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TECHNICAL REPORT

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Comparison of reflectometric interference spectroscopy with other instruments for label-free optical detection

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Abstract On the basis of kinetic measurements of biomolecular interactions, a reflectometric interference spectroscopy (RIfS) setup is compared with two commercial instruments. These instruments are based on evanescent wave techniques, surface plasmon resonance (SPR) (represented by BIAcore 2000) and resonant mirror (RM) technique (using IAsys plus). All methods allow a label-free and time-resolved optical detection of biomolecular interaction.

These methods are mainly used in biomolecular interaction analysis (BIA). They provide practical techniques for quantifying equilibrium constants and rate constants over several orders of magnitude.

The general parameters of the three detectors, namely baseline noise and drift as well as overall sensitivity and limits of detection were compared. The fluid handling and the related implications on the measurements have also been considered.

The interaction between thrombine and thrombine inhibitor (TI) was investigated as a test system with the three different methods and the kinetic rate constants were determined and compared. For this TI was immobilized on the surface and binding of thrombine was monitored time-resolved. Determination of the kinetic rate constants could prove that the RIfS set-up is comparable with SPR using BIAcore 2000 and RM technique represented by IAsys plus.

Keywords Label-free detection · Reflectometric interference spectroscopy · Biomolecular interaction · Thrombine · Thrombine inhibitor

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Introduction

Biomolecular interaction analysis (BIA) is a term which was established a few years ago by the Swedish Company Pharmacia Biosensors of Sweden (now Biacore). It comprises the investigation of bio-molecular binding events in real time without labelling by an optical detection system.

The advantage of the technology is that it provides a direct and rapid readout of both the affinity and the kinetics of interaction [1, 2]. The absence of labels means little or no time-consuming pre-treatment of the components. Optical sensors work continuously, reversibly and efficiently. Principles in optical detection and applications are well described in the literature [3, 4, 5].

Reflectometric interference spectroscopy (RIfS) as a direct optical detection method is compared with surface plasmon resonance (SPR) and resonant mirror (RM) techniques, represented by two commercially available instruments, BIAcore 2000 and IAsys plus.

Each of the three methods has been successfully used for time-resolved detection of biomolecular interactions as many references show, SPR using BIAcore [6, 7], RM using IAsys [8, 9] and also RIfS [10, 11, 12], even in detecting binding affinities of low molecular weight analytes [13].

The main objective in this study was to show that the RIfS set-up fits well into the context of BIA and that it is suitable for evaluation of biomolecular interactions and determining kinetic constants.

The main components, a sample handling unit, a transducer with a sensing layer and the detection device received attention throughout this paper. Fluid handling authoritatively determines the reproducibility of the measurements and the detection device influences the noise and consequently the limit of detection (LOD). The sensing layers should be suitable for the particular application and also highly sensitive and specific. Altogether each component can affect the merits of the instrument in a certain way and should be considered in this study.

A short description of the commercial instruments should show their advantages and limitations and the RIfS

detection method used will be described in a little bit more detail, because it is not as well known as the other principles.

The detection principle of the BIAcore instrument belongs, as do the RM techniques, to the refractometric techniques. Beyond the critical angle, light will be totally internally reflected at interfaces with different refractive indices. When this occurs, an electromagnetic field – an evanescent wave – passes from the interface to the lower refractive index medium, where it decays exponentially. The resonance angle at which the evanescent field is highly enhanced is extremely sensitive to the refractive index at the sensor surface.

Surface plasmons are excited at the surface of a thin metal layer (Au in the case of BIAcore) while the evanescent wave interacts with free oscillating electrons. Energy from the polarized incident light is lost to the metal film at a well-defined angle of incidence depending on the refractive index of the medium beyond the chip surface [14].

