1. Introduction

Protein interactions with small molecules ($M \leq 500$ Da) play an important role in regulatory biological processes, making small molecules potential candidates for future drugs. The action of the most prospective small molecule drugs is based on their ability to form stable complexes with therapeutic targets. Therefore, characterizing the binding kinetics of small molecule–protein complexes is important in pharmaceutical research. However, quantifying the affinity of the respective binding partners is rather challenging due to the intrinsically low concentration at which the complexes are formed.

Standard kinetic affinity methods may be categorized according to the phase state of the binding partners as heterogeneous or homogeneous. In heterogeneous measurements, one of the binding partners is coupled to a sensor surface, whereas homogeneous methods do not require the immobilisation of any of the binding partners for affinity measurements and are, in general, preferred over heterogeneous methods in protein-small molecule studies.

Perhaps the most prominent technique for homogeneous small molecule interaction studies is the electrophoretic mobility shift assay (EMSA). In EMSA the analyte is incubated with a fixed concentration of a radio-labelled probe and varying amounts of protein. The solutions are run on a native polyacrylamide gel to separate the bound from the free analyte. While EMSA is a very sensitive technique, the probes also have several disadvantages, including safety and environmental problems associated with their radioactivity.

Homogeneous methods generally do not allow the direct observation of binding events. Something that is more easily accomplished with heterogeneous affinity measurements is fluorescence resonance energy transfer (FRET). FRET exploits the energy transfer between two chromophores for sensing purposes. Binding reactions may be sensed with FRET by coupling one interacting agent with a ‘donor’ and its complementary binding partner with an ‘acceptor’, whose absorption spectrum overlaps with the donor’s emission spectrum. However, using FRET for small molecule sensing can be problematic, since the required labels are often quite large in comparison to the molecules to be investigated and could interfere with the binding activity of both partners.

Most heterogeneous label-free methods such as SPR require the immobilisation of the small interaction partner on the surface of a sensor for sensitive detection since it is easier to monitor the adsorption of the larger binding partner if the detection principle relies on a change in refractive index, for instance. The optical or acoustic signal as a function of the compound concentration can be analysed to obtain both kinetic rates and thermodynamic binding constants. Coupling of a small molecule to a sensor surface, however, may influence its binding ability due to steric reasons. Various workarounds to increase sensing sensitivity have been devised also with a focus on surface plasmon resonance (SPR) spectroscopy. Murthy et al. used a strategy to enlarge the detectable mass by adding micelles that display the epitopes of interest to the sensor surface. Alternatively, surface-enhanced fluorescence spectroscopy can be used to boost the detection level...
2. Experimental

2.1. Materials

Common chemicals as well as avidin from egg white and Atto488-Biotin were acquired from Sigma Aldrich (Deisenhofen, Germany). Aluminium with 99.999% purity was purchased from GoodFellow (Coraopolis, Pennsylvania, USA).

2.2. Preparation of Anodic Aluminium Oxide (AAO) membranes

2.2.1. Aluminium metal preparation and electrochemical polishing. Aluminium was annealed at 500 °C overnight to increase the self-organisation of the AAOs to be fabricated. The surface of the substrates was polished using a solution made from concentrated acids (H₂O/H₃PO₄/H₂SO₄, 1/1/1). The aluminium plates were warmed to 70 °C, and a constant voltage of 25 V was applied. The electrochemical dissolution of aluminium was continued until the surface became reflective (usually 3–5 min). The process was repeated until the surface was as close to mirror-like as possible, but was done at least 3 times for aluminium supports meant for AAO growth.

2.2.2. AAO membrane growth. The electrochemically polished aluminium plates were placed in an anodization chamber, filled with 0.3 M oxalic acid and cooled under stirring to 1.0 °C. The first anodisation step was carried out at 40 V for 3 h to pre-texture the surface. The oxide layer was dissolved using 5 vol% H₃PO₄ solution over 2–3 h. Anodisation was repeated under the same conditions for 75 min, resulting in 3 μm thick porous membranes. AAOs produced in 0.3 M oxalic acid at 40 V provide an interpore distance of 100 nm and a diameter of 25 nm. The pores were widened to diameters of 60 nm through slow dissolution of the pore material in 5 vol% of H₃PO₄ for 50 min. Functionalisation of the substrate was not necessary. Prior to measurement, the AAO chip was treated with oxygen plasma for 1 min.

