Imaging Ellipsometry: A Powerful Method for Biochip Development, QC and Evaluation

Martin Laging, Dirk Hoenig and Andreas Eing

Nanofilm Technologie GmbH, Goettingen, Germany

ABSTRACT

Ellipsometry as a well-established method in material research has substantially enriched the field of protein research: A special application known as SPR has widely been accepted as the major tool to characterize the interaction of proteins with all classes of molecules.
Taking advantage of the full potential of Imaging Ellipsometry provides us with a powerful tool not only allowing label-free kinetic measurements on multiple protein and DNA spots in situ: Imaging Ellipsometry allows the control of every step in the area of biochip experimentation, from development of prototype surfaces to a quality control of all fabrication steps, finally interaction experiments in a semi high throughput manner.

Keywords: Imaging Ellipsometry, Microarrays, Protein Arrays, Biochips, Kinetics

1. INTRODUCTION

Imaging ellipsometry is an optical method capable of measuring optical parameters of thin layer systems. Measurements are performed in a non-destructive and label-free manner, they are time resolved. These properties make imaging ellipsometry an ideal tool for biochip analysis. With a resolution in the picometer range in height and lateral resolutions down to 1 µm, spots of DNA or protein can directly be visualized. Precise quality control of the surface before and after spotting is possible. Furthermore, hybridization/binding can be followed in a time-resolved manner, extending applications from end point analysis to kinetic studies. This opens the field for large-scale protein-protein interaction studies on biochips.
Among current label-free methods to study protein-protein interactions, the Nanofilm imaging ellipsometer EP^3 offers an optical solution with several advantages:

1. It measures interactions on biochip surfaces without the need for large amounts of substances. Biochips are very versatile as well for small-scale experiments as for HTS.
2. In contrast to fluorescence-based related techniques, spots can be visualized at any step during fabrication, spotting and finally the binding experiment. This for the first time enables true quality control, eliminating several 'black boxes' in biochip experiments.
3. Today up to 150 regions of interest (ROI) can be analyzed simultaneously. In contrast to common SPR setups, the binding of a ligand to several receptors can be studied in one experiment [1]. This, together with real quality control, produces consistent biochip data.
4. On silica-based OptiSlides (Nanofilm) very high sensitivities are reached without the need for a gold layer as known from SPR setups. In fact, virtually any surface can be used as substrate. This allows the design of special chip surfaces: The biochips can be adapted to the specific requirements of all classes of proteins.

Here we show as a proof-of-principle the determination of kinetic constants for the binding of polyclonal anti-rabbit IgG to rabbit IgG. A rabbit IgG protein array was spotted and the binding of the antibody was recorded. Then kinetic constants (k_{on}, k_{off}, K_D) were derived.

2. MATERIALS AND METHODS

2.1 Materials

OptiSlides manufactured by UNAXIS (Balzers, Liechtenstein) were covered with a Dendrimer surface [2] and spotted with rabbit IgG at Chimera Biotec (Dortmund, Germany) (array layout in Figure 1). Three array sets per slide were spotted .

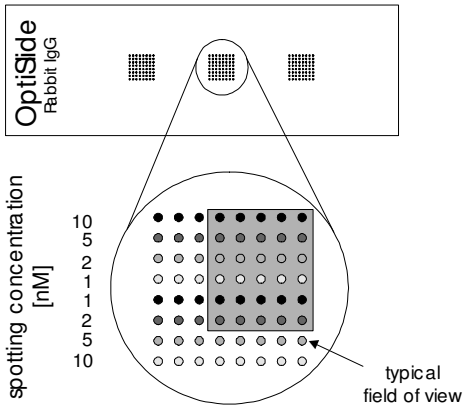

Figure 1: Setup of the rabbit IgG protein array. Spotting volume was 250 pl, resulting in spots of 200 µm diameter. Spacing between spots was 400 µm.

