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Study Small Molecule-Membrane Protein Binding Kinetics with Nanodisc and Charge Sensitive Optical Detection

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Abstract

Nanodisc technology provides membrane proteins with a native-like lipid bilayer and much needed solubility, and enables *in vitro* quantification of membrane protein binding with ligands. However, it has been a challenge to measure interaction between small molecule ligands and the nanodisc encapsulated membrane proteins, because the responses of traditional mass-based detection methods scale with the mass of the ligands. We have developed a charge sensitive optical detection (CSOD) method for label-free measurement of the binding kinetics of low molecular mass ligands with nanodisc encapsulated membrane proteins. This microplate compatible method is sensitive to the charge instead of the mass of a ligand, and is able to measure both large and small molecules in a potentially high throughput format. Use CSOD, we measured the binding kinetics between peptide and small molecule ligands and a nanodisc encapsulated potassium ion channel protein KcsA-Kv1.3. Both association and dissociation rate constants for these ligands are obtained for the first time. The CSOD results were validated by the consistent values with the reported binding affinities. In addition, we found that CSOD can tolerate up to 3.9% of DMSO, and up to 10% serum, which shows its compatibility with realistic sample conditions.

Graphical Abstract

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The authors declare no competing financial interests.

Supporting Information

The performance of the humidity control chamber, the method of nanodisc assembly and characterization, characterization of fiber modification, result of PEG-ShK - KcsA-Kv1.3 nanodisc interaction, details of data processing and analysis method, negative control results for the specificity of Kv1.3 ligands binding, DMSO effect on KcsA-Kv1.3 nanodisc, and result of low concentration compound 1 detection in serum. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Membrane proteins play key roles in various biological processes, including cell signaling, substance (ions and molecules) transport, and enzymatic activities. Additionally, membrane proteins are the targets of over 50% of therapeutic drugs¹ and account for ~30% of the human proteome². However, detailed biochemical and biophysical studies of membrane proteins have been hampered by their insolubility in aqueous buffer, and instability outside of the native lipid environment.³ Although detergents can improve membrane protein solubility by forming protein-detergent micelles, they may compromise membrane protein stability and functions.⁴ Moreover, detergents pose problems on structure determination and binding kinetics of membrane proteins.^{5,6} Recently, nanodisc technology has emerged as a powerful approach to enable membrane protein characterization in a detergent-free environment. Nanodiscs are self-assembled phospholipid bilayer nanoscale discs that are encirculated by membrane scaffold proteins (MSP),⁷ providing a native-like environment for the membrane proteins, as well as good solubility and stability, which are attractive for membrane protein studies.

Various label free methods, including isothermal titration calorimetry (ITC)⁸ and backscattering interferometry (BSI)⁹, have been used to determine the binding strengths and kinetics between small molecules and membrane proteins. However, ITC requires relative large sample amounts (~10 µg protein) and BSI can measure binding affinity but not the rate constants. A popular method to measure molecular binding kinetics is Surface Plasmon Resonance (SPR), which has also been applied to nanodiscs.¹⁰ However, SPR is mass sensitive, and the detection signal scales with the ligand mass, which makes it challenging to measure low molecular mass ligand binding, especially to nanodisc encapsulated membrane protein complexes that have a large foot print on the SPR sensor surface (low surface density).

To overcome these challenges, we have developed a Charge Sensitive Optical Detection (CSOD) method. CSOD is a label free technology that measures molecular binding via detecting charge changes, rather than mass changes. This unique feature allows the detection of both large and small molecules with CSOD. The molecular weight (MW) of a typical nanodisc is over 100 kDa, which is about 1000 folds larger than its small molecule ligands.

However, membrane proteins have at most dozens of net charges, which are only 10 folds more than those on small molecules. Because charge change is more significant than mass change upon binding, CSOD is particularly capable of measuring nanodisc-small molecule interaction that challenges the traditional techniques. We show here that CSOD can measure the binding kinetics and affinities of low molecular mass ligands with a nanodisc encapsulated membrane protein. The ligands include two peptides (ShK¹¹ and PEG-ShK¹²) and one small molecule (compound 1¹³), and the membrane protein is KcsA-Kv1.3⁹, which is a chimeric potassium ion channel protein. CSOD is the only method so far to reveal the details of the kinetic information of small ligands binding to KcsA-Kv1.3 channel. Studying the binding of these ligands to the ion channel protein is important for developing drugs for cardiovascular diseases, and also serves as a model system to establish CSOD as a label-free method for studying membrane protein binding kinetics with the nanodisc technology.

