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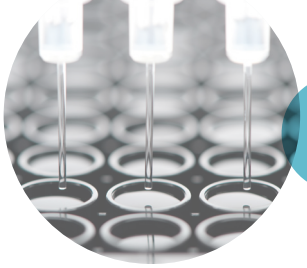

Characterizing Membrane Protein Interactions by Bio-Layer Interferometry (BLI)

Introduction

The cell membrane separates the intracellular components from the extracellular environment. Cell membranes consist of various components such as phospholipids, glycolipids, and cholesterol, in combination with integral and peripheral proteins. Membrane compositions vary significantly even within the same biological system, and the organization of the membrane components needs to be dynamic to mediate and modulate conformational changes, signaling, trafficking, and other cellular recognition events. The functional activity of membrane proteins further depends on the structure and environment surrounding the lipid molecules in the membrane. Investigating specific functions of membrane proteins *in vivo* has been historically challenging due to their complexity and heterogeneity.

In vitro characterization of membrane protein interactions requires the preservation of its native structural integrity and solubility in aqueous buffers. In most cases, membrane proteins are presented in their biologically relevant conformations by reconstituting into membrane-like environments such as liposomes[1-8], virus-like particles (VLP)[9-14] or nanodiscs[15-20] for analysis. Membrane proteins can also be presented by solubilizing in detergent environments[21-23]. In this case, the detergent and its concentration are critical for retaining protein activity. Detergent concentrations are typically maintained above their critical micelle concentrations (CMC) that will mimic membrane-like environments. However, determining optimal buffers and their concentrations can be less than straightforward, requiring screening of a multitude of buffer components and settings.

Bio-Layer Interferometry assays on the Octet platform offer several advantages for rapidly developing binding assays for membrane proteins. High-throughput, multi-channel configurations of Octet instruments can rapidly evaluate diverse assay conditions to speed assay development. Fluidics-free operation also allows for detection and characterization of interactions even using unpurified crude matrices, which might not be possible on systems that require microfluidics to handle samples. This provides a wide range of buffer compatibility and allows the reuse of valuable samples for further optimization and analysis when needed. In addition, ForteBio offers several ready-to-use biosensor chemistries that can be utilized for membrane protein interaction analysis.

Listed below are a few selected examples where membrane proteins are captured onto liposomes, VLPs, nanodiscs, or solubilized in detergent for characterization by BLI. Depending on the binding interaction system, membrane protein conformations can be immobilized to biosensors (ligand) or used as the analyte (see examples in Table 1). Capturing biotin labeled or affinity tagged membrane proteins to the biosensor is more commonly used as it allows increased immobilization specificity from protein preparations. Liposomes, nanodiscs, and VLPs preparations can also be captured by incorporating affinity tags or biotin moieties or can be captured using hydrophobic or electrostatic interactions onto APS biosensors. In addition to information available in the following examples, other common assay considerations for BLI kinetics assay development can also be found in ForteBio Applications Notes[24].

1. KINETIC ANALYSIS OF ANTIBODY BINDING TO AN EXPRESSED MEMBRANE PROTEIN ON CAPTURED LIPOPARTICLES[4]

CXCR4 is a G-protein coupled receptor in the CXC chemokine receptor family. It is widely expressed in lymph nodes and bone marrow and plays an important role in a number of biological processes such as organogenesis, hematopoiesis, and immune responses. In addition, it is also involved in activating several signaling pathways that aid tumor cell proliferation.

An Octet assay was developed to measure binding affinities and kinetics between a CXCR4 containing lipoparticle and an anti-CXCR4 antibody (Figure 1).

The application note details the assay development workflow including immobilization strategy and biosensor selection, tips for optimal assay performance, biosensor regeneration conditions and data analysis. The lipoparticle displaying CXCR4 was immobilized onto a streptavidin biosensor that was pre-immobilized with biotinylated wheat germ agglutinin (WGA) utilizing the high affinity interaction between lipoparticle carbohydrates and WGA. Additional assay considerations such as shake speed optimization, control reactions, assay buffers, and biosensor regeneration conditions are detailed. The Octet assay measured a 13 nM binding affinity between CXCR4 and anti-CXCR4 antibody.

