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Interfacing Lipid Bilayer Nanodiscs and Silicon Photonic Sensor Arrays for Multiplexed Protein-Lipid and Protein-Membrane Protein Interaction Screening

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Abstract

Soluble proteins are key mediators of many biochemical signaling pathways via direct interaction with the lipid bilayer and via membrane-bound receptors. Components of the cell membrane are involved in many important biological processes, including viral infection, blood clotting, and signal transduction, and as such, they are common targets of therapeutic agents. Therefore, the development of analytical approaches to study interactions at the cell membrane is of critical importance. Herein, we integrate two key technologies, silicon photonic microring resonator arrays and phospholipid bilayer nanodiscs, which together allow multiplexed screening of soluble protein interactions with lipid and membrane-embedded targets. Microring resonator arrays are an intrinsically multiplexable, label-free analysis platform that has previously been applied to studying protein-protein, protein-nucleic acid, and nucleic acid-nucleic acid interactions. Nanodiscs are protein-stabilized lipid assemblies that represent a convenient construct to mimic the native phospholipid bilayer, investigate the effects of membrane composition, and solubilize membrane-embedded targets. Exploiting the natural affinity of nanodisc-supported lipid bilayers for oxide-passivated silicon, we assembled single and multiplex sensor arrays via direct physisorption, characterizing electrostatic effects on nanodisc attachment. Using model systems, we demonstrate the applicability of this platform for the parallel screening of protein interactions with nanodisc-embedded lipids, glycolipids, and membrane proteins.

Keywords

silicon photonics; microring resonator; nanodiscs; lipid-protein interactions; membrane proteins

INTRODUCTION

The interactions of biomolecules at cell membranes are an essential component in ion and nutrient transport, cell-to-cell communication, endocytosis, and neurotransmission. Soluble proteins can interact with cells via proteins embedded within the phospholipid membrane or with the membrane lipid or carbohydrate components.^{1–3} One of the inherent challenges in studying signaling and interactions at the cell surface, however, is the need to adequately recapitulate the membrane environment in a soluble, non-native setting. This is especially challenging when membrane proteins are involved, due to their unique amphiphatic properties. Lipid bilayer nanodiscs serve as a convenient means to replicate the cell membrane for in vitro assays, serving to solubilize and stabilize membrane proteins in a native-like lipid environment.^{3–5} In comparison to other methods such as liposomes and

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detergent-stabilized micelles, nanodiscs offer unparalleled consistency and monodispersity.⁶ Liposomes form large lipid aggregates that limit the density of membrane proteins available for attachment to surfaces. Also, receptor stoichiometry is difficult if not impossible to control and only one side of the membrane protein is accessible for external interaction.⁷ Micelles have smaller particle sizes, but dynamic fluctuations in lipid content and the lack of a true bilayer are significant drawbacks. Additionally, required detergents can denature incorporated membrane proteins or disrupt native interactions.^{7–8}

Nanodiscs are made via a self-assembly process whereby lipid, membrane protein, and an amphipathic protein belt are combined to form nanometer scale planar phospholipid discs. The protein belt, termed the membrane scaffold protein (MSP), solubilizes the nanodiscs and precisely controls their size at optimized lipid ratios,⁹ enabling stoichiometric control over the number of receptors within each disc.¹⁰ Nanodiscs have been previously employed to study the mechanisms of blood coagulation,¹¹ binding of cholera toxin^{1, 12} and P450 enzymes,^{13–14} as well as the G protein coupled receptors.^{10, 15–17}

An appropriate construct for mimicking the phospholipid bilayer is only part of the solution for investigating important cell surface interactions, however. Analytical methodologies, capable of assessing multiple protein-membrane interactions on a single platform would significantly benefit studies of these model membranes. Optical microring resonators are well-served for these applications, providing a multiplexable platform on which nanodiscs, and thus nanodisc-solubilized biomolecules, can be immobilized for subsequent interrogation with soluble proteins. Not only is the ring resonator platform amenable to multiplexed assays while maintaining sensitivity,^{18–21} but the label-free nature of refractive index based sensing also obviates any need to chemically modify or tag the biomolecules of interest, preventing any alterations to their native and thus active conformations. Additionally, small sample volume requirements make this technology particularly amenable to nanodisc-solubilized membrane proteins, as these proteins are notoriously challenging to express and purify, often demonstrating low yields in comparison to soluble proteins.

