# QUANTITATIVE ANALYSIS OF MULTIPLE ANTIBODY-LIGAND INTERACTIONS IN A MICROCHIP USING FLUORESCENCE POLARIZATION ANISOTROPY

**K. Eyer, T. Robinson, P. Kuhn and P. S. Dittrich**

*Department of Chemistry and Applied Biosciences, ETH Zurich, Switzerland*

## ABSTRACT

We present a microfluidic device that allows quantitative analysis of protein interactions with fluorescently labeled ligands. Dissociation constants ($K_d$), binding classes (m) as well as number of binding sites (n) can be determined in the same experiment, even if protein mixtures are present.

**KEYWORDS:** Protein-ligand interaction, dissociation constant, binding studies

## INTRODUCTION

Antibodies are powerful agents for (bio)analytical studies and medical diagnostics. New therapeutics based on antibodies have recently been introduced into the drug market [1,2]. In this context, it is important to characterize binding capacities and nonspecific interactions of antibodies in buffer systems and in more complex environments. The gold standard for the determination of antibody-ligand binding strengths is surface plasmon resonance (SPR)[3,4]. The label-free technique senses the change of mass binding on a surface and allows characterization of global binding and dissociation events. However, this technique relies on a purified sample to properly measure interactions. Herein, we present a method based on microfluidic fluid metering and mixing, and binding analysis by fluorescence polarization anisotropy (FPA) measurements for a more thorough analysis of antibody-ligand interactions, that can even be used to distinguish different antibodies in polyclonal samples or complex matrices such as sera.

## THEORY

Since FPA is mass a sensitive measurement technique, binding of a fluorescently labeled moiety to a non-fluorescent binding partner can be observed as long as a significant difference in molecular mass is achieved when the complex is formed. In FPA, the measured value is a quotient of bound to unbound fluorescent moiety. Using this, we can calculate the free concentration of ligand ($c_u$) and the degree of binding (r), i.e. the number of molecules bound per protein, from the obtained value of anisotropy. Normally, r increases when $c_u$ increases and shows a saturation characteristic, where r is nearing the number of binding sites per protein (n). For further analysis, the data is fitted by non-linear regression binding models. For visualization, a linearization after Scatchard is done. The resulting graph shows the number of binding sites per protein as the intersection with the x-axis, whereas the intersection with the y-axis shows the number of binding sites multiplied with the association constant $K_a$, which can be easily converted to the dissociation constant, $K_d$. Normally these parameters are determined by preparing a dilution series of ligand and antibody. Here, our microfluidic device is preparing the dilution series by itself, so only two solutions with minimal volume (5 to 10 µl) need to be prepared.

## EXPERIMENTAL

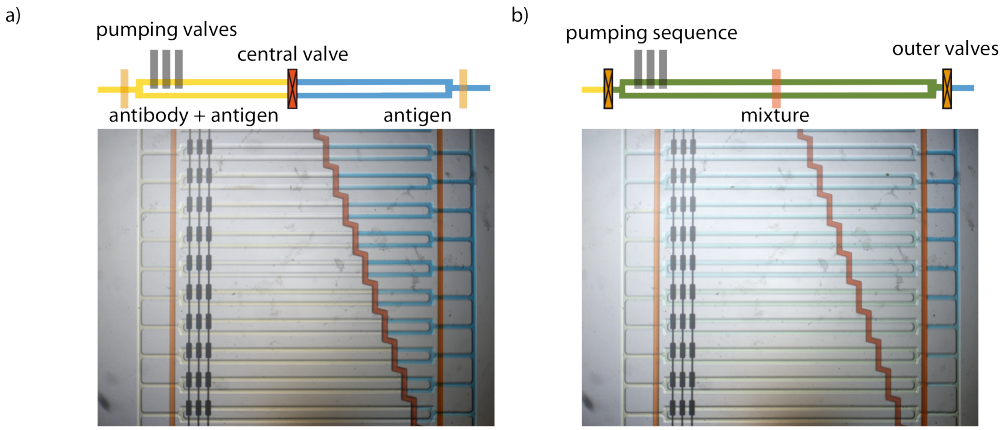


*Figure 1: Micrographs of the two-layer microfluidic device showing 10 out of 22 loops. a) The reactor channels are filled from either side, here with yellow and blue food dye solution, while the side valves (top layer, orange) are open and the central valve (pink) is closed. The position of the central valve determines the ratio of the two different solutions in the reactor channel. b) For mixing, the side valves are closed and the central valves are opened. For efficient mixing, we implemented three valves for pumping (shown in black).*