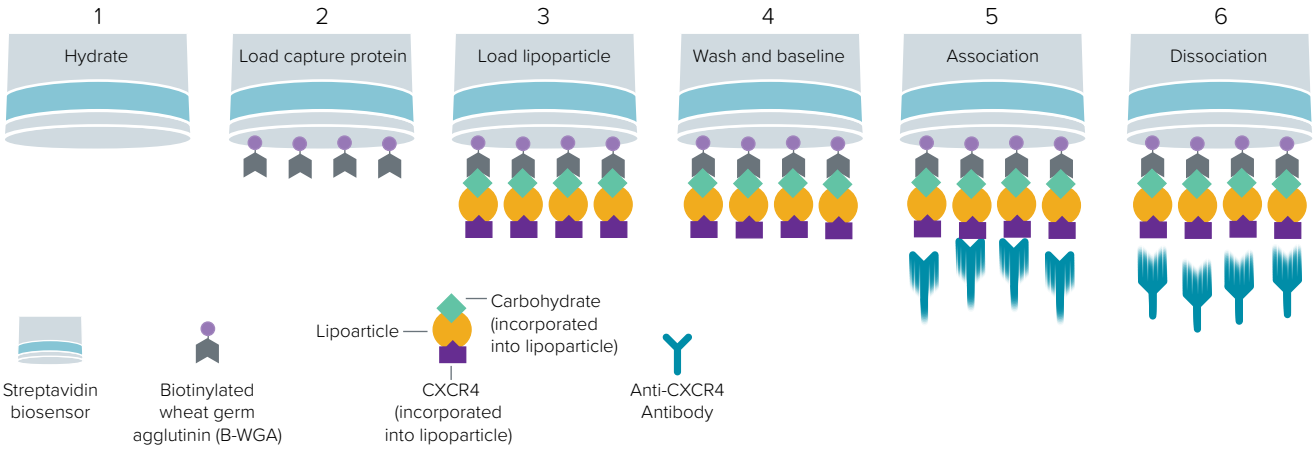

Figure 1: The schematic shows the workflow for kinetic characterization of the interaction between the CXCR4 membrane protein and an anti-CXCR4 antibody. Step 1: Streptavidin biosensor hydration, Step 2: Load biotinylated wheat germ agglutinin, Step 3: Immobilization of the CXCR4 containing lipoparticles, Step 4: Baseline in buffer, Step 5: Measure association kinetics between anti-CXCR4 antibody and lipoparticle-displayed CXCR4, Step 6: Measure dissociation kinetics between anti-CXCR4 antibody and lipoparticle-displayed CXCR4.

2. A CONFORMATION SPECIFIC NANOBODY BINDING TO ß2-ADRENERGIC RECEPTOR (ß2-AR) RECONSTITUTED INTO HDL PARTICLES [25]

In this study, Irannejad *et al.* employed a conformation specific nanobody (Nb80) to directly probe the activation of ß2-adrenoceptor (ß2-AR) triggered by agonist binding. To confirm Nb80 nanobody specificity towards ß2-AR conformations *in vitro*, an Octet binding assay was utilized. ß2-AR was incorporated into recombinant high-density lipoprotein rHDL particles using biotinylated apolipoprotein AI. Binding assays were performed using Streptavidin (SA) biosensors where biotinylated ß2-AR-rHDL particles were immobilized. Nb80 binding was assessed in presence and absence of the ß2-AR agonist isoprenaline in assay buffer. Results indicated that *in vitro* binding affinities were significantly favorable (>200 fold) towards the agonist-occupied ß2-AR structural conformation. *In vitro* observations were replicated *in vivo* where localization experiments indicated that Nb80 fused to GFP selectively binds the agonist (isoprenaline) occupied ß2-AR in the plasma membrane whereas in the absence of the agonist, Nb80-GFP was localized in the cytoplasm. Their data support the hypothesis that canonical GPCR signaling occurs from endosomes as well as in the plasma membrane.

3. LEAD CHARACTERIZATION AND VALIDATION OF NEUTRALIZING ANTIBODIES AGAINST CHIKUNGUNYA VIRUS (CHIKV) VLPs [11]

Chikungunya virus (CHIKV) is a member of the genus alphavirus. Like many other alphaviruses, CHIKV encodes two envelope glycoproteins E1 and E2 which are embedded in the viral membrane. In this work, a phage display campaign was carried out against structurally intact E1/E2 membrane glycoproteins displayed on virus-like particles (VLPs). Seven mAbs identified were selected for further characterization. An Octet assay was developed to measure binding affinities and kinetics between mAbs and E1/E2 presented in their native structural state displayed in VLPs. Purified VLPs were immobilized onto amine reactive (AR2G) biosensors via a CHIKV E1/E2 capture antibody that recognized a distinct epitope region from that is being characterized. mAbs were then assayed for binding against the immobilized VLPs. Measured affinities and kinetics from the Octet assays highly correlated with relative neutralization potencies in animal models.