PBS Buffer was prepared by dissolving tablets in ddH_2O, resulting in 10 mM phosphate buffer, 138m M NaCl, 2.7 mM KCl, pH 7.4 at 25 °C. Glycine buffer was prepared by dissolving glycine in ddH_2O, titrating to pH 2.2 and

adjusting to 10 mM. All chemicals were from Sigma (Taufkirchen, Germany).

2.2 Methods

Protein arrays were analyzed with a Nanofilm Imaging Ellipsometer EP³-SW (532nm), beam expander & 2x objective (field of view: 2.1 x 2.7 mm), kinetic/SPR cell with 60° BK7 prism (n=1.5), peristaltic pump using the EP³ View Software with a Kinetics Add On software module.

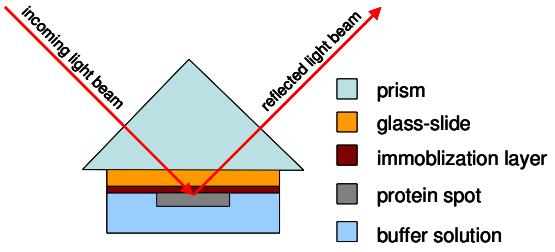

Figure 2: Experimental setup for kinetic binding studies on protein chips.

In the experimental setup (Figure 2), an array was placed in the kinetics cell in an inverted manner. The light beam passed a prism coupled to the glass slide with immersion oil and the reflection at the glass-liquid phase transition was measured. The flow cell was filled with PBS at a flow rate of 50 µl/min and then equilibrated at 1 ml/min for 10 minutes. An angle of incidence of 54 ° was chosen, the microscopic optics focussed and the auto procedure was used to adjust polarizer and analyzer for a high contrast live image. Regions of interest (ROIs) were placed on (signal) and around (background) the protein spots using the ROI array generation tool.

Then ellipsometric parameters delta and psi were recorded in steps of 10 seconds using a kinetic script. The technique to derive the ellipsometric parameters called nulling ellipsometry rotates polarizer and analyzer to find and fit for a signal minimum on the detector camera.
Different defined concentrations of anti-rabbit IgG (goat) ranging from 3 to 700 nM were then pumped through the flow cell in a cyclic manner (total volume 1.4 ml). Delta values were directly used to monitor binding curves and to derive kinetic constants with the Kinetics AddOn Software. The arrays were regenerated by washing with glycine buffer and subsequent reequilibration with PBS buffer.

3. RESULTS

A plot of delta values for one spot ROI (black) and a neighbouring background ROI (red) against time is shown in figure 3 (right). Subtracted curves (blue) were used for evaluation of kinetic constants. The corresponding ellipsometric image is shown on the left. No unspecific binding to the subtstrate (figure 4) or other proteins (goat IgG, BSA; data not shown) was observed.
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 kinetic constants a Langmuir model approach [3] has been applied:

1. The association was measured at various ligand concentrations.
2. The exponential rate constant τ for each concentration was derived with a fitting routine using the Kinetics AddOn following equation 1 (where $a \cdot t$ is a correction term for biphasic reactions).

