Applications of Lipid Nanodiscs for the Study of Membrane Proteins by Surface Plasmon Resonance

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Methods for the initial steps of surface plasmon resonance analysis of membrane proteins incorporated in lipid nanodiscs are described. Several types of Biacore sensor chips are available and require distinct strategies to immobilize proteonanodiscs on the chip surface. The procedures for immobilization on three of these chips (NTA, antibody coupled CM5, and L1) are described in this unit and results are demonstrated for a model system with cytochrome P4503A4 (CYP3A4) in nanodiscs binding to a polyclonal anti-CYP3A4 antibody. Advantages and disadvantages of each chip type are considered. © 2015 by John Wiley & Sons, Inc.

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INTRODUCTION

Methods for characterizing the structure and function of membrane proteins have lagged behind corresponding approaches for the study of soluble proteins. Despite significant advances in structural methods applied to membrane proteins (Topiol, 2014; Baker and Baldus, 2014; Huster, 2014; Moraes et al., 2014), it is often difficult to identify and implement the best conditions for purifying membrane proteins and for studying their functional interactions with ligands or other proteins. As a result many membrane proteins are studied in a diverse range of artificial membranes or detergents that are not optimal for all the studies desired for a complete characterization. The fundamental kinetic and thermodynamic parameters that describe the interaction of many protein complexes are required for accurate prediction of binding to cell surface receptors. However, the effects of receptor solubilization on these in vitro parameters remain uncertain and likely vary for different receptors. Even with a wide range of lipid systems or detergents available, many analytical methods applied to systems of this type remain difficult or impossible.

Surface plasmon resonance (SPR) has revolutionized the study of molecular interactions between soluble proteins and their partners or small molecule ligands. However, the application of SPR to membrane proteins is not always successful and may be significantly more susceptible to artifacts (Hahnefeld et al., 2004; Rich and Myszka, 2000;

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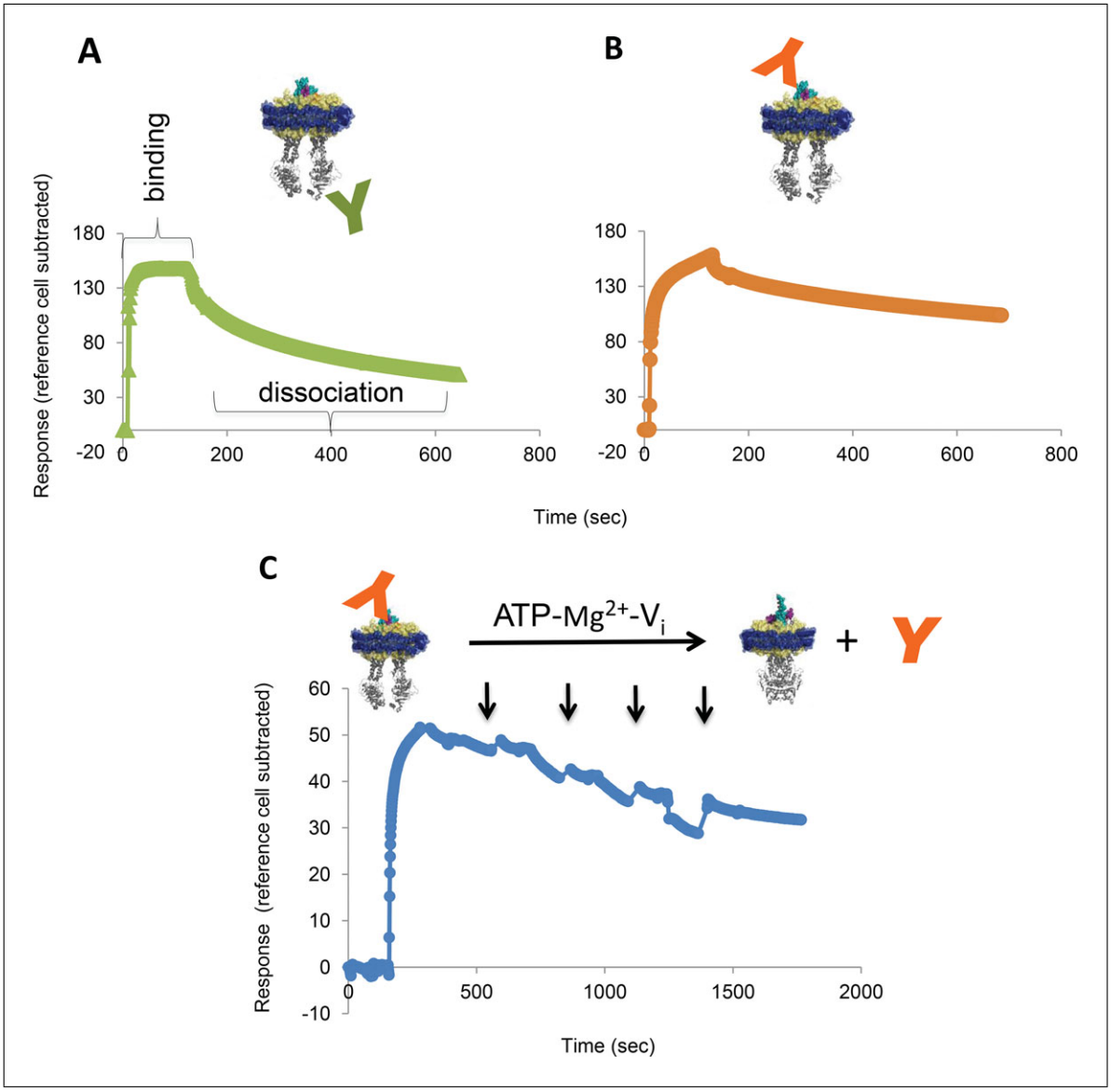

Figure 29.13.1 SPR can be used to interrogate membrane protein epitopes on either side of the membrane. P-glycoprotein (Pgp) embedded in MSP1D1E3/*E.coli* lipid nanodiscs were captured on L1 sensor chips. (**A**) Monoclonal antibody (M2) binding to the C-terminal FLAG tag on the cytosolic side of Pgp detected by SPR. (**B**) Monoclonal antibody (UIC2) to an extracellular epitope was detected by SPR. The results for k_{on} or k_{off}, which would require multiple analyte concentrations, have not been analyzed and for standard analytical procedures would require that the RU response reaches a saturable plateau. (**C**) UIC2 bound to Pgp nanodiscs on the L1 chip is eluted by increasing concentrations of ATP-Mg^{2+}-orthovanadate (injections depicted by arrows). ATP-Mg^{2+}-orthovanadate binds to the ATP- binding domain of Pgp, alters the conformation of Pgp and lowers the affinity for the UIC2 antibody. In panel C, by the fourth addition of ATP-orthovanadate-Mg^{2+}, the bulk refractive index change is large and results in a slightly different dissociation response. In these experiments, the reference cell contained empty nanodiscs captured to a similar level.

