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APPLICATION NOTE – JAGIELLONIAN CENTER OF INNOVATION

AFFINITY STUDIES OF IgG ANTIBODY AND ITS FRAGMENTS TO STAPHYLOCOCCAL PROTEIN A USING SPR AND MST METHODS

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ABSTRACT

Determination of dissociation constant (K_D) is an important in the evaluation of various affinity ligands including small molecules, proteins, artificial scaffolds and aptamers. We performed SPR (Surface Plasmon Resonance) and MST (MicroScale Thermophoresis) analysis of binding between Staphylococcal protein A and IgG1, protein A and the Fc region of IgG1 (Fcγ), and protein A and the Fab fragment of IgG1. Our results confirm that a complete IgG1 and its Fc fragment binds protein A with high affinity since the obtained K_D values were within a nanomolar range. The weak affinity of the Fab fragment to protein A was reflected in our experiments by the increase in the K_D values by at least two orders of magnitude.

INTRODUCTION

Protein A (42 kDa) from *Staphylococcus aureus* – possesses two distinct Ig-binding activities: it can bind Fcγ (the constant region of IgG involved in effect or functions) and Fab (the Ig fragment responsible for antigen recognition). The Fcγ binding site consist of the elbow region at the CH2 and CH3 interface of most IgG subclasses. The Fab binding site is localized on the variable region of the Ig heavy chain (Graille et al., 2000). The high affinity of Fcγ to protein A is commonly used for antibody and Fcγ-fusion proteins purification and labeling whereas a weak binding of Fab to protein A has been less investigated. Here we analyzed the affinity of complete IgG1, Fcγ and Fab to natural protein A from *Staphylococcus aureus* using two different approaches: SPR (Surface Plasmon Resonance) technology (Biacore 100X, GE Healthcare) and MST (MicroScale Thermophoresis) technology (NanoTemper, Monolith.NT115). The SPR experiments allowed us to obtain association rate constant (k_a), dissociation rate constant (k_d) and dissociation constant (K_D) while MST gave us K_D values without the kinetic parameters. The obtained results are in agreement to published data (Jansson et al., 1998; Tsukamoto et al., 2014).

RESULTS AND CONCLUSIONS

We first immobilized protein A on the surface of CM5 chip using amine coupling chemistry. The optimal buffer for the immobilization is 10 mM sodium acetate pH 5.0 since it gave a good pre-concentration on the CM5 chip surface and had the highest pH that should not affect protein A *(Figure 1).*

For the binding of the natural human Fab fragment to protein A immobilized on the surface of CM5, the following parameters were found: k_a of $4.28 \times 10^4 M^{-1}s^{-1}$, k_d of $8.72 \times 10^{-2}\ s^{-1}$ and K_D of 2.04×10^{-6} *(Figure 2).* A very rapid dissociation of the Fab fragment was determined by this experiment with the k_d approaching 1×10^{-1}, which is the detection limit of Biacore X100.

In the second experiment, we analyzed the binding of Fcγ to protein A immobilized on the surface of CM5 chip