Considering the fluid handling, BIAcore 2000 has a sophisticated, automated microfluidic system which is an integral part of the biosensor. It has a very low dead volume and rapidly takes the sample to the sensing surface, a constant and highly reproducible flow driven by highly precise syringe pumps. It allows a consumption of sample between 30 and 80 μL/cycle by a microfluidic cartridge system. Up to four channels can be used, so one of the channels is mostly utilized as an in-line reference. Samples containing macromolecular aggregates or entire cells could cause problems by plugging up the microchannels.

BIAcore is provided with a variety of surfaces for different applications. The most recommended and used surface for the commercial systems is the carboxymethylated dextran layer. It is a hydrophilic, three-dimensional, high surface area matrix, bearing charged derivatizable carboxylate groups. It is used for most of the kinetic measurements and evaluation of rate constants described in the literature [15, 16]. These binding sites within the three-dimensional layers provide different accessibility for ligands and therefore no homogeneously distributed binding kinetics.

RM, represented by IAsys plus, uses a dielectric coupling layer of high refractive index on an integrated prism as a waveguide. It is separated from the prism by a low index layer, which allows a coupling into the resonant layer for certain incident angles. The angles of excitation are sensitive to changes in refractive index [17].

IAsys plus operates with a special cuvette system which contains sample container and sensing layer. To avoid diffusion limitation and ensure rapid sample mixing it includes a microstirrer. Its sample consumption is about 150 μL/measurement. Also, “dirty” samples can be applied without plugging the chamber. The cuvette includes two chambers, so one could be used as a reference cell.

A disadvantage could be the user-dependent sample handling in case of the lack of an autosampler. The sample has to be injected manually into the cuvette.

Considering the available surfaces, the same sensing layers as the BIAcore instrument supplies are provided and recommended.

Both evanescent field methods are strongly affected by slight changes in temperature, so a highly sophisticated instrumentation for referencing and temperature stabilisation is required.

RIfS, as a very simple and successful approach, supplies a very specific method giving cheap, robust and reliable sensor elements. This reflectometric technique determines the apparent optical thickness ($n \times d$) of a thin layer by measurements of interference of white light caused by partial reflection at interfaces with a distance of approximately 1 μm. Any change in optical thickness causes a shift in the resulting interference pattern [18].

In detail, a thin transparent film passed by a light beam will cause at each of the interfaces a multitude of reflected beams. A light beam passing a weakly reflecting thin film will be reflected in part at each of the interfaces. 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, as shown in Fig. 1.

The use of a simultaneous spectrometer allows fast acquisition of the reflectance spectra. Any change in optical thickness will lead to a change in the interference spectrum. A resolution of optical thickness ($n \times d$) of about 0.5 pm has been achieved [18]. Drift measured with the set-up normally ranges smaller than 10 pm/h.

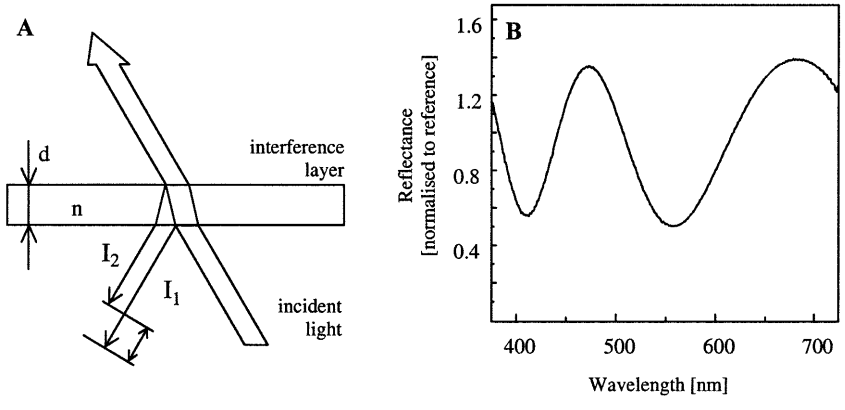

Fig. 1 **a** Principle of the detection of affinity interactions by reflectometric interference spectroscopy (RIfS) (*n* and *d* are the refractive index and the physical thickness of the layer, I_1 and I_2 intensities of the light beams, reflected at the interfaces of the layer). **b** Spectral reflectance pattern due to constructive and destructive interference of the reflected radiation

In comparison to the described refractometric methods, the reflectometric detection method selectively identifies physically attached material on a surface [19] with no need of referencing or temperature control unit.