Rich and Myszka, 2010; de Mol, 2012). These experiments often include immobilization of a soluble protein, or solubilized portion of a membrane protein, on a sensor chip with subsequent analysis of a soluble binding partner as an analyte. In principle, there could be value in immobilizing full-length membrane proteins in a native-like membrane environment using soluble proteins or small molecule ligands as analytes. Attempts have been made to use liposomes as the membrane in this experimental approach (Maynard et al., 2009; Kinouchi et al., 2013; Zeng et al., 2014). However, liposomes are not obviously generalizable to many SPR analyses. In short, traditional membrane platforms have limitations when used in SPR analyses. For the purpose of this unit, it is assumed

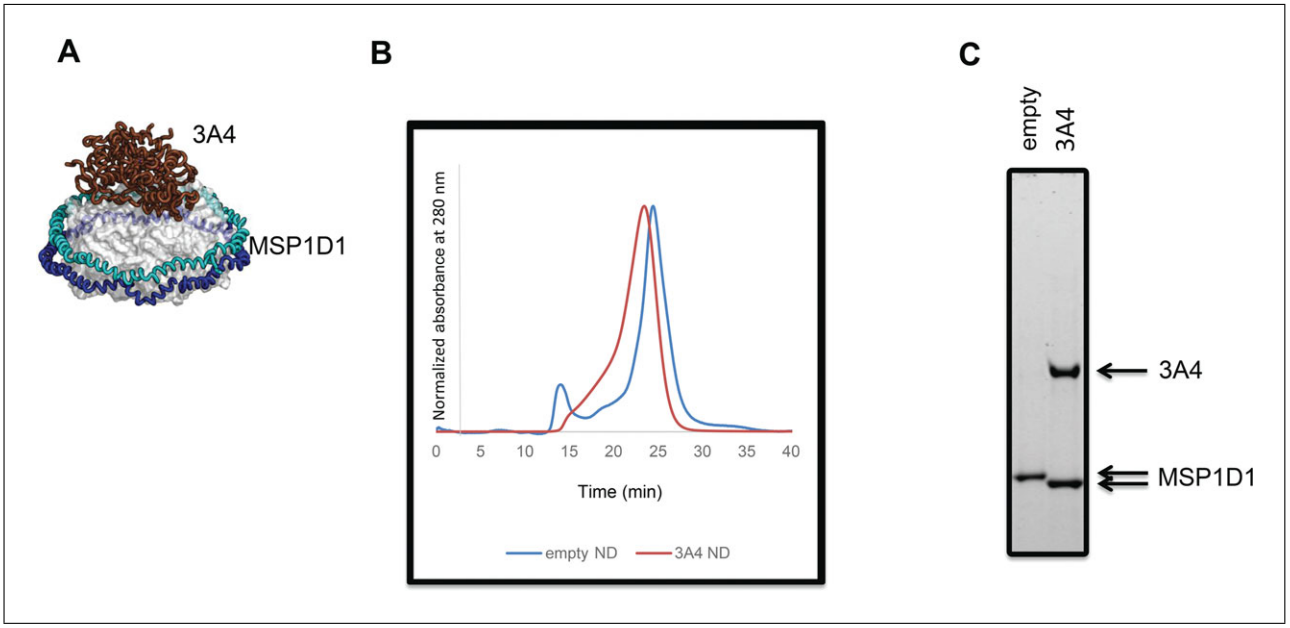

Figure 29.13.2 The model proteonanodisc platform. (**A**) A model of cytochrome P450 3A4 (CYP3A4) proteonanodisc with two molecules of the scaffold protein, MSP 1D1, surrounding a POPC lipid bilayer. (**B**) Trace from size-exclusion chromatography (SEC) purifications of the empty nanodisc and the CYP3A4 proteonanodisc. (**C**) Peak fractions 20 to 27 for 3A4 proteonanodiscs or 22 to 28 for empty nanodiscs, from the SEC step were concentrated analyzed by SDS-PAGE using a 4% to 20% gradient gel. The two species of MSP1D1 represent the full length histidine-tagged protein used to generate empty nanodiscs and the histidine-tag cleaved protein used to prepare the CYP3A4 proteonanodiscs.

that the reader will have basic familiarity with the theory of SPR, and a practical guide to start experiments and optimize the sensor chip surface to detect subsequent interactions with proteins is provided.

A recently developed model membrane platform may have advantages for the study of membrane proteins by some methods, including SPR. Lipid membrane nanodiscs have been used to capture several membrane proteins in lipid bilayer particles of defined, monodisperse size, and stoichiometry (Denisov et al., 2004; Nath et al., 2007; Ritchie et al., 2009; Grinkova et al., 2010; Tavoosi et al., 2013). The nanodiscs are self-assembled particles comprised of a lipid bilayer of defined composition circumscribed by engineered membrane scaffold proteins (MSPs) of defined length, and a target membrane protein. The MSPs are variants of human apolipoprotein A1 and the availability of a range of MSPs of varied lengths translates into the possibility of generating membrane discs of varying diameters. Target proteins successfully incorporated into nanodiscs include peripheral, integral, and full transmembrane proteins. Many nanodisc-protein particles are well characterized and demonstrate the versatility of this model membrane system in many applications. A useful feature of nanodisc-incorporated transmembrane proteins is that epitopes on both sides of the membrane are available for analysis. This is demonstrated in Figure 29.13.1—an example where two different antibodies can be observed to bind to human P-glycoprotein incorporated into nanodiscs by SPR. A second example with P-glycoprotein-nanodiscs demonstrates that both sides must be available in at least some particles. Here ATP and VO_4^- bind to the cytosolic nucleotide-binding domains and cause a conformational change that releases the UIC2 antibody, known to bind at the external side of P-gp in its ligand-free form. Such a result would not be possible with liposomes where both nucleotide and the UIC2 antibody could not reach their binding sites on a single P-gp. Nanodiscs provide a potential means of studying complex interactions via SPR with some unique advantages.

There are only a few examples of the application of nanodiscs to study membrane proteins by SPR (Goluch et al., 2008; Das et al., 2009; Glück et al., 2011; Ritchie et al., 2011; Proverbio et al., 2013) and the limited experience with this combination of technologies already suggests that many experimental parameters must be considered and optimized. Therefore, the purpose of this unit is to provide protocols for the initial parameterization of SPR experiments in which a membrane protein or receptor incorporated in a nanodisc is immobilized on SPR chips for analysis of binding to a soluble partner protein. The protocols provided here do not include the actual analysis of rates or equilibria for binding, which has been reviewed previously (Hahnefeld et al., 2004). Rather, methods to prepare different sensor chips with immobilized nanodiscs and proteonanodiscs, using the membrane protein cytochrome P4503A4 (CYP3A4) nanodiscs as a model, are provided. It is nearly certain that the optimal immobilization strategies will be different for different proteonanodiscs, so the purpose of this unit is to provide strategies for this optimization. The purity of the CYP3A4 nanodiscs used here is shown in Figure 29.13.2, as a benchmark for the level of homogeneity of proteonanodiscs that yields the best results. In addition, a polyclonal anti-CYP3A4 is used as a model analyte to demonstrate some non-standard SPR results. These atypical results are instructive as described within.

STRATEGIC PLANNING

The first decision to be made, and the focus of these protocols, is the type of sensor chip and corresponding immobilization strategy for the protein to be analyzed. Three types of sensor chips discussed here are schematized in Figure 29.13.3, and they include NTA chips for capture of his-tagged molecules, CM5 chips with amine-coupled antibody, and L1 chips, which capture via hydrophobic interactions with the lipid phase of the nanodisc.