2.3. Performing small molecule binding experiments

2.3.1. Setup. The binding studies were carried out with a home-made RIfS set-up. This type of instrumental assembly has been described in the literature previously. A tungsten halogen lamp (LS-1, Ocean Optics, Dunedin, Florida, USA) is used as the light source, from which radiation is coupled into a y-shaped optical fibre (also purchased from Ocean Optics), and guided to a measurement chamber holding a transducer chip, which is irradiated from the top perpendicular to the surface (Fig. 1). The optical fibre consists of six illuminating fibres placed concentrically around one collecting fibre, resulting in a sensing area of 1 mm². The reflected light is gathered by the collecting optical fibre and guided to a spectrometer (Nanocalc-2000-UV/VIS, Ocean Optics).

The measurement chamber was built to allow the exchange of fluids during experiments. For this purpose, a flow channel was milled (3 × 10 × 1 mm³; volume 30 μl) into an acrylic glass cover. A continuous flow was generated via a peristaltic pump (Perimax, Spetec, Erding, Germany). The flow rate was usually set to 1.2 ml min⁻¹.

2.3.2. Adsorption of avidin within AAO membranes. A chip was placed in the measurement chamber of the RIfS set-up and a baseline was recorded in buffer (20 mM HEPES, 100 mM Na₂SO₄, pH = 7.0). After approximately 5 min, a 0.1 mg ml⁻¹ solution of avidin was added and allowed to circulate until the...
adsorption reaction of avidin to the pore walls of the substrate reached equilibrium (Fig. 3). Buffer was flown through the system until the measured signal stabilized again and the RIfS measurement was terminated.

2.3.3. Monitoring of biotin–avidin interaction. The evaluation software was set to track the reflectivity values of 501 nm radiation (the absorption maximum of Atto488-Biotin). A baseline was recorded 10 min before Atto488-Biotin was added in increasing concentrations (0.5 nM–15 nM) and the system was left to equilibrate after each analyte addition (Fig. 4A). Eventually, the system was rinsed with buffer for 30 min. To determine the equilibrium dissociation constant, measurements were done in triplicate.

2.4. Data processing

2.4.1. Spectrum analysis. All reflectivity spectra recorded during avidin immobilisation and biotin binding were evaluated with RIfS software to track the changes in optical thickness. Additionally, the reflectivity changes for 501 nm radiation, the absorption maximum of the employed fluorescent dye, were read-out. The RIfS evaluation was executed using wavelengths ranging from 550 nm to 700 nm, in order to avoid the analysis being compromised by the absorption effects of the atto dye. A typical reflectivity spectrum is shown in Fig. 2C.

2.4.2. Influence of light-absorbing molecules on reflectivity. Filling the pores with an absorbing medium such as a liquid with dissolved dyes changes the reflectivity of the effective medium comprising the porous matrix (alumina oxide) and the liquid in the pores. It is, however, not possible to detect small amounts of absorbing molecules dissolved in buffer (μM–nM regime) as detailed below. Therefore, adding a solution with dissolved dyes to the porous alumina oxide below 1 mM remains almost invisible to the reflectometric sensor. Only molecules accumulating at the pore walls change the reflectivity of the thin film significantly enough to be detected by a change in reflectivity. The Fresnel coefficients for normal incidence are

$$r_{12} = \frac{n_1 - n_2}{n_1 + n_2}, \quad (1)$$

where $n_1$ indicates the refractive index of the ambient medium (water) and $n_2$ is the refractive index of the AAO membrane. $n_2$ becomes a complex quantity $n_2 = n_{eff} - ik_{eff}$ if an absorbing substance is present in the porous film. $n_{eff}$ and $k_{eff}$ may be calculated according an effective medium model published by Garahan et al. The model predicts the optical properties of nanoporous thin films with horizontally aligned cylindrical nanopores with a specified diameter and porosity.

$$n_{eff}^2 = \frac{1}{2} \left( A + \sqrt{A^2 + B^2} \right),$$

$$k_{eff}^2 = \frac{1}{2} \left( -A + \sqrt{A^2 + B^2} \right), \quad (2)$$

with

$$A = f(n_d^2 - k_d^2) + (1-f)(n_m^2 - k_m^2) \quad (3)$$

and

$$B = 2n_d k_d f + 2n_d k_m (1-f) \quad (4)$$

$f$ denotes the filling factor of domains (pores) present in the substrate, $n_m$ and $k_m$ are the real and imaginary parts of the refractive index of the substrate material, while $n_d$ and $k_d$ are the real and imaginary parts of the refractive index of the material forming the domains, respectively. Having calculated the refractive index of the porous layer and the Fresnel coefficients of the system, its reflectivity $R$ may be calculated by