The liquid handling for the RIfS system, providing a single flow through cell, suffices because there is no necessity of signal referencing. The continuous flow is gained by a robust fluidic system with either syringe pumps or peristaltic pumps, which means a sample consumption of 200 µL and 500 µL respectively. Agglomerated samples are not supposed to cause any problems by plugging up the cell. A disadvantage could be the dead volume of 20 µL causing dispersion of the sample plug.

Also, there are suitable sensing layers for various applications. For kinetic measurements a plain, two dimensional sensing layer is recommended, which shows homogeneous accessibility of binding sites and for that reason a homogeneous kinetic behavior over the whole detection area [20].

The data obtained with BIA can be used to derive unambiguous answers to many qualitative questions; the quantitative kinetic analysis is often difficult [21]. In some cases there were multiphasic association and dissociation sections found. The deviations were attributed to heterogeneity of the binding sites [22, 23]. So the sensing layer of the transducer plays an important role.

In this study the interaction of thrombine and a thrombine inhibitor (TI) is observed. TI was immobilized to the surface and the binding of thrombine was monitored resolved in time. Comparing general parameters such as noise and drift and the evaluation of kinetic rate constants should prove that determination of kinetic rate constants obtained from the RIfS set-up is comparable with those received from SPR and RM instruments.

Material and methods

Devices

For the SPR-based measurements a BIAcore 2000 device with autosampler of the company Biacore (Uppsala, Sweden) was used; Sensorchips CM5 (carboxylate dextran surface) were also delivered from there.

The RM technique was performed by IAsys plus of the company Labsystems (Affinity Sensors division). Carboxylate dextran (CMD)-modified cuvettes have been supplied from there.

For the RIfS set-up components from several companies were used:

1. Flow injection analysis device ASIA from Ismatec, Wertheim.
2. Diode array spectrophotometer SPEKOL, Analytik Jena, Germany, modified according to [10] with a halogen reflector lamp from Welch Allyn, New York, USA, and a polymer fibre of PMMA 1 mm diameter from Ratioplast, Optoelectronics, Löhne, Germany.
3. Optical transducers consisting of float glass coated with an interference layer system (10 nm Ta_2O_5/330 nm SiO_2), obtained from Schott, Mainz, Germany. The term "transducer" provides the additional information, that a physical property of the used surface, here the interference-layer, is used to obtain the physical measurement parameter. Therefore the word "chip" is not used in this context; nevertheless it could be used as a synonym.
4. The flow cell has the following dimensions 4×1×0.05 mm^3, < 200 nL.
5. Microcal Origin, version 5.0, Northampton, UK, was used to evaluate kinetic data.

Materials

Chemicals and biochemicals were either from Sigma (Deisenhofen, Germany) or Fluka (Neu-Ulm, Germany). The TI (MW 393 g/mol) was kindly put at our disposal by Dr. Friedrich, BASF, Ludwigshafen, Germany. Polyethyleneglycol (MW 2000 Da) was from Rapp Polymere, Tübingen.

GA (Glutaric anhydride) was used to synthesize diamino-polyethyleneglycol (DA-PEG)-GA surfaces by carboxylation of DA-PEG-modified surfaces. Phosphate buffered saline (PBS) consisted of 150 mM sodium chloride and 10 mM potassium phosphate at pH 7.4. Ultrapure water (18.2 MOhm·cm, SG, Barsbüttel, Germany) was used throughout this study.

