

# Direct Immobilization of Antigen for Kinetic Analysis Poster

Common  
Revision A.01

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## Introduction

Affinity capture is a popular kinetic assay format as it ensures directed immobilization of the ligand. Directed immobilization avoids losses in binding activity and reduces steric hindrance by preventing excessive cross-linking of the ligand with the surface. Unfortunately a suitable affinity capture reagent may not be readily available.

Excessive cross-linking is common where direct coupling of the ligand to a hydrogel is employed. Ligand that is immobilized in this way is differentially accessible and may be considered a heterogeneous ligand surface. Such heterogeneity distorts the kinetic binding interaction curves causing poor fits to the simple 1:1 interaction model. Proteins that are immobilized by random amine coupling to a hydrogel surface are more likely to suffer this effect as there are usually a high number of surface amine groups through which many covalent linkages may form. A linkage chemistry that targets a functional group that is remote from the binding site would be ideal but no such group exists for most proteins. In a limited number of cases, a thiol group or a carbohydrate moiety may be available.

Here we present two kinetic data sets for the interaction of monoclonal antibodies with antigens that were immobilized by random amine coupling to a planar carboxylated surface. The data conforms to the ideal model and shows no trace of ligand heterogeneity. It is reasonable to conclude that the two dimensional surface chemistry greatly limits the number of cross-linkages a ligand can form with the surface compared to a three dimensional hydrogel. The highly mobile polymer chains of a hydrogel can easily “wrap-up” a protein and form a large number of linkages. In contrast, a protein that is brought onto an activated planar surface will usually interact with the surface over a limited area thereby limiting the number of linkages that can form.

## Experimental

The running buffer for all experiments was HBS buffer, pH 7.4, containing 10mM HEPES, 150mM NaCl, 3.4mM EDTA, and 0.005% (v/v) Tween 20. The flow rate was held constant at 50 $\mu$ L/min and the temperature was 25 $^{\circ}$ C.

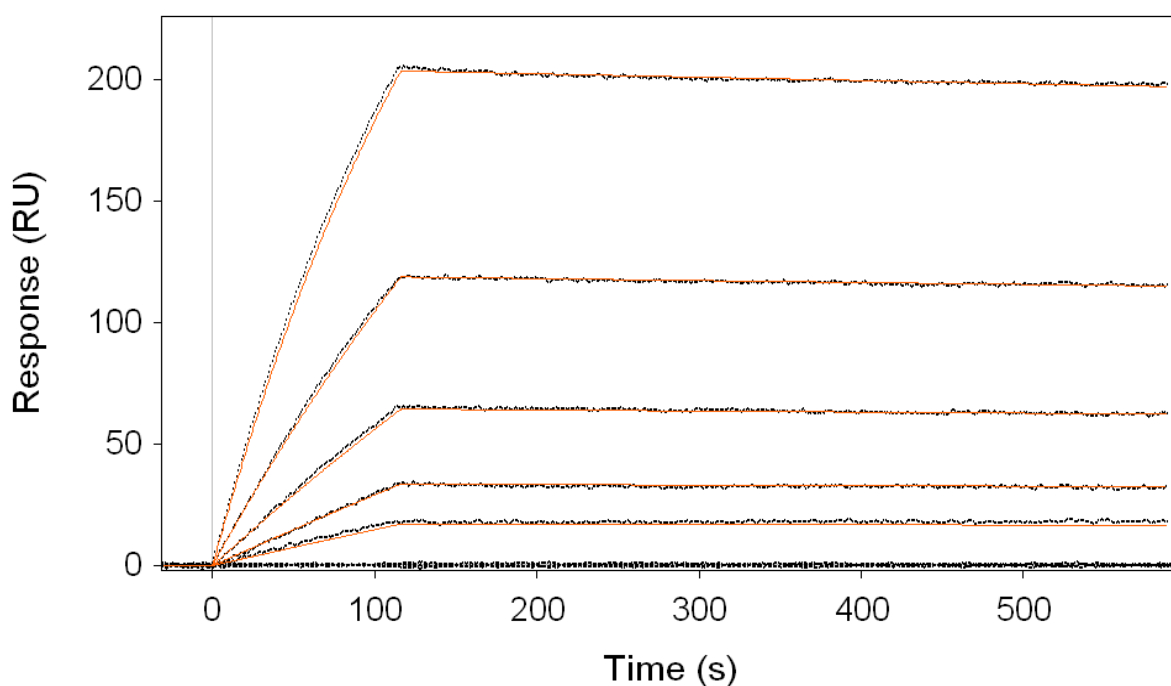
**Ligand Immobilization:** The first channel of the carboxylated sensor surface was activated by injecting a 1:100 dilution of activation solution (i.e. 0.2M EDC/0.05M NHS mixture in deionized water).

The ligand was prepared at 10 $\mu$ g/ml in 10mM acetate buffer, pH 4.5 and injected for ~10 min (may vary in order to control yield). Excess activated groups were capped by

injecting 1M ethanolamine pH 8 for 2 min. Surface regeneration was performed by injection of 50mM phosphoric acid for 1 min to remove affinity bound antibody.