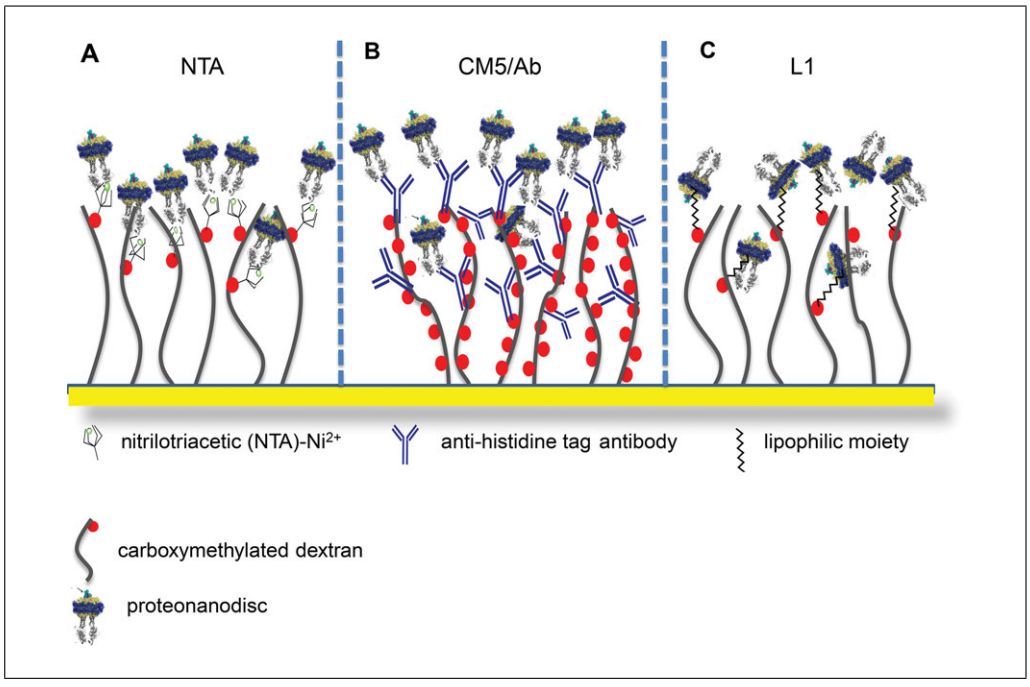

Figure 29.13.3 A schematic of the SPR sensor chips and their capture strategies. Depicted are three Biacore sensor chips that were used for capturing nanodiscs. All chips have a gold layer coated with a carboxymethylated dextran matrix. The first two chips, NTA and CM5/Ab, exploit the histidine tag on the nanodisc for capture. The third, the L1 chip, uses the lipid bilayer for capture. (**A**) The nitrilotriacetic acid (NTA) chip is charged with nickel ions that chelate the NTA and the histidine-tagged nanodisc. (**B**) The CM5 chip uses amine coupling to covalently link an anti-histidine tag antibody to the chip surface that binds to the histidine-tagged nanodisc. (**C**) The L1 chip has a proprietary lipophilic substituent on the dextran that inserts into the lipid bilayer. This chip does not require histidine-tagged nanodiscs.

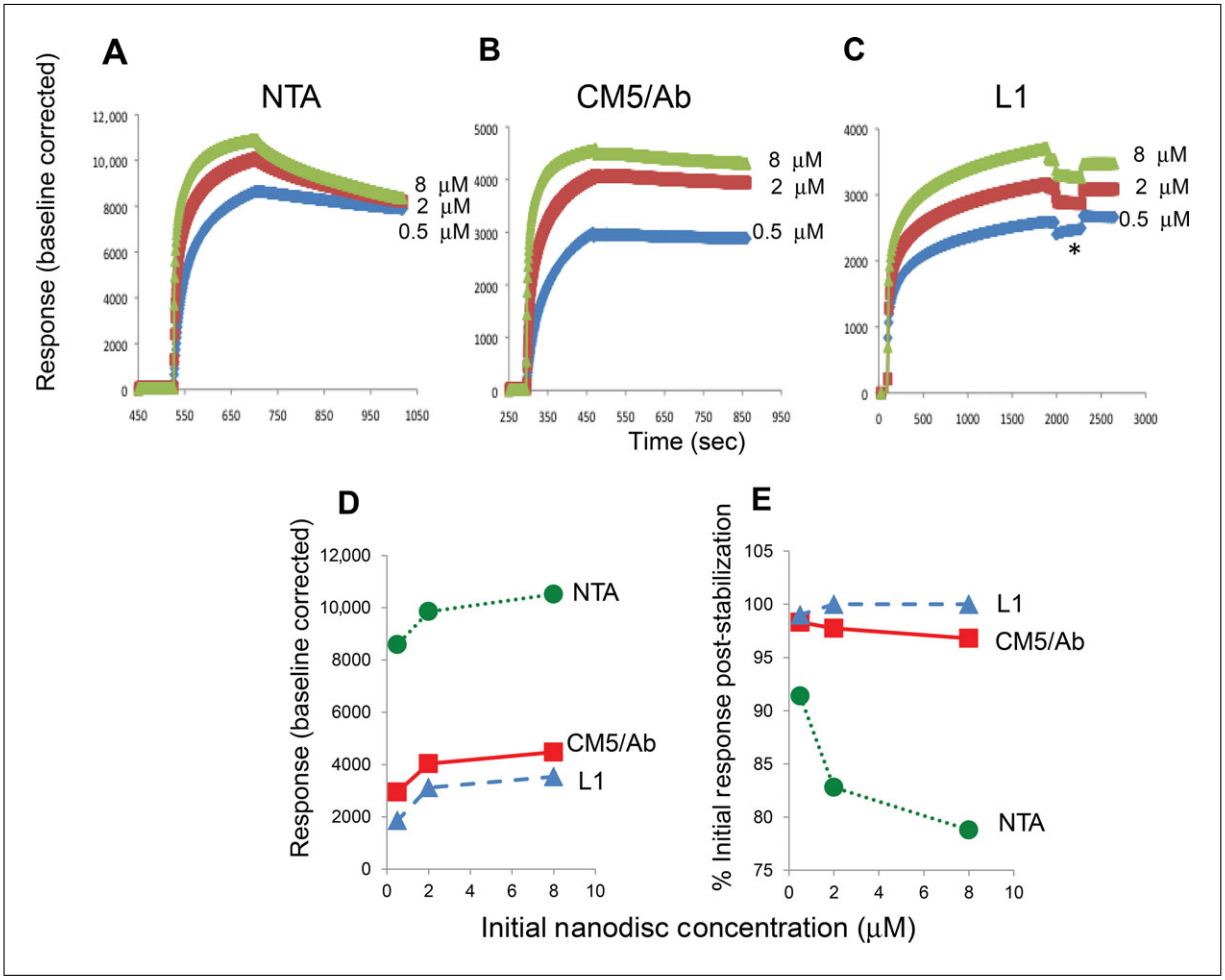

Figure 29.13.4 Capture and stability of nanodiscs on NTA, CM5 anti-histidine antibody and L1 sensor chips. Three concentrations of histidine-tagged MSP1D1-POPC empty nanodiscs (8, 2, and 0.5 mM) were captured on three different sensor chips. (**A**) The NTA chip surface was charged with nickel then loaded with the indicated concentrations of nanodiscs for 3 min followed by a 5-min stabilization period. (**B**) The CM5 chip was activated with EDC/NHS to amine couple the anti-6-his antibody then loaded with nanodiscs for 3 min followed by a 5-min stabilization period. (**C**) The L1 chip was conditioned with brief injections of 20 mM CHAPS, washed with buffer then loaded with nanodiscs for 30 min, tested for saturation with BSA, followed by a 5-min stabilization period. The decline seen (noted with an asterisk) is the bulk shift effect due to the BSA injection. All chips were regenerated in between cycles of increasing nanodisc concentrations. (**D**) The capture capacity of the different chips derived from the data in A) to C as a function of the initial nanodisc concentration used for capture. (**E**) The percentage of the captured nanodisc response remaining after the 5-min stabilization period as a function of the initial nanodisc concentration used for capture.

