protein interactions

tech note 5948

# Analysis of Membrane Protein Interactions Using Lipoparticles Bound to the Surface of the ProteOn™ XPR36 Sensor Chips

Laura Moriarty[1] and Joseph Rucker[2],

[1]Bio-Rad Laboratories, Inc., Protein Function Division, Life Science Group, 2000 Alfred Nobel Drive, Hercules, CA 94547

[2]Integral Molecular, Inc., 3701 Market Street, Suite 416, Philadelphia, PA 19104

## Introduction

Optical biosensors are valuable tools in basic and applied research for measuring the affinity, specificity, and kinetics of molecular interactions. Biosensor experiments involve immobilizing molecules of interest (ligands) on a chip surface and assessing the binding of interacting molecules (analytes) that are flowed across the chip. Bio-Rad's biosensor, the ProteOn XPR36 protein interaction array system can perform these experiments in high throughput as it measures 36 interactions simultaneously.

For soluble proteins, biosensor methodologies are well developed. However, this technology has not been widely employed with membrane proteins due to their functional requirement of an intact lipid bilayer and such membrane structures are difficult to attach to biosensor chip surfaces. Whole cells and membrane preparations are too large and heterogeneous for application to biosensors. Other types of lipid-based structures, such as liposomes, can also be difficult to attach due to potential deformation of the lipid bilayer and inactivation of the embedded membrane proteins. Integral Molecular's lipoparticle technology makes biosensor applications accessible to membrane proteins by presenting them in a format that is readily amenable to surface attachment, using validated protocols. In conjunction with the array-based ProteOn biosensor, lipoparticles enable a convenient platform for screening membrane protein interactions with analytes such as antibodies.

Lipoparticles from Integral Molecular are stable, nanoscale (~150 nm diameter) membrane particles derived directly from cells using retroviral structural proteins. They are engineered to incorporate high concentrations of a specific membrane protein on their surface at concentrations 10–100 fold higher than those found in cells or membrane preparations. Lipoparticles can be directly attached to biosensor chip surfaces via amine coupling. Alternatively, they can be indirectly attached to biosensor chip surfaces using a capture antibody against native surface proteins on the lipoparticle surface, or using wheat germ agglutinin (WGA) lectin coupling. All three methods permit reproducible attachment of lipoparticles without compromising the structural integrity of target membrane proteins. Since the attachment methods are common for all lipoparticles, an entire array-based biosensor chip can be rapidly prepared with a single attachment protocol for the analysis of diverse membrane proteins.

Bio-Rad ProteOn XPR36 sensor chips (GLC, GLM, and GLH) are composed of a modified carboxylated alginate which is easily activated for general amine coupling and amenable to WGA and antibody-based capture methods. In this tech note we describe how the binding of therapeutics antibodies to membrane proteins incorporated into lipoparticles can be investigated on the ProteOn XPR36 system.

## Materials and Methods

### Preparation of Biosensor Surface, Antibody Immobilization, and Lipoparticle Capture

GLC chips were initialized using glycerol and preconditioning was performed by sequential injections of 0.5% SDS, 100 mM HCl, and then 50 mM NaOH, each for 60 sec at 25 µl/min. The surface was then activated using a 1:1 mixture of EDAC and Sulfo-NHS, diluted to 1:5 in $dH_2O$, injected for 5 min at 25 µl/min. Lipoparticle capture antibody JS-81 (anti-CD81, BD Biosciences) 50 g/ml in 10 mM sodium acetate, pH 5.0, was injected in the vertical direction over the desired channels for 10 min at 25 µl/min, giving ~2,000 RU of immobilized antibody. The remaining activated carboxyl groups were blocked with a 3 min injection of 1 M ethanolamine at 25 µl/min in the vertical direction. The chip was washed with PBS until a stable baseline was achieved. The running buffer was then switched to PBS with 1 mg/ml BSA (0.2 m filtered) (PBS-B). After equilibrating the chip with PBS-B, diluted lipoparticles (1:15 in PBS-B) containing the membrane protein(s) of interest, or lipoparticles without the membrane protein(s) of interest ('null' lipoparticles as negative control) were captured in the vertical direction using two 18 min injections at 25 µl/min. Buffer injections were used between lipoparticle injections to allow for surface stabilization. The instrument was then washed with running buffer until the baseline stabilized.

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#### One-shot Kinetics™ Experiment

To obtain kinetic data using a One-shot Kinetics protocol, a concentration series of the analyte of interest (monoclonal antibody [mAb]), was injected over the captured lipoparticles. Injection of the antibody (analyte) diluted in PBS-B was done in the horizontal direction at 30 µl/min for 2–3 min. An equivalent buffer or a matched antibody control injection was used for reference subtraction. The dissociation time was set to 5–10 min. Injection rates during the association and dissociation phases may be varied depending on the analyte's kinetics parameters.

