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Specific binding of low molecular weight ligands with direct optical detection

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Abstract: The characterization of low molecular weight ligand interaction with receptor molecules is of importance for the investigation of biological processes and for drug research. We report on the investigation of the binding of low molecular weight ligands to immobilized receptors by label-free detection. Reflectometric interference spectroscopy, an optical transducer which allows the monitoring of a few picograms per square millimetre changes in surface coverage, was used to study two model systems. In both cases detection of the binding event was successful. High affinity binding of biotin to immobilized streptavidin was clearly detectable at receptor surface concentrations as low as $1–2 \times 10^{10}$ binding sites/mm^2. Linear correlation between the receptor surface concentration and the response to biotin binding was observed. Using immobilized DNA, we investigated the binding of common intercalators with respect to kinetics and thermodynamics by evaluation of the association and the dissociation part of the binding curve. Bi-exponential increase and decrease of intercalator loading was observed, indicating complex interaction kinetics. The four structurally different intercalators showed significant distinction in binding kinetics and equilibrium signals. Improvement of experimental parameters is required to obtain more reliable kinetic data. © 1997 Published by Elsevier Science Limited

Keywords: direct optical affinity probe, low molecular weight ligands, DNA, intercalators

INTRODUCTION

Specific ligand–receptor interactions play an outstanding role in biological signalling and recognition. Toxic or therapeutic activity mainly bases on competitive interaction with those specific affinity interactions. Frequently, low molecular weight ligands (i.e. < 1000 g/mol) are involved, e.g. hormones, neurotransmitters, toxic or cancerogenic compounds and therapeutic drugs. Typical receptor molecules that bind low molecular weight ligands are not just of the class proteins but are frequently DNA and related compounds

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(Wang, 1987; Waring, 1981). The investigation of binding processes involving low molecular weight compounds is therefore of particular interest.

Studies of ligand binding at a receptor are usually carried out with labelled compounds. After a separation step the amount of ligand bound to the receptor is determined by quantifying the label. Thermodynamical characterization of the affinity interaction is achieved by such procedures. While labelling with dyes or with enzymes is possible for macromolecular ligands, for low molecular weight ligands radioactive isotopes are frequently required. Alternatives for such costly and tedious procedures are demanded.

During the last decade methods for label-free detection of affinity interactions have been studied. Various transducer principles have been investigated. Several of them are based on optical detection of changes in local refractive index due to the binding of matter at the transducer surface (e.g. Liedberg *et al.*, 1983; Lukosz, 1991; Cush *et al.*, 1993; Gauglitz *et al.*, 1993). A few commercial systems are available to date (BIAcore/Pharmacia Biosensor (now Biacore AB), IAsys/Fisons Affinity Sensors and BIOS-1/ASI). These direct detection methods allow assessment of thermodynamical and kinetical information of an interaction by monitoring of the binding event (Karlsson *et al.*, 1991).

As the optical methods are based on the change in refractive index the absolute amount of matter bound to the surface is the limiting factor. Therefore the molecular mass of the molecules detected plays an important role for the performance. Detection limits in the range of 1‰ of a monolayer (i.e. several picograms per square millimetre) and below have been reported for macromolecules (proteins, DNA, polymers >10·000 g/mol). The detection of low molecular weight ligand binding to an immobilized receptor is usually critical. The surface concentration of receptor binding sites determines the maximum loading by the low molecular weight ligand. The ratio between the molecular mass per binding site (20·000–100 000 g/mol) and the molecular mass of the ligand (100–1000 g/mol) is about 10^2–10^4. Only a few picograms per square millimetre are therefore expected to be bound by a receptor layer of several nanograms per square millimetre, which is close to the detection limit of such systems. Hence, interactions with low molecular weight ligands are mainly investigated by an indirect test format (inhibition assays): interaction between receptor and ligand in the liquid phase is probed by receptor binding at an immobilized ligand. This test format is efficient for assessment of affinity constants (Piehler *et al.*, 1997), but kinetic information is, if retrieved at all, retrieved indirectly (Karlsson, 1994). Avoiding such indirect test schemes is therefore an attractive opportunity for the characterization of low molecular weight ligand binding.

