Molecular size of interacting partners is not critical in the determination of binding affinities with the ConSense™ Analyzer and brightness-based analysis with ASFS

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Abstract

Molecular interactions lead to changes in the photo-physical properties of fluorescence labeled molecules. This effect can be readily exploited by analyzing the brightness of the molecules involved in binding events. Here, we demonstrate the applicability of this concept for the determination of dissociation constants for different molecules and sizes in solution.

Introduction

The setup of molecular interaction assays —whether in solution or on surfaces— is often governed by the size of the inter-acting molecules. It is usually preferable to immobilize or label the smaller reaction partner, whose binding behavior is more likely influenced by such a treatment than the bigger molecule. fluIT´s ASFS offers more flexibility for the assay setup and circumvents possible artifacts due to immobilization or labeling.

Materials and method

Four interaction systems with different sizes of reacting molecules were tested for their binding affinities:

No.	Labeled tracer (size)	Reaction partner (size)	Mass ratio
1	Protein G (22 kDa)	Antibody (150 kDa)	1:7
2	Antibody 1 (150 kDa)	Antibody (150 kDa)	1:1
3	Fusion Protein (76 kDa)	Protein (20 kDa)	4:1
4	Antibody 2 (150 kDa)	Antigen (28 kDa)	5:1

Atto532 was used as the fluorescence label for Protein G, the fusion protein and the antibody 2. Antibody 1 was labeled with Alexa dye 532. All solutions were prepared in PBS buffer.

Stock solutions of antibodies and proteins were diluted with reaction buffer in a final volume of 250 µl, mixed and allowed to react to equilibrium at room temperature. Three µl of each mixture were transferred into ConSense Type A microchips.

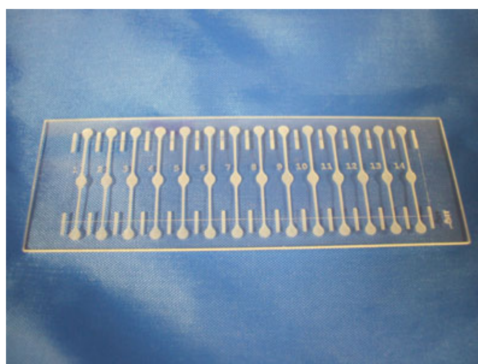

Fig. 1: 14-channel standard glass chip, sample volume 3 µl.

Instrumentation

Measurements were conducted with the confocal ConSense Analyzer using the 532 nm single mode, single frequency laser for excitation. Excitation power was 160 µW. The read time for each mixture was 60 seconds, allowing the determina-tion of one dissociation constant in less than 15 minutes.

Assay setup

For each interaction study the tracer was kept at a constant concentration in the sub-nanomolar range (0.1 nM for No. 1 and 2 and 5.6 nM for No. 3 and 4). The reaction partner was then added in a titration series from low concentrations of 0.01 nM to reach final concentrations between 10 to 100.

For all interactions the binding of the reaction partners could be observed by change in the molecular brightness of the bound vs. the unbound tracer.

No.	Brightness tracer (kcps)	Brightness bound tracer	Mass ratio
1	1.2	1.5	1: 7
2	62	43	1:1
3	32	45	4:1
4	35	51	5:1

In three cases an increase of the molecular brightness could be detected, that means that the bound tracer pro-duces more fluorescence light than the unbound tracer alone. The antibody-anti-body interaction shows a different pic-ture. Here quenching, a reduction of mo-lecular brightness, occurs upon binding.

The characteristic binding curves for these test systems and dissociation constants have been determined using a fit algo-rithm that is implemented in fluIT´s virtual lab software, assuming a 1:1 binding stoichiometry. The results were in-line with the values determined with surface plasmon resonance.

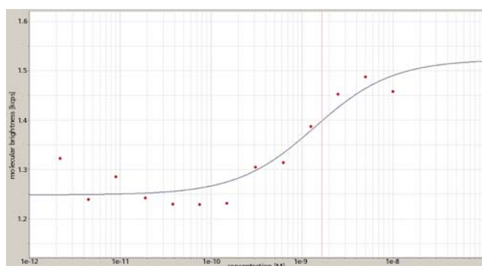

Fig. 2: Interaction of a protein G (22 kDa) with a specific antibody (150 kDa). The calculated dissociation constant is 1.7 nM.

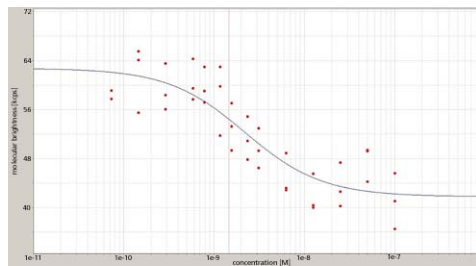

Fig. 3: Interaction of two antibodies (each 150 kDa). The calculated dissociation constant is 1.4 nM. Results of three replicates are shown.

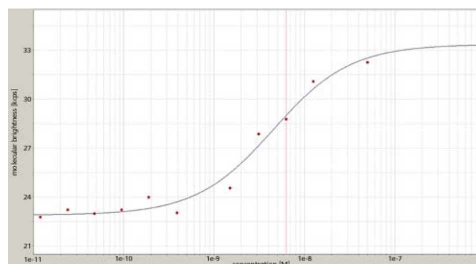

Fig. 4: Interaction of a fusion protein (76 kDa) with a second protein (20 kDa). The calculated dissociation constant is 6.3 nM.

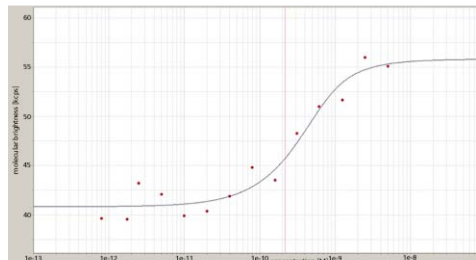

Fig. 5: Interaction of an antibody (150 kDa) to its antigen (28 kDa). The calculated dissociation constant is 0.2 nM.

In all figures the molecular brightness is plotted against the concentration of the non-labeled binding partner.