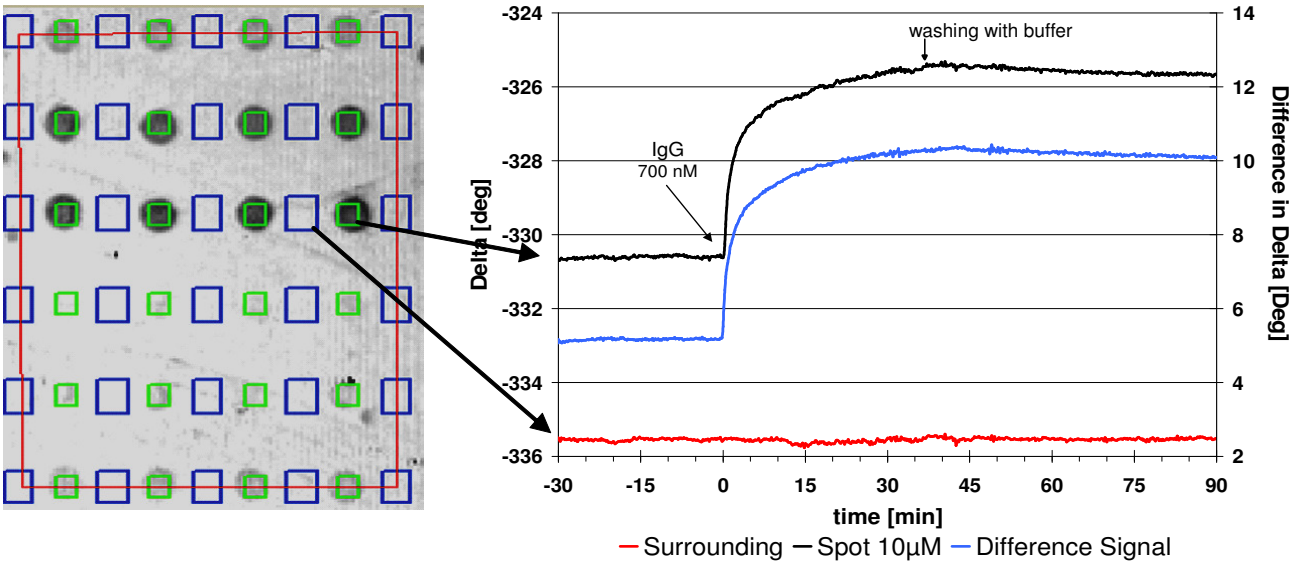

Figure 3: Ellipsometric live image with ROIs on rabbit IgG spots and reference area (left) and plot of delta values against time for one spot (black), one reference ROI (red) and resulting corrected signal (blue) (right).

NSTI-Nanotech 2005, www.nsti.org, ISBN 0-9767985-0-6 Vol. 1, 2005

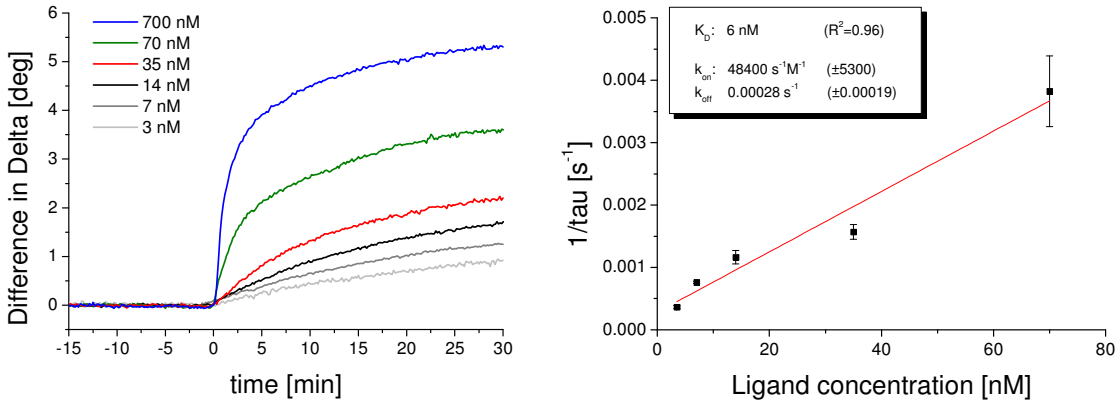

Figure 4: Delta vs. time plot (left) of anti-rabbit IgG binding at various concentrations. 1/τ vs. ligand concentration plot (right). Derived kinetic constants are given in the box.

3. $1/\tau$ is plotted against the ligand concentration $c_{L,0}$. The association rate constant k_{on} and the dissociation rate constant k_{off} are derived as the slope and the intercept with the y-axis, respectively (equation 2).
4. The binding constant K_D is the quotient of k_{off} and k_{on} (equation 3).

