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Affinity Detection of Low Molecular Weight Analytes

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In this paper we report attempts to detect directly the binding of a low molecular weight substance to a protein binding site. An optical transducer based on reflectometric interference spectroscopy (RIFS) was used to detect the binding of biotin (244 g/mol) to a thin silica film surface coated with streptavidin. RIFS allows measurement of changes in the optical thickness of thin transparent films with high resolution. During immobilization of streptavidin, an increase in layer thickness of about 5 nm was detected. Subsequent incubation with biotin (4 μM) resulted in a thickness increase of about 70 pm. Repeated incubation with biotin gave no further increase in layer thickness. The lowest biotin concentration showing significant effects was 40 nM. Incubation with benzoic acid (40 μM) gave no thickness change. The setup allowed significant detection of thickness increases of 2 pm and above. Therefore, the thickness effects observed in the study could be unambiguously and clearly identified.

The interaction of small molecules (<1000 g/mol) with protein receptor structures plays an important role in biological signaling and recognition. The investigation of such binding processes is of paramount importance in elucidating biological processes and in the search for therapeutic drugs. In the field of analytical chemistry, antibodies that bind selectively a particular analyte are used to detect and quantify organic substances.

Typically, the physical effects of the binding process itself are only minute. Binding assays, therefore, rely on various indirect methods. If a biological receptor structure is in its natural environment, then the detection of biological effects is feasible. The interpretation of the resulting data depends on the knowledge of the entire system. Binding effects can be monitored by the use of tracers.[1] While there is a variety of labeling schemes for high molecular weight substances, the labeling of small molecules is, in many cases, restricted to the use of radioactive isotopes (e.g., ^{1}H ^{14}C, ^{32}P, ^{35}S). After separation of bound and free analyte, the amount of radioactivity bound allows estimation of the binding behavior of the substance under investigation.

Within the last years, techniques for the direct detection of binding effects in biological systems have emerged. These techniques are based on the physical effects accompanying the binding of organic matter to a transducer surface. Therefore, no additional label is required. As binding effects are detected at the surface of a transducer, all such devices operate in a heterogeneous format. A list of transducers that can be used for the direct monitoring and quantification of binding processes is given in Table 1.

The quartz microbalance responds directly to mass deposited at the transducer surface. Other transducers quantify a physical parameter that varies with increasing concentration of organic matter in the typically aqueous sample matrix. Optical transducers can detect binding of organic matter by changes in local refractive index. The formation of a protein film with a refractive index different from that of the sample matrix can also be detected as the formation and growth of a thin transparent layer.

To date, most work with direct affinity transducers has been directed at the detection of macromolecules with considerable molecular weight (>10 000 g/mol).[2,5,10] Small molecules are typically detected in a competitive assay format where surface-immobilized and free analyte molecules compete for the binding sites of free receptor molecules.[11] Recently, a commercial manufacturer of an optical affinity transducer system reported on the direct detection of low molecular weight substances. Biotin binding could be detected at a concentration of 400 μM.[12] In this study, we report results from the detection of binding of a small organic molecule (biotin) to surface-immobilized receptor protein with the RIFS transducer. The expected effects can be estimated from the ratio of molecular weights of receptor and ligand and are about 2 orders of magnitude below effects observed on protein binding.

We have worked for several years on reflectometric interference spectroscopy (RIFS) at thin films as an optical transducer for affinity interactions. The basic effects are due to interference occurring at thin transparent films.[6,10] A light beam passing a weakly reflecting thin film will be reflected in part at each of the interfaces (Figure 1). As the two reflected beams travel different optical paths, a phase difference is introduced. If the film thickness is small (a few micrometers), the difference in optical paths is minute, and even for short coherent (white) light sources, interference effects can be observed. These lead to a modulation of reflected light intensity due to constructive and destructive interference. In the case of perpendicular incidence of light, the reflectance R of a thin nonabsorbing layer as a function of the wavelength is given by[6]