#### Surface Regeneration

After the final antibody/control injections, the channels were regenerated back to JS-81 capture antibody baseline at least 5 times by removal of the lipoparticles. To remove the lipoparticles, two 30 sec pulses of 1% Empigen (Sigma Aldrich) at 50 µl/min were run followed by two pulses of 100 mM $H_3PO_4$. The procedure was repeated twice.

#### Data Analysis

Equilibrium and rate constants were calculated using ProteOn Manager™ software. These analyses may be performed globally or grouped by channel (ligand surface). A local $R_{max}$ value may also be used. Data analysis also requires a choice of binding models. While the Langmuir (1:1) model is usually the default choice, additional models (Langmuir with mass transfer, bivalent analyte, or Langmuir with drift) could also be used if appropriate.

## Results and Discussion

#### Visualization of Lipoparticle Binding to the Sensor Chip

Lipoparticles containing either green fluorescent protein (GFP) or red fluorescent protein (dsRed) were attached to alternate channels on a ProteOn GLC chip using the antibody JS-81, which recognizes a native protein (CD81) on the surface of the lipoparticles. The biosensor chip was then visualized by fluorescence microscopy (Figure 1).

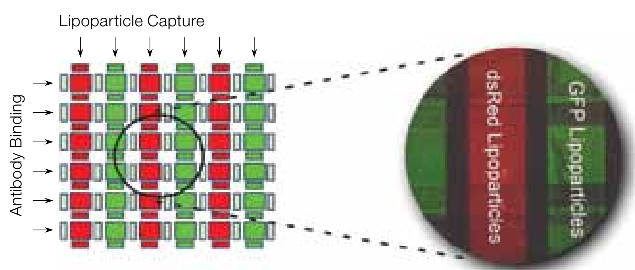


**Fig. 1. Binding of lipoparticles to the surface of a ProteOn sensor chip.** GFP and dsRed containing lipoparticles were captured in alternate vertical channels of a GLC sensor chip by JS-81 antibody capture. Sensor chip was imaged by fluorescence microscopy.

#### Liporaticle Capture to the Sensor Chip Surface

Capture of lipoparticles containing the membrane protein CXCR4 to a GLC chip using either antibody capture or the WGA coupling method was measured in real-time. Approximately 1,000 and 4,000 RU of binding was achieved, respectively (Figure 2). Within each channel, similar levels of attachment were obtained for all six interaction spots. Multiple injections could be used to increase the amount of captured lipoparticles. Importantly, the membrane proteins within the lipoparticles retained native conformation after attachment, as measured by binding of conformationally-sensitive antibodies. Antibody capture and WGA coupling are preferred attachment strategies for lipoparticles since they allow the biosensor chip to be more easily regenerated for re-use.

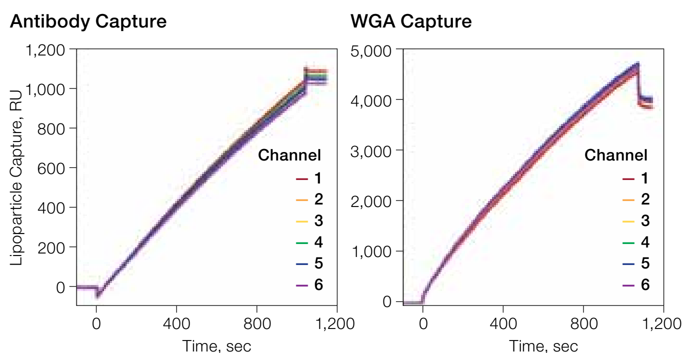


**Fig. 2. Kinetics of lipoparticle capture on ProteOn GLC chip.** CXCR4 containing lipoparticles were captured on a ProteOn GLC chip containing either a JS-81 capture antibody or with WGA lectins. The six sensorgram traces in each figure represent measurements taken at each of the six interaction spots in the channel.

#### One-shot Kinetics: Antibody Binding to Lipoparticle Proteins

To evaluate the specificity and relative affinity of mAbs against various membrane proteins, lipoparticles containing four different membrane proteins (CXCR4, CXCR3, CCR5, and 5HT-1a) were immobilized on the GLC sensor chip surface. Six different concentrations of the analyte (mAb) were simultaneously injected in the orthogonal channels of the sensor chip. Interaction kinetics were measured for each protein/mAb concentration, resulting in a complete kinetic profile for each binding pair. Figure 3 shows typical binding curves generated with CXCR4-containing lipoparticles immobilized on a chip and analyzed with different concentrations of an anti-CXCR4 antibody. Upon fitting the binding curve for each concentration of antibody, kinetic parameters such as $k_a$, $k_d$ and $K_D$ were determined (Figure 3).