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

$$\frac{1}{\tau} = k_{on}c_{L,0} + k_{off} \qquad (2)$$

$$K_D = \frac{k_{off}}{k_{on}} \qquad (3)$$

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

As mentioned in the introduction, delta and psi values can be recorded in parallel for up to 150 ROIs. Kinetic constants have been derived from a series of 16 spots in parallel. A record of delta values for one binding experiment (7 nM anti-rabbit IgG) plotted against time is shown in figure 5. Even for the spots of lowest spotting concentration a calculation of kinetic constants is possible (see 0.5 µm spots curves in figure 5, right.).

With an Imaging Ellipsometer, each pixel can serve as a detector. Subsequently, in a mapping mode ellipsometric parameters delta and psi can be determined for each pixel. These delta and psi maps can be transferred via optical modelling into thickness maps, where every "pixel" now reflects a real nanometer value (instead of greyscale information). This mapping feature is a very powerful tool for quality control on biochips. Figure 6 shows one spot of the rabbit IgG array at a high magnification at different timepoints during a binding experiment.

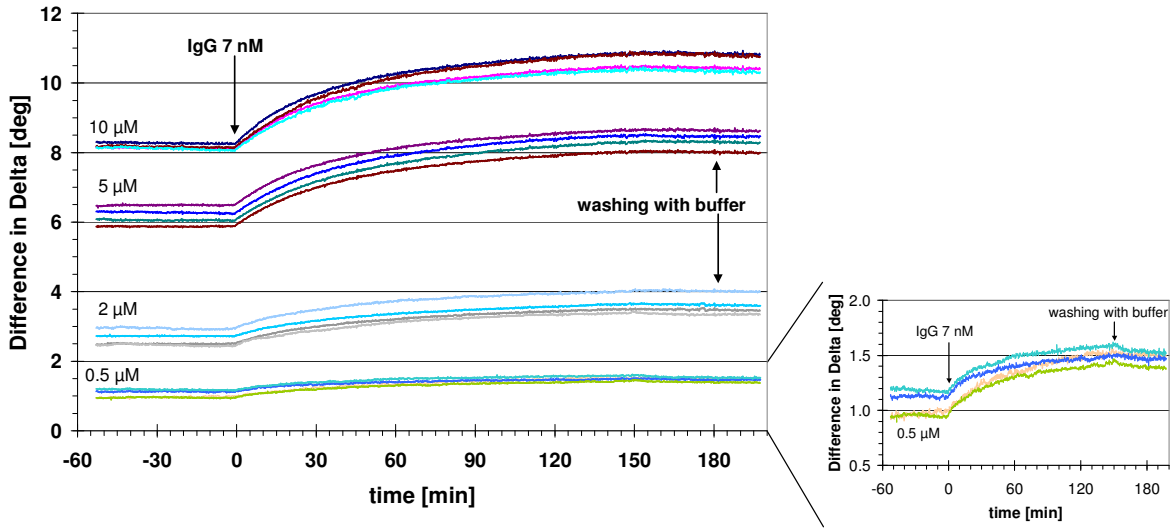

Figure 5: Binding curves for 16 rabbit IgG spots (concentrations of spotting solutions between 0.5 µm and 10 µm as depicted) when treated with 7 nM anti-rabbit IgG. A clear signal can even be observed for 0.5 µm spots as shown by the curves with adjusted scalings (right).