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Table 1. Techniques for Direct Detection of Binding Effects (Mass Deposition) at a Transducer Surface[a]

transducer	working principle	ref
surface plasmon resonance	change in coupling conditions (angle, wavelength) for excitation of surface plasmons (longitudinal electron density fluctuations) in a thin metal film with change in refractive index above the film (microrefractometer)	2, 3
film waveguide grating coupler	change of coupling condition (angle, wavelength) for light into film waveguide at a phase grating with change in refractive index above the film (microrefractometer)	4
film waveguide prism coupler	change of coupling condition (angle, wavelength) for light into film waveguide by a prism coupler with change in refractive index above the film (microrefractometer)	5
thin film interference	change of reflectance pattern from a thin film due to changes in its thickness or refractive index	6, 7
ellipsometry	change in the ratio of reflectance for light polarized parallel and perpendicular to plane of incidence	8
quartz microbalance	decrease in oscillation frequency of a quartz resonator with increasing surface coverage	9

[a] For the optical transducers, coupling conditions can be probed by change in angle of incidence or by variation of wavelength.

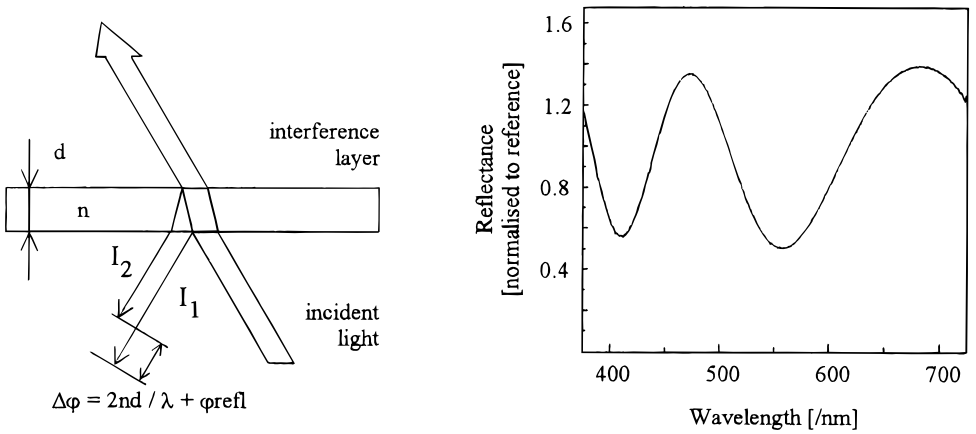

Figure 1. (Left) Light reflection at weakly reflecting thin film. At thin films (~1 μm), the reflected partial beams I_1 and I_2 show interference. Reflectance varies according to the phase difference $\Delta\varphi$ between I_1 and I_2.. (Right) Resulting reflectance spectrum.

$$R = R_1 + R_2 + 2(R_1R_2)^{1/2}\cos(4\pi nd/\lambda + \varphi_{\text{refl}})$$

where R_1 and R_2 are the Fresnel reflectances of the film interfaces, n is the refractive index of the film, d is the physical thickness of the film, λ is the wavelength of incident light, and φ_{refl} is the phase change occurring upon reflection. Reflection at a medium of higher refractive index gives a phase shift of π.

If dispersion effects are neglected, the reflectance pattern can be described by a cosine function in the wavenumber space. Any change in thickness of the thin film will cause a change in the reflectance spectrum. Changes in the thickness of the thin film can be determined from the reflectance spectrum with high resolution. The use of the RIFS principle for monitoring dynamic processes at interfaces became feasible with the development of simultaneous (diode array) spectrometers. These devices allow the acquisition of complete spectra with considerable speed and with high wavelength reproducibility, as no moving parts are involved. Currently, miniaturized diode array spectrometers are emerging that further stimulate the use of spectroscopic techniques.