DETECTION PRINCIPLE

The schematic of the CSOD system is shown in Figure 1. The details of the CSOD detection principle has been described previously.¹⁴ Briefly, a glass optical fiber with an etched tip (diameter of ~15 μm and length of ~8 mm) is dipped into a microplate well filled with buffer or analyte solution. A periodic voltage is applied to two platinum electrodes to create an oscillating electric field perpendicular to the fiber, which drives the optical fiber, if charged, into oscillation. The oscillation amplitude is proportional to the surface charge of the optical fiber, allowing us to measure molecule binding induced charge changes on the optical fiber, and to determine the binding kinetics by monitoring the oscillation amplitude in real time. To measure the oscillation amplitude precisely, light is coupled into the optical fiber, and emitted from the tip. By detecting the emitted light with an optical microscope equipped with a CCD camera, and analyzing the optical signal with a differential algorithm, CSOD can detect fiber oscillation as small as 1 nm.

CSOD requires charge changes, and most drugs and biologically significant ligands are either charged or contain charged groups. For example, only 10 out of 1611 of FDA approved small molecule drugs are completely uncharged.¹⁵ Even in the case when the ligand has no charge, binding of the ligand to the receptor on the optical fiber may change the conformation and charge status of the receptor molecules, and induce a charge density changes on the fiber that can be detected by CSOD.

To study molecular binding, the optical fiber tip is functionalized with a molecular receptor. Because the fiber is made of silica glass, silica-based surface chemistry, such as APTES (3-aminopropyl) triethoxysilane), can be readily applied to immobilize molecular receptors and reduce non-specific adsorption. In this work, the tip was functionalized with KcsA-Kv1.3 nanodisc with a procedure described in Scheme 1.

MATERIALS AND METHODS

Materials

125 μm diameter optical fibers were purchased from Thorlabs, Inc. Hydrofluoric acid was from Avantor Performance Materials. Sulfo-(N-Hydroxysuccinimide (NHS))-Biotin and

Streptavidin were from Thermo Scientific. KcsA-Kv1.3 nanodisc, empty nanodisc, compound 1, ShK, PEG-ShK, and standard nanodisc buffer (20 mM Tris, 100 mM NaCl, 0.5 mM EDTA, pH 7.4) were obtained from Amgen Inc. Horse serum was purchased from ATCC. Other chemicals were from Sigma-Aldrich. DI water with a resistivity of 18.2 MΩ/cm, filtrated with 0.45 μm filter, was used in all experiments.

The CSOD Setup

An inverted microscope (Olympus IX70 with 40X objective) with a CCD camera was used for recording fiber oscillation. A 96-well microplate with two platinum electrodes (1 cm × 0.5 cm, and 0.5 cm distance) inside the wells was mounted on the stage. The amplitude and frequency of the applied voltage were 4 V and 40 Hz, respectively. An internally developed MATLAB program was used to record the position of fiber tip, and control the movement of stages for switching the fiber among wells. When switching the fiber tip from one well to another, the fiber was pulled out of the first well vertically by a motorized arm (A-LSQ075B, ZABER), and then the plate was moved horizontally with a motorized microscope stage (MAC 6000 Modular Automation Controller, Ludl Electronic Products Ltd) so that the second well was under the fiber tip, followed by moving the fiber tip downward into the second well. A plastic chamber, covering the microplate and the optical fiber, with a continuous supply of water vapor, was used to minimize water evaporation from the fiber tip during the transfer. The humidity stability of the chamber is shown in Figure S1.

