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

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Abstract

Methods for the initial steps of surface plasmon resonance analysis of membrane proteins incorporated in lipid nanodiscs are described. Several types of BiacoreTM 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 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.

Keywords

membrane protein; surface plasmon resonance binding analysis; antibody-receptor binding; lipid nanodisc; biosensor

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 (Baker et al., 2014; Huster, 2014; Moraes et al., 2014; Topiol, 2013), 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

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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 (Rich et al., 2000; Rich et al., 2010; de Mol, 2012; Hahnefeld et al., 1975). 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. Indeed, attempts have been made to use liposomes as the membrane in this experimental approach (Maynard et al., 2009; Zeng et al., 2014; Kinouchi et al., 2013). 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 chapter, it is assumed that the reader will have a basic familiarity with the theory of SPR, and we provide only a practical guide to start experiments and optimize the sensor chip surface to detect subsequent interactions with proteins.

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 (Ritchie et al., 2009; Nath et al., 2007; Denisov et al., 2004; 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 length 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 nanodiscs-protein particles are well characterized and demonstrate the versatility of this model membrane system in many applications. An interesting and 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 1 with an example in which 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. Clearly, nanodiscs provide a potential means of studying complex interactions via SPR with some unique advantages.

However, there are only a few examples of the application of nanodiscs to study membrane proteins by SPR (Ritchie et al., 2011; Goluch et al., 2008; Proverbio et al., 2013; Das et al., 2009; Gluck et al., 2011) 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 chapter is to provide protocols for the initial parameterization of SPR experiments in which a membrane protein or receptor incorporated in a nanodiscs 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, 1975). Rather we provide methods to prepare different sensor chips with immobilized nanodiscs and proteonanodiscs, using the membrane protein cytochrome P4503A4 (CYP3A4) nanodiscs as a model. It is nearly certain that the optimal immobilization strategies will be different for different proteonanodiscs, so the purpose of this article is to provide strategies for this optimization. The purity of the CYP3A4 nanodiscs used here is shown in Figure 2, as a benchmark for the level of homogeneity of proteonanodiscs that yields the best results. In addition, we use a polyclonal anti-CYP3A4 as a model analyte in order 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 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 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 1 and 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 need to 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. $R_{\max}$ needs to be large for small molecule analytes, which requires 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 section). 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 4, the NTA chips easily load a high level of his-tagged nanodiscs with nearly 12000 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 μ M), 21% of the mass was lost during the five minute 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. We have not included that protocol here, but suggest that it 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 much lower than the NTA chip. This level is certainly adequate for analysis of protein or large molecule binding, as shown in Figure 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 μ M 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. In short, the data in Figures 4 and 5 lead to the suggestion that multiple chips should be compared for their interaction with the specific proteonanodiscs and the corresponding empty nanodiscs in order 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 again in Anticipated Results.

In light of this discussion 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, discussed above (Rich et al., 2011) However, this method could become cost prohibitive due to the need to use new chips for each experiment.

BASIC PROTOCOL 1: ANTIBODY BINDING TO NANODISCS ON THE NTA CHIP

Most membrane proteins are currently expressed *in vitro* with an affinity capture handle, such as histidine-tags or FLAG-tags. Conveniently, the same handle can be used in subsequent SPR work, to attach the membrane protein that has been incorporated in a nanodisc on the sensor chip surface. We provide a protocol for histidine-tagged proteins,

which should be adaptable for membrane proteins with other affinity handles. The NTA surface is charged with Ni^{2+} allowing the capture of histidine-tagged proteins.

Materials

Running Buffer (see recipe)

0.5 mM NiCl_2

350 mM EDTA pH 8

3mM EDTA pH 8

Purified empty and CYP3A4 nanodiscs (diluted in Running Buffer, centrifuged at $16,000 \times g$ for 30 min at 4°C to remove any particulates)

100 $\mu\text{g}/\text{ml}$ of 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 the chip and sample compartment is set at room temperature (25°C). The Running Buffer must not contain divalent cations such as Mg^{2+} or Ca^{2+} .

