Kinetic Binding Studies using OptiSlides

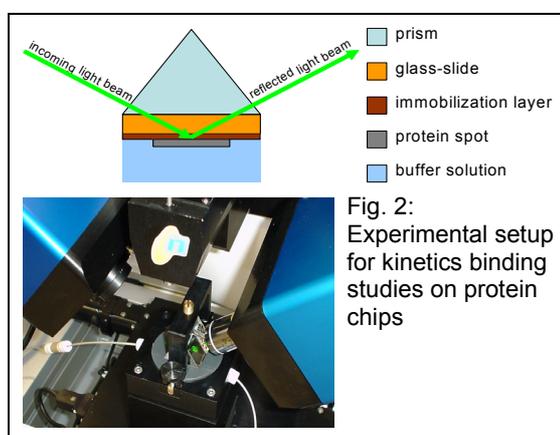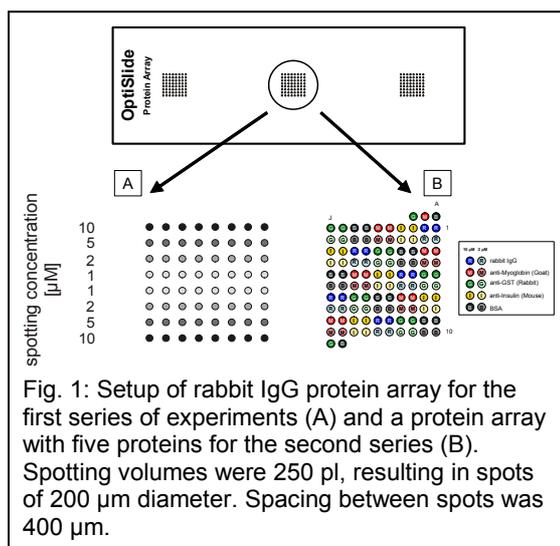## Characterization of polyclonal anti-rabbit IgG

Introduction

In the field of proteomics, one has to deal with a class of molecules with diverging and in several aspects still unpredictable properties. It is quite undesirable to have to modify proteins with labels before being able to characterize them. Obviously, under such circumstances label-free detection methods are strongly favoured. Surface plasmon resonance spectroscopy is the most common label-free technique. However, a gold layer is needed on the solid support which has to be produced under highest standards. Current applications are in most cases restricted to a few regions of interest. Protein arrays on standard glass slides cannot be read out or even be characterized kinetically.

The quantification of the affinity of an antibody to its partners and the evaluation of its unspecific binding is an important issue. With the Imaging Ellipsometer EP³ the interaction of an antibody to immobilized antigens on a microarray can be observed in a time resolved, label-free and highly parallel manner [1]. Today, up to 150 channels, measured at so called 'regions of interest' (ROI), can be analyzed simultaneously. Different from common SPR setups, the binding of a ligand to several receptors can be studied in one experiment and thus consistent biochip data is obtained. Using Nanofilm´s proprietary OptiSlides, a very high sensitivity can be reached. Here we show the determination binding constants of polyclonal anti-rabbit IgG to immobilized rabbit IgG and study the specificity of the polyclonal antibody.

Sample

OptiSlides manufactured by UNAXIS (Balzers, Liechtenstein) were covered with a Dendrimer surface and spotted with rabbit IgG (first series) and five proteins

Fig. 1: Setup of rabbit IgG protein array for the first series of experiments (A) and a protein array with five proteins for the second series (B). Spotting volumes were 250 pl, resulting in spots of 200 µm diameter. Spacing between spots was 400 µm.

Fig. 2: Experimental setup for kinetics binding studies on protein chips

(second series of experiments) at Chimera Biotec (Dortmund, Germany; array layout in fig.1). Phosphate buffered saline (0.01 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) with 0.005% Tween was used as running buffer.