Recently, the feasibility of direct detection of low molecular weight ligand binding at an immobilized receptor by optical detection systems has been reported (Karlsson & Stahlberg, 1995; Piehler *et al.*, 1996). In this study we report on two different systems of low molecular weight ligands binding to an immobilized receptor. Our basic goal is the development of parallel detection systems for high throughput screening using label-free detection approaches. For this application the principal capabilities and limitations of those methods is a current topic of investigation.

The interaction between biotin (240 g/mol) and streptavidin (60 000 g/mol) was chosen as a model system for high affinity ligand binding to an immobilized protein. The affinity constant of the avidin–biotin interaction (10^{25} l/mol) ensures almost irreversible binding of the ligand. A simple, non-covalent immobilization method is feasible as a streptavidin molecule can bind four biotin molecules. A part of these binding sites can therefore be used for the attachment of the protein to the surface.

As a rather different system of low molecular weight ligand binding the interaction of intercalators with immobilized DNA was investigated. DNA intercalation is an interaction of medium affinity (10^5–10^6 l/mol) along the double strand with repetitive binding sites. The maximum loading of the DNA by an intercalator is therefore much higher than for the typical ligand–protein interaction. For this reason, inhibition assays are not practical and displacement assays are common (Pandey & Weetall, 1995). The information retrieved from such assays is complex as the interaction parameters of the displaced compound have to be taken into account. Furthermore, different binding sites exist for different types of interaction which can not be investigated in a single displacement assay. Direct detection of intercalator binding to DNA allows characterization of the interaction kinetics and thermodyn-

amics by monitoring the surface coverage during the incubation period.

MATERIALS AND METHODS

Materials

Polymerized streptavidin (pSA) and thermally treated conjugate of biotin and bovine serum albumin in a ratio of 15:1 (BSA-Bi) was kindly supplied by Boehringer Mannheim (Germany) (Berger *et al.*, 1993).

Salmon sperm DNA, nogalamycin, branched polyethylenimine (PEI, 50·000 g/mol) and biotin were purchased from Sigma, (Deisenhofen, Germany), actinomycin D, doxorubicin and sanguinarine from Fluka (Neu-Ulm, Germany). Poly (vinyl sulphate) (PVS) was purchased from Aldrich. The structures of the intercalators investigated are shown in Fig. 1.

Sheared DNA was prepared by sonication (Labsonic U, B. Braun) of DNA in solution (2 mg/ml) twice for 3 min. From agarose gel electrophoresis, the length of the DNA strands was determined to be between 300 and 3000 bp.

Transducer slides (10 nm Ta_2O_5 and 500 nm SiO_2 on a glass substrate) were produced by Schott (Mainz, Germany) in a PICVD process.

Detection system

Reflectometric interference spectroscopy (RIfS) is a highly sensitive transducer for label-free monitoring of binding events at immobilized receptor molecules. This technique and the experimental set-up have been reported previously in detail with respect to various applications (Gauglitz *et al.*, 1993; Brecht *et al.*, 1994; Piehler *et al.*, 1996).

White light interference at a thin silica layer is detected spectrally by a diode array spectrometer (MCS 410, Carl Zeiss, Jena, Germany). The changes of the optical thickness of the interference layer during biospecific interaction with an immobilized receptor are determined from the shift of the interference pattern. A change of 1 nm optical thickness corresponds to approximately 1 ng/mm^2 protein (Brecht *et al.*, 1996). A baseline noise below 1 pm r.m.s. has been demonstrated (Piehler *et al.*, 1996), allowing a detection limit of less than 3 pg/mm^2 protein. Sample handling in a flow through system was carried out by flow injection analysis (ASIA system from Ismatec, Wertheim-Mondfeld, Germany).