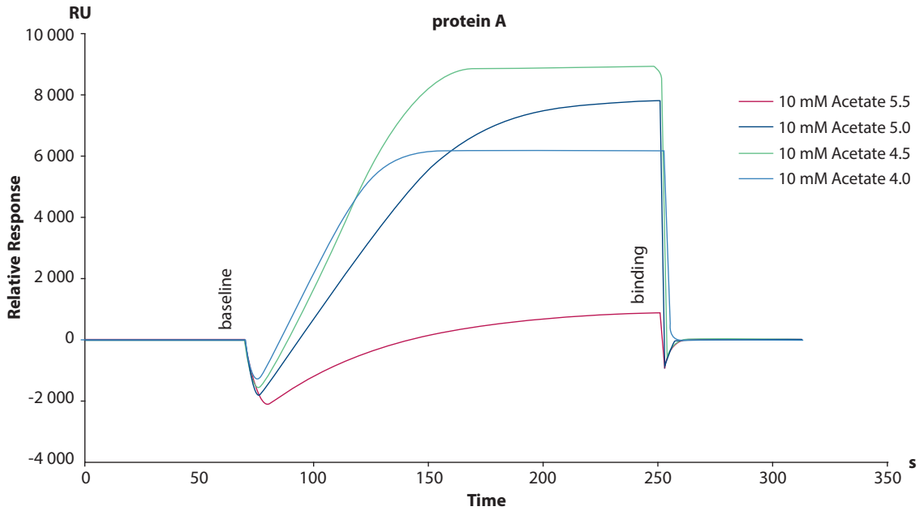

Figure 1. The immobilization pH scouting procedure. *Sensorgrams show the SPR responses generated by the ligand protein A injected in immobilization buffer pH 5.5, pH 5.0, pH 4.5, pH 4.0, respectively.*

(Figure 3). Since Fcγ forms a dimer via hydrophobic interaction between CH3 domains and two covalent disulphide bridges in the hinge region, we determined the kinetic parameters using the bivalent analyte model assuming a two-step interaction between Fcγ and protein A whose density on the chip was relatively high (800 RU). Such analysis gave the k_{a1} of 1.35 x $10^{4}M^{-1}s^{-1}$ and k_{d1} of 8.56 x $10^{-3}s^{-1}$, yielding a dissociation was 9.98 x 10^{-9}M. As expected, Fcγ binds with a high affinity to protein A.

Similarly to the binding of Fcγ to protein A, the interaction between complete IgG and protein A was analyzed with the bivalent anylate kinetic model *(Figure 4).* Such analysis gave the k_{a1} of 1.19 x 10^{5} $M^{-1}s^{-1}$ and k_{d1} of 8.34 x $10^{-5}s^{-1}$, yielding a dissociation constant K_{D1} of 7.01 x 10^{-10}M for the initial

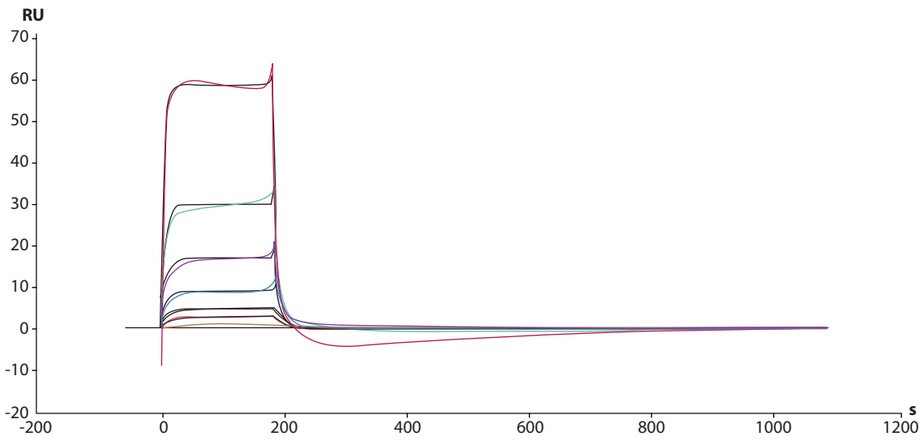

Figure 2. Binding of Fab to protein A on SPR. *An overlay plot of sensorgrams obtained from injection of different concentrations of natural human Fab fragment ranging from 15.6 nM to 2.5 µM. Analysis of these experimental data was performed using the Langmuir 1:1 kinetic model.*

constant K_{D1} of 5.9 x 10^{-7}M for the initial 2:1 interaction. The dissociation rate constants for the second step of this binding were k_{a2} of 7.15 x 10^{-6} $RU^{-1}s^{1}$ and k_{d2} of 3.96 x $10^{-4}s^{-1}$. For comparison, the K_D obtained for this SPR experiment using the Langmuir 1:1 model the K_D

2:1 interaction. The association and dissociation rate constants for the second step of this binding were k_{a2} of 4.68 x $10^{-5}RU^{-1}s^{1}$ and k_{d2} of 2.00 x $10^{-2}s^{-1}$. For comparison, the K_D obtained for this SPR experiment using the Langmuir 1:1 model was