Conditioning the NTA chip

- 1 Dock a new chip. Prime the instrument with Running Buffer.
- 2 Set flow rate to $30 \mu\text{l}/\text{min}$; flowpath = 2-1.
- 3 Inject 350 mM EDTA for 60 sec.
- 4 Inject Running Buffer 2×60 sec.
- 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 a cycle of 1×60 sec of 350 mM EDTA, at a flow rate of $30 \mu\text{l}/\text{min}$. Indicate an “extra wash” step with Running Buffer.

Charging the NTA chip with Nickel

- 6 Set flow rate to $10 \mu\text{l}/\text{min}$.
- 7 Inject 0.5 mM NiCl_2 for 60 sec.
- 8 Indicate an “extra wash” step with 3mM EDTA
- 9 Allow a stabilization time of 60 sec.

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

Nanodisc (Ligand) Binding

Choose empty nanodiscs as the reference cell (for example flow cell 1) and proteonanodiscs as the experimental cell (for example flow cell 2). Reference subtracted sensorgrams can be obtained as Flow cell 2- 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).

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

Antibody (Analyte) Binding

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

15 Inject 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.

Regeneration

16 Select both flow cells. Set flow rate to 30 µl/min.

17 Inject 350mM EDTA for 60 sec.

18 Indicate an “extra wash” step with Running Buffer.

BASIC PROTOCOL 2: NANODISCS ON THE 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 the 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-dimethylaminopropyl)-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
10 mM Sodium Acetate pH 4.5
10 mM Glycine-HCl pH 1.5
Purified empty and CYP3A4 nanodiscs (diluted in Running Buffer, centrifuged at 16,000 x g for 30 min at 4°C to remove any particulates)
Running Buffer (see recipe)
100 µg/ml of affinity-purified Rabbit polyclonal anti-CYP3A4 antibody in Running Buffer
Biacore™ T200
Series S Sensor Chip CM5 (GE Healthcare Life Sciences)
Biacore™ rubber caps
Biacore™ plastic vials

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

Immobilizing anti-6-histidine antibody on the CM5 Chip

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

- 1 Dock a new chip. Prime the instrument with HBS-EP buffer.
- 2 Set the flow rate to 10 µl/min.
- 3 Inject mixture of EDC/NHS (1:1) for 10 min.
- 4 Inject anti-6-histidine antibody for 5 min.
- 5 Inject ethanolamine for 7 min.
- 6 Inject HBS-EP buffer for 2 × 60 sec., or until the 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.

Stabilizing baseline before capturing of nanodiscs

- 7 Start manual run. Switch to Running Buffer and prime.
- 8 Inject 10 mM Glycine-HCl for 2 × 20 sec.
- 9 Inject Running Buffer 2 × 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.

Nanodisc (Ligand) Binding

Choose empty nanodiscs as the reference cell (for example flow cell 1) and proteonanodiscs as the experimental cell (for example flow cell 2). Reference subtracted sensorgrams can be obtained as Flow cell 2- Flow cell 1.

- 10 Inject Running Buffer 3×60 sec to stabilize and clean surface of residual regeneration solution.
- 11 Set flow rate to 10 $\mu\text{L}/\text{min}$; 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.

Antibody (Analyte) Binding

- 15 Increase flow rate to 50 $\mu\text{L}/\text{min}$ 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 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.

Regeneration

- 17 Set flow rate to 30 $\mu\text{L}/\text{min}$.
- 18 Inject 10 mM Glycine-HCl pH 1.5 2×20 sec. If response unit does not return to baseline, inject a third time.
- 19 Inject Running Buffer 3×60 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 time may improve efficiency of the regeneration process.

