Human IgG Quantification and Affinity Analysis

Summary

SKi Pro, a label-free biomolecular interaction analysis platform, is used to determine the equilibrium dissociation constant, K_D, for an antibody-antibody interaction. Using a standard 96-well microtiter plate and the 8-well strip SKi Sensors, quantitative antibody association data can be generated without labeling the proteins.

Introduction

As antibodies increase in importance as both diagnostic agents and therapeutic targets, the need for research and diagnostic tools with ever increasing ease of use, sensitivity and economy continues to grow. To meet that need the SKi Pro system, which uses nanoPore Optical Interferometry technology (nPOI) is presented here (Fig. 1). This analytical system measures the strength of antibody-antibody interactions without the use of protein labels.

Figure 1: The SKi Pro system used in this work. A standard 96-well microtiter plate is placed into the instrument together with several 8-well strips using carboxyl capped SKi Sensors. The instrument monitors both covalent and non-covalent protein binding in real-time.

Two strips of SKi Sensor, carboxyl biochips—each containing 8 porous Silicon (poSi) biochips—are used to monitor anti-human IgG interacting with IgG. Anti-IgG is first immobilized within the pores and various concentrations of IgG are then allowed to interact with this receptor. The amount bound is monitored in real time by measuring the change in optical path difference (ΔOPD) and is then plotted against IgG concentration. A non-linear least squares fit of concentration vs. ΔOPD is performed to determine K_D.

Method

The carboxyl groups on the insides of the pores (Fig. 2) are modified as sulfo-succinimide esters using EDC chemistry during an initial activation step (**C**). 100 μL of activation buffer (400 mM EDC, 100 mM sulfo-NHS solution buffered at pH 6.0 with 100 mM MES and 0.9% NaCl) is titrated into each of 16 wells. The chips are then activated for 15 minutes in this solution and transferred into immobilization buffer (pH 4.5, 20 mM acetate, 1 mM EDTA) and washed twice.

Next, strips are placed into wells, each containing 75 μL of immobilization buffer and OPD baseline is taken for 10 minutes. A solution of anti-IgG (Goat Anti-Human IgG, Mouse Adsorbed, Caltag Laboratories H10500) in immobilization buffer is then added to each chip to obtain a final working concentration of 1.3 μM in 150 μL of solution per chip. The immobilization then proceeds for 30 minutes (**D**). Following immobilization, blocking buffer (100 mM ethanolamine-HCl, pH 5.0) is applied for 15 minutes to deactivate any unreacted succinimide esters in preparation for specific target binding (**E**). Resulting in a surface ready for affinity monitoring (**F**).

75 μL of binding buffer (100 mM PO_4^{3-}, 150 mM NaCl, pH 7.2) is then added to each of the wells and 10 minutes of baseline OPD monitored. Human IgG (Human IgG, Reagent Grade, Sigma I4506) is added to achieve working concentrations of 6.0, 15, 30, 110, 180,