Surface chemistry

To remove the polymer coating layer on the optical fiber, a section (18 mm) of the fiber was first soaked in acetone for 1 min, then rinsed with DI water and dried with N₂. The coating was then stripped off by an optical fiber stripper. The bare fiber was etched by soaked in 47% hydrofluoric acid for 30 minutes and 15 seconds to reach a diameter of ~15 μm. The etched fiber was rinsed with DI water, dried with N₂ flow, and cut to ~8 mm in length. At last, these tips were cleaned with oxygen plasma for 3 minutes to remove surface contaminants. The etched fibers were stored in desiccators filled with N₂ to minimize contamination.

The etched fibers were modified with APTES in gas phase to create an amine functionalized surface. The gas phase modification took place in a desiccator, in which 500 μL APTES was added into a petri dish placed at the bottom, and fibers were placed above the petri dish. A quick evacuation was then applied to the desiccator, and the fibers were kept in this near vacuum environment for 18 h. After this surface reaction, the fibers were rinsed with toluene and ethanol 3 times to remove any unbound APTES,¹⁶ and baked in an oven at 110 °C for 1 h to condense bound APTES.¹⁷

The amine-coated fibers were incubated in NHS-Biotin solution for 2 hours to allow linking of biotin. The NHS-Biotin solution was prepared by dissolving 0.2 mg Sulfo-NHS-Biotin in 1.5 ml DI water. The biotin-coated fibers were then rinsed with DI water and dried with N₂ flow. A layer of streptavidin was immobilized on the surface by dipping fibers in 12 μg/ml streptavidin solution (in 1X phosphate buffered saline (PBS)) for 1 hour. The fibers were rinsed with nanodisc buffer followed by incubation in 30 nM KcsA-Kv1.3 nanodisc or

empty nano-disc solution (in nanodisc buffer) for 1 hour, to allow immobilization of nanodisc. The method of assembling nanodisc is described in Supporting Information, and the function of the assembled KcsA-Kv1.3 nanodisc has been validated previously.⁹ To block non-specific binding sites on nanodisc, the functionalized fibers were dipped in 40 times diluted nanodisc buffer containing blocker for at least 1 hour. The concentration of blocker was optimized according to each analyte, 0.01% casein for compound 1, 0.0001% BSA for ShK, and 0.01% BSA for PEG-ShK. Surface functionalized optical fibers were stored in this blocking buffer before use. Characterization of surface chemistry is described in Supporting Information.

RESULTS AND DISCUSSION

Measurement of KcsA-Kv1.3 nanodisc-ShK binding kinetics

We first carried out kinetic and equilibrium analysis for a medium sized biomolecule, ShK (MW = 4055 Da), binding to the pore region of KcsA-Kv1.3 nanodisc. ShK, a 35-amino acid peptide from the sea anemone *Stichodactyla helianthus*, is a Kv1.3 inhibitor with potent pico-molar activity and of great interest to autoimmune diseases.¹⁸ Recent studies of ShK are focused on discovering more specific and potent ShK derivatives,^{19–21} which can benefit from kinetic measurement combined with nanodisc technology. In CSOD measurement, the KcsA-Kv1.3 modified fiber tip was initially dipped into the well of a 96-well microplate containing buffer, and its oscillation amplitude was recorded over time to establish a baseline. The fiber tip was subsequently switched from the buffer well to a sample well containing ShK to measure the binding or association process of ShK to KcsA-Kv1.3 nanodisc. ShK has 6 positive charges, and the binding changed the surface charge of the fiber tip, which was measured from the oscillation amplitude change. Four minutes after the association started, the fiber tip was switched back to the original buffer well to study the dissociation of ShK from KcsA-Kv1.3 in the nanodisc. Figure 2a shows the binding kinetics of ShK to KcsA-Kv1.3 nanodisc at four different concentrations, where the response is the oscillation amplitude change. The red and blue arrows in Figure 2, 3&4 mark the start points of the association and dissociation, respectively. By fitting of the kinetic curves at different concentrations globally with the first order kinetics, the association rate constant (k_a) and dissociation rate constant (k_d) were determined to be $6.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $1.3 \times 10^{-2} \text{ s}^{-1}$, respectively, and the equilibrium constants (K_D) was determined to be 203 pM ($\text{Res } sd = 0.48$) using the relation $K_D = k_d/k_a$. These results are in good agreement with those in the literature (0.20 nM⁹). Note that K_D was also determined from the equilibrium analysis, which plots the equilibrium response vs. ShK concentration (Figure 2b) with 3 data points per concentration ($R^2 = 0.97$). The corresponding K_D was found to be 196 pM by fitting the equilibrium curve, which is consistent with that obtained from the kinetic analysis.