Instrumentation

Standard Imaging Ellipsometer EP³-SW (532 nm), beam expander & 2x objective, field of view: 2.1 x 2.7 mm, Kinetics / SPR cell with 60° BK7 prism (n=1.5), peristaltic pump, EP³ View Software V2.03 with Kinetics AddOn module
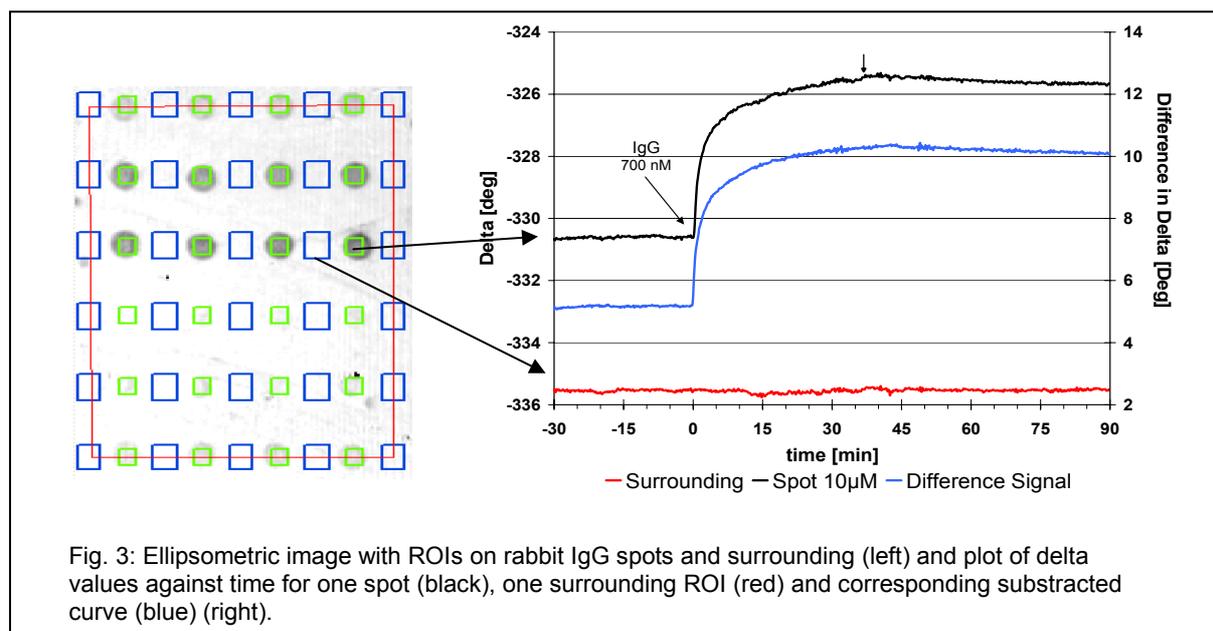
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Fig. 3: Ellipsometric image with ROIs on rabbit IgG spots and surrounding (left) and plot of delta values against time for one spot (black), one surrounding ROI (red) and corresponding substracted curve (blue) (right).

Task

In the first part the determination of kinetic constants for binding of polyclonal anti-rabbit IgG to immobilized Rabbit IgG is examined and in the second part the specific and unspecific binding of it to 5 immobilized antigens is evaluated.

Steps of evaluation

1. Inserting the slide into the Kinetics /SPR cell.

2. Filling the cell with the running buffer. The buffer is pumped with a flow rate of 1 ml/min in a cyclic manner (volume: approx. 1.5 ml).

3. Visualization of the spots at 54° angle of incidence, adjustment of the optical components to get a good contrast.

4. Adjustment of the x/y position to spots of interest.

5. Definition of regions of interest (ROI) on the spots and around them as reference channels.

6. Measurement of the ellipsometric parameter delta on the spots and their surrounding. The difference between information for calculating thickness or mass (fig. 3).

7. For the kinetics experiments defined concentrations (3 - 700 nM) of anti-rabbit IgG (goat) were pumped through the flow cell containing IgG array (fig. 1A) in a cyclic manner (total volume here: approx. 1.5 ml) and binding was monitored by re cording delta values in 10 s steps. For each concentration a new array was used. The data of 16 individual spots were chosen for evaluation.

8. For the analysis of specificity 50 nM anti-rabbit IgG (goat) solution was pumped through the flow cell containing the array shown in fig. 1B. After washing with buffer to examine the offrate of the polyclonal anti-rabbit IgG the array was regenerated with glycine buffer (pH 2.2) and PBS-Tween.

Results

Kinetics experiments

As shown in fig. 3, the binding of anti-Rabbit IgG is significant and quick, indicating a high k_{on} value. On the other hand, dissociation is very slow (see slope during the washing step). As a consequence, though calculation of k_{off} is possible and has been shown, for more accurate calculation of kinetics constants the following approach has been applied:

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1. The association was measured at various ligand concentrations.