Microring resonator technology has been previously employed for screening protein-protein interactions,^{18, 22–23} quantifying proteins^{24–27} and nucleic acids^{20, 28–29}, as well as determining binding kinetics^{30–31} in both single- and multi-plex assay formats. Herein, we further extend the analytical capability of this platform by presenting a straightforward approach to multiplexed immobilization of nanodiscs, thus enabling characterization of biomolecular interactions with cell membrane model systems. This represents the first application of the microring resonator technology to membrane-bound targets.

By directly immobilizing nanodiscs to the silica microring substrate via physisorption, the effect of lipid composition on nanodisc loading was examined. The binding of a protein toxin to a nanodisc-solubilized glycolipid receptor demonstrated the utility of the platform to quantitatively evaluate concentration-dependent binding of soluble proteins to membrane-embedded receptors. The platform was then functionalized with four different nanodisc systems in order to examine the binding specificity of interactions between soluble proteins and nanodisc components in a multiplexed assay format.

EXPERIMENTAL

Materials

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] (POPS), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), N-(biotinoyl)-1,2-dipalmitoyl-sn-glycer-3-phosphoethanolamine

(biotin-DPPE), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from Avanti Polarlipids Inc. (Alabaster, AL, USA). MSP1D1 was expressed and purified as described previously.⁴ Streptavidin was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). An antibody to human cytochrome P450 3A4 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Annexin V, cholera toxin B subunit, Amberlite XAD-2 hydrophobic beads, and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and used as received. Buffers were prepared with 18 M. deionized water and sterile filtered prior to use.

Solution Preparation

Nanodisc solutions were prepared in a Tris-HCl buffer (20 mM Tris-HCl, 100 mM NaCl, 0.01% (w/v) NaN₃, pH 7.4). During nanodisc immobilization, samples were diluted from stock solutions with either Tris-HCl buffer or 10 mM phosphate buffered saline, pH 7.4 (PBS), prepared from Dulbecco's phosphate buffered saline packets (Sigma Aldrich, St. Louis, MO). Annexin binding studies were performed in a HEPES-Ca²⁺ buffer (10 mM HEPES, 150 mM NaCl, and 2.5 mM CaCl₂, pH 7.4). Soluble proteins (annexin, CTB, streptavidin, and anti-CYP3A4) were typically injected at 1–2 µg/mL concentration unless otherwise indicated in figure captions. In studies that involved annexin, including the multiplexed assay, soluble proteins were diluted to the desired concentrations in HEPES-Ca²⁺ buffer. In the CTB binding assay, the entire experiment was run in Tris-HCl buffer.

Nanodisc Self-assembly and Purification

Procedures for nanodisc preparation have been described in detail previously for each of the systems investigated herein. Briefly, lipids were solubilized in chloroform, dried under nitrogen, and resuspended in a sodium cholate buffer. For mixed lipid systems of POPC:POPS,² DMPS:GM1,¹² and DPPC:biotin-DPPE,² the lipids were combined in the appropriate ratios by mixing their chloroform stocks prior to drying. Cholate-solubilized lipids were combined with scaffold protein (MSP1D1). Detergent solubilized CYP3A4^{5, 32} was combined with the lipids and MSP at this step if applicable. Next, detergent removal was accomplished with porous polystyrene beads (Amberlite XAD-2) and subsequent centrifugation to remove beads prior to purification. The assembled nanodisc samples were purified via a Superdex 200 prep grade column (1.6 × 30 cm) prior to use. Nanodisc stock solutions were quantified via the MSP absorbance at 280 nm.

Chip Design, Instrumentation, and Data Analysis

Ring resonator optical scanning instrumentation, control software, and sensor chips were obtained from Genalyte, Inc. (San Diego, CA) and have been described previously.^{26, 33} Briefly, arrays of silicon photonic microrings are fabricated into the top layer of silicon-on-insulator substrates. Each 6 × 6 mm microchip contains 32 microrings (30 µm diameter) with adjacent linear waveguides. The input and output diffractive grating couplers at each end enable independent measurements to be made at each ring using a tunable, external cavity diode laser (centered at 1560 nm). These silicon photonic microring resonator arrays have previously been employed for label-free and multiplexed detection of a variety of biomolecules including DNA,²⁸ RNA,^{20, 29, 34} and proteins.^{23–27, 31} This technique is responsive to very small changes in refractive index (RI) that result from molecular interactions at the ring surface. Light is coupled from an adjacent linear waveguide into the microring only at wavelengths that meet an interference-based resonance condition: $m\lambda = 2\pi r n_{eff}$ where, m is an integer, r is the microring radius, and n_{eff} is the effective RI sampled by the optical mode. Specific biomolecular binding events at the ring surface cause a change in refractive index that is measured as a corresponding shift in the resonance wavelengths. This approach permits real-time monitoring of surface functionalization as

well as biomolecular binding events, and the magnitude of the wavelength shift correlates quantitatively to the amount of bound analyte.