The RIFS transducer measures changes in the thickness of a thin transparent film. Binding of analyte molecules to the surface of the interference film leads to the formation of an adlayer of organic matter. The formation of this adlayer can be monitored with the RIFS transducer if the refractive indexes of the forming adlayer and the interference film are similar. Most organic substances, including proteins, have a refractive index higher than that of water ($n_D^{20} \approx 1.333$). Thin and stable transparent films can be made from polymers or inorganic substances. For example, silica films with $n_D^{20} \approx 1.45$ can be easily produced. The binding of an analyte molecule to the surface of such a thin film leads to a localized and discrete change in thickness. Despite this fact, a smooth increase in thickness will be observed as long as the size of the binding entities is less than the wavelength of incident light.

EXPERIMENTAL SECTION

As model receptor ligand system, biotin (244 g/mol) and streptavidin (~60 000 g/mol) were chosen. One mole of streptavidin binds four moles of biotin, with a very high affinity constant, 10^{15} L/mol. This system was chosen for two reasons: (1) the high affinity constant ensures almost irreversible binding of the ligand to the receptor and (2) the availability of four binding sites per receptor molecule allows noncovalent immobilization of streptavidin by biotin−avidin interaction. This provides a rapid and facile preparation of high-affinity surfaces with high binding capacity and avoids tedious and laborious covalent surface chemistry. Biotin, biotin−protein conjugate (bovine serum albumin conjugate, ~15 mol of biotin per mole of protein), and polymerized streptavidin were obtained from Boehringer Mannheim.[13]

Binding curves were recorded by using a setup (Figure 2) consisting of an infrared filtered tungsten light source (20 W, stabilized) and a diode array spectrometer (Zeiss MCS 410, 512 diodes, 335−779 nm) connected by a 2:1 fiber-optic coupler (1 mm PMMA, MicroParts, Dortmund, FRG). Interference films (500 nm SiO_2 as interference layer deposited on top of 10 nm Ta_2O_5 for reflection enhancement) were deposited by Schott/

(13) Berger, M.; Deger, A.; Maler, J. Eur. Patent 0 331 127 B1, 1993.

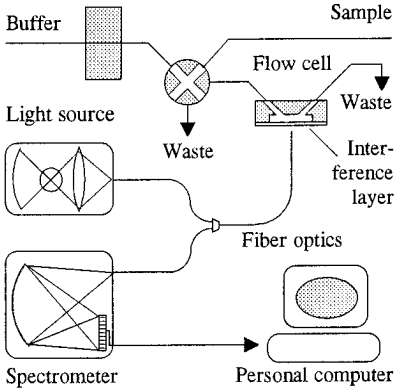

Figure 2. Setup for monitoring binding events with RIFS.

Table 2. Protocol for Ligand Binding Assays

time (s)	action
0−150	rinsing of flow cell with PBS; recording of prerun baseline
150−200	rinsing of flow cell with sample solution; recording of binding event
200−350	rinsing of flow cell with PBS; recording of postrun baseline
350	start of next incubation cycle or end of measurement

Mainz on 1 mm float glass by plasma-induced chemical vapor deposition. The coated glass was cut into pieces of 15 × 15 mm^2 prior to use. Glass carriers were mounted on a flow cell with a depth of 50 μm and a volume of 150 nL, with the interference layer facing the cell lumen. Sample handling was carried out with an Ismatec ASIA-FIA (500 μL injection loop). Process control, acquisition of reflectance spectra, and data evaluation were done with software we wrote on a PC running under Windows.

For each point of the binding curve, 15 spectra were acquired at an integration time of 290 ms and summed. The position of the reflectance minimum at 550 nm was determined by polynomial regression and converted to an optical thickness value. Thickness values were determined every 5 s.