Regeneration buffer used with BIAcore was TMM (2%Triton, MgCl2 : Maleate:Tris, 1:1:1, 1 M NaCl), pH=6.8. Sodium acetate buffer (NaAc) consisted of 10 mM sodium acetate in ultrapure water at pH 4.6. For the activating solution 100 mM *N*-hydroxysuccinimide (NHS) (MW 115.1 g/mol) was diluted in ultrapure water and 400 mM *N*-ethyl-*N'*-dimethylaminopropylcarbodiimide hydrochloride (EDC) (MW 191.7 g/mol) was diluted in ultrapure water also. For activating surfaces both solutions were freshly mixed, 1:1. Blocking solution was ethanolamine in water 1 M, pH 8.0.

Surface chemistry for RIfS transducers

Pre-treatment of surfaces. All transducer chips (1.2×1.2 cm^2) were cleaned and modified with a reactive silane derivative by the same procedure. Chips were first cleaned in NaOH in an ultrasonic bath for 2 min, then washed with tap water. The surface was mechanically cleaned and dried with Kim Wipes. Freshly prepared Piranha solution (concentrated H_2SO_4 and H_2O_2 (30%), mixed 7+3) in an ultrasonic bath for 15 min was used as the final cleaning step and to ensure a surface rich in reactive silanol groups (security advice: freshly prepared Piranha solution is extremely hot and aggressive. Use a fume cupboard and appropriate security means). Afterwards, transducers were washed thoroughly with ultrapure water and dried in a nitrogen stream. To generate a surface provided with amino-groups for coupling, the transducers were covered first with 10 µL 3-glycidyloxypropyl-trimethoxysilane (GOPTS) for 1 h, followed by cleaning with dry acetone and drying in a nitrogen stream, and immediately after this pre-treatment 30 µL of a solution of DA-PEG in dimethylsulfoxide (4 mg/1 mL) was placed on each transducer and melted at 70 °C overnight. Afterwards the transducers were rinsed thoroughly with ultrapure water and dried. For converting amino functions into carboxylate groups, 10 µL of a GA/*N,N*-dimethylformamide (DMF) solution (2 mg/1 µL) were put onto each transducer and than a second transducer was used to cover (sandwich technique). The reaction needed 6 h within a DMF saturated chamber. Then the transducers were washed with DMF and ultrapure water and were dried.

Immobilization of TI on the carboxylic surfaces on the RIfS transducer. On the carboxymethylated surfaces, 10 µL of a TI/DMF/diisopropylcarbodiimide solution (1 mg/10 µL/1.5 µL) were placed and sandwiched with a second transducer. After 6 h in a DMF saturated chamber the transducers were rinsed with DMF and ultrapure water and dried.

Characterization of immobilized TI with RIfS. Transducers were characterized for maximum specific binding of thrombine by a sample concentration of 50 µg/ml and reached a maximum signal of 500 pm, which is very low. A low capacity of binding sites is an advantage to kinetic measurements, because measuring near the limit of capacity reduces the probability of rebinding. Determination of dissociation rate constants is more precise.

For non-specific binding of another protein (below 10 pm), ovalbumin from chicken egg was injected. For proof of the stability of the immobilized TI layer,l several regenerations were carried

out. RIfS as a label-free optical detection technique allows the monitoring of real time changes in the optical thickness (physical thickness d×refractive index n) of a thin transparent interference layer coated to a glass carrier. The interference layer was exposed to the sample, while it was illuminated with white light through the glass carrier from the back side. Interference effects lead to a characteristic spectral modulation of reflected intensity, which was recorded by a diode array spectrometer. Changes in the reflectance pattern allow on-line detection of binding events [24, 25, 26], with a resolution down to some picograms per millimetres squared [10, 27]. The set-up used for RIfS-measurements consisted of a 20 W tungsten halogen reflector lamp (Welch Allyn, New York), a bifurcated HCP silica fibre coupler (600 μm fibre diameter) and a diode array spectrometer MMS Spekol (Analytik Jena, Jena, Germany). The transducer chips were mounted to a flow cell (4×1×0.05 mm^3, <200 nL) and matched to the fibre with glycerol. A FIA system (ASIA, Ismatec, Zürich, Switzerland) was used for sample handling.