Sensor chips are loaded into a custom-built microfluidic chamber that features channel(s) defined by a 0.007" thick Mylar gasket (RMS Laser, El Cajon, CA, USA). These gaskets contain 1, 2, or 4 channels that define the fluidic flow across the chip and divide the sensor rings into distinct regions for multiplexed sample delivery. Each channel on the gasket is 500 μm wide. The channel length depends on the configuration employed in a given experiment, but for a 2-channel gasket, each channel has a 250 nL volume. All data was analyzed using Origin Pro 8 and Microsoft Excel. Net shifts were quantified once saturation of the nanodisc or protein sample was observed.

Microchip Functionalization

Prior to use, chips were cleaned with a freshly prepared piranha solution (3:1 H_2SO_4 :30% H_2O_2) for 30 s, and then rinsed with water and dried with N_2 . (*Caution! Piranha solutions are extraordinarily dangerous, reacting explosively with trace quantities of organics.*). Nanodiscs were immobilized on the microchip substrate via 1-, 2-, or 4-channel microfluidic gaskets²⁶ depending on the experiment. Nanodisc solutions were diluted from stock solutions to 250 or 500 nM and typically flowed across the sensor surface and allowed to physisorb for 10 minutes, or until a saturated signal was observed. Any exposed regions of the substrate were then blocked by flowing across a 2% bovine serum albumin (BSA) solution to prevent nonspecific binding to the microchip surface. Subsequently the soluble proteins of interest were flowed across the sensor surface. All experiments were performed at a flow rate of 10 $\mu\text{L}/\text{min}$.

RESULTS AND DISCUSSION

Similar to other phospholipid bilayer systems,^{35–36} nanodiscs have been shown previously to physisorb directly to silicon oxide,^{2, 4} the same surface presented on the Si waveguides, natively passivated by $\sim 2\text{nm}$ of SiO_x . Herein we exploit the native affinity of phospholipids for silica as a means to immobilize nanodiscs on the microring resonator surface. Nanodiscs containing either phosphocholine (POPC) or a mixture of POPC and phosphoserine (POPS; the mixed discs referred to as POPC:POPS) were flowed across the sensor array, and their attachment to microrings was visualized in real-time as positive shifts in resonance wavelength. As shown in Figure 1, nanodisc binding to the sensor surface was observed, demonstrating the general utility of direct physisorption as a means for immobilizing nanodiscs onto microring resonator arrays. The differential loading between POPC and POPC:POPS is explored further below.

In order to assess the ability to monitor the interactions between soluble proteins and nanodisc cargo, the lipid-binding protein annexin was flowed across the nanodisc-functionalized microring array. Annexins are known to interact with anionic phospholipids (including POPS) in a calcium dependent fashion.^{37–41} As an annexin V solution was flowed across the POPC and POPC:POPS nanodisc array, binding was only observed at the mixed POPC:POPS-functionalized microrings (Figure 1), consistent with the fact that POPS carries a net negative charge whereas POPC is neutral. Furthermore, we confirmed that this interaction is calcium-dependent as demonstrated by immediate dissociation of the bound annexin V from the POPS-containing nanodiscs when the flowed buffer is switched to a calcium-free PBS buffer.

The data presented in Figure 1 clearly establish the ability to load nanodiscs onto microring arrays and to observe subsequent biomolecular interactions. However, it also raised questions regarding the role that lipid composition plays in nanodisc loading via

physisorption. To further investigate this effect, the immobilization of nanodiscs containing varying ratios of POPC and POPS was examined. As shown in Figure 2a–b, nanodisc adsorption decreased as the POPS content was increased from 0 up to 50%. This result reflects a profound role that electrostatics plays in nanodisc loading onto native silica surfaces. The pK_a of SiO_x is $\sim 2-4$,^{42–43} and thus the microring surface is negatively charged at physiologically-relevant pHs, such as the buffered systems employed here. This adds a repulsive element to the dynamics of physisorption, and consequently, nanodiscs made with anionic lipids have a lower affinity for the resonator surface.