2. The exponential rate constant τ for each concentration was estimated with a fit ting routine (implemented in the Kinetics AddOn software) following

$$\Delta f(t) = \Delta f_{equ}\left(1 - \exp\left(-\frac{t}{\tau}\right)\right) + a \cdot t$$

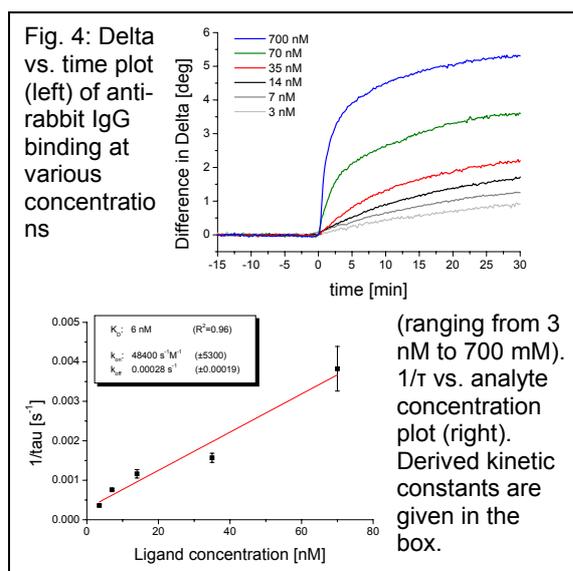where $a \cdot t$ is a correction term for biphasic reactions.

3. $1/\tau$ is plotted against the analyte concentration $c_{L,0}$. The slope of the curve is the association rate constant k_{on}, the intercept with the y-axis is the dissociation rate constant k_{off}:

$$\frac{1}{\tau} = k_{on}c_{L,0} + k_{off}$$

4 The binding constant K_D is the quotient of k_{off} and k_{on}:

$$\frac{1}{\tau} = k_{on}c_{L,0} + k_{off}$$

A series of binding curves, the corresponding plot of $1/\tau$ and derived kinetic constants are shown in fig. 4.

Fig. 4: Delta vs. time plot (left) of anti-rabbit IgG binding at various concentrations

(ranging from 3 nM to 700 mM). $1/\tau$ vs. analyte concentration plot (right). Derived kinetic constants are given in the box.
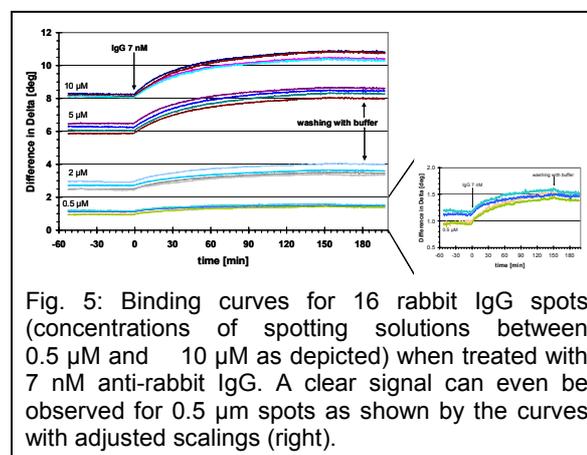
As mentioned above, kinetics can be followed for each single protein spot, independently from the amount of protein spotted. This has been shown by calculating kinetic constants for a series of 16 spots arranged as in fig. 1 and 3. A record of delta values for these spots bound by 7 nM IgG plotted against time is shown in fig. 5. One can see that even for the spots of lowest concentration a calculation of kinetic constants is possible (see also 0.5 µM spots curves in fig. 5, right).
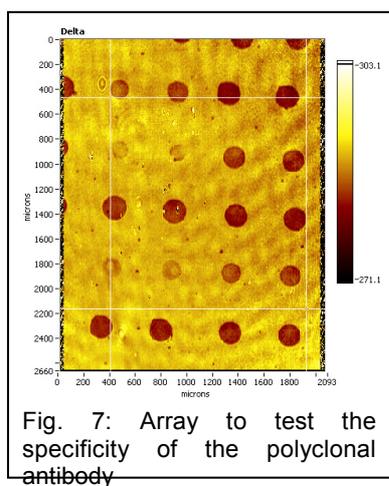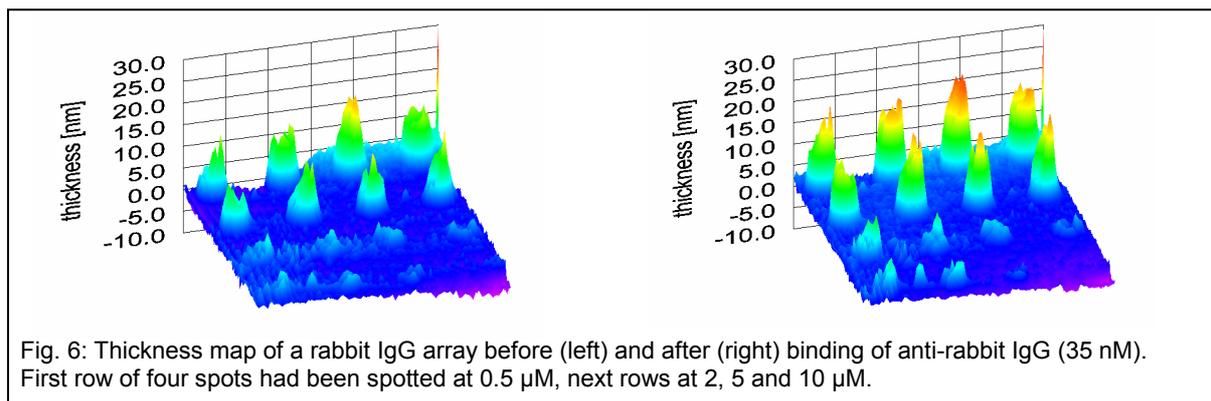
Optical modeling allows the determination of the refractive index of any material analysed. Here the thickness of individual spots for a three-dimensional visualization of the chip surface was determined. Fig. 6 shows a rabbit IgG array before and after binding of anti-rabbit IgG.