$$R = \frac{\left( r_{12} + t_{12}^2 e^{-2\delta} \right)^2}{1 - \frac{t_{12}^2 t_{21}^2 e^{-2\delta}}{1 - r_{12}^2 r_{21}^2 e^{-2\delta}}}, \quad (5)$$

with the phase difference

$$\delta = \frac{2\pi}{\lambda} dn_2 \quad (6)$$

between the light-beam reflected on the surface of the substrate and the light-beam refracted into the AAO film. $r_{12}, t_{21}, t_{23}, t_{12},$ and...
\( I = \lambda_0 e^{\alpha z} \)

where \( \alpha \) is the extinction coefficient, \( \lambda_0 \) is the wavelength, and \( z \) is the distance traveled through the material.

2.4.3. Equilibrium dissociation constant. The reaction of Atto488-Biotin binding to avidin can be described as

\[
B + A_{\text{surf}} \rightleftharpoons BA_{\text{surf}},
\]

where \( A_{\text{surf}} \) represents the avidin adsorbed in the porous film, \( B \) represents the free biotin in solution and \( BA_{\text{surf}} \) represents the bound complex. The equilibrium dissociation constant \( K_D \) can be obtained from the corresponding Langmuir equation:

\[
\Delta R = \frac{\Delta R_{\text{max}} [B]}{1 + [B]/K_D},
\]

where \( \Delta R_{\text{max}} \) represents the change in reflectivity obtained when all binding sites on the surface are occupied and \( \Delta R \) is the steady state signal for any given biotin concentration. \( K_D \) is obtained by fitting the parameters \( K_D \) and \( \Delta R_{\text{max}} \) of eqn (7) to a graph with \( \Delta R \) as a function of concentration.

3. Results and discussion

As a sensing principle for this small molecule binding assay we used a variation of reflectometric interference spectroscopy (Fig. 2). If a substance adheres to the walls of a porous AAO film, its refractive index changes. The refractive index may be influenced in two ways. First, the deposition of the material alters the real part of the refractive index of the porous film, increasing its refractive number and thus its optical thickness, which becomes visible as a red-shift of the interference pattern in the reflectivity spectrum of the film (Fig. 2B and C: red to green curve). This effect was used to measure the adsorption of avidin in the porous layer.

Secondly, another way to influence the refractive index of the porous film is to deposit a small dye-labelled agent on the pore walls. This leads to a change of the extinction coefficient of the porous film. In other words, the reflectivity of the AAO film is attenuated at the absorption maximum of the immobilised agent, which becomes visible as a drop in the interference pattern of the reflectivity spectrum (Fig. 2B and C: red vs. green curve). We utilised this phenomenon to sense the binding of Atto488-Biotin to immobilised avidin as it is more sensitive than monitoring a shift in optical thickness.

Fig. 3 depicts the changes in optical thickness during the deposition of avidin in the porous matrix. The wavelength range used to calculate \( \Delta \theta \) is indicated by the brackets in Fig. 2C. The adsorption of avidin on the pore walls of the AAO film is clearly visible in this graph as an increase in \( \theta \) of 48 nm immediately after protein addition at point ‘a’. Avidin adsorbs on AAOs via electrostatic interaction without the help of any surface functionalisation at a pH higher than 3.0. The average surface concentration of avidin \( \langle J(t) \rangle \) as a function of time is at low values of time proportional to the bulk concentration \( C_0 \) of avidin and the ratio between pore radius \( R_{\text{pore}} \) and thickness of the AAO membrane \( d \) leading to \( \langle J(t) \rangle \approx R_{\text{pore}} C_0 t \).

Excess protein in solution was removed from the system causing the measured signal to drop by 5 nm (point ‘b’). Since avidin was not covalently attached to the pore walls, the continuous flow of liquid through the measurement chamber might affect desorption of avidin molecules, which could be the cause of the constant drift of 1.3 nm h\(^{-1}\) seen in the graph. Once the signal stabilized, Atto488-Biotin was added in increasing overall concentration.