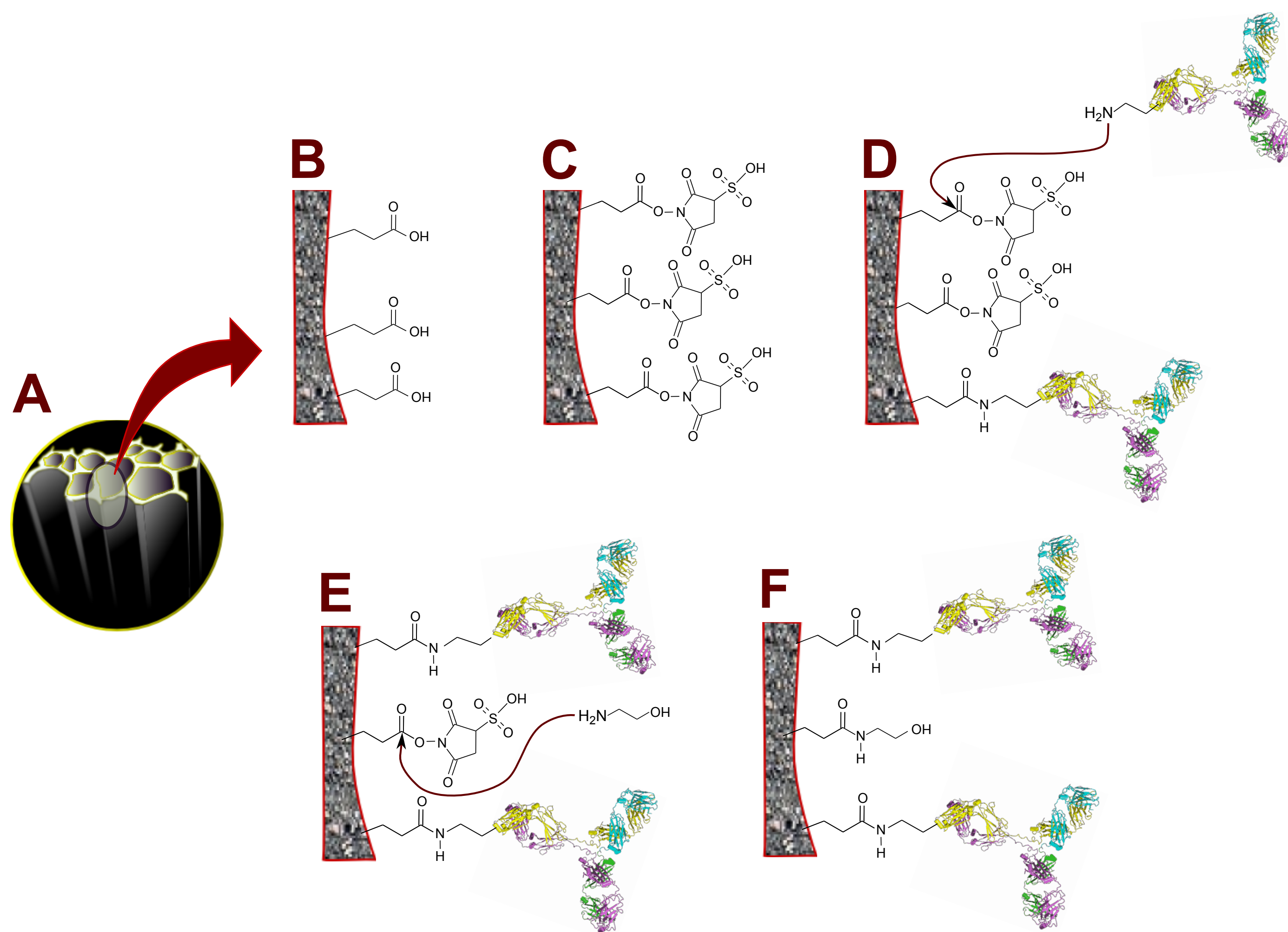

Figure 2: Biomolecules are bound to the insides of the nano-pores (**A**) through their exposed carboxyl groups (**B**). These carboxyl units are actived using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in the presence of sulfo N-hydroxy succinimide (sulfo-NHS) to form succinimide esters throughout the pores (**C**). Primary amines on proteins from e.g. lysines, will react with the succinimide esters to form stable peptide bonds (**D**) covalently linking the protein to the surface. Unreacted succinimide esters which are blocked by reacting with ethanolamine (**E**). The resulting surface is ready for monitoring specific binding events (**F**).

490, 1,200 and 3,400 nM protein using 150 μL per chip. Before addition, concentrations were verified using reverse phase HPLC. The binding of IgG with anti-IgG is monitored by its OPD shift over the course of 30 minutes.

Results

SKi Report software automatically generates real-time binding curves, corrects for reference—where there is no receptor—and blank—where there is no target. After subtraction of these, the software carries out binding and affinity analyses. The measurements performed here are logically divided into activation→immobilization→blocking→binding steps. During both the immobilization and binding steps OPD data is taken in real-time and is presented below.

The immobilization of anti-IgG shown in Figure 3 indicates covalent binding of the receptor protein to the pore surface. Shown are data taken simultaneously from each of the two well strips after using SKi Report to remove any effects from the buffer baseline. Between 18–22 nm of OPD shift are seen for the immobilization which is a number consistent with about 12% of a monolayer.[1] Experiments have shown this to

[1] Based on calculation using the expected characteristic diameter and refractive index of anti-IgG together with the SEM measured pore size of the chips.

be a reasonable amount of receptor immobilization for affinity measurements.