Anti-6-histidine antibody coupled CM5 chip can be stored up to 3 months at 4°C in a 50ml Falcon 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 (Herberg et al)

BASIC PROTOCOL 3: NANODISCS ON THE 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 at 16,000 x g for 30 min at 4°C to remove any particulates)

0.2 mg/ml BSA in Running Buffer

Series S Sensor Chip L1 (GE Healthcare Life Sciences)

100 µg/ml of affinity-purified Rabbit polyclonal anti-CYP3A4 antibody in Running Buffer

Biacore™ T200

Biacore™ rubber caps

Biacore™ plastic vials

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

Conditioning the L1 chip

- 1 Dock the chip and prime the instrument with Running Buffer.
- 2 Set flow rate to 10 µl/min; flowpath = flow cell 2-1
- 3 Inject 20 mM CHAPS for 2 × 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 for 2 × 30 sec, 1 × 60 sec; chip is now ready to load on nanodiscs.

Nanodisc (Ligand) Binding

Choose empty nanodiscs as the reference cell (for example flow cell 1) and proteonanodiscs as the experimental cell (for example flow cell 2). Reference subtracted sensorgrams can be obtained as Flow cell 2- 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 (Hodnick and Anderluh, 2010).

- 11 Inject Running Buffer for 120 sec

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 would be required.

- 12 Increase flow rate to 50 μ l/min in flow path 2-1

We have varied flow rate depending on the antibody. In cases where mass transfer limited binding is suspected increase flow rate to 100 μ l/min.

- 13 Inject antibody for 120 sec

We have varied time 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

Regeneration

- 15 Set the flow rate to 10 μ l/min

- 16 Inject 20 mM CHAPS for 3×30 sec.

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

- 17 Inject Running Buffer for 2×60 sec.

Store the L1 chip in a 50ml Falcon tube containing a few ml of distilled water at 4°C. We have used the same L1 chip for over 15 experiments with consistent capture and regeneration profiles.

REAGENTS AND SOLUTIONS

Running Buffer

100 mM Potassium Phosphate pH 7.4

50 mM NaCl

0.22 μ m filter sterilize

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 microfluidics system coupled with 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 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 (20–100 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 molecule 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 our 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

1. Purity of Nanodiscs—It is critical that nanodisc preparations be as pure as possible. We recommend removing empty nanodiscs from a his-tagged proteonanodisc preparation by nickel chromatography followed by size exclusion chromatography. 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.

2. Desired Nanodisc Capture Level—This 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:

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

High molecular weight analytes will result in greater responses so lower capture levels are adequate. Low molecular weight analytes requires greater capture levels to achieve a detectable $R_{\max}$. In addition some analytes may demonstrate mass transfer limited binding requiring low capture levels (see point 4). The L1 chip requires more nanodiscs due to longer capture time (15–30 min) compared to CM5 and NTA chip (1–2 min).

3. 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. We recommend using empty nanodiscs as

the reference surface. They are convenient to make alongside the proteonanodiscs and are a good control for all the chips we tested. In order to capture similar levels of empty and full nanodiscs, factor in the relative molecular weights of the discs. For our studies we need a two-fold molar excess of the empty discs 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 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.

4. 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 on rate for the analyte. To minimize possible mass transfer limitations for kinetic experiments, lower capture levels of nanodisc (ligand) on the surface and fast flow rates (30 – 100uL/min) of analyte are recommended. Both 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 5B and 5C 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 1, are required to obtain accurate on rates or off rates for the analyte. Importantly, 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 great caution in fitting the data to any kinetic models. It is also possible that the apparent linear response in Figures 5b and 5C 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.

5. 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 we have 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 vs. quantitative answers.

The L1 chip has been observed to provide the most stable baseline of captured nanodiscs compared to the CM5/Ab chip and NTA chip. Although both the NTA and CM5 chip

demonstrate significant drift at high capture level, lowering the capture level can improve the stability of response on the surface, especially for NTA chip.

6. 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 the nanodiscs, such as the deactivated surface or anti-6-his antibody surface for 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 500mM NaCl), BSA (up to 2 mg/ml), or EDTA (250 uM). Detergents should be avoided because they can affect the lipid bilayer. If the level of non-specific binding is unacceptable (see Figure 5A for the NTA chip) try a different sensor chip chemistry. For the results shown in Figure 5A illustrate a clear example of nonspecific binding of the analyte.

7. 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.