Following immobilization of nanodiscs with variable POPC:POPS content, annexin V was flowed across the 4-plex array at a constant concentration, and the net shifts in resonant wavelengths are shown in Figure 2c. Although the linear relationship between annexin V binding and POPS content is not observed in the raw data, this correlation is clearly evident when the annexin response is normalized by nanodisc loading, as shown in Figure 2d. This agrees with previous studies demonstrating increased annexin V binding to nanodiscs with higher POPS content.²

In addition to examining electrostatic interactions between soluble proteins and anionic phospholipids, the ability to monitor protein binding to a glycosylated lipid receptor was also examined. Nanodiscs containing the glycolipid receptor, monosialotetrahexosyl ganglioside (GM1), were immobilized to the sensor surface. GM1 is responsible for binding and internalizing the virulent protein secreted by the bacterium *Vibrio cholera*.^{44–46} In particular it is the B subunit of cholera toxin (CTB) that interacts with GM1 presented on the surface of cells. Following immobilization of 5% GM1 nanodiscs, varying concentrations of CTB were flowed across the sensor surfaces, and the relative shifts were determined. As can be seen in Figure 3, CTB binding was linear with respect to concentration, over the range tested. Unlike with the POPS-annexin system, normalization of the CTB net shift to the amount of GM1 nanodiscs immobilized on the sensor surface was unnecessary since the nanodisc composition and hence loading was uniform.

Although this response was linear over the concentration range tested, the observed error was larger than expected. Although we do not fully understand the origin of this behavior, it may be the result of receptor clustering on the sensor surface. It was shown previously, in studies employing supported phospholipid membranes, that GM1 can cluster within phospholipid membranes precluding the multivalent CTB interaction.⁴⁷ While this is not expected within the defined nanodisc constructs, it is possible that physisorbed nanodiscs form multilayered structures which simulate the clusters previously shown in supported membranes. This multilayering could be unevenly distributed across the array of sensors, thus leading to increased measurement error. Multilayering, and any potential receptor clustering, could also explain the consistently observed sigmoidal responses to CTB binding on account of complex, cooperative binding interactions known to exist between this analyte and GM1.⁴⁸ Another possible explanation for the sigmoidal CTB binding response is the depletion of the analyte through absorption onto the tubing leading from the stock solution to the microring sensor array. This second explanation would seem to be supported by the continual increase in binding response after switching back to buffer, during which CTB would be progressively released from the tubing and once again able to bind to the GM1 nanodiscs. A more detailed study of such phenomena will be necessary in the future. However, this demonstration does further establish the ability of nanodisc-functionalized microring resonators to quantitatively probe binding interactions between soluble proteins and model membrane systems.

In addition to label-free operation, the scalable fabrication and inherent multiplexing capability of silicon photonic microring resonators are key attributes of the technology. To

demonstrate the ability to perform multiplexed protein-nanodisc interaction screening, we constructed a proof-of-principle array consisting of four uniquely-prepared nanodisc systems immobilized on a single sensor chip via direct physisorption. Specifically, in addition to the POPC:POPS and GM1 nanodiscs described previously, binding interactions with immobilized nanodiscs containing biotinylated lipids (biotin-DPPE) as well as cytochrome P450 3A4 (CYP3A4) enzyme were also investigated. A previously described¹⁸ four-channel fluidic gasket allowed for distinct regions of the chip to be functionalized with each of the four nanodisc systems via direct physisorption. Following a blocking step, annexin V, CTB, streptavidin, and anti-CYP3A4 were flowed across the entire chip surface in a sequential manner, and their specific binding to POPS-, GM1-, biotin-DPPE-, and CYP3A4-containing nanodiscs was observed. As seen in Figure 4 orthogonal binding of each soluble protein interacting with the appropriate nanodisc construct was observed with no observable cross-reactive responses. This key data demonstrates the applicability of the microring resonator platform for multiplexed investigations involving multiple nanodisc-embedded cargo.