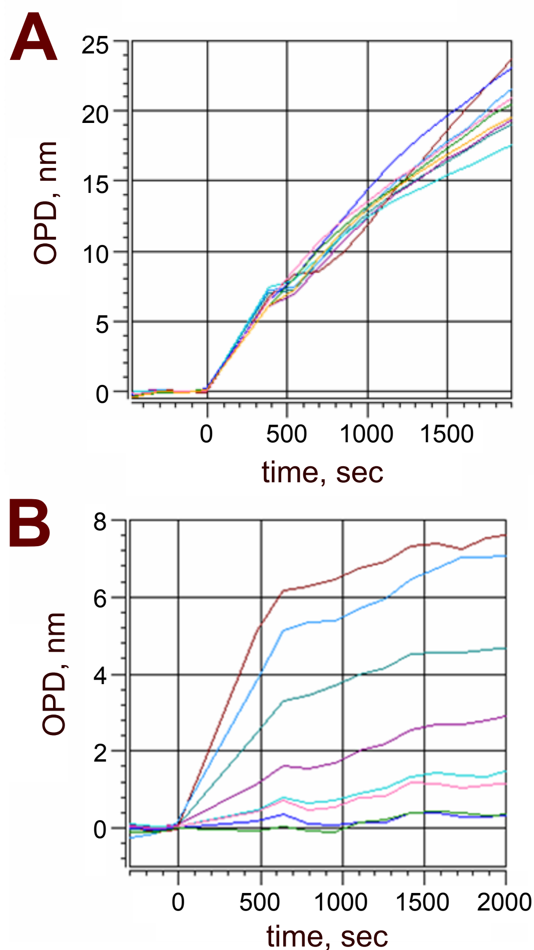

Figure 3: Screen shots from SKi Report showing the immobilization and binding results. Anti-IgG is immobilized at 1.3 μM in **A**. The time resolved data show the optical path difference (OPD) increase as the protein covalently binds to the activated carboxyl groups within the pores. The wells all increase to approximately the same level. IgG at 6.0–3,400 nM is shown binding to the anti-IgG in **B**. As IgG interacts and binds with the immobilized anti-IgG, the OPD increases proportionately to the IgG concentration.

Following a blocking step in which unreacted sulfo-succinimide esters are passivated, the chips are ready for binding. The several concentrations of IgG which are used for the affinity determination show a time resolved increase in OPD which is proportional to the amount of protein introduced into the wells (Fig. 3). The figure shows the results of this binding after subtracting a blank solution.

The amount of OPD change increases monotonically until reaching a plateau. The analysis of the data which follows is based on the equilibrium amount bound at the several concentrations, however it can be seen that, at the higher concentration, wells come to equilibrium faster than those at lower concentration, consistent with a bimolecular rate constant.

Discussion

The current work aims to determine the dissociation equilibrium constant (K_D) of the IgG/anti-IgG interaction without the use of protein labels. As equilibrium binding data is available at a variety of concentrations, computing K_D can be automated using the SKi Report software.

The binding data of Figure 3 is averaged between 1,500 and 2,000 seconds and plotted against the HPLC-verified target concentrations as shown in figure 4. The OPD data are plotted against target concentration ($[T]$) with a semi-log scale, but are fitted using a non-linear least squares algorithm with the two state-model:

$$OPD = OPD_{\circ}\frac{({}^{1}/_{K_D})[T]}{1+({}^{1}/_{K_D})[T]} + C$$

Where $OPD_{\circ}$ and C are fitting constants not related to the affinity analysis.

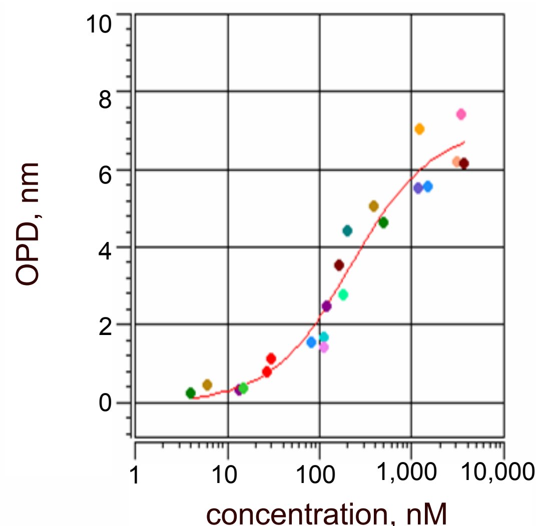

Figure 4: K_D determination of the IgG/anti-IgG interaction. SKi Report automatically performs a non-linear, two-state fit to the equilibrium OPD shift. Several repeats of the experiment performed are shown together with the best fit line through the data, corresponding to a K_D of 220 nM.

The K_D of 220 nM computed from this fit implies a reasonably strong interaction between the two molecules and corresponds to values in the literature.

The system and approach described here can be used to measure interactions of antibodies in a straightforward fashion using standard well-plates and a minimum amount of time. As opposed to luminescent or radiometric methods, no time needs to be spent labeling the protein and there will never be a doubt as to whether the labeling step was completed successfully.

The carboxyl chip chemistry described allows any antibody molecule to be placed within the pores and, together with its target, quantified with respect to K_D.

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