Surface modification

Prior to modification the transducer slides were cleaned in a freshly prepared mixture of 60% v/v

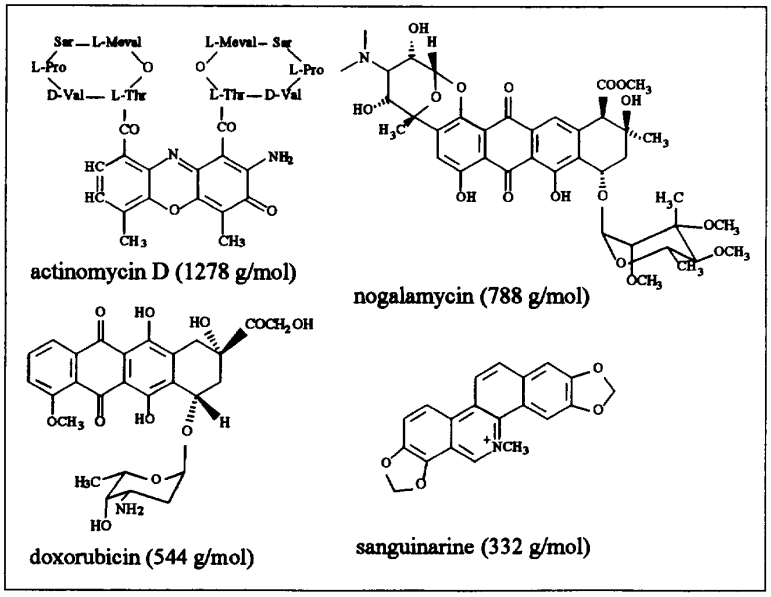

Fig. 1. Structures of the intercalators investigated.

concentrated H_2SO_4 and 40% v/v H_2O_2 and rinsed thoroughly with ultrapure water. Direct adsorption of DNA at these hydrophilic surfaces gave poor and instable loadings of less than 1 ng/mm^2. DNA is a negatively charged molecule under buffer conditions (pH 7·4) due to deprotonation of the PO_4^- units. We took advantage of this polyanionic property of the DNA molecule for the immobilization: the cleaned surface was pre-treated by adsorption of PEI (50 μg/ml), a highly charged polycation that binds strongly to the anionic silica surface (SiO^-). DNA (50 μg/ml) was subsequently adsorbed to the cationic modified surface. The incubation of both PEI and DNA was carried out in the flow cell and monitored by RIfS for estimation of the surface loading. As a reference layer of comparable charge properties the polyanion PVS was immobilized in the same way as DNA.

The immobilization of pSA was achieved by specific binding at adsorbed BSA-Bi. The cleaned surface was pre-treated by silanization with 2% dichlorodimethylsilane in 1,1,1-trichlorethane. BSA-Bi (50 μg/ml) was incubated to the surface for 30 min. The incubation of 50 μg/ml pSA was carried out in the flow cell and monitored by RIfS for the determination of the surface loading. By decreasing the incubation times, the surface coverage of pSA was systematically reduced for the investigation of low receptor surface concentrations.

All samples, including the polyions, were prepared in phosphate buffered saline at pH 7·4.

Incubation protocols

The incubation periods for the immobilization of the receptor molecules were adjusted to reach maximum coverage during the incubation step. PEI and DNA were incubated for approximately 100 s, pSA for approximately 500 s. Prior to the injection of the sample, the baseline was recorded for 120 s. The intercalators were incubated at a flow rate of approximately 70 μl/min for 4·5 min. Biotin (400 nM throughout all experiments) was incubated with a flow rate of 100 μl/min for 50 s. The flow rates were maintained during the injection of the sample to avoid interference with the sample handling.

RESULTS AND DISCUSSION

DNA–intercalator interaction

The immobilization of DNA at the PEI modified surface and the subsequent binding of the intercalator doxorubicin (10 μg/ml) are demonstrated in Fig. 2. For DNA a reproducible loading of 2·2 ± 0·2 nm was observed for this immobilization protocol. This corresponds to a surface concentration of 2 ng/mm^2 DNA or 2×10^2 base pairs/mm^2 (600 g/mol) assuming the sensitivity to changes in surface coverage as for proteins.