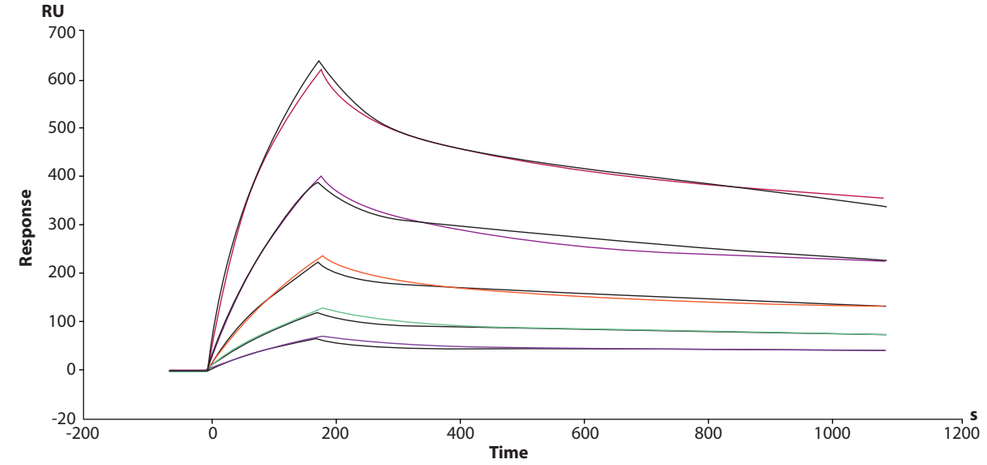

Figure 3. Binding of Fcγ to protein A on SPR. *An overlay plot of sensorgrams of obtained from injection of different concentrations of of human IgG Fc fragmentranging from 9.375 nM to 150 nM. Analysis of these experimental data were performed using thebivalent anylate kinetic model.*

3.85 x 10^{-10}M. Notably, a very slow dissociation of Ig from immobilized protein A was observed. Microscale thermophoresis experiments were performed using fluorescently labeled protein A through lysines with red fluorophores. The concentration of labeled protein A was kept constant at 1.5 nM, while the concentration of ligands was varied. The initial fluorescence did not vary more than 10%. The shape of shoulders of

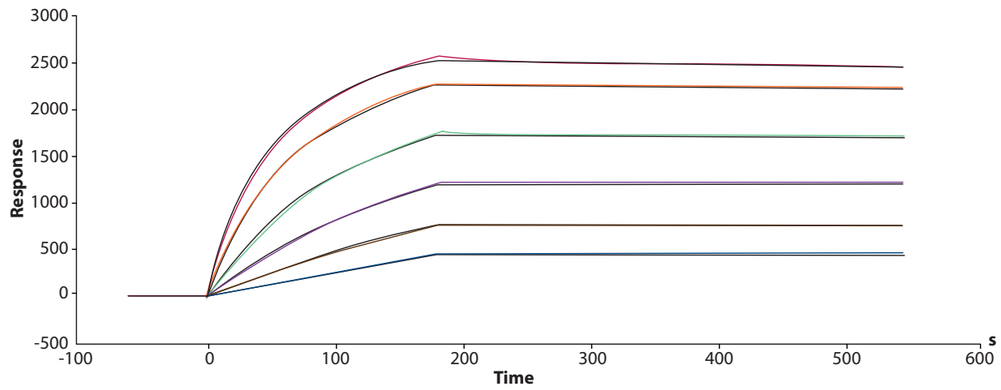

Figure 4. Binding of IgG1 to protein A on SPR. *An overlay plot of sensorgrams of obtained from injection of different concentrations of human IgG ranging from 6.25 nM to 150 nM. Analysis of these experimental data were performed using the bivalent analyte kinetic model.*

the curves from capillary scan were similar, meaning that the studied proteins does not form aggregates in experimental conditions.

The K_D was calculated at 4.57 nM *(Figure 5)* for IgG1. The K_D was fitted based on average of three

independents mesurements. The K_D was calculated at 17.11 nM *(Figure 6)* for Fc. The K_D was fitted based on average of three independent mesurements. The highest concentration of Fab for MST measurements was 26.6 µM. It was imposibble to estimate K_D value in evaluated concentration range.