Figure 29.13.4 shows results for the capture of empty nanodiscs on each of these surfaces. Note the difference in the maximal response for each surface, which is due to differences in functional group density, steric restrictions, or other undefined characteristics. These differences are optimized empirically as described below.

The data in Figures 29.13.1 and 29.13.5 illustrate some critical components in the strategic planning process for nanodisc-based SPR experiments, and they provide a comparison of advantages and disadvantages of different approaches. Three important parameters must be optimized, and they are not independent—the amount of nanodisc that is captured on the sensor chip surface, the stability of captured ligand, and the degree of nonspecific analyte binding. The extent of nanodisc capture determines the sensitivity of the experiment and detection of small molecule binding requires high loading. R_{max} is the theoretical maximal response that is possible for analyte binding based on the amount of immobilized ligand, proteonanodisc in this case. R_{max} must be large for small

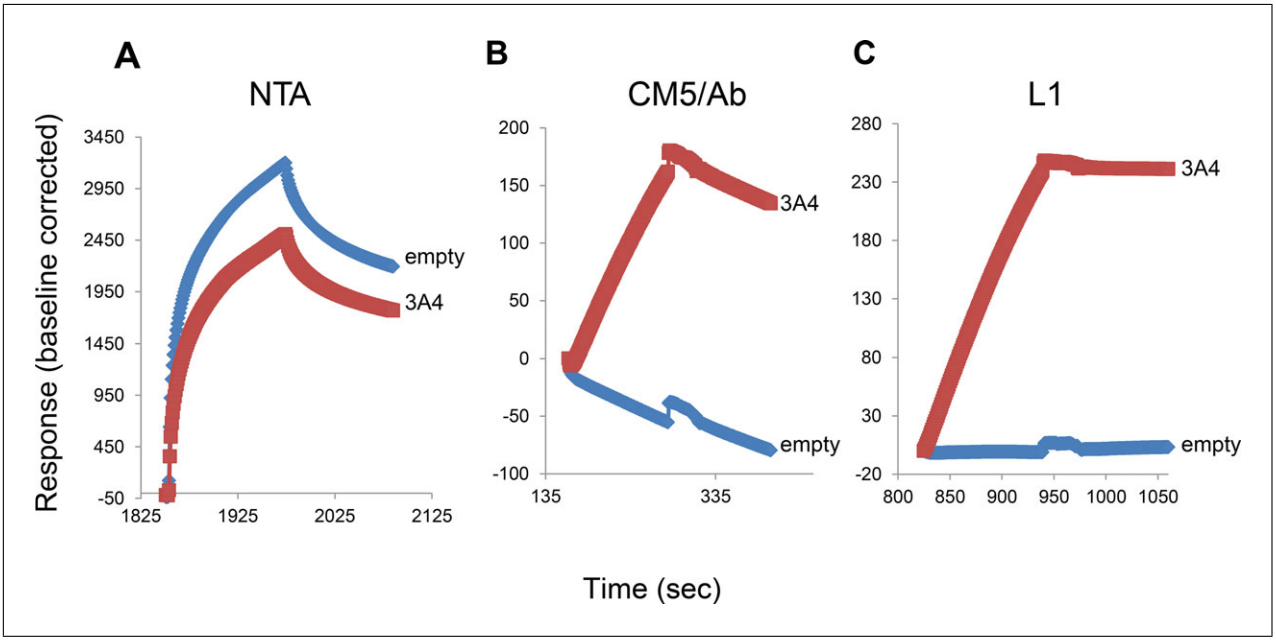

Figure 29.13.5 Antibody binding to nanodiscs captured on the NTA, CM5/Ab, and L1 chips. CYP3A4 proteonanodiscs (experimental cell) and empty nanodiscs (reference cell) were captured on (**A**) NTA chip (~300 RU captured for each), (**B**) CM5/Ab chip (~1000 RU captured for each), or (**C**) L1 chip (~2700 RU captured for each). A rabbit polyclonal anti-3A4 antibody (100 μg/ml) was injected for 120 sec over each surface and a 120-sec dissociation phase recorded.

molecule analytes, which require a high level of capture. However, a high level of capture can lead to increased nonspecific binding, which requires more rigorous referencing (see Critical Parameters). The stability of the surface determines the baseline drift, and thus the ability to properly 'reference' the binding reaction against nonspecific interactions. As seen in Figure 29.13.4, the NTA chips easily load a high level of his-tagged nanodiscs with nearly 12,000 RUs on the surface at the highest concentration of nanodiscs, and this is significantly higher than the loading achieved with either L1 or CM5/anti-6-his antibody surfaces. However, as shown, the NTA capture method does not yield a stable surface—with the highest initial level of nanodiscs (8 μM), 21% of the mass was lost during the 5-min stabilization period. This loss of nanodiscs from the NTA surface during the minutes following loading is obviously a problem for subsequent binding analysis. Methods for stabilizing protein-NTA surfaces have been reported (Rich et al., 2011). Specifically, after capturing the nanodisc on the nickel-activated NTA chip, the surface is treated with EDC/NHS. This results in covalent amine coupling of the proteins, or proteonanodiscs providing a stable surface. That protocol is beyond the scope of this unit, but it should be considered for analysis of small molecule binding to proteonanodiscs (Rich et al., 2011). One disadvantage to this NTA/amine coupling approach is cost. The resulting surfaces with covalently captured proteins are not reusable.

The CM5/antibody-nanodisc and L1-nanodisc chips capture similar levels of nanodiscs, although both are much lower than the NTA chip. This level is adequate for analysis of protein or large molecule binding, as shown in Figure 29.13.5. Although both L1-nanodisc and CM5/antibody-nanodisc surfaces are significantly more stable than the NTA chips (with no covalent capture), the L1-nanodisc surface is more stable than the CM5/antibody-nanodisc. For example, with the top nanodisc concentration of 8 μM the CM5/antibody lost 3.2% of the captured nanodisc mass during stabilization, while the L1 chips had no detectable loss. Even a low level of instability can be a serious impediment if the expected R_{max} values for analyte binding are very small. Thus, L1 chips should be considered for work with proteonanodiscs. The data in Figures 29.13.4 and 29.13.5 suggest that multiple chips should be compared for their interaction with specific

proteonanodiscs and the corresponding empty nanodiscs to determine the best choice for each specific experiment. Differences in loading arise from inherent differences in the chip surfaces, including functional group density and different bulk refractive indices for different chips.

Nonspecific binding results from weak off-target interactions between the surface and the analyte and can occur at the level of the actual chip surface or ligand molecules attached to it. As a result, it varies significantly with different combinations of ligand and analyte and is best assessed empirically. This is discussed further in Anticipated Results.

It is clear that the ability of SPR to detect small ligand binding to membrane proteins in nanodiscs will be limited to cases where nonspecific binding is negligible and the expected R_{max} is much greater than the observed drift. This is most likely achievable with the capture-coupling method that uses NTA chips, as discussed above (Rich et al., 2011). However, this method could become cost-prohibitive due to the need to use new chips for each experiment.