Fast and strong binding of the intercalator doxorubicin to the immobilized DNA is observed and saturation is reached within 1 min. After the incubation, fast dissociation is observed during a rinsing period, reaching the original baseline after 5 min. An intercalator loading of 300 pg/mm^2 ($\approx 3 \times 10^{11}$ molecules/mm^2) is estimated from the equilibrium signal during the incubation period. The maximum number of binding sites corresponds to the number of base pairs. The loading by doxorubicin indicates that more than one-tenth of these binding sites is occupied.

A significant problem in direct affinity detection is non-specific binding to the surface that can not be discriminated from specific interactions. As both polyanionic (PEI) and polycationic (DNA) molecules were attached to the surface, non-specific adsorption may become a critical point. Therefore, the specificity of the intercalator binding detected on the immobilized DNA was investigated by reference measurements at surfaces modified by adsorption of a polyanion of different structure. At PVS layers of about 1·2 ng/mm^2 immobilized in the same way as the DNA, no significant binding of doxorubicin was found (Fig. 3).

The binding effects for the four intercalators actinomycin D, doxorubicin, nogalamycin and sanguinarine at a concentration of 10 μg/ml are compared in Fig. 4.

All intercalators investigated bound reversibly to the immobilized DNA. Different equilibrium loadings are observed that are due to different molecular mass of the intercalators and interaction affinity constants. Differences in the dissociation kinetics of the different intercalators are significant. The feasibility of interaction kinetics analysis by monitoring of the intercalator binding is demonstrated in Fig. 5 for the binding of 10 μg/ml doxorubicin.

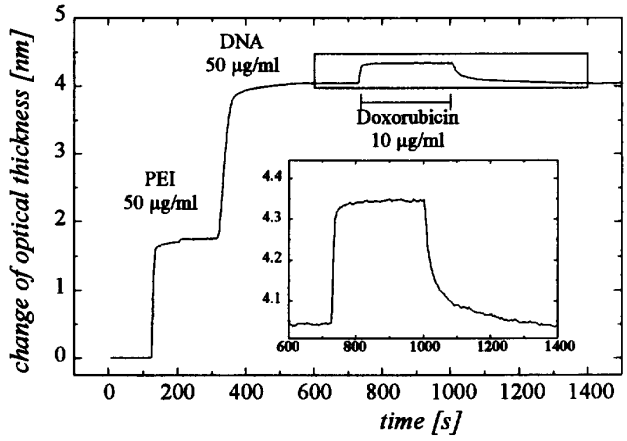

Fig. 2. Adsorption of PEI and DNA at a hydrophilic silica surface and reversible binding of the intercalator doxorubicin. The enlarged binding curve of the intercalator is shown in the inset.

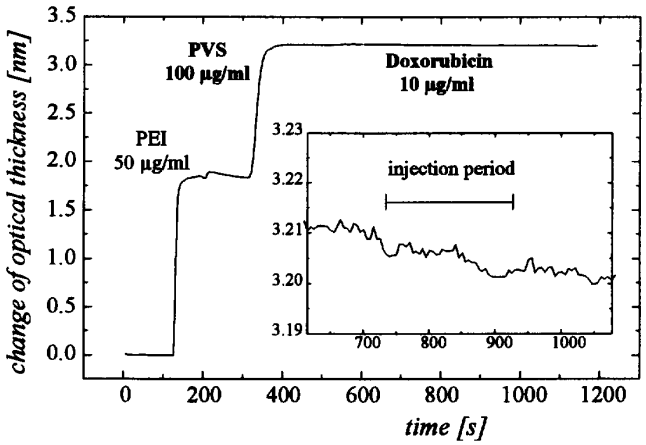

Fig. 3. Reference measurement at immobilized PVS. No binding of the intercalator doxorubicin is observed.