Interference films were cleaned in freshly prepared, hot H_2SO_4/H_2O_2 (6:4) and excessively rinsed with water. A hydrophobic surface was generated by treatment with dichlorodimethylsilane (1% in 1,1,1-trichloroethane). Biotin was introduced to the surface by adsorption of a biotin−protein conjugate for 1 h from a solution containing 100 μg/mL conjugate in phosphate buffered saline pH 7.4 (PBS). Subsequently the layers were rinsed with PBS and mounted on the flow cell. Streptavidin binding to the surface was monitored online with the RIFS method. To ensure a high amount of binding sites at the surface, polymerized streptavidin (poly-SA) was used. Binding of this polymerized protein leads to cross-linking of the biotin conjugates adsorbed in the first step, thus increasing the stability of the protein layer at the surface. At the same time, an excess of biotin binding sites is ensured. Poly-SA was injected at a concentration of 100 μg/mL and a flow rate of 35 μL/min for a period of 500 s. Afterward, the flow cell was rinsed with PBS for 200 s.

All incubations of the resulting surface with low molecular weight analytes were carried out at increased flow rate (100 μL/min) and followed the scheme given in Table 2.

Between experiments, the system was cleaned by subsequent injection of 500 μL of methanol/water (1:1) and 500 μL of streptavidin (100 μg/mL in PBS). Without these cleaning steps, we failed to record biotin binding effects. Biotin was injected at concentrations from 4 nM to 4 μM in PBS. As a nonspecific ligand of comparable size and charge, benzoic acid was injected at a concentration of 40 μM.

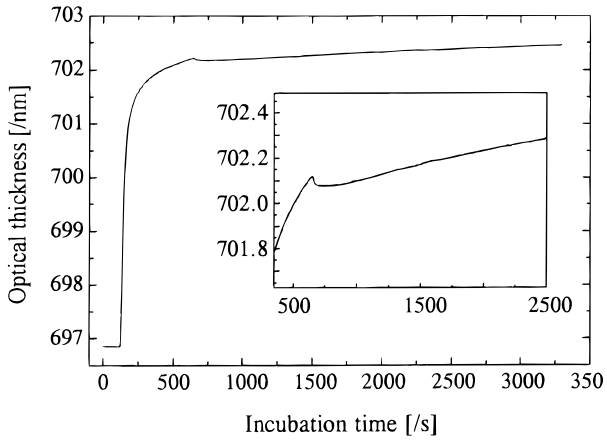

Figure 3. Binding of poly-SA to an interference film pretreated with biotin−BSA. The enlarged inset shows the effects after rinsing of the flow cell.

RESULTS AND DISCUSSION

The loading of the surface with poly-SA resulted in a fast increase in optical thickness by about 5 nm under the conditions used (Figure 3). The binding rate decreases quickly with increasing surface coverage, but saturation of the surface was not reached within the incubation period. After the incubation period, the rinsing of the cell with buffer leads to a transient decrease (15 pm) of optical thickness, followed by a long-lasting drift of the optical thickness to higher values (~0.1 pm/s). The washout effect at the onset of rinsing can be ascribed to a small refractive index effect of the poly-SA and the removal of protein adsorbed loosely to the poly-SA surface. The long-lasting drift was only observed for the system under investigation. The binding of high-affinity antibodies to hapten-modified surfaces resulted always in a stable baseline after binding and subsequent rinsing of the cell (data not shown). It is assumed that the drift effect is due to the multivalent nature of the interacting molecules. The poly-SA layers show high affinity for biotinylated compounds. Therefore, it is clear that biotin binding sites remain unoccupied during the loading of the surface. The same will hold for a fraction of the biotin residues from the biotin−BSA conjugate adsorbed in the first step. After the fast initial binding of poly-SA, the reaction of the remaining reaction partners is restricted by sterical hindrance in the cross-linked layer. Slow reorientation processes in the protein layer allow further binding events to happen. In consequence, the layer becomes more cross-linked and therefore more compact. This leads to reduced physical thickness of the layer and increased refractive index. While the optical thickness (physical thickness times refractive index) is not affected by this process, the increasing refractive index leads to better matching of the optical constants of the protein layer and the silica layer. In consequence, the RIFS transducer detects a higher fraction of the real optical thickness of the protein layer.[14] Two further observations are in agreement with this theory. (1) The drift process was slowed down or stopped upon incubation of the poly-SA layer with high concentrations of biotin. (2) The biotin binding

(14) Brecht, A.; Gauglitz, G. *Fresenius J. Anal. Chem.* **1994**, *349*, 360−366.