Troubleshooting

Troubleshooting for nanodiscs on L1 Chip		
Problem	Cause	Solution
Regeneration cycle does not return baseline to initial RU level	20 mM CHAPS insufficient	Inject 10–30 sec of 2:3 isopropanol : 50 mM NaOH followed by 3 × 60 sec buffer washes
Bulk shift obscures effect	refractive index difference between running buffer and analyte	More closely match the Running Buffer to the 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 see 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 High capture level may cause some non-specific accumulation on the surface	Reduce captured level or increase the length of stabilization period (up to 60 min); Increase the length of his-tag on protein may reduce drift; consider NTA chip for a more stable baseline at lower captured level Stabilize baseline before sample injections
Troubleshooting for nanodiscs on NTA chip		
Problem	Cause	Solution
Significant baseline drift of captured his-tagged nanodiscs	A high amount of nanodiscs is captured (> 6000 – 10000 RUs)	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)

Troubleshooting for nanodiscs on L1 Chip

Problem	Cause	Solution
Significant nonspecific binding	Possible interactions of antibody with NTA chip due to surface His or Cys residues	Use either L1 or CM5- antibody chip

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 (Figure 4A). However at these high capture levels the stability is extremely poor (Figure 4E). When the capture level was reduced (to 300 RU, Figure 5A) we had unacceptable levels of non-specific binding to the anti-CYP3A4 antibody. This is due to surface-exposed metal chelating residues such as His or Cys (Knecht et al. 2009). Notably we also observe binding of the antibody to the unactivated NTA surface because which requires a different mechanism. 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 Figure 4B and C), however this is accompanied by slightly less stability. Comparing the stability of similar capture levels of nanodiscs (~3000 RU; the 2 μ M curve for L1 and the 0.5 μ M curve for CM5) we see that 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 (Figure 4C). When levels less 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 4E. We have detected very little non-specific 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

It takes about a week to obtain pure proteonanodiscs from purified membrane scaffold proteins and protein of interest (Ritchie et al., 2009). Once nanodiscs are prepared, loading of nanodiscs on the L1 chip take approximately 40 min each with conditioning and stabilization. Empty nanodiscs titration (with at least 3 concentrations) will take over 2 hours with conditioning and regeneration of chip. To capture and stabilize nanodiscs for analyte binding, ~ 1.5 hours will be required before the start of analyte injections.

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

NTA chip requires ~ 4 min of conditioning with EDTA and charging with Ni^{2+} , with extra washes in between. Empty nanodiscs titration (with at least 3 concentrations) will take around 30 min in total. Capturing and stabilizing nanodiscs for analyte binding will take 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.

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

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Figure 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. Note we have not analyzed the results for k_{on} or k_{off} , which would require multiple analyte concentrations, 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-Mg²⁺-orthovanadate (injections depicted by arrows). ATP-Mg²⁺-orthovanadate binds to the ATP- binding

domain of Pgp, alters the conformation of Pgp and lowers the affinity for the UIC2 antibody. In these experiments the reference cell contained empty. In panel C, by the 4th addition of ATP-orthovanadate-Mg²⁺, the bulk refractive index change is large and results in a slightly different dissociation response. nanodiscs captured to a similar level.

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Figure 2.

The model proteonanodisc platform. **(A)** A model of Cytochrome P450 3A4 (CYP3A4) proteonanodisc with 2 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 20–27 for 3A4 proteonanodiscs or 22–28 for empty nanodiscs, from the SEC step were concentrated analyzed by SDS-PAGE using a 4–20 % 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.

Figure 3.

A schematic of the SPR sensor chips and their capture strategies. Depicted are three BiacoreTM sensor chips that we have 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 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 μ M) 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 three 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 *) 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)–(C) as a function of the initial nanodisc concentration used for capture. (E) The percentage of the captured nanodisc response remaining after the five min stabilization period as a function of the initial nanodisc concentration used for capture.

Figure 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) or L1 chip (~2700 RU captured for each). A rabbit polyclonal anti-3A4 antibody (100 μ g/ml) was injected for 120 seconds over each surface and a 120 sec dissociation phase recorded.