CONCLUSIONS

Interactions at the cell membrane, whether with the lipids in the bilayer or with proteins immobilized within the bilayer, represent an important step in biomolecular signaling that is characterized here with optical microring resonator arrays. Lipid bilayer nanodiscs are immobilized on the resonator sensor surface through direct physisorption, a simple and quick immobilization strategy that requires minimal sample preparation and no modification to the nanodisc, nanodisc-embedded targets, or the membrane scaffold protein. Following immobilization, the nanodiscs were shown to bind soluble proteins, and the effect of lipid electrostatics on nanodisc loading was characterized using annexin V binding to POPS-containing nanodiscs as a model interaction. Concentration-dependent binding of cholera toxin to nanodiscs containing the glycolipid receptor GM1 demonstrated the utility of this platform for the quantitative determination of a soluble protein, based on its interaction with a nanodisc-solubilized biomolecule. Four nanodisc systems containing biotin-DPPE, POPS, GM1, and CYP3A4 were then probed simultaneously to examine the specificity of binding between streptavidin, annexin, cholera toxin, and an anti-CYP3A4 antibody and the appropriately functionalized rings in a multiplexed assay.

The studies presented within this paper demonstrate the fusion of two key technologies, namely nanodiscs and microring resonators, and represent a critical development towards examining biomolecule-membrane interactions in a multiplexed manner. The multiplexing capabilities of the platform can be further advanced by utilizing spotting technologies to create more complex nanodisc arrays. This advance will have the added benefit of reagent conservation, which might be particularly important when interrogating systems with difficult to express components, such as membrane-bound proteins. Future work will develop arrays of nanodisc-solubilized receptors on a single chip, enabling quantitation and kinetic determinations of multiple signaling proteins, toxins, or pharmaceuticals of interest in particular disease states or pathogenic mechanisms.

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Figure 1. POPC-only (blue) and POPC:POPS (red) nanodiscs immobilized directly onto optical microring resonator surface. Dashed lines indicate time points of sample introduction. At t=18 minutes, nanodiscs are immobilized onto the sensor surface. At t=60 minutes, binding of 10 $\mu\text{g/mL}$ annexin is observed only on rings presenting POPS-containing discs. Returning the system to calcium-free PBS buffer, annexin dissociates from the immobilized nanodiscs. Asterisks on the graph indicate a buffer change either back to PBS (**) at t=40 and t=78 minutes or to HEPES- Ca^{2+} buffer (*) at t=57 and t=67 minutes.

a. Real Time Nanodisc Immobilization

b. Net Shift Nanodisc Immobilization

c. Net Shift Annexin Binding

d. Normalized Annexin Binding

Figure 2.

Nanodiscs with increasing amounts (0–50%) of POPS exhibit differences in the loading capacity on microring resonators as observed through the real time relative shift data (a) and the net shift observed once saturation is achieved (b). The dashed line indicates nanodisc sample introduction at $t=10$ min. The asterisk at $t=20$ min indicates the switch back to buffer (a). This difference in nanodisc loading also affects the binding of the soluble protein annexin (c). Only when the annexin binding (net shift) is normalized to the nanodisc net shift is an increase in annexin binding with increasing phosphoserine content observed (d). Net shifts for nanodisc immobilization and annexin binding are calculated over a 5 minute period once saturation on the sensor surface is achieved. The data is then plotted as the average net shift observed on multiple rings (5 rings per experiment) across multiple microchips (3 chips in total), $n=15$ rings. Error bars correspond to the standard deviation of the calculated net shift (again, $n=15$).

Figure 3.

Concentration-dependent response of CTB on nanodiscs with 5% GM1 content. Data is shown from representative sensor rings for each concentration tested. The dashed line labeled CTB indicates the time point of CTB injection at t=11 min. The dashed line labeled with an asterisk indicates the switch back to buffer (Tris-HCl) at t=15 min (a). Net shifts were again calculated over a 5 minute period once steady state was reached. Based on net shift, a linear response is observed with changing CTB concentration, data shown is from 5 different microchips, approximately 18 rings per concentration, and the error bars are calculated as the standard deviations (b).

Figure 4.

Analysis of four orthogonal nanodisc systems that do not exhibit cross-reactivity with one another. Red boxes indicate the on-target nanodisc-protein interactions, while off-target interactions are in grey. For simplicity, data in each row is a representative ring functionalized via direct physisorption with nanodiscs containing 30% POPS, biotin-DPPE, GM1, and CYP3A4. Data in each column exhibits the shifts upon flowing annexin, streptavidin, CTB, and anti-CYP3A4 sequentially across the entire chip surface. Dashed lines indicate sample introduction, and the asterisks indicate the switch back to buffer.