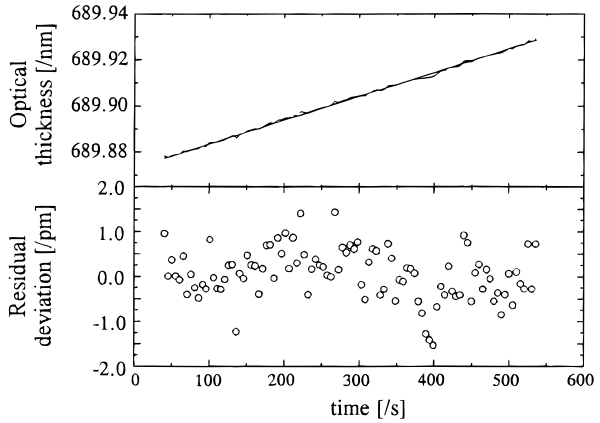

Figure 4. Baseline measurement and residual deviation (noise) in optical thickness after linear regression.

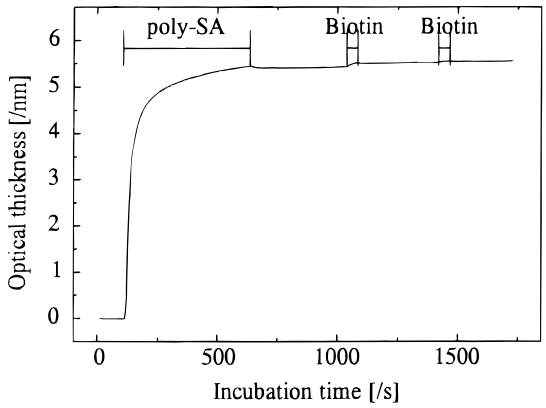

Figure 5. Full test cycle. Loading of the surface with poly-SA (100 g) and two subsequent injections of ligand (400 nM biotin).

capacity of the poly-SA layers decreased with time. This indicates that biotin binding sites are involved in the drift effect. As the drift was almost linear for limited intervals of time, a simple linear approximation was sufficient to account for this effect.

The quality of the thickness data was estimated from the thickness versus time curve obtained from a chip after the binding of poly-SA (cf. protocol given above) under the same flow conditions used for ligand binding experiments. The curve was corrected for drift by linear regression, and the residual noise in optical thickness was calculated. The residuals plot (Figure 4) shows only a little systematic variation and a peak to peak noise of <3 pm. A statistical analysis results in a root-mean-square baseline noise of 0.56 pm.

Ligand binding was investigated according to the protocol given above. By injection of a salt solution with different refractive index, the concentration profile of the ligand in the flow cell was estimated. Six seconds after the beginning of the injection period, the concentration in the flow cell began to rise. Six seconds after the end of the injection period, the concentration reached a peak and returned within 45 s to the baseline value (data not shown). The resulting net incubation time is about 90 s.

The binding of biotin was investigated at concentrations ranging from 4 μM (1 mg/L) down to 4 nM (1 μg/L). A binding curve from a typical experiment is shown in Figure 5. The effects caused by ligand binding are small compared to the streptavidin effect. Therefore, in all subsequent figures, only the ligand incubation is shown. Biotin at a concentration of 4 μM caused a net thickness increase of about 70 pm (Figure 6). After the first biotin incubation, the drift is significantly reduced. During the

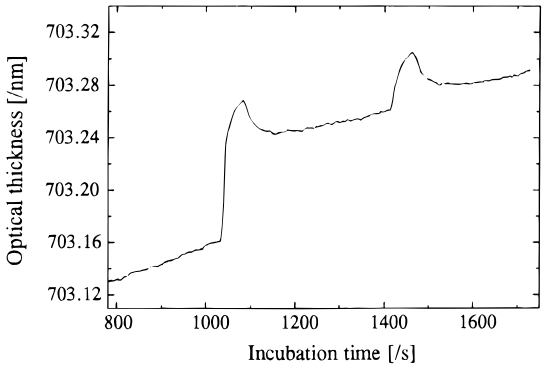

Figure 6. Binding of biotin (4 μM) to poly-SA surface (two incubations shown).

