Ligand-Receptor Binding Constant Determination Using Separation by Asymmetrical Flow Field-Flow Fractionation

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Introduction

Ligand-receptor interactions are important controls for diverse biological processes where binding affinity and kinetics are critical parameters in regulation of receptor functions. Therefore, development of new bio-therapeutics requires measurement of the binding behaviors under physiological conditions. However, such a binding environment could be met by many mainstream methods for affinity measurement. Our group is the first to apply Asymmetrical Flow Field-Flow Fractionation (AF4) to measure the affinity between biomolecules [1], which provides the advantages of negligible damage to the non-covalent complex, compatibility with physiological buffers, and capability in separation of large biomolecules.

This study aims to show the feasibility of using AF4 as a singular incubation and separation instrument for developing binding assays by determining the binding constant between protein and aptamer, using IgE and its aptamer developed by Wiegand et al. as the example [2]. Such an assay will be valuable for determination of the binding characteristics of new aptamer-based drugs and applicable to other interaction biosystems.

Asymmetrical Flow Field-Flow Fractionation (AF4)

In AF4, fractionation is induced in a narrow, ribbon-like channel by the counteraction of a cross-flow separation force against the Brownian motion of the particles or molecules introduced. Under equilibrium conditions, the particles align in the different velocity streams of the parabolic flow profile of the channel according to their hydrodynamic size. This results in the elution of the particles in size order from small to large (Figure 1) [2].
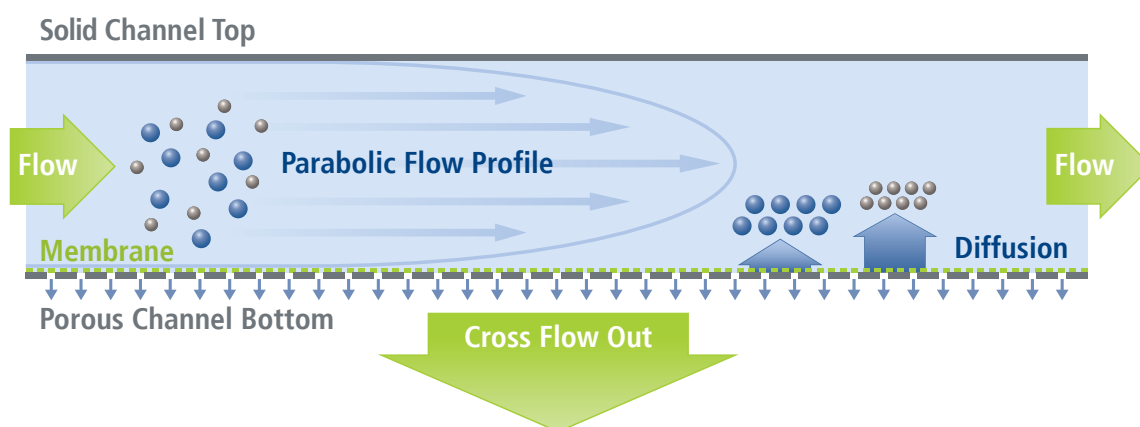

Figure 1: AF4 separation principle.

AF4 Optimization

A suitable ligand-receptor pair for study in AF4 would have large differences in the diffusion coeffecients of the molecules. This would create baseline separation of the free and bound aptamer peaks for affinity calculation. In our example system, the Mw difference between IgE and the Wiegand aptamer is approximately 162 kDa. Baseline separation is highly possible, because AF4 has been known to be able to resolve analytes with size difference > 20 %.

Considering the size of the Weigand aptamer (~ 28 kDa) and that of IgE (190 kDa), we believe the free and bound aptamers can be resolved well. Affinity calculations can be achieved using peak height or peak area using an online detector capable of quantifying the bound aptamer.

A 10 kDa regenerated cellulose (RC) membrane was selected due to the size of the Weigand aptamer, and the low level of interactions RC membranes have towards IgE and single stranded DNA. The method was optimized to achieve baseline peak separation while minimizing run times. The focusing time was increased to allow sufficient incubation times. In this study, an incubation time of 6 minutes was added to the previously optimized band focusing time of 4 minutes. The running buffer was selected based on the ligand, receptor, and membrane. Some additives, like 5 mM $MgCl_2$, were added to help stabilize the analytes and the protein-aptamer binding conditions. Additionally, the aptamer sample was heated to 90°C for 5 minutes to denature the hairpins, then rapidly cooled to 4°C to avoid dimer and agglomerate formation before binding.

Data Collection and Results

Data was collected using an online PN3410 fluorescence detector. Fluorescently labeled aptamers were used for better detection and higher specificity over UV-Vis detection. Prior to injection, 10 fmol of aptamer was mixed with varying amounts of protein (0.100 µg to 20 µg) and injected (Figure 2).

The chromatograms were analyzed on Origin Pro 8 and the two peak areas (free and bound) were compared to generate the fraction of bound aptamer. Plotting this against the concentration allows the IgE binding curve (Figure 3) to be generated using a Hill Equation fitting.

The K_d of the interaction estimated by the fit is 1.93 ± 0.03 µM.
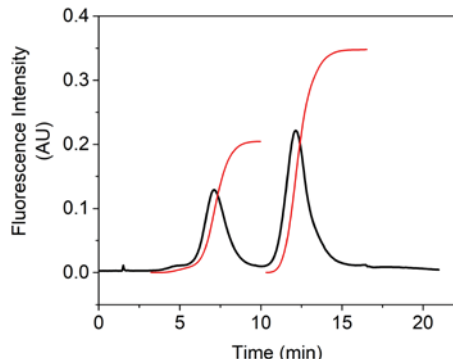

Figure 2: 10 µg injection of IgE showing the bound and unbound peaks. The peak areas were used to generate the bound ratios. The red lines are the integrals for the area of each peak.
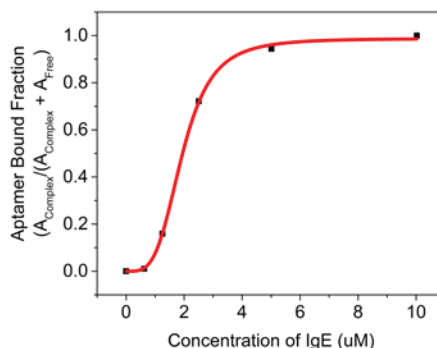

Figure 3: Plot of the bound ratio against the different amounts of protein injected. K_d can be estimated at the concentration in which half of the free aptamer is bound by the protein.

Conclusion

A K_d was successfully calculated in a simple and straightforward separation-based strategy. The high diffusion coefficient of an aptamer compared to the larger protein allows easy separation of the two peaks. This AF4 technique can be applied to any ligand-receptor pair that has sufficient difference in diffusion coefficients, allowing for efficient baseline separation of the peaks and calculation of K_d.

References

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