Two applied methods gave similar binding affinities constants. Using SPR approach we can study binding kinetics (k_a; k_d), meanwhile MST enables interaction analysis in solution. Other advantages of MST approach include sample handling, low material consumption, and fast analysis.

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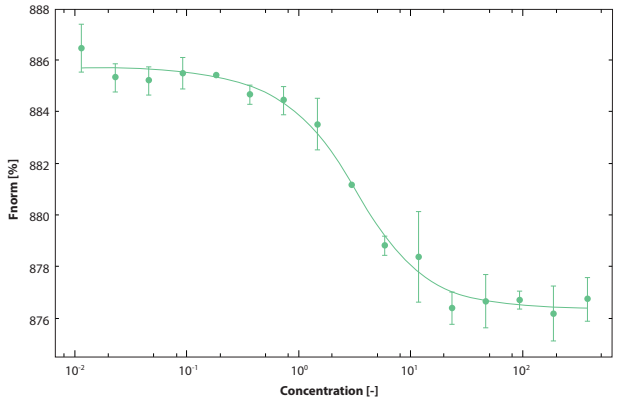

Figure 5. Binding of IgG1 to protein A on MST. *The fluorescently labeled protein A was used at the constant concentration of 1.5 nM with varying concentrations of IgG1.*

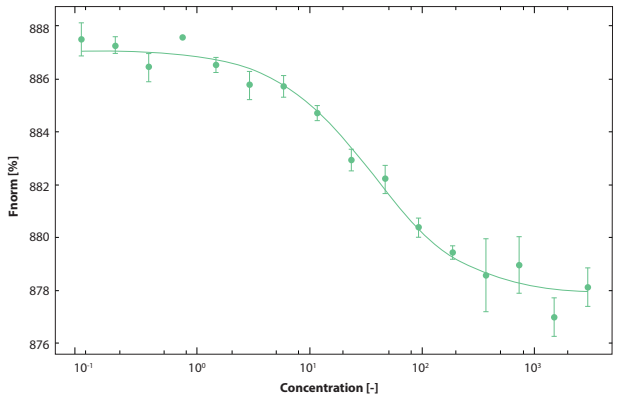

Figure 6. Binding of Fc to protein A on MST. *The fluorescently labeled protein A was used at the constant concentration of 1.5 nM with varying concentrations of IgG1.*

MATERIAL AND METHODS

SPR ANALYSIS

800 (for IgG1 and Fcγ experiments) or 200 (for Fab experiments) resonance units (RU) of protein A were immobilized on the surface of CM5 chip (GE Healthcare) using amine coupling chemistry in 10 mM sodium acetate pH 5.0. The optimal pH for immobilization was determined by a pH scouting experiment prior to the immobilization step. A binding kinetics assay was performed in HBS-EP+ buffer pH 7.4 (GE Healthcare). 50 mM NaOH was used for chip regeneration. The binding data was fitted to a bivalent analyte model for IgG1 and Fcγ and the Langmuir 1:1 binding model for Fab using Biacore X100 Evaluation software. The SPR experiments were performed using GE Biacore X100.

MST ANALYSIS

Protein A was stained with red fluorescent dye using NT-647-NHS kit (Nanotemper). The kit uses NHS-ester chemistry, which reacts efficiently with primary amines of proteins to form highly stable dye-protein conjugates. Primary amine groups are found on lysine residues which are usually solvent accessible and therefore suitable for labeling rections. NT-647-NHS dye labeled proteins show fluorescence excitation and emission of approximately 650 nm and 670 nm, respectively. The fluorescently labeled protein A was used at the constant concentration of 1.5 nM with varying concentrations of ligands. After 20 minutes of incubation, samples were loaded into Premium Coated MST capillaries and capillary scan was performed. Then, MST analysis were performed at 20% MST power and 50% LED power. The MST experiments were performed at Nanotemper Monolith NT.115 instrument. Results were analysed using NT. Control software (Nanotemper).

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