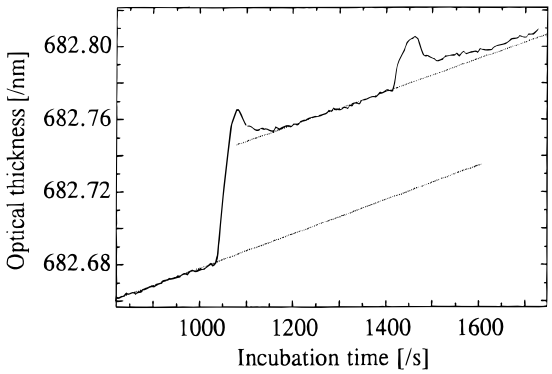

Figure 7. Binding of biotin (400 nM) at poly-SA surface (two incubations shown).

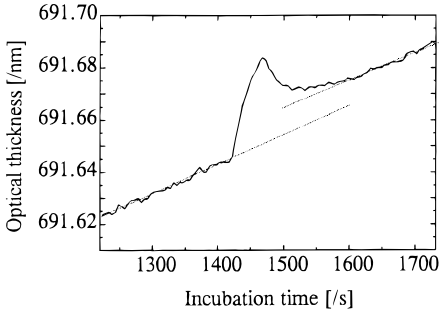

Figure 8. Binding of biotin (40 nM) at poly-SA surface (one incubation shown).

incubation, a transient increase in thickness occurs. Thorough investigations indicate that this is caused by differences in the composition of the ligand solution and the working buffer. Substances not yet identified seem to be introduced from glassware during sample handling. The second biotin incubation causes only the transient increase in thickness, but no net effects were observed. The incubation of a poly-SA layer with 400 nM biotin resulted in a net increase in thickness of about 60 pm (Figure 7). No significant change in baseline drift was introduced. A second injection of biotin (400 nM) caused another slight increase in thickness of about 5 pm. The lowest concentration of biotin resulting in a significant net change in thickness was 40 nM (Figure 8). However, only about a 10 pm thickness increase was observed at that concentration. At 4 nM biotin, only a transient effect was observed. To prove that the thickness increase observed during biotin incubation was a specific effect, benzoic acid was injected as another small organic compound with a free carboxylic acid moiety (Figure 9). At 40 μM benzoic acid, no net binding effect was observed, indicating that the biotin

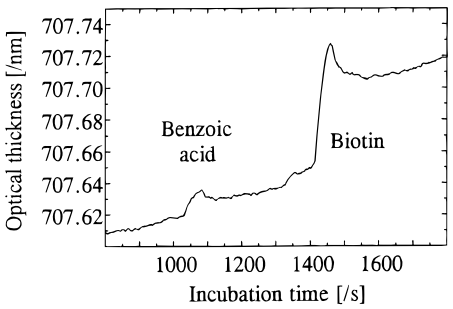

Figure 9. Incubation of poly-SA layer with benzoic acid (40 μM) and biotin (400 nM).

effects are due to specific molecular interaction. Biotin binding was not affected by the incubation with benzoic acid.

The maximum increase in optical thickness observed during biotin binding was about 70 pm after binding the equivalent of 5 nm poly-SA. Assuming identical effects on refractive index for biotin and protein, the effects can be compared on the basis of the ratio of the molecular masses. Streptavidin has a molecular mass of about 15 000 g/binding site. The molecular mass of biotin is about 1.6% of this value. The maximum biotin binding effect observed corresponds to 1.4% of the poly-SA value.