Fig. 4A displays the change in reflectivity recorded at 501 nm, close to the adsorption maximum of the attached fluorescent dye Atto488 (as indicated in Fig. 2C). The original data shown in grey were smoothed by averaging over 5 data adjacent points and fit with a monoeponential decay to illustrate the suitability of the Langmuir description. All further data analyses were done with the original data. Once it was confirmed that the reflectivity at the wavelength is stable, the addition of labelled biotin in very low concentrations was started. The binding of Atto488-Biotin to avidin in the AAO membrane is clearly visible in the graph as a decrease of its reflectivity (Fig. 4A). An adsorption isotherm was measured by increasing the concentration of Atto488-Biotin stepwise (concentrations are shown in the graph) and waiting for equilibrium to establish after each successive addition (see ESI, Fig. 1†).
In order to prove that the labelled biotin binds to the physically adsorbed avidin and not the pore wall itself, Atto488-Biotin in a concentration of 100 nM was allowed to circulate over an untreated AAO chip (data not shown). No significant change of its reflectivity was observable upon addition of the labelled compound, indicating that the observations during the actual experiment can safely be attributed to the specific binding of biotin to avidin. Biotin without a label did not produce any noticeable change in reflectivity.

The dissociation constant \( K_D \) of the avidin–biotin complex can be determined from adsorption isotherm measurements as shown in graph B of Fig. 4 by fitting the parameters of the Langmuir equation (Experimental section, eqn (7)) to the acquired data. A value of \( 1.6 \pm 0.6 \) nM was found (the mean value of three independent measurements). This value differs significantly from the dissociation constant of the complex in solution \( (K_D = 1 \times 10^{-15} \text{ M}) \), but is comparable with other surface based techniques, which also reported values in the nanomolar regime.\(^{14-16}\)

There are two possible explanations for the difference of the \( K_D \) value determined in solution and the \( K_D \) values determined with surface-based sensors.

In 2000, Ben-Tal \textit{et al}. reported that for affinity measurements on surfaces, an entropy loss upon adsorption of one interacting agent on a surface needs to be considered, resulting from the loss of translational degrees of freedom of the said interaction partner that decrease the free energy of binding of the complex by around \( 1.5 k_B T \) per degree of freedom.\(^{25}\) This accounts for higher \( K_D \) values determined from surface based techniques compared to those measured in solution. This argument is further strengthened by a study published by Zhao and Reichert that showed the binding kinetics of streptavidin interacting with immobilised biotin differ, if biotin is directly attached to the surface or a spacer is present.\(^{26}\) Another interesting study was conducted by Sheehan and Whitman in 2005.\(^{27}\) They calculated the detection limits for nano- and microscale biosensors and were able to show a significant dependence on surface geometry and analyte flow. They deduced that a flat sensor surface is least suitable for low analyte concentration sensing and furthermore that even with ideal geometry, detection schemes based on static or conventional microfluidic flow systems are unlikely to exceed the femtomolar range. We have to come to the same conclusion as Ben-Tal \textit{et al}. that dissociation constants found for surface-based reactions are usually considerably higher than those measured in solution.

The dynamic range of the sensor scheme lies in between 0.1 and 10 nM for biotin to avidin. The lower concentration regime is limited by the signal to noise ratio that is inherently related to the differences in refractive index and absorbance of the substrate and adsorbent.\(^{22}\)

4. Conclusion

On the basis of the avidin–biotin model system for high affinity small molecule interaction, we were able to demonstrate the high sensitivity of a new sensing approach which allows for the direct observation and quantification of small molecule binding events. Using fluorescently labelled biotin, we were able to quantify its binding to surface immobilised avidin by sensing the change in reflectivity of the transducer chip at the absorption maximum of the fluorescent dye. Even though, the technique also relies on labelled compounds for detection purposes, which might be considered as a drawback, it offers advantages compared to other small molecule binding assays. The labelling itself is not restricted to rather bulky fluorescent dyes. The only criterion the label would have to meet is that its absorption maximum lies in between the medium UV and the near infrared spectrum to produce an appreciable absorption peak in the reflectivity spectrum. This allows for the use of smaller compounds than the conventional fluorescent dyes. Another advantage compared to assay formats based on fluorescence is that the measurements are not notably affected by photo-bleaching. Furthermore, employing an anodic alumina oxide membrane as a transducer chip, a well established substrate for biosensing, opens the possibility of various applications for this binding assay, since a library of surface functionalisation is readily available. Yet another advantage of this sensor compared to other techniques based on planar surfaces is that it offers the opportunity to directly observe binding events and to measure equilibrium dissociation constants in the low nanomolar regime with a very simple and inexpensive instrumental
arrangement. In the future, it is conceivable to combine the AAO chip with other more sensitive optical transducers.  

Acknowledgements

We gratefully acknowledge the invaluable help of Michaela Klingebiel by preparing AAO membranes. Financial support was granted by the DFG through grant SFB 937 (A08).

References