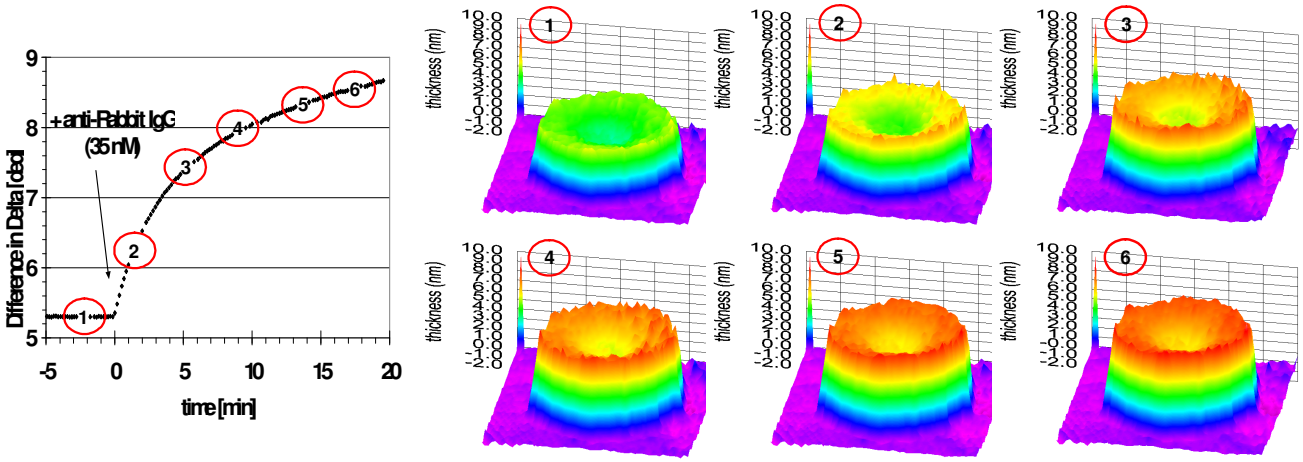

Figure 6: Binding of anti-rabbit IgG to rabbit IgG spots. At different time points (see binding curve, left) delta maps were recorded and transformed into thickness maps via optical modeling. This series for the first time shows the shape of proteins during a binding experiment. It can clearly be seen that the initial donut shape is preserved during binding of the antibody.

4. DISCUSSION

In the field of proteomics, one has to deal with a class of molecules with diverging and in many cases unpredictable properties. It is quite undesirable to have to modify proteins before knowing their native behaviour. A label might change a proteins conformation, stability and affinity to ligands. A label-free detection methods is thus strongly favoured.

Other label-free methods, as calorimetry, chromatography or analytical ultracentrifugation, consume large amounts of protein. Often very highly concentrated samples are needed, which for many proteins cannot be produced in a soluble and native manner.

Surface plasmon resonance is the most related technique, enabling kinetic analysis with very high detection sensitivities. However, current systems are restricted to some fiew regions of interest (or channels). Furthermore, a gold layer is needed on the solid support, which has to be produced with highest standards. Protein arrays on standard glass slides or specially adapted surfaces cannot be analyzed via SPR.

Today read-outs of protein or DNA arrays can be performed only with fluorescence-based techniques. As discussed above, the fluorescent label is not desirable.

Imaging ellipsometry as a label-free detection method avoids the disadvantages of fluorescence based techniques. It is a non-destructive technique and allows real time data acquisition. The ability to measure on 150 ROI simultaneously enables kinetic studies on protein and DNA arrays. Furthermore, imaging ellipsometry for the first time allows real quality control of biochip experiments. All steps can be effectively monitored, starting with surface control of the slide, control of the immobilization layer, and judging the quality of each single spot. This should eliminate to a significant extend the problems with biochips observed today regarding sensitivity, error rates and reproducibility.

This publication shows the use of imaging ellipsometry for kinetic analysis on a protein chip. The interaction of a soluble ligand with a receptor immobilized on a silica-based support has been characterized. It has been shown that each individual protein spot can be evaluated to achieve consistent kinetic constants. Different protein array samples have been used in this study, leading to consistent data sets indicating a high degree of reproducibility. No unspecific interaction with the support matrix has been observed (not shown). Interestingly, no detergent had to be added to the buffer at any time. Addition of detergent (e.g. Tween20) increases the sensitivity of the system, but for some proteins one might want to avoid these kinds of additives for ligand and/or receptor stability reasons. The reproducibility, together with real quality control options, opens the field of biochip technology for new challenges.

ACKNOWLEDGEMENT

We would like to thank Unaxis for their efforts to co-develop the OptiSlides and Chimera Biotec for supplying the samples.

LITERATURE

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

[2] Angenendt et al., *Journal of Chromatography A*, 1009 (**2003**) 97-104

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