Bi-exponential curves were fitted to the association and dissociation parts of the curve because single exponential curves showed systematical deviations from the data points. Apparently, the binding kinetics of the intercalator at the immobilized DNA is not first order with a single rate constant. This effect can be explained either by a more complex binding process or decrease of the binding rate constant with increasing intercalator loading due to sterical hindrance. During the dissociation kinetics, rebinding processes are probable because of the high surface concentration of binding sites. The design of the experiments shown here does not allow interpretation of the rates determined for the lower time constants of the bi-exponential fit. The rate constants determined for the slower process are listed for four different intercalators in Table 1.

More detailed investigation of the binding process requires optimization of the experimental parameters: improvement of the flow conditions by higher flow rates, higher time resolution of detection (1 Hz) and, in particular, lower intercalator concentrations to reduce the binding rate. Thermodynamical characterization of the interaction can be carried out by the determination of equilibrium surface loading for various intercalator concentrations (adsorption isotherms). The affinity constant of the intercalation is then obtained from a Scatchard plot.

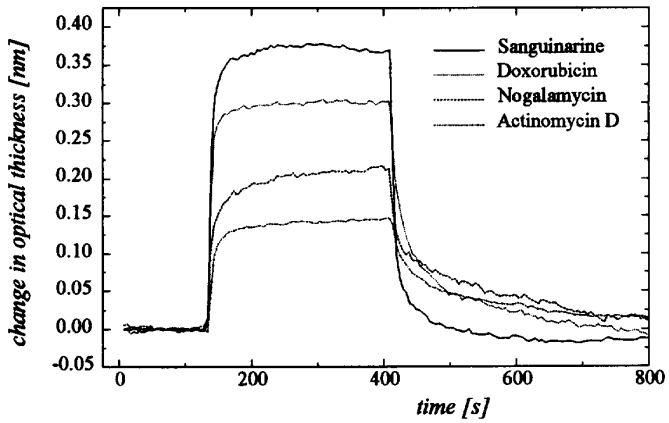

Fig. 4. Binding of various intercalators at immobilized DNA.

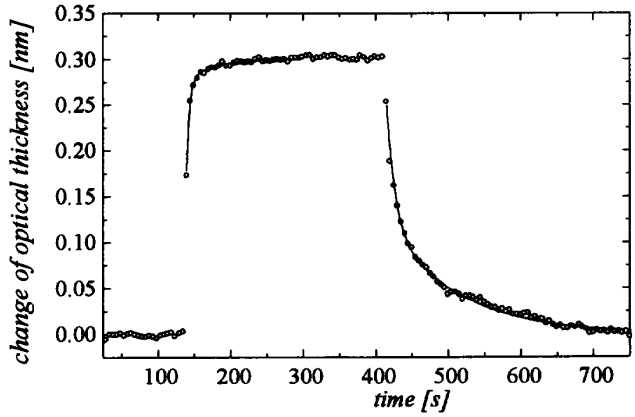

Fig. 5. Binding kinetics analysis of doxorubicin binding curve by a bi- exponential fit to the binding curve.

TABLE 1 Association and dissociation rate constants obtained for intercalator binding at immobilized DNA

Intercalator	*Association*			*Dissocation*
	k_1	c (μM)	k_{ass} ($mol^{-1}\,s^{-1}$)	k_{diss} (s^{-1})
Doxorubicin	0.024	17	820	0.010
Actinomycin D	0.018	30	370	0.007
Nogalamycin	0.018	89	150	0.005
Sanguinarine	0.030	95	80	0.022

Biotin binding at immobilized pSA

Binding of pSA at surfaces pre-treated with BSA-Bi is shown in Fig. 6. Rapid binding is observed due to the strong avidin–biotin interaction. After rinsing, a reproducible drift is observed, probably caused by the rearrangement of the pSA layer on the first protein layer (Piehler *et al.*, 1996). Linear correction of this drift within the short time-scales of the biotin incubations was adequate for the determination of the biotin binding. After the first incubation of biotin a significant, non-transi-