A negative control experiment was performed using an empty nanodisc (no protein was encapsulated) functionalized optical fiber tip as the sensor probe. The control fiber tip was dipped in 86 nM ShK and no response was observed (Figure 2c), which confirms that the oscillation amplitude change shown in Figure 2a was due to the specific binding of ShK to the KcsA-Kv1.3 ion channel proteins. To demonstrate the specificity of the ShK ligand we carried out another negative control experiment using IgG to replace ShK (Figure S6a) and

the result showed no binding of IgG to KcsA-Kv1.3 nanodisc. We also performed a positive control experiment using a PEG analog of ShK, and observed a similar binding kinetics to that of the native ShK peptide (Figure S5).

Measurement of KcsA-Kv1.3 nanodisc-small molecule binding kinetics

To show the measurement of small molecule binding to nanodisc encapsulated membrane proteins with CSOD, a small molecule, compound 1 (4-(2-ethylpiperidin-1-yl)-2-methyl-6-phenyl-5H-pyrrolo[3,2-d]pyrimidine, MW = 320 Da), was measured with KcsA-Kv1.3 nanodisc coated optical fiber probes. Compound 1 is not only a drug candidate for treating obesity through binding to Y5 receptor,^{13,22} but also a blocker of the pore region of Kv1.3 channel.⁹ Studying compound 1 and Kv1.3 interaction provides evidence for its potential side effects and demonstrates the capability of CSOD for small molecule drug screening. Figure 3a shows the kinetic curves at four concentrations of compounds 1, as well as global fittings of the data with the first order kinetic model (solid curves). The kinetic constants determined from the global fitting were $k_a = 1.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_d = 4.1 \times 10^{-2} \text{ s}^{-1}$, and $K_D = 22 \text{ nM}$ ($\text{Res } sd = 0.75$). Note that K_D from the equilibrium analysis shown in Figure 3b is 66 nM ($R^2 = 0.97$). The results are comparable with the values in literature (30 nM).⁹ One interesting observation is that although compound 1 has only one positive charge, it produces similar amplitude change upon binding compared with ShK, which has six positive charges. This is probably because KcsA-Kv1.3 adopts different conformations after bound with compound 1 and ShK, and changes its surface charge. Figure 3c shows no measurable binding signal in a negative control experiment using empty nanodisc coated fiber dipped in 2.8 μM compound 1. We also included a small molecule anti-cancer drug, imatinib (MW = 494 Da) as a negative control (Figure S6b) and observed no binding either. Comparing with ShK and PEG-ShK measurements, though compound 1 was much smaller in size, the signal did not diminish. This result demonstrates that the CSOD signal does not depend on the molecular mass, making CSOD suitable for small molecule applications. Note that the solubility of compound 1 was poor in water, and thus it was originally dissolved in DMSO. An additional control experiment was carried out to measure the oscillation change caused by DMSO (Figure S7). In this control experiment, a KcsA-Kv1.3 nanodisc coated fiber was dipped in a well filled with 40 times diluted nanodisc buffer, and DMSO were injected several times with increasing concentrations (up to 3.9%). Since there was no obvious amplitude change, we concluded that DMSO had no impact on CSOD.

Small molecule binding kinetics in serum

For many applications, it is desirable to measure protein interactions in serum samples. To demonstrate this capability, we measured compound 1 interaction with KcsA-Kv1.3 nanodisc in horse serum. Serum contains various proteins and salts, which are known to lead to non-specific adsorption. This has been a serious challenge to most detection technologies, particularly label-free technologies, such as SPR.²³ We evaluated the impact of serum to the CSOD detection of nanodisc encapsulated membrane proteins in the serum.