The maximum specific binding capacity of transducers was characterized with solutions of 50 μg/mL of thrombine in PBS: 1 mL of sample solution was loaded into the sample loop (0.8 mm inner diameter, 800 μL) of the liquid handling system and injected with an initial flow rate of 240 μL/min (10 s) followed by a flow rate of 100 μL/min (200 s). Subsequently the flow cell was rinsed with PBS at 240 μL/min (125 s). For the regeneration of the transducer surface it was rinsed with a pulse of low pH (0.01 M HCl, 250 s, 100 μL/min). Non-specific interactions were checked using the same injection protocol with 100 mg/mL of ovalbumin.

During all steps of the incubation the reflectance spectra were recorded continuously and averaged over time intervals of 5 s. From the resulting interference spectra the apparent change in optical thickness was calculated on-line. For binding experiments the signal shift from the pre-run baseline to the signal after binding and rinsing was determined. For proteins a change in apparent optical thickness of 1 pm corresponds to a surface loading of about 1.6 pg/mm^2, which is calibrated by measurements of the specific interaction between immobilized biotin and ^{125}I-labelled streptavidin. A publication regarding this is still in preparation.

Immobilization of TI on BIAcore chips. Immobilization of TI on BIAcore chip CM5 was carried out on-line. The chip has a carboxylate-modified surface as described above. TI (2.1 mg) was dissolved in 100 μL NaAc buffer. After activation of the surface by a 200 μL pulse of EDC/NHS (1:1), the injection block was washed with running buffer. TI solution (80 μL) was injected at a flow rate of 10 μL/min. Subsequently the surface was blocked by a 70 μL pulse of ethanolamine solution. After this treatment the chip was regenerated with 20 μL of 1 M NaCl solution.

The chip was given the ability to immobilize targets on four different spots. Every spot can be accessed individually during immobilization. During measurements any desired spot can be switched in a row and used in parallel. So the first spot was chosen for the reference measurement and the second one used for immobilisation of the considered compound. So every sample hit the reference spot first and afterwards the measuring point in order not to reduce the concentration of the sample before measuring.

Immobilization of TI in IAsys cuvettes. Every cuvette consists of two measuring chambers. One was used as reference cell and the other as measuring cell. For referencing the signal in both cells the same liquid was injected but only in the measuring cell TI was immobilized.

For measurements of thrombine kinetics with RM a CMD cuvette was used. In one of the two cuvette cells the immobilization was accomplished. The other was used as reference cell. In order to change the liquid in the cell completely every injection was carried out three times. The liquid (always 50 μL) was handled with a pipette and the cuvette was sucked dry automatically. Stirrer speed used was always 100 times /min and the immobilization was carried out on-line.

After activating with EDC/NHS within 7 min the surface was washed with PBS and afterwards changed to acetate buffer. Ten microlitres of TI solution (1 mg/ml in acetate buffer) was added for 5 min and washed with PBS buffer. Subsequently the surface was blocked by injection of ethanolamine for about 3 min. Then the cuvette was rinsed twice with NaCl (1 M in utrapure water) and in between with PBS.

Measurement protocols

RIfS measurement protocol. For binding measurements of thrombine to immobilized TI several sample concentrations were injected into the flow cell. During all these measurements an autosampler was used and the measurements were carried out by program. After a 120 s baseline measurement with PBS buffer the sample was injected for 180 s at a flow rate of 100 μL/min. After that association period, buffer was injected for a dissociation period of 150 s. Afterwards the surface was regenerated by a 250 s pulse of low pH (0.01 M HCl, 30 μL/min) and washed with buffer for 120 s.