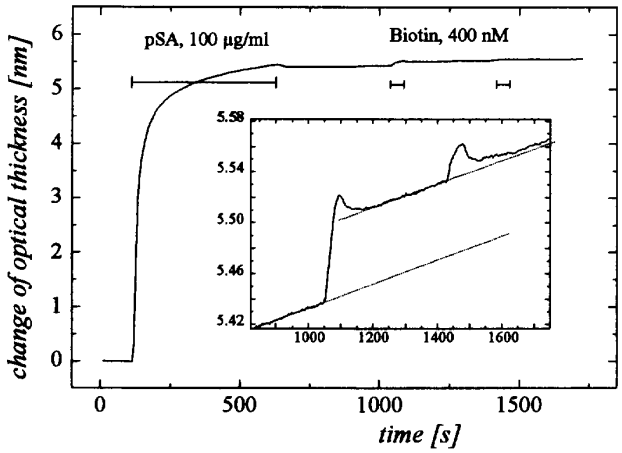

Fig. 6. Binding of pSA at adsorbed BSA-Bi and two sequential incubations of biotin. The response during interactions with biotin are enlarged in the inset.

ent change of the optical thickness is observed due to nearly irreversible binding of biotin to the streptavidin binding sites.

Further investigations of this ligand–receptor system were carried out by reducing the surface concentration of the receptor. The response due to binding of biotin at a pSA concentration of 1 ng/mm^2 (1 × 10^{10} molecules/mm^2) is shown in Fig. 7. The increase in optical thickness of 7 pm is clearly above the detection limit of the experimental set-up.

We found a linear correlation between the loading by pSA and the response by biotin binding with a slope of 6·4 pm biotin response per nm pSA response. Assuming the same sensitivity for pSA and biotin mass loading, an average number of 1·5 molecules biotin (240 g/mol) per streptavidin molecule (60 000 g/mol) was calculated from this slope. This indicates that two to three of the four binding sites per streptavidin molecule are not accessible by biotin, probably because they are occupied during the immobilization step.

SUMMARY AND CONCLUSIONS

The results presented in this paper demonstrate that detection of low molecular weight ligand binding and investigation by RIfS is feasible. Two very different systems have been studied.

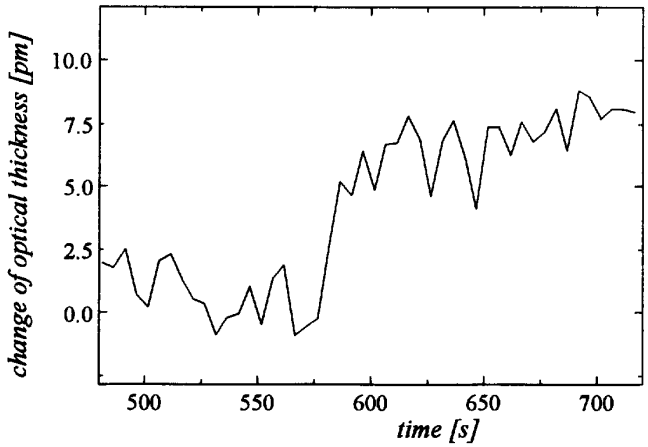

Fig. 7. Binding of 400 nM biotin at a receptor concentration of approximately 1 ng/mm^2 pSA.

Low affinity binding of various intercalators at immobilized DNA was detected. Monitoring of intercalator binding allow kinetic and thermodynamical characterization of the interaction. Biotin binding was detected down to receptor concentration of 1–2 × 10^{10} binding sites/mm^2 (i.e. one binding site per 100 nm^2). This is significantly below the surface concentrations of binding sites expected for a typical monolayer of protein receptor molecules (e.g. IgG).

At these low signals, interferences by sample composition (changes in pH or refractive index) can be critical and have to be avoided by careful sample handling. If this is properly taken into account we think that direct detection of low molecular weight ligands is also feasible in a parallel screening set-up. As the diffusion rates of these compounds exceed the diffusion rates of macromolecules significantly, sample handling under stopped flow conditions is suitable. This opens up a new and interesting field in direct affinity interaction analysis.

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