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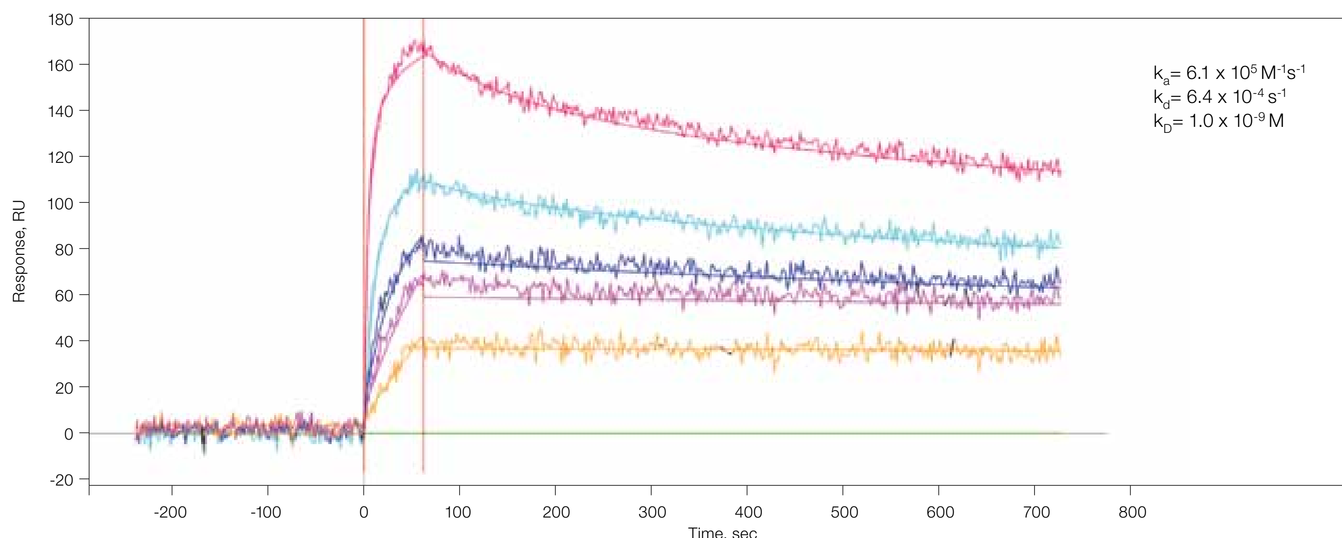


**Fig. 3. One-shot Kinetic analysis of CXCR4 antibody binding.** ProteOn sensorgrams showing association and dissociation of an anti-CXCR4 antibody to CXCR4-containing lipoparticles are shown. An antibody concentration range of 50 to 0.08 µg/ml was used in this One-shot Kinetic assay. Specific responses were calculated by subtracting the responses to null lipoparticles. — 50 µg/ml; — 10 µg/ml; — 2 µg/ml; — 0.4 µg/ml; — 0.08 µg/ml.

**One-shot Kinetics Analysis of Binding to Membrane Protein Variants**

The contributions of specific CXCR4 epitope residues to the binding kinetics of the 12G5 mAb were assessed by creating a panel of lipoparticles containing CXCR4 proteins with point mutations in the charged contact residues (E179V, D181V, D182V, R183G, and D187V) (Figure 4). The lipoparticles were immobilized on a ProteOn GLC sensor chip for the simultaneous assessment of binding kinetics. Analysis of binding kinetics using a One-shot Kinetics analysis indicated that some mutations such as D181V led to marked changes in the binding profile with 12G5 whereas other mutations had minimal effects on the binding kinetics of the mAb.

Sensorgrams for all mutants were curve fitted to determine $k_a$, $k_d$, and $K_D$ values as shown in an isoaffinity plot (Figure 5). Overall, these data reveal that many of the charged contact residues studied are critical for the rate of association ($k_a$) of 12G5 with CXCR4, but they are less critical to the rate of dissociation ($k_d$), implying the importance of long range electrostatic steering interactions for the initial phase of antibody binding. Understanding the energetic contributions of amino acids that constitute mAb binding sites within receptors is important for developing structural models of mAb–receptor interactions, and for the development of more effective therapeutic antibodies.