4. CELL SURFACE GALACTOSYLCERAMIDE (GALCER) CONTAINING LIPOSOMES BINDING TO HIV-GP [3]

Mucosal epithelial cell surface galactosylceramide (Galcer) is known to be a receptor for HIV-1 envelope interactions with mucosal epithelial cells. In this publication, Dennison *et al.* investigated whether the HIV-1 interaction with the receptor can be blocked using an anti-Galcer antibody. The Galcer-antibody interaction affinities and blocking efficiency of receptor by the antibody were analyzed using two Octet assays. The binding of HIV envelope protein Gp140 to Galcer was measured on aminopropylsilane (APS) biosensors. APS biosensors offer a convenient way to immobilize ligands through hydrophobic or electrostatic interactions. APS biosensors however can only be used for membrane proteins where detergents are not required to maintain protein activity or solubility. Galcer containing POPC liposomes were loaded onto APS biosensors. After blocking the remaining protein binding sites in the APS biosensor with BSA, gp140 binding affinities to Galcer was measured. A second Octet assay was developed, where the competition between HIV gp140 and anti-Galcer antibody binding to Galcer was evaluated. Galcer containing liposomes were immobilized onto APS biosensors followed by binding of saturating concentrations of anti-Galcer antibody. The Galcer-antibody complex was then exposed to HIV receptor gp140 indicating that conditions employed and antibody recognition was sufficient to prevent gp140 binding to Galcer proteins.

5. BIOLAYER INTERFEROMETRY OF LIPID NANODISC RECONSTITUTED YEAST VACUOLAR H^+-ATPase[18]

In this work, Sharma and Wilkens described a method for determining kinetics and affinities of membrane protein-protein interactions using an Octet assay by reconstituting membrane proteins into lipid nanodiscs. Vacuolar H^+-ATPase (V-ATPase) is an ATP dependent proton pump present in all eukaryotic organisms. V-ATPase is a multi-subunit protein complex that consists of cytoplasmic and membrane integral components. V-ATPase mis-regulation is implicated in a number of disease states and has been evaluated as a drug target for therapeutic purposes. One of the main challenges to biophysical investigations of multi-subunit complexes such as the V-ATPases and their binding interactions is the inability to retain complex stability and protein activity in the presence of detergents that is required to maintain membrane proteins solubility. To overcome these challenges, V-ATPase was reconstituted into lipid nanodiscs using native vacuolar lipid membranes scaffold proteins engineered to contain a N-terminal biotin acceptor to biotinylation in *E. coli*. The biotin tag was used to immobilize the nanodisc onto streptavidin biosensors (SA) for kinetic studies. After confirming the activity of lipid nanodisc-reconstituted V-ATPase complex, they showed that Octet experiments can be successfully used to investigate the kinetics and affinities of interactions between V-ATPase and cellular factors known to bind to the ATPase enzyme. The monitoring of nucleotide dependent dissociation of the complex in real-time was also demonstrated.

Immobilized ligand	Analyte	Membrane protein environment	Biosensor	Ref
HIV-1 env glycoprotein trimer	IgGs	Glycoprotein incorporated liposomes	SA	1,5
Hornerin protein	Peptide	Analyte peptide-conjugated liposomes	SA	2
Various liposome formulations	Cytochrome C	Liposomes captured onto biosensors by sterically stabilized micelles	SA	7
HIV 1 gp41	IgG	gp41 fragments conjugated to liposomes	APS	8
Heparin	Bacteriophage Qß	VLP	SA	10
GLUT4 transmembrane protein	mAb	VLP	SA	12
HPV-VLP	RNA	VLP	AR2G	13
RNA	HPV-VLP	VLP	SA	13
BRAF-RBD protein	K-RAS4B	K-RAS4B Nanodisc	GST	16
Amyloid-ß protein structures	Ganglioside GM1 nanodiscs	Nanodisc	SA	19
Various compositions of lipid nanodiscs	α-Synuclein	Nanodisc	AR2G	20
Small molecule inhibitor (MMV007839)	pfFNT	Detergent solubilized	SA	21
CLDN1 protein	Fab	Detergent solubilized	SA	22
DNA Aptamers	ß2-Adrenoceptor	Detergent solubilized	SA	23

Table 1: Examples of membrane protein characterization by bio-layer interferometry (BLI). For additional assay development guidance and considerations please read ForteBio Applications Note 14[24].

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