ANTIBODY BINDING TO NANODISCS ON NTA CHIP

BASIC PROTOCOL 1

Most membrane proteins are currently expressed in vitro with an affinity capture handle, such as histidine-tags or FLAG-tags. The same handle can be used in subsequent SPR work to attach the membrane protein that has been incorporated into a nanodisc on the sensor chip surface. A protocol for histidine-tagged proteins is provided, which should be adaptable for membrane proteins with other affinity handles. The NTA surface is charged with Ni^{2+}, allowing capture of histidine-tagged proteins.

Materials

Running buffer (see recipe)
350 mM and 3 mM EDTA, pH 8
0.5 mM $NiCl_2$
Purified empty and CYP3A4 nanodiscs (diluted in running buffer, centrifuged 30 min at 16,000 × *g*, 4°C, to remove particulates)
100 μg/ml affinity-purified rabbit polyclonal anti-CYP3A4 antibody in running buffer

Series S sensor chip NTA (GE Healthcare Life Sciences)
Biacore T200
Biacore rubber caps
Biacore plastic vials

NOTE: Temperature of chip and sample compartment is set to room temperature (25°C). The running buffer must not contain divalent cations such as Mg^{2+} or Ca^{2+}.

Condition NTA chip

1. Dock a new series S sensor chip. Prime Biacore T200 instrument with running buffer.
2. Set flow rate to 30 μl/min; flowpath = 2-1.
3. Inject 350 mM EDTA for 60 sec.
4. Inject running buffer two times for 60 sec each time.
5. Indicate an "extra wash" step with running buffer.

The Biacore software provides a method run for conditioning the chip with EDTA and charging with Ni^{2+}. Set a conditioning assay step with one cycle of 60 sec of 350 mM EDTA, at a flow rate of 30 μl/min. Indicate an "extra wash" step with running buffer.

Charge NTA chip with nickel

6. Set flow rate to 10 μl/min.
7. Inject 0.5 mM $NiCl_2$ for 60 sec.
8. Indicate an "extra wash" step with 3 mM EDTA.
9. Allow a stabilization time of 60 sec.

Nickel charging gives an increase of ~40 resonance units (RU) at baseline.

Perform nanodisc (ligand) binding

Choose empty nanodiscs as the reference cell (e.g., flow cell 1) and proteonanodiscs as the experimental cell (e.g., flow cell 2). Reference subtracted sensorgrams can be obtained as flow cell 2 minus flow cell 1.

10. Set flow rate to 10 μl/min; flowpath = flow cell 1.
11. Inject empty nanodiscs for 180 sec (3 min).

The injection time will depend on the specific capture needs (see Critical Parameters) and concentration of nanodiscs.

12. Change to flowpath = flow cell 2.
13. Inject proteonanodiscs for 180 sec (3 min).

The captured proteonanodisc level should match the empty nanodisc level for good referencing and to minimize experimental artifacts.

Perform antibody (analyte) binding

14. Increase flow rate to 50 μl/min in flow paths 1 and 2.
15. Inject affinity-purified rabbit polyclonal anti-CYP3A4 antibody for 120 sec. Wait 120 sec for dissociation.

For double referencing, include a running buffer (matched to any additives that may be in the antibody solution) injection for 120 sec and wait for 120 sec. The buffer control can be subtracted post-hoc.

Regenerate

16. Select both flow cells. Set flow rate to 30 μl/min.
17. Inject 350 mM EDTA for 60 sec.
18. Indicate an "extra wash" step with running buffer.

BASIC PROTOCOL 2

NANODISCS ON CM5 CHIP IMMOBILIZED WITH ANTI-6-HISTIDINE ANTIBODY

This approach also exploits the histidine tag on the nanodisc. An anti-6-histidine antibody is immobilized on the surface via amine coupling allowing capture of histidine-tagged proteins.

Materials

HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.0005% v/v surfactant P20)
0.4 M 1-ethyl-3-(3-dimethylaminoprolyl)-carbodiimide (EDC)
0.1 M N-hydroxysuccinimide (NHS)
Mouse anti-6-histidine antibody (86.7 μg/ml in 10 mM sodium acetate, pH 4.5)
1.0 M ethanolamine-HCl, pH 8.5
Running buffer (see recipe)

10 mM glycine-HCl, pH 1.5
Purified empty and CYP3A4 nanodiscs (diluted in running buffer, centrifuged 30 min at 16,000 × *g*, 4°C to remove particulates)
100 μg/ml affinity-purified rabbit polyclonal anti-CYP3A4 antibody in running buffer

Series S sensor chip CM5 (GE Healthcare Life Sciences)
Biacore T200
Biacore rubber caps
Biacore plastic vials

NOTE: Temperature of chip and sample compartment is set at room temperature (25°C).

NOTE: Buffers used for antibody immobilization must not contain amine derivatives (e.g., Tris, sodium azide), which may interfere with the amine coupling process.

Immobilize anti-6-histidine antibody on CM5 chip

1. Dock a new chip. Prime the Biacore T200 instrument with HBS-EP buffer.
2. Set flow rate to 10 μl/min.
3. Inject 1:1 mixture of EDC/NHS for 10 min.
4. Inject mouse anti-6-histidine antibody for 5 min.
5. Inject ethanolamine-HCl for 7 min.
6. Inject HBS-EP buffer two times for 60 sec, or until baseline is stable.

Select the Immobilization Wizard Template from Biacore T200 control software to accomplish the immobilization process. This protocol typically results in ~13,000 RU of immobilized antibody.

Stabilize baseline before capturing nanodiscs

7. Start manual run. Switch to running buffer and prime.
8. Inject 10 mM glycine-HCl two times for 20 sec.
9. Inject running buffer two times for 60 sec.

Typically, there will be a small decrease in baseline response units after the first regeneration injection (10 mM glycine-HCl, pH 1.5). This is due to the removal of loosely bound antibody on the surface. Subsequent regeneration cycles should yield a consistent baseline.

Perform nanodisc (ligand) binding

Choose empty nanodiscs as the reference cell (e.g., flow cell 1) and proteonanodiscs as the experimental cell (e.g., flow cell 2). Reference subtracted sensorgrams can be obtained as flow cell 2 minus flow cell 1.

10. Inject running buffer three times for 60 sec to stabilize and clean surface of residual regeneration solution.
11. Set flow rate to 10 μl/min; flowpath = flow cell 1.
12. Inject empty nanodiscs for 180 sec (3 min).
13. Change to flowpath = flow cell 2.
14. Inject proteonanodiscs for 180 sec (3 min).

Captured proteonanodisc level should match empty nanodisc level for good referencing and to minimize experimental artifacts.

Perform antibody (analyte) binding

15. Increase flow rate to 50 μl/min in flow paths 1 and 2.

    For double referencing, inject running buffer matched to any additives that may be in the antibody solution for 120 sec and wait for 120 sec. The buffer control can be subtracted post-hoc.

16. Inject affinity-purified rabbit polyclonal anti-CYP3A4 antibody for 120 sec. Wait 120 sec for dissociation.

    To obtain quantitative results from kinetic and affinity analyses, inject increasing concentrations of the antibody in a step-wise manner without regeneration in between.