From protein binding studies with radiolabeled protein, a protein mass sensitivity for the optical thickness detected with the RIFS transducer of about 1 pm change in optical thickness (pg of protein/mm^2 of transducer surface) can be estimated.[15] A thickness increase of 70 pm corresponds to 70 pg/mm^2 or 2.9×10^{-13} mol of biotin/mm^2. The estimated area per available binding site is about 6 nm^2. Assuming a spherical diameter of 5 nm for streptavidin and a packing density of 1, a footprint of the molecule at a surface of 20 nm^2 results. As each streptavidin molecule offers up to four binding sites, this corresponds reasonably well to the surface area estimated per biotin binding site.

The decrease of the net thickness effect with decreasing biotin concentration may have several causes. At low concentrations, the equilibrium surface coverage for a ligand will fall below unity, a certain amount of ligand may be lost in the sample handling system, and only a fraction of the analyte reaches the transducer surface due to the limited residence time in the flow cell. The binding constant K for biotin to streptavidin is reported to be 10^{15} L/mol. Assuming Langmuir adsorption behavior, the equilibrium surface coverages Θ given by

$$\Theta = c/(c + 1/k)$$

at concentrations c of 10^{-15} and at 10^{-14} M biotin should be 0.5 and 0.9, respectively. The lowest concentration used is far above these values. Therefore, mass transport and sample handling must be responsible for the decreased binding observed. The total amount of biotin brought to the system at 4 nM, a flow rate of 100 μL/min, and an incubation time interval of 50 s is 3×10^{-13} mol. If all of this material was bound, one-third of the maximum thickness increase observed would be expected. Inevitably, some poly-SA will be adsorbed in the flow system during loading of the transducer surface. Therefore, the amount of biotin delivered to the flow cell at the lowest concentration is further diminished. The laminar flow rate of the analyte in the flow cell is about 17 mm/s. The residence time of a volume element in the flow cell is only about 100 ms, and only a fraction of the analyte in the sample will reach the transducer surface. From these considerations, it seems realistic to make the loss of analyte and the flow regime responsible for the empirical limit of detection found between 4 and 40 nM of biotin.

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CONCLUSION AND OUTLOOK

The investigation of biomolecular interaction and recognition processes is an important aspect of work in analytical chemistry and biochemistry. Of particular importance is the detection of binding events between an immobilized receptor compound of high molecular weight and a low molecular weight ligand. We have demonstrated that the RIFS transducer allows one to monitor binding effects during such an interaction. The maximum effects observed during the binding of biotin to a poly-SA layer exceeded clearly the limit of detection for the RIFS transducer. In this study, a root-mean-square noise level of 0.56 pm was reached. Thus, thickness effects below 2 pm can be resolved. This corresponds to surface coverages of a few picograms of organic matter per square millimeter. The lowest concentration of analyte giving an observable effect was about 1 order of magnitude above the theoretical limit. This is due to loss of substance and limited mass transport to the transducer.

A noncovalent modification strategy for the surface gave access to immobilized receptor layers with reproducible test performance and without delicate surface chemistry. The ratio of poly-SA binding and biotin binding effects is in good agreement with molecular weights and dimensions of the reactants. The atypical drift behavior of the poly-SA layers could be ascribed to slow reorientation and binding processes in the layer system.

The stability of the transducer system was excellent. All measurements could be done without any referencing. As the RIFS transducer responds only weakly to refractive index effects, no temperature control was required. Further improvements can be expected from improvements in the RIFS system and the sample handling. The RIFS performance will benefit from advanced spectrometers with increased dynamic range. Permanent referencing of the light source may also help to achieve even higher resolution. Improvements in sample handling will be aimed at the reduction of the transient injection effects. This will help to better discriminate binding effects from artifacts introduced by sample handling.

The level of performance achieved for optical transducers in the field of biospecific interaction analysis opens the door for a new and exciting chapter in research on molecular interaction effects of small molecules.

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