Peripheral membrane proteins are key regulators of signal transduction pathways at cell membranes. These signaling pathways are activated by conformational changes that occur in peripheral membrane proteins upon ligand binding. Currently, the ability to measure ligand induced conformational change in peripheral membrane proteins under physiological conditions is difficult. Here, we discuss a novel approach for detecting and measuring conformational change in peripheral membrane proteins when attached directly to physiologically relevant supported lipid bilayers.

Introduction

Cell membranes are fluid lipid bilayers composed of a variety of lipids and associated integral and peripheral membrane proteins. Structurally, bilayer membranes are composed of two monolayers of lipids, also known as leaflets, in which the hydrophobic fatty acids tails are oriented toward the center of the bilayer between the hydrophilic lipid head groups. The most abundant class of lipids in the bilayer is the phospholipid family. Phospholipids are distinguished from one another through the incorporation of different lipid head group modifications and commonly include lipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). Interestingly, membranes have an asymmetric distribution of lipids in the inner and outer leaflet of the bilayer that differentiates and specializes each leaflet for important physiological processes. In particular, PS and PI are both found primarily in the inner cytoplasmic leaflet where they recruit peripheral membrane proteins via interaction with the lipid head groups to the inner membrane. Once recruited to the inner membrane and activated by extracellular cues, these proteins regulate diverse signal transduction pathways that control cellular processes such as transcription, cell division, differentiation, and apoptosis.

To better understand the role of lipid bilayers in these cellular processes, model lipid bilayer systems have been developed in vitro. One such model system is the supported lipid bilayer (SLB). In the SLB system, small unilamellar vesicles (SUVs) are deposited onto a glass substrate in an aqueous solution where they spontaneously form fluid lipid bilayers. Biodesy recently developed a commercial system for the investigation of protein conformational changes by second-harmonic generation (SHG) that uses an integrated immobilized metal affinity capture (IMAC) Ni-NTA lipid for the tethering of His-tagged proteins to SLBs. A major advantage of this approach is that the SLBs are biomimetic, maintain native protein structure and function, and they have been used extensively in many biochemical and cell biology experiments.

Second-harmonic generation (SHG) is a nonlinear optical technique in which two photons of equal energy are combined by a nonlinear material or molecule to generate one photon with twice the energy. As SHG is a surface selective technique, it requires tethering of proteins to a surface in an oriented manner. Once tethered to a surface, a labeled second-harmonic-active protein irradiated by a fundamental light source produces an SHG signal whose intensity depends sensitively on the tilt angle of the dye with respect to the surface normal. When the protein undergoes a conformational change upon ligand binding, this causes a change in the time- and space-averaged orientation of the second-harmonic-active moiety leading to a change in the intensity of light. This yields a real-time measurement that reports directly on a probe’s change in orientation at one or more labeled residues in a protein with high angular sensitivity.

We were interested in the potential role of lipid bilayer specialization to capture labeled proteins to SLBs for the purpose of conformational change measurement by SHG. To explore this possibility, Biodesy developed new bilayer formulations that contain specific phospholipid classes to directly capture full-length peripheral membrane proteins to SLBs without the need for a His-tag and Ni-NTA lipids.
We demonstrate that once bound to the SLBs, the full-length peripheral membrane proteins are competent to bind ligands and undergo conformational change as detected by SHG and the Biodesy Delta system. This new approach should be broadly applicable for both drug discovery and mechanistic studies of peripheral membrane protein function under physiologically relevant conditions.

**Results**

To explore the ability of the Biodesy SLB platform to capture proteins without the use of IMAC techniques, we began by testing the ability of physiologically mimetic bilayer formulations to capture untagged full-length peripheral membrane proteins to the SLB surface. In collaboration with a pharmaceutical company, our first target was a clinically relevant peripheral membrane protein that has previously been shown to associate specifically at membranes containing phosphatidylinositol phosphate (PIP) but not at membranes containing PI. We modified our commercial SUV formulations, including replacement of our Ni-NTA lipids with either PIP lipids or control lipids containing PI. Using these new SUV formulations, we prepared modified SLBs on Biodesy plates, deposited full-length peripheral membrane protein on both the PIP and control PI bilayers, and measured SHG signal using the Biodesy Delta system to evaluate protein capture on the SLB surface. As shown in Figure 1, SLBs containing PIP or PIP2 captured the full-length peripheral membrane protein whereas the control, non-phosphorylated PI bilayer, was unable to capture the protein. These data demonstrate the specific capture of the untagged, full-length peripheral membrane protein to the Biodesy SLB surface through the addition of PIP lipids to the SUV formulation.

As a control for the specificity of the protein binding to the surface, we performed an additional experiment in which a truncated version of the protein lacking the N-terminal lipid-binding domain (∆N-Protein) was deposited onto lipid bilayers containing either PI and PIP. As seen in Figure 2, addition of the truncated protein to either surface did not produce an increase in the SHG signal intensity compared to the bilayer-only controls. In combination with the previous results, these data suggest that the full-length peripheral membrane protein binds the Biodesy surface specifically through an interaction between the N-terminal lipid-binding domain and PIP-containing lipids on the SLB membrane surface.