Regenerate

17. Set flow rate to 30 μl/min.

18. Inject 10 mM glycine-HCl, pH 1.5, two times for 20 sec. If response unit does not return to baseline, inject a third time.

19. Inject running buffer three times for 60 sec.

    Start with milder conditions before moving to harsher conditions to completely remove bound nanodiscs while preserving the antibody surface. Sometimes, a lower pH with fewer injections and shorter injection times may improve efficiency of the regeneration process.

    The anti-6-histidine antibody–coupled CM5 chip can be stored up to 3 months at 4°C in a 50-ml tube with little loss in binding capacity. A similar antibody has been shown to lose only 20% of binding capacity after 80 cycles of regeneration (see Hahnefeld et al., 2004).

BASIC PROTOCOL 3

NANODISCS ON L1 CHIP

The L1 chip contains hydrocarbon groups linked to the dextran layer that can intercalate into lipid membranes. This exploits the lipid bilayer of the nanodisc and does not require tagged proteins.

Materials

Running buffer (see recipe)
20 mM CHAPS in running buffer
Purified empty and CYP3A4 nanodiscs (diluted in running buffer, centrifuged 30 min at 16,000 × *g*, 4°C, to remove particulates)
0.2 mg/ml BSA in running buffer
100 μg/ml affinity-purified rabbit polyclonal anti-CYP3A4 antibody in running buffer

Series S Sensor Chip L1 (GE Healthcare Life Sciences)
Biacore T200
Biacore rubber caps
Biacore plastic vials

NOTE: Temperature of the chip and sample compartment is set at room temperature (25°C).

Condition L1 chip

1. Dock chip and prime Biacore T200 instrument with running buffer.

2. Set flow rate to 10 μl/min; flowpath = flow cell 2 – flow cell 1.

3. Inject 20 mM CHAPS two times for 30 sec. If baseline continues to decrease on second injection, add a third.

   CHAPS is a mild detergent generally useful for cleaning the surface.

4. Inject running buffer two times for 30 sec and one time for 60 sec; chip is now ready to load on nanodiscs.

Perform nanodisc (ligand) binding

Choose empty nanodiscs as the reference cell (e.g., flow cell 1) and proteonanodiscs as the experimental cell (e.g., flow cell 2). Reference subtracted sensorgrams can be obtained as flow cell 2 minus flow cell 1.

5. Set flow rate to 10 μl/min; flowpath = flow cell 1.
6. Inject empty nanodiscs for 1800 sec (30 min).

   The injection time will depend on the specific capture needs (see Critical Parameters) and concentration of nanodiscs.

7. Change to flowpath = flow cell 2.
8. Inject protein embedded nanodiscs for 1800 sec (30 min).
9. Change to flow path 2 – 1.
10. Inject 0.2 mg/ml BSA in running buffer for 300 sec (5 min).

    BSA will bind to any exposed L1 surface and provides an indication of the degree of chip saturation with the captured nanodiscs. A fully saturated surface will not bind BSA (Hodnik and Anderluh, 2010).

11. Inject running buffer for 120 sec.

Perform antibody (analyte) binding

To obtain quantitative results for kinetic and affinity analyses, a few well-chosen concentrations of the antibody with replicates and good model fitting is required.

12. Increase flow rate to 50 μl/min in flow path 2 – 1.

    Flow rates have been varied depending on the antibody. In cases where mass transfer limited binding is suspected, increase flow rate to 100 μl/min.

13. Inject affinity-purified rabbit polyclonal anti-CYP3A4 antibody for 120 sec.

    Time has been varied depending on the antibody. In cases where mass transfer limited binding is suspected, decrease contact time to 60 sec.

14. Wait 120 sec for dissociation.

Perform regeneration

15. Set the flow rate to 10 μl/min.
16. Inject 20 mM CHAPS three times for 30 sec.

    If baseline continues to decrease on third injection, add a fourth injection. Alternatively, consider a more harsh regeneration solution as discussed in Troubleshooting.

17. Inject running buffer two times for 60 sec.

    Store the L1 chip in a 50-ml tube containing a few milliliters of distilled water at 4°C. The same L1 chip has been used for >15 experiments with consistent capture and regeneration profiles.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****.*

Running buffer

100 mM potassium phosphate, pH 7.4
50 mM NaCl
Filter sterilize using a 0.22-μm filter
Store up to 6 months at 4°C. Filter sterilize before next use.

COMMENTARY

Background Information

Since the 1980s, SPR and related evanescent wave technologies have been exploited to study biomolecular interactions. From what began as a means to measure affinities, the development of a microfluidics system coupled with a sensor-on-a-chip interface became an additional valuable tool to obtain kinetic data. By detecting a mass or refractive index change on the chip surface, SPR is one of the "label-free" technologies that provides real-time monitoring of interactions between immobilized receptors and their binding partners without the need for a fluorescent or radioactive label. Compared to other approaches such as calorimetry, rapid mixing techniques, NMR, and mass spectrometry, which also allow the detection of binding interactions, SPR has advantages in terms of low protein requirement (20 to 100 μg protein), minimal sample preparation or clean-up, as well as the ease and speed of analysis (suitable for high-throughput screening). Application of SPR in the studies of membrane proteins, small molecules, and weak affinity binding interactions remains as a challenging, state-of-the-art endeavor. There is a variety of, vendor-specific, sensor chips to choose from. For the authors' work with his-tagged proteonanodiscs, the NTA chip (see Basic Protocol 1) and CM5 chip immobilized with anti-6-histidine antibody (see Basic Protocol 2) present three possible approaches for docking his-tagged proteins. The L1 chip (see Basic Protocol 3) is also described because the hydrophobic surface is excellent for capturing lipid bilayer nanoparticles.

Critical Parameters and Troubleshooting

Purity of nanodiscs

It is critical that nanodisc preparations be as pure as possible. Removing empty nanodiscs from a his-tagged proteonanodisc preparation by nickel chromatography followed by size-exclusion chromatography is recommended. For the L1 chip, it is important to purify the discs by size-exclusion chromatography because liposomes and various lipid-protein complexes can also be captured via the lipophilic substituent.

Desired nanodisc capture level

The desired nanodisc capture level depends on many factors, including the specific analyte (high molecular weight versus small molecule) as well as the goal of the experiment (quantitative kinetics or qualitative response). In either case, first calculate the R_{max}, the theoretical maximal response obtainable for the analyte, as follows:

$$R_{max}(\text{RU}) = (\text{analyte mol. wt./ligand mol. wt.}) \times \text{immobilized ligand (RU)} \times \text{stoichiometry}$$

High-molecular-weight analytes will result in greater responses so lower capture levels are adequate. Low-molecular-weight analytes require greater capture levels to achieve a detectable R_{max}. In addition, some analytes may demonstrate mass transfer limited binding requiring low capture levels (see Mass Transfer Limitations below). The L1 chip requires more nanodiscs due to longer capture time (15 to 30 min) compared to CM5 and NTA chip (1 to 2 min).

Choosing a reference surface

A well-matched reference surface is critical to deal with artifacts arising from possible refractive index changes in the analyte solution (the bulk effect), injection noise, and non-specific binding. Using empty nanodiscs as the reference surface is recommended. They are convenient to prepare alongside the proteonanodiscs and are a good control for all chips tested. To capture similar levels of empty and full nanodiscs, factor in the relative molecular weights of the discs. For the studies mentioned in this unit, a two-fold molar

excess of the empty discs is needed to achieve similar capture levels to the CYP3A4 proteonanodiscs. If empty nanodiscs are unavailable for the reference cell, an anti-6-his antibody–immobilized CM5 chip or a deactivated CM5 surface (capped with ethanolamine) can be used. In the case of the NTA chip, the reference cell can be the bare NTA surface (uncharged with Ni^{2+}), a charged surface is not recommended due to issues with non-specific binding.

