).

20. P450-POPC Nanodiscs can be stored at 4 °C for 1-2 weeks. For long-term storage, add 15-20% of ultrapure glycerol, flash-freeze and store at −80 °C.

3.2 Self-Assembly of P450 in Nanodiscs Without Using Cholate

1. Perform steps 1—3 of Method 3.1.

2. Add 1960 μl of disc buffer to the tube containing dried lipids, and seal with laboratory film. Resuspend the lipids by three cycles of vortexing for 30 seconds, immersing the tube in liquid N_2 and thawing the frozen solution at room temperature (*see* Note 17).

3. Deliver 115 μl of 20% Triton X-100 to the tube (*see* Note 18).

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[12]Choice of the incubation temperature depends on the phase-transition temperature of the lipid being used, with the goal of assembly of a slightly higher temperature. For POPC, this is 270 K [71].

[13]For efficient recovery of protein solution wash spent beads with 2-3 equal volumes of cold buffer and add it to the filtrate recovered earlier.

[14]Use 1 ml of Ni-NTA resin for every 5-6 mg of protein sample.

[15]Wash until there is no protein in the flow-through. To verify, mix 2 μl of Commassie reagent with 100 μl of the flow-through. The appearance of a blue color indicates the presence of a protein. Typically, washing with 4-6 column volumes of the wash buffer is sufficient.

[16]The retention time of P450-embedded Nanodiscs is only 0.3-0.5 minute less (depending on the sample volume) than that for empty Nanodiscs of same MSP-lipid pair.

[17]Ensure that after vortexing all POPC has dislodged from the surface of the tube. At the end of this process the solution will possess a cloudy white appearance.

[18]The amount of Triton X-100 in this preparation is an important determinant of the final yield of Nanodisc-associated P450. Ideally, only enough detergent to fully solubilize the POPC will be present in the mixture before addition of Amberlite XAD-2. We have observed that detergent excesses result in the formation of large aggregates and result in extremely low yields. However, failure to completely solubilize the lipids will similarly interfere with the assembly of Nanodiscs. For protein stock solutions containing ~ 50 μM P450 and 0.02-0.2% Emulgen 913, we have determined that the optimal molar ratio of Triton X-100 to POPC is 1.15:1 at this step.

4. Seal the tube with laboratory film and sonicate for 30 minutes in a heated bath sonicator set to ~60°C. Transfer the solution to an ice bucket and allow 5 minutes of cooling before proceeding. The solution should now be slightly more translucent than observed in step 2.
5. Add 2000 μl of 250 μM MSP and 500 μl of 50 μM detergent-solubilized P450.
6. If the solution remains cloudy after 1 minute, add 5-10 μL of 20% Triton X-100 and gently mix with a transfer pipette. Repeat this step until the solution has completely clarified.
7. Perform steps 11—20 of Method 3.1.

3.3 Co-incorporation of P450 and Cytochrome P450 Reductase (CPR) into Nanodiscs

1. In order to self-assemble Nanodiscs containing both P450 and CPR, mix the two proteins in a ratio of 1:2, preserving the detergent concentration that the membrane P450 is stored in.
2. Incubate this mixture for one hour at (room temperature).
3. Add this mixture to Nanodisc components consisting of cholate-solubilized POPC and MSP1E3D1(−). For MSP1E3D1 POPC Nanodiscs, the optimal ratio of lipids to MSP is 120:1 per Nanodisc.
4. Incubate the mixture at room temperature for 45 minutes and then on ice for an additional hour.
5. Perform steps 12—16 of Method 3.1.
6. Load the Ni-NTA eluent onto a 2′-5′-ADP-agarose column equilibrated with buffer of choice (*see* Note 19).
7. Wash the column with 3-5 column volumes of equilibration buffer. 8. Elute the protein with 2-3 column volumes of equilibration buffer containing 2.5 mM 2′-AMP.
8. Dialyze the eluent against 1000-fold volume of disc buffer, with three exchanges every 3-4 hours.
9. Load the solution on to a calibrated Superdex-200 10/300 column (GE Life Sciences) pre-equilibrated with column buffer.
10. Perform steps 20—21 of Method 3.1.

Functional CPR: P450 complexes with different CPR to P450 ratios can also be prepared by adding full-length CPR to the solution of P450 preassembled into Nanodiscs at 37 °C. The complexes thus formed are stable and can be separated by size-exclusion chromatography. Functional properties for P450-CPR complexes assembled in this manner depend on the molar ratio of P450 and CPR used. For CYP3A4, NADPH oxidation and testosterone

[19]Nanodiscs containing both P450 and CPR will bind to the 2′,5′-ADP column, whereas those containing only P450 would flow-through.

hydroxylation have been found to depend on the CPR:CYP3A4 ratio peaking at tenfold molar excess of CPR [67].

More specific details can be found in original publications, including supplemental materials in references [11, 13, 30, 65].

In conclusion, we provide a list of cytochrome P450 studies that used Nanodiscs and a brief summary of results. Most of them were done with the most abundant xenobiotic-metabolizing human CYP3A4; however, recently, we initiated systematic mechanistic studies of steroidogenic human enzymes CYP19 and CYP17. Different aspects of substrate binding have been addressed in [20, 27, 32, 36-37, 72-74], dynamics and kinetics in [20, 28, 31, 35, 37, 75-76], catalytic activity in [29, 33, 67] and redox properties of cytochrome P450 [28, 65, 75] and CPR in Nanodiscs [66]. We also applied cryogenic spectroscopy to study unstable oxygenated intermediates in CYP19 [34, 76] and in CYP3A4 [16], and documented the EPR spectra and annealing of the peroxo-intermediate in CYP19, obtained using cryoradiolytic reduction [34].

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