Our two-layer microfluidic device consists of 22 microfluidic loops (Fig.1); each of which can be filled from either side with the antibody (represented by yellow food dye) and the antibody-ligand solution (blue food dye), respectively. In the end, the antigen concentration is constant throughout all the loops, whereas the antibody concentration varies. During the introduction of the binding partners, the central valve that passes over the loops at different positions is closed, thereby defining the ratio of the two binding partners. By doing so, the concentration of antibody is constant throughout all the loops, whereas the concentration of binding partner varies by a factor of around 12.

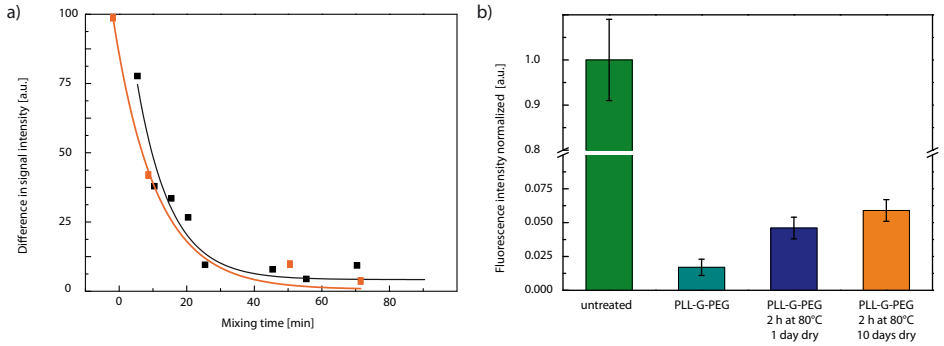


*Figure 2. a) Characterization of the mixing performance. Water and a solution of either fluorescein (black) or streptavidin-FITC (orange) are introduced and pumped around the loops (valves operate at 5 Hz). The graphs represent the normalized difference of the fluorescence intensity measured during one passage. Complete mixing is achieved after about 40 minutes, independent on the molecular size of the molecule. b) Reduction of nonspecific surface binding of the antibody, here shown for FITC-labeled IgG. When treated with PLL-g-PEG, surface binding is greatly reduced to about 1.5 % compared to a non-treated surface. The treatment was sufficiently good even after a few days, when the chip was dried for storage.*

This enables parallel measurements and greatly reduces the required sample volume to 5-10 µL, instead of around 200 µL needed for SPR experiments. After closing the side valves and opening the central valves, a microfluidic pump (adapted from [5,6]) is used to enhance the speed of mixing in the circuits (Fig. 2a). As expected, mixing times do not seem to correlate with molecular size of the analyte, but rather with the frequency of the mixing. When mixing is complete and biding equilibrium is reached, the ratio of free to bound ligand can be conveniently measured by FPA. To minimize signal noise originating from nonspecific surface binding, the surface was coated with PLL-g-PEG (Fig. 2b). Conveniently, the surface can be treated with the agent directly after plasma bonding. Afterwards, the solution is exchanged to distilled water and the chip is dried at 80°C. The dried chip can be stored up to 10 days without affecting its surface repelling characteristics, as measured with a fluorescently labeled antibody.

