Rapid Optimization of Immobilization and Binding Conditions for Kinetic Analysis of Protein-Protein Interactions Using the ProteOn™ XPR36 Protein Interaction Array System

Vered Bronner,1 Tsafrir Bravman,1 Ariel Notcovich,1 Dana Reichmann,2 Gideon Schreiber,1 and Kobi Lavie1
1 Bio-Rad Laboratories, Inc., Gutwirth Park, Technion, Haifa 32000, Israel
2 Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

Introduction
The characterization of protein-protein interactions is a fundamental need in the rapidly growing field of proteomics. Surface plasmon resonance (SPR) technology is widely used for kinetic studies of protein-protein interactions. In the design of SPR experiments, however, determining the optimal conditions for ligand immobilization and analyte binding can often require some trial and error.

The ProteOn XPR36 protein interaction array system is an SPR biosensor with a multichannel module and interaction array sensor chip for analysis of up to 36 protein interactions in a single injection step. The parallel sample processing capability of the ProteOn XPR36 system can facilitate the determination of optimal reaction conditions. Because multiple conditions can be tested in parallel and in a single injection step, the effects of each condition on an interaction can be viewed in context of all the conditions simultaneously. Thus, the information needed to make progress toward a successful result is generated more quickly and with more flexibility than is possible with serial sample analysis. Additional details on this array-format system are provided in Bronner et al. (2006).

In this tech note, we demonstrate an efficient, rapid protocol for determining the optimal ligand immobilization and analyte binding conditions for a model protein interaction using the ProteOn XPR36 system. We then use these conditions to perform extensive kinetic and equilibrium analyses of protein-protein binding. The entire experiment is performed in just two ligand immobilization and analyte binding cycles using just two ProteOn sensor chips. In the first cycle, multiple conditions are tested, and in the second cycle, a detailed kinetic analysis is performed under the conditions found to be optimal in the first cycle.

Methods
Instrumentation, Reagents, and Experimental Model
Experiments were performed using the ProteOn XPR36 protein interaction array system and two ProteOn GLC sensor chips. ProteOn phosphate buffered saline with 0.005% Tween 20, pH 7.4 (PBS/Tween) was used as running buffer, and all experiments were performed at 25°C.

The experimental model used consisted of wild-type TEM1 β-lactamase (TEM1, pI = 5.0) and a mutant form of β-lactamase inhibitor protein (BLIP-F142A) (Albeck and Schreiber 1999, Bush 1989).

Optimization of Ligand Immobilization and Analyte Binding Conditions
In the first injection cycle, the optimal pH for TEM1 immobilization was investigated. TEM1 protein was immobilized onto five of the six ligand channel surfaces using the amine coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, 133 mM) and N-hydroxysulfosuccinimide (sulfo-NHS, 33 mM) (components of the ProteOn amine coupling kit). The sixth channel was not modified and served as a reference channel. TEM1 samples (2 µM) were prepared in 10 mM ProteOn acetate buffer with pH values of 3.0, 3.5, 4.0, 4.5, and 5.0. The five TEM1 samples (180 µl) were injected in parallel at a flow rate of 30 µl/min. To deactivate remaining surface groups in the five activated channels, 1 M ethanolamine HCl, pH 8.5 (ProteOn amine coupling kit) was then injected. A sample of running buffer was included in each step for injection into the reference channel.

For analyte (BLIP-F142A) binding, the multichannel module directed flow into the six parallel analyte channels orthogonal to the six ligand (TEM1) channels. Six solutions of BLIP-F142A were prepared at concentrations of 600, 300, 150, 75, 37.5, and 18.8 nM by serial dilution in PBS/Tween. The six samples (150 µl) were injected for 90 sec at a flow rate of 100 µl/min.
Optimization of Analyte Binding Conditions

The 36 sensorgrams generated in the first TEM1/BLIP-142A injection cycle were grouped into six sets of six, with five sets corresponding to the interaction of the BLIP-F142A concentration series with each immobilization condition (Figure 1).

Kinetic rate constants were calculated for the four immobilization conditions that yielded measurable responses (pH 3.0–4.5). These constants were similar, with association rate constants \(k_a\) of \(6.7–7.9 \times 10^4\) M\(^{-1}\)sec\(^{-1}\), dissociation rate constants \(k_d\) of \(1.56–1.74 \times 10^{-2}\) sec\(^{-1}\), and equilibrium dissociation constants \(K_D\) of 200–250 nM.

Ligand activity and maximum analyte binding differed among these four immobilization conditions. The maximum response of 600 nM BLIP-F142A occurred upon interaction with TEM1 immobilized at pH 4.0, the pH value corresponding to the highest TEM1 ligand density and highest ligand activity (%\(R_{max}\)) achieved (Figure 2).

Ligand activity expresses the percentage of immobilized TEM1 capable of binding BLIP-142A. TEM1 ligand activity was calculated as %\(R_{max}\), the ratio of the measured \(R_{max}\) of 300 nM BLIP-F142A with the expected \(R_{max}\). The expected \(R_{max}\) was determined by multiplying the TEM1 ligand density by the ratio of the molecular weights of BLIP-142A and TEM1 (assuming 1:1 binding); this value represents the level of response expected if 100% of the TEM1 molecules were to bind BLIP-142A. The maximum ligand activity observed here was in the range of 20% (Figure 2), a level sufficient for kinetic analysis and one that can be difficult to attain for many classes of proteins.