Specificity of the polyclonal Antibody

In a second series of experiments the specificity of the polyclonal anti-rabbit antibody has been examined. An array with five proteins, four antibodies and serum albumin in two concentrations, has been printed like shown in fig. 1. With an EP³ equipped with a 2x objective, an area of 2 x 3 mm can be examined, corresponding 4 x 5 spots (fig. 7).The anti-rabbit IgG bound specifically to rabbit antibodies (fig. 8). No binding could be observed to anti-myoglobin IgG from goat, anti-insulin IgG from mouse or to bovine serum albumin.

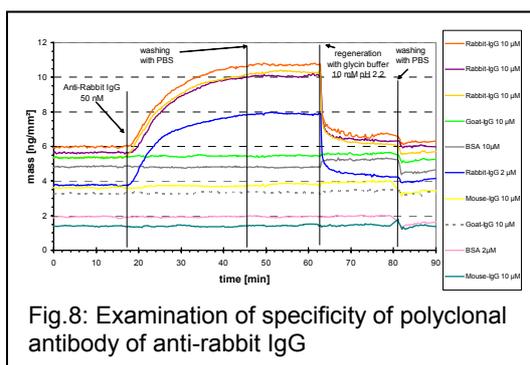Fig. 5: Binding curves for 16 rabbit IgG spots (concentrations of spotting solutions between 0.5 µM and 10 µM as depicted) when treated with 7 nM anti-rabbit IgG. A clear signal can even be observed for 0.5 µm spots as shown by the curves with adjusted scalings (right).

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Fig. 6: Thickness map of a rabbit IgG array before (left) and after (right) binding of anti-rabbit IgG (35 nM). First row of four spots had been spotted at 0.5 µM, next rows at 2, 5 and 10 µM.

Fig. 7: Array to test the specificity of the polyclonal antibody

Conclusion

The Imaging Ellipsometer EP^3 is an attractive alternative for label-free kinetic analysis. The interaction of a soluble ligand with immobilized receptors on a protein array has been demonstrated. Using OptiSlides as a substrate overcomes limitations of SPR based systems in terms of linker chemistry. It has been shown that each individual protein spot can be evaluated achieving consistent kinetic constants. Different protein array samples have been used in this study, leading to consistent, reproducible data sets.

Acknowledgement

We would like to thank Unaxis for their cooperation developing the OptiSlides and Chimera Biotec for supplying the samples.

Fig.8: Examination of specificity of polyclonal antibody of anti-rabbit IgG

Literature

[1] Vaupel et al., in "*Microarray Technology and Its Applications*", Müller & Nicolau (Eds.), Springer, Berlin **2005**, ISBN 3-540-22931-0, 181-207

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[3] Yasui, in "*Real-Time Analysis of Biomolecular Interactions*", Nagata & Handa (Eds.) Springer, Tokyo **2000**, ISBN 4-431-70289-X

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