## RESULTS AND DISCUSSION

First, we started with a test system including fluorescently labeled biotin and avidin. The avidin-biotin interaction is well characterized, and literature values suggest a dissociation constant of around $10^{-15}$ M and a degree of binding of 4 [7]. After data analysis and fitting, we found a degree of binding of 3.72 and a $K_d$ of $8.5*10^{-14}$. Next, we wanted to analyze the binding of the ligand cAMP (labeled with the fluorescent dye Alexa 488) and a specific monoclonal antibody against cAMP. First, we measured in the presence of antibody alone and found a degree of binding of 2 with a $K_d$ of around 1.46 nM. The $K_d$ value was confirmed by bulk measurements as well as by measuring the on- and off-rates (see table 1). Afterwards, we wanted to see weather we can extract the same parameters when a mixture of proteins is present. To create such a mixture, bovine serum albumin (BSA) was added, which is one of the most abundant proteins in blood. Analysis of the data revealed a dissociation constant of 1.2 nM for the antibody-cAMP interaction. Resolved due to the mass sensitivity of FPA, we could further extract the non-specific interactions of the cAMP with BSA with a dissociation constant of 250 nM. Similar dissociation constants for the BSA cAMP-Alexa 488 interaction were also found when the experiment was performed in a fluorescence spectrophotometer (see table 1).

*Table 1. Summary of measured protein binding parameters*

| **Binding moiety** | **Protein binding parameters** | | | | |
|---|---|---|---|---|---|
| | **On-chip** | | | **flow cell** | **Cuvette** |
| | **$K_d$ [nM]** | **m** | **n** | **$K_d$ [nM]** | **$K_d$ [nM]** |
| Avidin | $8.5*10^{-5}$ | 1 | 3.72 | n.d. | n.d. |
| Anti cAMP mAB (alone) | 1.46 | 1 | 2 | 2.56 | 1.15 |
| In mixture | 1.2 | 1 | 2 | n.d. | n.d. |
| BSA | 250 | 1 | n.d. | n.d. | 162 |

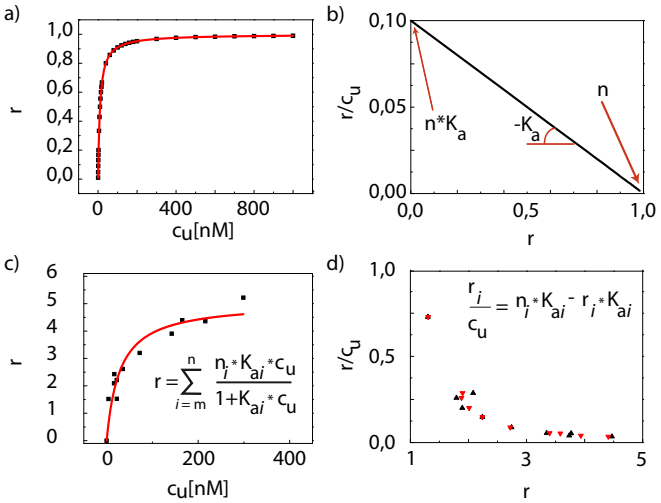


*Figure 3. a) Simulated curve for a binding event with one binding site (n) per protein and a dissociation constant of 10 nM. b) Scatchard plot from calculated experiment with one binding class and one binding site. c) Measured curves for the experiment with various cAMP antibody concentrations with added BSA concentrations and constant cAMP-Alexa 488 concentrations. When fitting only with a single binding event taking place, i.e. the antigen only interacts with the antibody, but not with the BSA, one would suggest a $K_d$ of around 25 nM for the antibody in the mixture (red curve). Formula for fitting is shown in the graph with r as the binding degree and $c_u$ as the free concentration of ligand d) Non-linearity of the data after linearization reveals the presence of two binding partners with different dissociation constants. The resulting curve is the sum of two linear curves. Black: experimental values, red: fitted.*

## CONCLUSION

Our herein presented microfluidic device allows quantitative analysis of protein interactions with fluorescently labeled ligands. The gathered data shows good agreement with bulk experiments (see table 1). Protein ligand interactions were successfully determined and parameters describing the interactions such as dissociation constants ($K_d$), binding classes (m) as well as number of binding sites (n) could be determined in the same experiment.

In summary, our device allows a detailed analysis of interactions between antibodies and ligands. Due to the parallel approach, assay time was short and sample consumption was low, which is of great interest during the development of new therapeutic antibodies. Future studies will focus on the characterization of polyclonal antibodies, therapeutic antibodies and the use of more complex mixtures such as sera.

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## CONTACT

Petra S. Dittrich. Phone: +41 44 633 68 93. Mail: dittrich@org.chem.ethz.ch