The dependence of TEM1 ligand density and activity, and the BLIP-F142A analyte response on the immobilization conditions (buffer pH) follow bell-shaped curves. That each of these parameters attains its optimal value when TEM1 is immobilized at pH 4.0 indicates the protein is preconcentrated most efficiently at this pH, and also maintains maximum activity and binding capacity.

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Kinetic and Equilibrium Analyses

The 36 sensorgrams generated in the second injection cycle were also grouped into six sets of six, with five of the sets corresponding to the interaction of a BLIP-F142A concentration series with each TEM1 immobilization level (Figures 3 and 4) and the sixth to the reference channel. Equilibrium dissociation constants ($K_D$) were obtained from these sensorgrams by both kinetic and equilibrium analysis methods.

For kinetic analysis, each set of six sensorgrams was globally fitted to a 1:1 bimolecular kinetic model (Figure 3) to obtain the association rate constant ($k_a$) and dissociation constant ($k_d$); $K_D$ is given by the ratio $k_d/k_a$. For equilibrium analysis, the same sensorgrams were used, and the response at equilibrium of the interaction was measured in each sensorgram for each of the six analyte concentrations. These equilibrium response levels ($R_{Eq}$) were then fitted to a simple bimolecular equilibrium model (Figure 4), from which $K_D$ was derived as that concentration producing a response equal to 50% of the response at saturation.

The kinetic rate constants and equilibrium dissociation constants obtained for all experiments by both analysis modes are shown in Table 2. Note that similar equilibrium dissociation constants were obtained by both modes of analysis.
Table 2. Summary of TEM1/BLIP-F142A kinetic rate constants. TEM1 was immobilized at pH 4.0. The equilibrium dissociation constant value determined by kinetic analysis was calculated from $k_d/k_a$.

<table>
<thead>
<tr>
<th>Ligand Density (RU)</th>
<th>$k_a$ (M⁻¹sec⁻¹)</th>
<th>$k_d$ (sec⁻¹)</th>
<th>$K_d$ (nM)</th>
<th>$K_o$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,020</td>
<td>$8.55 \times 10^4$</td>
<td>$1.27 \times 10^{-2}$</td>
<td>148</td>
<td>140</td>
</tr>
<tr>
<td>1,023</td>
<td>$8.06 \times 10^4$</td>
<td>$1.25 \times 10^{-2}$</td>
<td>155</td>
<td>131</td>
</tr>
<tr>
<td>789</td>
<td>$8.75 \times 10^4$</td>
<td>$1.30 \times 10^{-2}$</td>
<td>148</td>
<td>126</td>
</tr>
<tr>
<td>789</td>
<td>$9.41 \times 10^4$</td>
<td>$1.30 \times 10^{-2}$</td>
<td>138</td>
<td>135</td>
</tr>
<tr>
<td>609</td>
<td>$7.90 \times 10^4$</td>
<td>$1.12 \times 10^{-2}$</td>
<td>141</td>
<td>143</td>
</tr>
</tbody>
</table>

Average: $8.53 \times 10^4$, $1.25 \times 10^{-2}$, 146, 135

Standard error: $6.00 \times 10^2$, $7.46 \times 10^{-2}$, 6.6, 6.9

CV (%): 7.03, 5.98, 4.5, 5.1

**Discussion**

Determining the optimal immobilization conditions for different proteins can often be tedious and time-consuming. When embarking on an investigation of a protein-protein interaction, one needs to first optimize various parameters of ligand immobilization, including ligand and analyte concentrations, injection times, flow rates, and immobilization buffer characteristics. The optimal pH for the immobilization buffer balances the electrostatic attraction of the protein to the chip surface while minimizing protein deactivation. In this study, the optimal pH for immobilization was determined to be that pH that yielded the highest ligand density while maintaining optimal ligand activity and maximum analyte response.

Testing a range of ligand immobilization and analyte binding conditions is essential, even when the pI of the ligand is known. The optimal pH can often be predicted to be about 1 pH unit below the pI, as was found here. However, many factors influence immobilization level and binding response, including protein degradation, changes in protein configuration, and even mutation of a single residue, which can significantly alter a protein’s pI.

The capability of the ProteOn XPR36 protein interaction array system to assess multiple immobilization conditions minimizes the time and resources needed to achieve optimal results. Sensor chip usage is minimized by simultaneous measurement of 36 interactions on a single chip. Reagent consumption can be kept low by using a short injection time during preliminary screening, and time is saved by seeing the results of testing in parallel.
Fig. 4. Equilibrium analysis of the TEM1/BLIP-F142A interaction for determination of the equilibrium dissociation constant, $K_D$. Left panels, sensorgrams for the five levels of TEM1 immobilization. BLIP-F142A concentrations in all panels are 600, 300, 150, 75, 37.5, and 18.8 nM (traces from top to bottom). Black lines represent the equilibrium response level ($R_{eq}$) for each analyte concentration. Right panels, plots of $R_{eq}$ as a function of concentration, with the curves fit to a 1:1 equilibrium model for determination of $K_D$ at 50% saturation response. See Table 2 for the equilibrium dissociation constant values derived from these data.
Conclusions
In this study, the ProteOn XPR36 6 x 6 interaction array was applied both to rapidly optimize binding conditions and to perform a detailed kinetic and equilibrium analysis of the TEM1/BLIP-F142A interaction. Optimization and analysis were each accomplished in a single ligand-analyte injection cycle, demonstrating the power of the ProteOn XPR36 system.

References
Bush K, Classification of β-lactamases: Groups 1, 2a, 2b, and 2b′, Antimicrob Agents Chemother, 33, 264–270 (1989)

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