Mass transfer limitations

The transfer of the analyte from the bulk flow to the sensor surface is a diffusion-controlled process. When mass transfer is limiting, the resulting 'binding phase' does not yield an accurate rate for the analyte. To minimize possible mass transfer limitations for kinetic experiments, lower capture levels of nanodisc (ligand) on the surface and increase flow rates (30 to 100 μl/min) of analyte, both of which help reduce the analyte consumption and diffusion distance. Mass transfer limitation can be checked by using different flow rates for the analyte injection. If mass transfer limitation occurs, the apparent rates of both association and dissociation are decreased. The results in Figure 29.13.5B,C are illustrative of a possible mass transfer limited process, because the binding phase is 'linear' rather than exponential. Exponential binding and dissociation, as shown in Figure 29.13.1, are required to obtain accurate on or off rates for the analyte. The diagnostic test of changing flow rates can result in a false negative. That is, it is possible that mass transfer may still be operative even if changing flow rate has no effect on the sensorgram. In fact, for this experiment, changing flow rates did not change the appearance of the sensorgram (data not shown). However, the appearance of the sensorgram alone should prompt the user to exercise caution in fitting the data to any kinetic model. It is also possible that the apparent linear response in Figure 29.13.5B,C is due to the use of a polyclonal antibody as the analyte and a high capture level of CYP3A4-nanodiscs. However, the observed binding still reflects specific interactions between CYP3A4 and the antibody, so experiments that yield data with such an appearance still have value as a qualitative tool for screening.

Baseline drifts with different chips

Baseline drift, the dissociation of captured nanodiscs from the surface of the chip, complicates the analysis of analyte binding especially in cases where the level of drift approaches the analyte response level. In addition, different rates of drift between the experimental and reference cell must be taken into account. For example, it has been found that the drift, when nanodiscs are captured via the histidine tag, on the nanodisc scaffold protein is less than when capture is via the tag on the CYP3A4 protein. This will likely vary with individual proteins and can be tested. Ultimately, the amount of tolerable drift will be determined by the requirements of the user and the utility of qualitative versus quantitative answers.

The L1 chip has been observed to provide the most stable baseline of captured nanodiscs compared to CM5/Ab and NTA chips. Although the NTA and CM5 chips demonstrate significant drift at high capture level, lowering the capture level can improve the stability of response on the surface, especially for the NTA chip.

Nonspecific binding

Initially test for non-specific binding by injecting analyte over the reference surface of empty nanodiscs. If binding is detected, determine the source by performing control experiments injecting analyte over surfaces without nanodiscs, such as the deactivated surface or anti-6-his antibody surface for the CM5 chip, the NTA or Ni-NTA surface for NTA chip, or the exposed hydrophobic layer on the L1 chip. In some cases, the running buffer can be optimized to reduce the non-specific binding by adding: soluble carboxymethyl dextran (NSB reducer, GE Healthcare), high salt (up to 500 mM NaCl), BSA (up to 2 mg/ml), or EDTA (250 μM). Detergents should be avoided because they can affect the lipid bilayer. If the level of non-specific binding is unacceptable (see Fig. 29.13.5A for the NTA chip), try a different sensor chip chemistry. The results shown in Figure 29.13.5A illustrate a clear example of nonspecific binding of the analyte.

Surface equilibration

Although typical protocols have specific times for washing and surface equilibration, it is critical to ensure the baseline is stable before adding the next reagent or attempting analysis. It may be necessary to extend wash times to remove, for example, ethanolamine before beginning the next step.

Table 29.13.1 Troubleshooting the Use of Lipid Nanodiscs for the Study of Membrane Proteins by SPR

Troubleshooting for nanodiscs on L1 chip

Problem	Cause	Solution
Regeneration cycle does not return baseline to initial RU level	20 mM CHAPS insufficient	Inject 10 to 30 sec of 2:3 isopropanol/50 mM NaOH followed by three times, 60 sec each time, with buffer washes
Bulk shift obscures effect	Refractive index difference between running buffer and analyte	More closely match running buffer to analyte buffer composition or dialyze analyte into running buffer

Troubleshooting for nanodiscs on CM5 chip

Problem	Cause	Solution
His-tagged protein nanodiscs does not bind to anti-6-histidine antibody immobilized on CM5 chip	Presence of glycerol His-tag on protein of interest is inaccessible once incorporated into nanodiscs Inactive protein	Protein nanodiscs should have a low percent of glycerol (<4%) Perform activity assay to see if protein nanodiscs are active before use Perform a western blot to determine if protein nanodiscs can bind to anti-6-histidine antibody
Significant baseline drift of captured nanodiscs	Gradual dissociation of His-tagged protein or His-tagged uncleaved empty nanodiscs from anti-6-histidine antibody	Reduce captured level or increase the length of stabilization period (up to 60 min); increasing the length of His-tag on protein may reduce drift; consider NTA chip for a more stable baseline at lower captured level
	High capture level may cause some non-specific accumulation on the surface	Stabilize baseline before sample injections

Troubleshooting for nanodiscs on NTA chip

Problem	Cause	Solution
Significant baseline drift of captured His-tagged nanodiscs	High amount of nanodiscs is captured (>6000 to 10,000 RU)	Try lower concentrations, shorter injection time, lower flow rates, or a combination. If higher density of nanodiscs is required, try capture-coupling method (Rich et al., 2011).
Significant nonspecific binding	Possible interactions of antibody with NTA chip due to surface His or Cys residues	Use either L1 or CM5-antibody chip

Troubleshooting

Table 29.13.1 describes some problems commonly encountered with lipid nanodiscs along with explanations of possible causes and recommendations for overcoming or avoiding these problems.

Anticipated Results

NTA chip

The nickel-activated NTA surface captures significantly more nanodiscs than either the CM5/Ab or L1 chips—over 10,000 RU captured at the highest ligand concentration (Fig. 29.13.4A). However, at these high capture levels, the stability is extremely poor (Fig. 29.13.4E). When the capture level was reduced (to 300 RU, Fig. 29.13.5A), unacceptable levels of nonspecific binding to the anti-CYP3A4 antibody were obtained. This is due to surface-exposed metal-chelating residues such as His or Cys (Knecht et al., 2009). Binding of the antibody to the unactivated NTA surface, which requires a different mechanism, was also observed. Nonspecific binding may

also result from heterogeneity of the analyte (Nieba et al., 1997).

CM5 chip

The anti-6-his antibody–coupled CM5 chip has a slightly higher capture level than the L1 chip (compare Fig. 29.13.4B,C); however, this is accompanied by slightly lower stability. Comparing the stability of similar capture levels of nanodiscs (~3000 RU; the 2 μM curve for L1 and the 0.5 μM curve for CM5), material on L1 shows no loss during the stabilization period, yet 1.7% of the initial capture is lost from the CM5 chip (49 RU).