The baseline signal of CSOD remained stable in serum solution, regardless of serum concentration, which indicates no serious non-specific adsorption took place on the optical fiber probe. However, the oscillation amplitude decreased with increasing serum

concentration, which was due to ionic screening that reduced the effective surface charge on the fiber tip (Figure 4a). We measured KcsA-Kv1.3 nanodisc and compound 1 interaction in 4, 10, 20, and 40 times diluted serum (Figure 4b), and found that CSOD was able to measure the binding kinetics up to 10% serum. Both of the association and dissociation rate constants increased with dilution of the serum. However, the equilibrium constant decreased (Figure 4c). Note that the 40 times diluted buffer has the lowest ionic concentration (3 mM, compared to ~140 mM for serum) among these solutions. Compared with measurement in buffer, the discrepancy in kinetic constants can be attributed to two factors: 1) Serum has more ion species and elevated concentration, which can affect the binding kinetics through the interaction of ions with the target protein.²⁴ 2) Some serum proteins may interact with compound 1, and therefore compete with KcsA-Kv1.3 nanodisc and affect free ligand concentration and the kinetic measurement.²⁵ Nevertheless, our results demonstrated that CSOD can be combined with the nanodisc technology to study membrane protein in complex and realistic media, which is important for unraveling physiological activities of membrane proteins and for developing drugs that target membrane proteins. Taking advantages of microplate compatibility and programmed moving of the probe, it is promising to develop CSOD into a multiplexed format with multiple probes working simultaneously, realizing high throughput detection.

CONCLUSION

The nanodisc technology provides membrane proteins a native-like amphiphilic environment, and the CSOD technology is a label-free detection technology, capable of detecting small molecule binding kinetics with the membrane proteins. By combining the two technologies, we measured small molecule binding kinetics with membrane proteins. We have studied the interactions between KcsA-Kv1.3 nano-disc and its inhibitors, including two peptides (ShK and PEG-ShK) and a small molecule (compound 1). We have also found that CSOD can tolerate up to 3.9% of DMSO without significant loss of sensitivity, and can measure small molecule binding in the presence of up to 10% serum. Since CSOD is compatible with the standard microplate technology, the nanodisc-CSOD combination promises a high throughput tool for drug discovery.

The major limitations of CSOD technologies are: 1) Insensitive to molecular binding events that not induce any net charge change (although this is uncommon); and 2) the measurements need to be done in low ionic buffer, which may introduce complications to the molecular interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of CSOD. a) Illustration of the setup. b) A zoomed-in figure of the circle in a) shows the measurement of a binding process.

Figure 2.

CSOD measurement of KcsA-Kv1.3 nanodisc-ShK binding kinetics and equilibrium analysis. a) CSOD response to ShK. Solid lines are global fitting of the data to the first order kinetics. b) Equilibrium analysis, which displays the equilibrium response vs. concentrations. Note: each concentration was repeated three times to create the error bars. c) Negative control experiment with empty nanodisc fiber probe dipped into the well of 86 nM ShK. The nanodisc buffer was 40 times diluted with 0.0001% BSA and pH 7.4.

Figure 3.

CSOD measurement of KcsA-Kv1.3 nanodisc- compound 1 binding kinetics and equilibrium analysis. a) CSOD response to compound 1. b) Equilibrium analysis, each concentration was repeated three times to create the error bars. c) Negative control experiment with empty nanodisc fiber probe dipped into 2.8 μ M compound 1 solution. The nanodisc buffer was 40 times diluted with 0.01% casein and pH 7.4.

Figure 4.

Small molecule detection in serum. a) The oscillation amplitude of KcsA-Kv1.3 nanodisc coated fiber increased with the dilution of the serum. Three optical fibers were measured in diluted serum with 1 (no dilution), 2, 4, 8, 16, 32, and 64 times dilution. b) CSOD response to 2.8 μ M compound 1 in 4, 10, 20 and 40 times diluted serum. c) Kinetic constants measured in 10, 20 and 40 times diluted serum and 40 times diluted nanodisc buffer.

Scheme 1.

Surface functionalization of CSOD optical fiber with nano-disc.