### Interleukin 4 -Antibody Interaction

The amount of immobilized ligand is reduced in order to prevent bivalent binding of the injected antibody. In this way the interaction can be modelled as a 1:1 binding interaction. Figure 1 shows a set of binding interaction curves for the interaction of immobilised interleukin 4 with an anti-interleukin 4 monoclonal antibody over a range of concentrations from 1.25nM to 20nM.



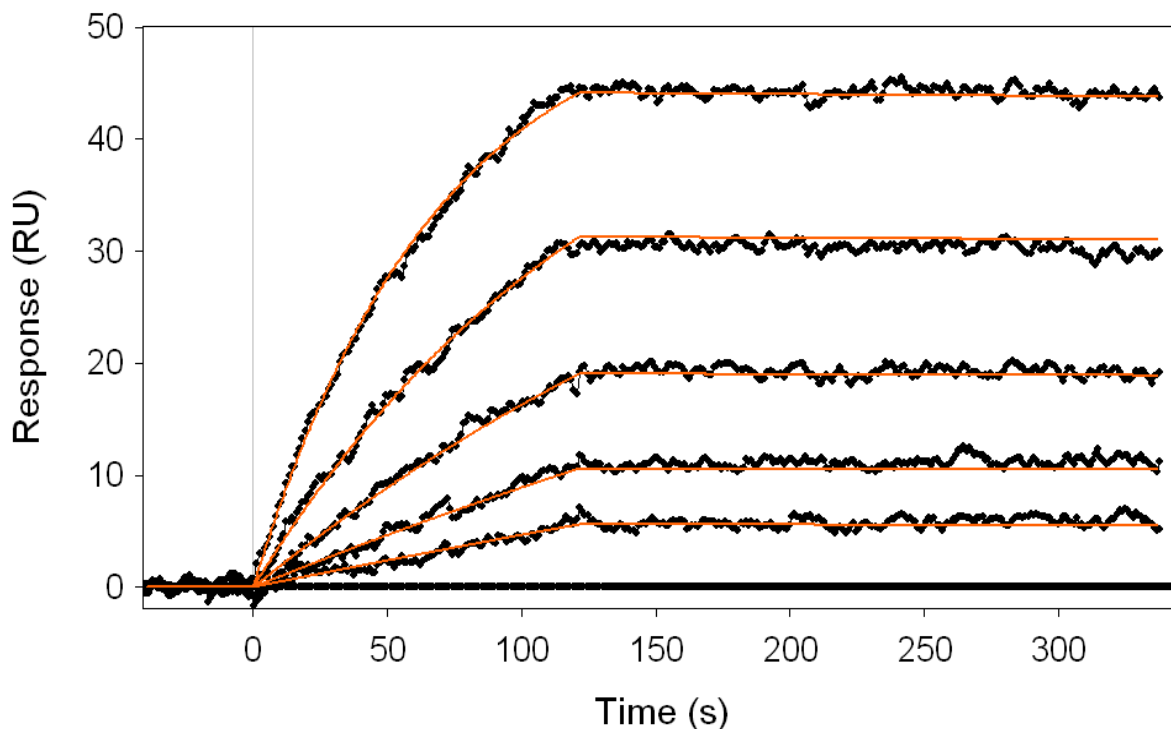
The data set was fitted to a 1:1 interaction model with all parameters (i.e.  $k_a$ ,  $k_d$  and  $R_{max}$ ) constrained to a single global value over the set of curves.

$k_a$	$2.9 \times 10^5 \text{ (M}^{-1}\text{s}^{-1}\text{)}$
$k_d$	$6.8 \times 10^{-5} \text{ (s}^{-1}\text{)}$
$K_D$	0.23 nM
$R_{max}$	415 (RU)
ResSD	0.94 (RU)

### Interleukin 6-Antibody Interaction

Rat recombinant Interleukin-6 was immobilized by random amine coupling. Figure 2 shows a set of binding interaction curves for the interaction of immobilised interleukin 6 with an anti-interleukin 6 monoclonal antibody over a range of concentrations from 200nM to 12.5nM.

The data was globally fitted to a 1:1 interaction model.



$k_a$	$7.3 \times 10^4 \text{ (M}^{-1}\text{s}^{-1}\text{)}$
$k_d$	$3.8 \times 10^{-5} \text{ (s}^{-1}\text{)}$
$K_D$	0.5 nM
Rmax	53 (RU)
ResSD	0.62 (RU)

### Ligand Regeneration

Both interactions were found to be “high affinity” interactions where regeneration of the surface was required. In both examples, the saturation response (Rmax) was fitted globally thereby indicating that regeneration with 50mM phosphoric acid did not significantly denature the immobilized ligand during these assays. Immobilization of antigen is usually a more robust format because the binding epitope is usually not significantly affected by slight conformational changes caused by regeneration. In

contrast, a monoclonal antibody-coated surface may often lose as much as 90% of its binding capacity after a single regeneration.

### **Conclusion**

The excellent agreement of the fitted curves (red) with the actual data (black) for a fully constrained global fit provides a high degree of confidence in these kinetic analyses. This validates the use of a planar surface chemistry with direct immobilization of the antigen and confirms the absence of significant ligand heterogeneity. The absence of such artifacts reduces the level of expertise required to perform successful experiments.