We next wanted to explore whether the peripheral membrane protein, when bound to a physiologically relevant biomimetic bilayer surface, would undergo a conformational change upon the addition of appropriate compounds. Using the Biodesy Delta system, compounds were injected at saturating doses and the change in SHG intensity was monitored five minutes after injection. As shown in Figure 3, the addition of each compound to the peripheral membrane protein sample resulted in a change in SHG intensity that was significantly different than the injection of the vehicle alone (Student’s t-test, p< 0.05). As the intensity of the SHG signal is highly dependent on the average angular orientation of the second-harmonic-active probe relative to the membrane plane, these results suggest that the measured changes in SHG intensity correspond to conformational change induced by compound binding at the attachment site of the second-harmonic-active probe on the protein. In addition, these results demonstrate that the new capture methodology is amenable to the study of protein conformational change with the Biodesy Delta system.

As another demonstration of the power of this approach,
we developed a membrane-binding assay using the Biodesy Delta system to measure the effects of compound binding on the ability of the protein to associate with the SLB surface. Inhibition of the association of the target protein with PIP membranes is of clinical importance, and therefore we developed an assay that could directly interrogate the effect of each compound on the target under physiologically relevant conditions. In this assay, each compound is incubated with the target protein, and then the sample is deposited on the SLB surface. After sample incubation, unbound material is washed out and SHG intensity is quantified in each well using the Biodesy Delta. As shown in Figure 4, when compared to both vehicle and a known inhibitor (positive control), Compound C clearly inhibits the association of the target protein to the PIP SLB surface.

In order to further validate the ability of physiologically relevant SLBs to capture proteins without requiring a His-tag and IMAC tethering, we performed, in collaboration with a pharmaceutical company, experiments on a membrane-associated kinase previously shown to bind to membranes containing the anionic lipid phosphatidylserine (PS). Similar to our previous approach, we modified our commercial SUV formulation, including replacement of our Ni-NTA lipids with PS lipids. SUVs containing PS lipids were then prepared on Biodesy plates, protein was deposited onto the PS SLBs, and SHG signal was measured on the Biodesy Delta. As shown in Figure 5, addition of the full-length kinase to the PS SLB surface results in an enhancement of the SHG signal over the control PS bilayer-only signal, demonstrating capture of the kinase to the SLB surface in the absence of IMAC tethering.

We next sought to validate that the SLB captured membrane protein was competent to undergo conformational change when bound to its physiologically relevant bilayer surface. We performed experiments in which either an activator or inhibitor of kinase activity was injected onto samples in which the kinase target was tethered to the PS SLB surface. As can be seen in Figure 6, the addition of a known activator ligand and a known inhibitor compound each resulted in a significant change in the SHG intensity compared to control vehicle injection. Moreover, as the change in SHG intensity, or “conformational signature,” of the ligand is distinct from the conformational signature of the inhibitor in terms of magnitude and sign of the change relative to baseline, these data indicate that the binding of each molecule to the kinase induces a distinct conformation of the target protein that results in either the up- or down-regulation of kinase activity.

### Discussion

We have previously demonstrated SHG to be a powerful technique for measuring protein conformational change in real time under physiological conditions. Previous results with SHG have used, with great success, IMAC techniques to tether His-tagged proteins to Ni-NTA SLB surfaces, and this approach is now widely available as part of the Biodesy Delta system. While this His/Ni-NTA approach is preferable for cytosolic proteins, the direct capture of peripheral membrane proteins to SLB surfaces through interactions between the protein target and the lipid head group can offer advantages.
One advantage of the direct capture approach is in the ability to conduct experiments with the native protein under physiologically relevant conditions, such as assessing interaction between the protein and the lipid bilayer under various conditions and in the presence of a variety of compounds. Specific interactions between lipid head groups and peripheral membrane proteins are well documented in the literature and in many cases these interactions are required for the full activity of the protein. Interaction with the bilayer may induce functional protein conformations that may not be present in solution or in other assays. Another advantage of this approach is the ability to study the full-length, untagged target protein, which may allow for the discovery of more selective inhibitors due to the identification of novel binding pockets for small molecules that are not present in truncated versions of the target protein. As capture occurs through direct interaction between the target protein and the SLB, recombinant affinity tags can be removed to prevent the potential for unwanted interactions.

In summary, we have shown that we can modify the formulation of our proprietary lipid bilayer surface to bind peripheral membrane proteins directly, specifically, and in a biologically relevant manner as an alternative to traditional affinity capture techniques. Further research is underway to validate other combinations of membrane proteins and lipid bilayer surfaces. Although our custom lipid bilayer surfaces are not currently widely commercially available, please contact Biodesy if interested.

**Figure 6** Quantification of the Change in SHG Intensity of a Membrane Associated Kinase protein upon compound injection. Differences in magnitude and sign of the change indicate that each compound induces a distinct conformation upon binding the kinase protein, consistent with different mechanisms of action. DMSO was used as the vehicle.

References
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