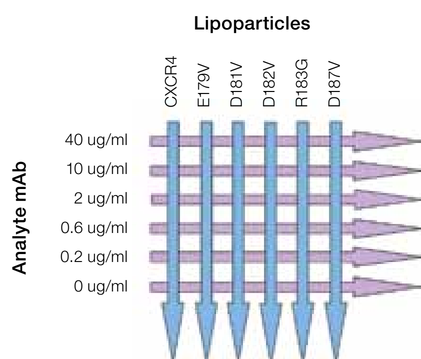


**Fig. 4. Schematic representation of an interaction array.** Lipoparticles with CXCR4 membrane protein containing point mutations were immobilized using a capture antibody on the surface of the vertical channels. Various concentrations of mAb analytes were flowed in the horizontal channels over the attached particles. Interaction kinetics were measured at the intersection points between perpendicular channels.

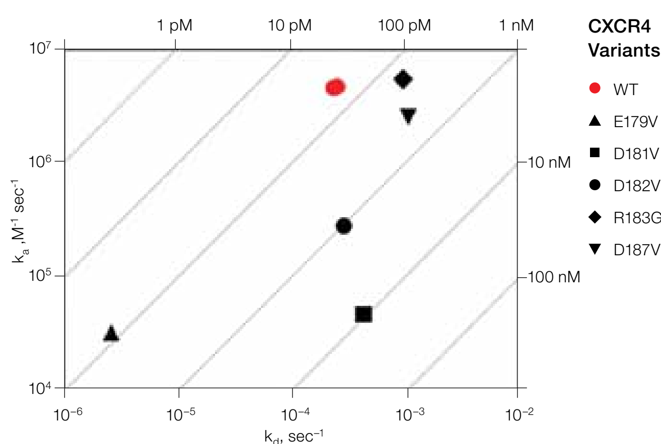


**Fig. 5. Isoaffinity plot of 12G5 mAb binding to various CXCR4 mutants.** Binding sensorgrams from biosensor experiments were curve fitted using One-Shot Kinetics to calculate rate constants $k_a$ and $k_d$. Downward and rightward shifts represent lower affinity interactions, whereas points on the same line have the same overall affinity ($K_D$). A dilution series of 12G5 antibody (40, 10, 2, 0.6, 0.2, 0 µg/ml) was used for this experiment.

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**One-shot Kinetic Analysis of mAbs Binding to Chemokine Receptor CCR5**

To analyze membrane protein interaction kinetics, four mAbs of known reactivity were simultaneously screened against the chemokine receptor CCR5. In an alternative assay format using lipoparticles as analytes, the capture antibody was amine coupled to a ProteOn GLC sensor chip, and the CCR5 mAbs were captured on parallel chip channels. Lipoparticles incorporating either wild type CCR5 or an HA–tagged CCR5 were simultaneously flowed across the chip in orthogonal channels. Binding responses indicate mAb binding in channels containing CCR5 or HA-CCR5 lipoparticles, but not in control channels containing null lipoparticles or buffer alone. Using these biosensor responses obtained with the four different mAbs, the binding of each mAb could be quantitated and compared to identify high and low affinity mAbs. Masking of the N-terminus structure by the HA tag led to decreased affinity (Figure 6).

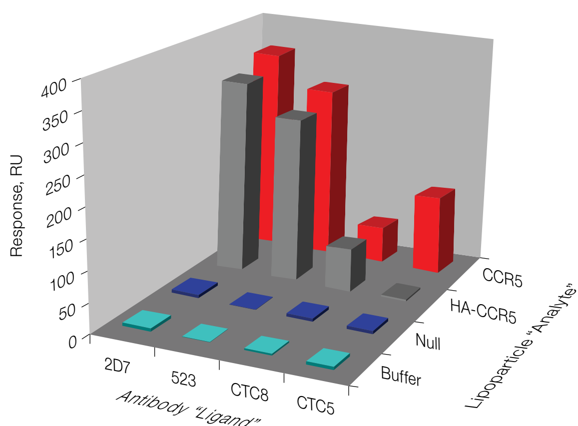


**Fig. 6. Binding of mAb to CCR5.** Data indicating specific binding of mAbs to CCR5 lipoparticles. Responses were observed in channels challenged with CCR5 and HA-CCR5 lipoparticles, but not in control null lipoparticles or with buffer alone. Each mAb was tested for specificity and relative affinity against CCR5 and HA-CCR5 presented within lipoparticles.

## Conclusion

Analysis of the binding characteristics of membrane proteins to specific ligands, such as monoclonal antibodies, is now feasible by using proteins embedded in lipoparticles that can be easily attached to the surface of the ProteOn sensor chips while preserving the native conformation of the proteins. Alternatively the lipoparticles can be utilized as analytes. This flexible, innovative system gives the researcher the perfect method for analyzing membrane protein interactions with therapeutics antibodies in a high-throughput manner to identify and characterize binding kinetics.

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For more information on lipoparticles contact info@integralmolecular.com

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