L1 chip

The L1 chip saturates at ~3500 RU of empty nanodiscs (Fig. 29.13.4C). When levels lower than 3000 RU are captured a response is detected from the BSA injection; for capture levels around 2000 RU expect an additional ~100 RU of BSA. The baseline post-nanodisc capture should be stable as depicted in Figure 29.13.4E. We have detected very little nonspecific binding with antibody as the analyte. Because the L1 surface captures the lipid bilayer we have not seen significant differences in baseline drift between proteonanodiscs and empty nanodiscs.

Time Considerations

To obtain pure proteonanodiscs from purified membrane scaffold proteins and a protein of interest requires ~1 week (Ritchie et al., 2009). Once nanodiscs are prepared, loading of nanodiscs on the L1 chip takes ~40 min each with conditioning and stabilization. Empty nanodisc titrations (with at least three concentrations) will take >2 hr with conditioning and regeneration of the chip. To capture and stabilize nanodiscs for analyte binding, ~1.5 hr will be required before the start of analyte injections.

For the CM5 chip, immobilization of anti-6-histidine antibody will take ~30 min. Capturing time for nanodiscs varies between 3 and 5 min. Empty nanodisc titrations (with at least three concentrations) will take ~20 min with conditioning and regeneration of the chip. Capturing and stabilizing nanodiscs for analyte binding would take another 10 min.

The NTA chip requires ~4 min of conditioning with EDTA and charging with Ni^{2+}, with extra washes in between. Empty nanodisc titrations (with at least three concentrations) will take ~30 min in total. Capturing and stabilizing nanodiscs for analyte binding will require another 10 min.

Depending on the number of analytes, concentrations, duration of injection/dissociation as well as replicates, the time required for binding assays will vary. Each analyte (antibody) binding event described in the Basic Protocols takes 4 min for association and dissociation.

Priming the instrument after switching buffers takes ~7 min. Docking (or undocking) chips takes 1.5 min each time. Performing a desorb procedure after each run as part of regular instrument maintenance takes ~15 min, and system check takes ~21 min.

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Literature Cited

Baker, L.A. and Baldus, M. 2014. Characterization of membrane protein function by solid state NMR spectroscopy. *Curr. Opin. Struct. Biol.* 27C:48-55.

Das, A., Zhao, J., Schatz, G.C., Sligar, S.G., and Van Duyne, R.P. 2009. Screening of type I and II drug binding to human cytochrome P450-3A4 in nanodiscs by localized surface plasmon resonance spectroscopy. *Anal. Chem.* 81: 3754-3759.

de Mol, N.J. 2012. Surface plasmon resonance for proteomics. *Methods Mol. Biol.* 800:33-53.

Denisov, I.G., Grinkova, Y.V., Lazarides, A.A., and Sligar, S.G. 2004. Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size. *J. Am. Chem. Soc.* 126:3477-3487.

Glück, J.M., Koenig, B.W., and Willbold, D. 2011. Nanodiscs allow the use of integral membrane proteins as analytes in surface plasmon resonance studies. *Anal. Biochem.* 408:46-52.

Goluch, E.D., Shaw, A.W., Sligar, S.G., and Liu, C. 2008. Microfluidic patterning of nanodisc lipid bilayers and multiplexed analysis of protein interaction. *Lab Chip* 8:1723-1728.

Grinkova, Y.V., Denisov, I.G., and Sligar, S.G. 2010. Engineering extended membrane scaffold proteins for self-assembly of soluble nanoscale lipid bilayers. *Protein Eng. Des. Sel.* 23:843-848.

Hahnefeld, C., Drewianka, S., and Herberg, F.W. 2004. Determination of kinetic data using surface plasmon resonance biosensors. *Methods Mol. Med.* 94:299-320.

Hodnik, V. and Anderluh, G. 2010. Capture of intact liposomes on Biacore sensor chips for protein-membrane interaction studies. *Methods Mol. Biol.* 627:201-211.

Huster, D. 2014. Solid-state NMR spectroscopy to study protein-lipid interactions. *Biochim. Biophys. Acta* 184:1146-1160.

Kinouchi, H., Onishi, M., and Kamimori, H. 2013. Lipid membrane-binding properties of daptomycin using surface plasmon resonance. *Anal. Sci.* 29:297-301.

Knecht, S., Ricklin, D., Eberle, A.N., and Ernst, B. 2009. Oligohis-tags: Mechanisms of binding to Ni^{2+}-NTA surfaces. *J. Mol. Recognit.* 22:270-279.

Maynard, J.A., Lindquist, N.C., Sutherland, J.N., Lesuffleur, A., Warrington, A.E., Rodriguez, M., and Oh, S.H. 2009. Surface plasmon resonance for high-throughput ligand screening of membrane-bound proteins. *Biotechnol. J.* 4:1542-1558.

Moraes, I., Evans, G., Sanchez-Weatherby, J., Newstead, S., and Stewart, P.D. 2014. Membrane protein structure determination—The next generation. *Biochim. Biophys. Acta* 1838:78-87.

Nath, A., Atkins, W.M., and Sligar, S.G. 2007. Applications of phospholipid bilayer nanodiscs in the study of membranes and membrane proteins. *Biochemistry* 46:2059-2069.

Nieba, L., Nieba-Axmann, S.E., Persson, A., Hämäläinen, M., Edebratt, F., Hansson, A., Lidholm, J., Magnusson, K., Karlsson, A.F., and Plückthun, A. 1997. Biacore analysis of histidine-tagged proteins using a chelating NTA sensor chip. *Anal. Biochem.* 252:217-228.

Proverbio, D., Roos, C., Beyermann, M., Orbán, E., Dötsch, V., and Bernhard, F. 2013. Functional properties of cell-free expressed human endothelin A and endothelin B receptors in artificial membrane environments. *Biochim. Biophys. Acta* 1828:2182-2192.

Rich, R.L. and Myszka, D.G. 2000. Advances in surface plasmon resonance biosensor analysis. *Curr. Opin. Biotechnol.* 11:54-61.

Rich, R.L. and Myszka, D.G. 2010. Grading the commercial optical biosensor literature—Class of 2008: The Mighty Binders. *J. Mol. Recognit.* 23:1-64.

Rich, R.L., Errey, J., Marshall, F., and Myszka, D.G. 2011. Biacore analysis with stabilized GPCRs. *Anal. Biochem.* 409:267-272.

Ritchie, T.K., Kwon, H., and Atkins, W.M. 2011. Conformational analysis of human ATP-binding cassette transporter ABCB1 in lipid nanodiscs and inhibition by the antibodies MRK16 and UIC2. *J. Biol. Chem.* 286:39489-39496.

Ritchie, T.K., Grinkova, Y.V., Bayburt, T.H., Denisov, I.G., Zolnerciks, J.K., Atkins, W.M., and Sligar, S.G. 2009. Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. *Methods Enzymol.* 464:211-231.

Tavoosi, N., Smith, S.A., Davis-Harrison, R.L., and Morrissey, J.H. 2013. Factor VII and protein C are phosphatidic acid-binding proteins. *Biochemistry* 52:5545-5552.

Topiol, S., Moraes, I., Evans, G., Sanchez-Weatherby, J., Newstead, S., and Stewart, P.D. 2014. Membrane protein structure determination—The next generation. *Biochim. Biophys. Acta* 1838:78-87.

Zeng, S., Baillargeat, D., Ho, H.P., and Yong, K.T. 2014. Nanomaterials enhanced surface plasmon resonance for biological and chemical sensing applications. *Chem. Soc. Rev.* 43:3426-3452.