Measurement protocol for SPR. During measurements with BIAcore, the autosampler was used and a flow rate of 10 μL was steadily run. After 120 s of buffer pre-run the sample was injected for 180 s. Subsequently the surface was washed with running buffer for 270 s and after that dissociation period the thrombine was removed off the chip by a 180 s pulse of TMM solution. Afterwards the surface was rinsed for 100 s with buffer. Spot 1 was used as reference and spot 2 as measuring spot.

Measurement protocol using RM technique. Measurements with IAsys plus were carried out without the use of an autosampler. All injections were done by hand using an Eppendorf pipette. The instrument gave the ability to suck dry the cuvette. Every exchange of liquid was done three times in order to ensure a complete removal of the previous liquid.

After 2 min pre-run with buffer the sample was injected and stayed in the cuvette for 5 min. Afterwards it was washed with PBS buffer for 3 min and thrombine removed by low pH (0.01 M HCl) for 2 min. After that the surface was washed with buffer for 2 min. Every step was carried out in both cells in parallel.

Results and discussion

Biomolecular interaction between immobilized TI and thrombine using different techniques will be compared. Kinetic data achieved from measurements with RIfS set-up, SPR (using BIAcore 2000) and RM (using IAsys plus) will be evaluated and compared.

Short description of the methods used

RIfS is one of the reflectometric techniques that have been applied to direct immunosensing. The basic effect is white light interference at thin transparent films. The principle is extensively discussed elsewhere [11, 24].

SPR is also a direct optical method that is used in detection of solid phase interactions. The basis of this technique is reflection of incident radiation at an interface between media with different refractive indices. Beyond a critical angle the radiation will not pass through the interface but will be totally reflected, the light is guided. An evanescent field exists close to the interface in the medium of lower refractive index and/or the transmittance. The electric field of the reflected radiation depends on the

refractive index, so any changes in these properties influence the evanescent field.

Surface plasmon waves are concerted oscillations of electrons at a surface of a guiding material. The impact of incident photons (only the parallel polarized part of the radiation, TM mode) can excite such surface plasmons to oscillate in resonance with the frequency of the radiation.

In general, a prism is used to excite plasmons in a second interface (a metal like Au, Ag) which is coated on the prism. The metal is used as a guiding material, incident radiation penetrates this metal layer and excites the plasmons at the interface to the analyte. These plasmons exhibit resonance in the visible range. Under conditions of resonance, the intensity of reflected light is reduced like an “absorption peak” at the scale of the angle of incidence or wavelength of the exciting radiation.

So this technique gives a highly sensitive probe for detecting changes in refractive index while binding to the surface. But it needs a highly precise temperature controlling system in order to detect only changes in refractive index caused from binding, not from media.

The third method used in this study is a RM technique, which resembles the SPR sensor in its construction [17]. Light is totally reflected from the sensing surface by means of a prism. At the sensing surface, the metal layer is replaced by a dielectric resonant layer of high refractive index. It is separated from the prism by a low index layer thin enough that light may couple into the resonant layer via evanescent field. Efficient coupling occurs only for certain incident angles where phase matching between the incident beam and the resonant modes of the high index layer is achieved. At the resonance point, light couples into the high index layer and propagates some distance along the sensing layer interface before coupling back into the prism. Resonance happens for TE and TM polarization and is observable as fine structure in reflected light. The angle of excitation is sensitive to changes at the sensing layer caused by changes of refractive index during interactions at the surface. Light is reflected at any incident angle, but a shift in the phase of reflected light during resonance can be detected.

Liquid handling

In flow-through systems, time resolution of the detection is limited by sample handling, for example by the time which is necessary for exchange of solution in the flow cavity. In this study there were only two flow-through systems used for sample handling.

In order to evaluate data for determination of kinetic rate constants it is necessary to know the region of maxi-

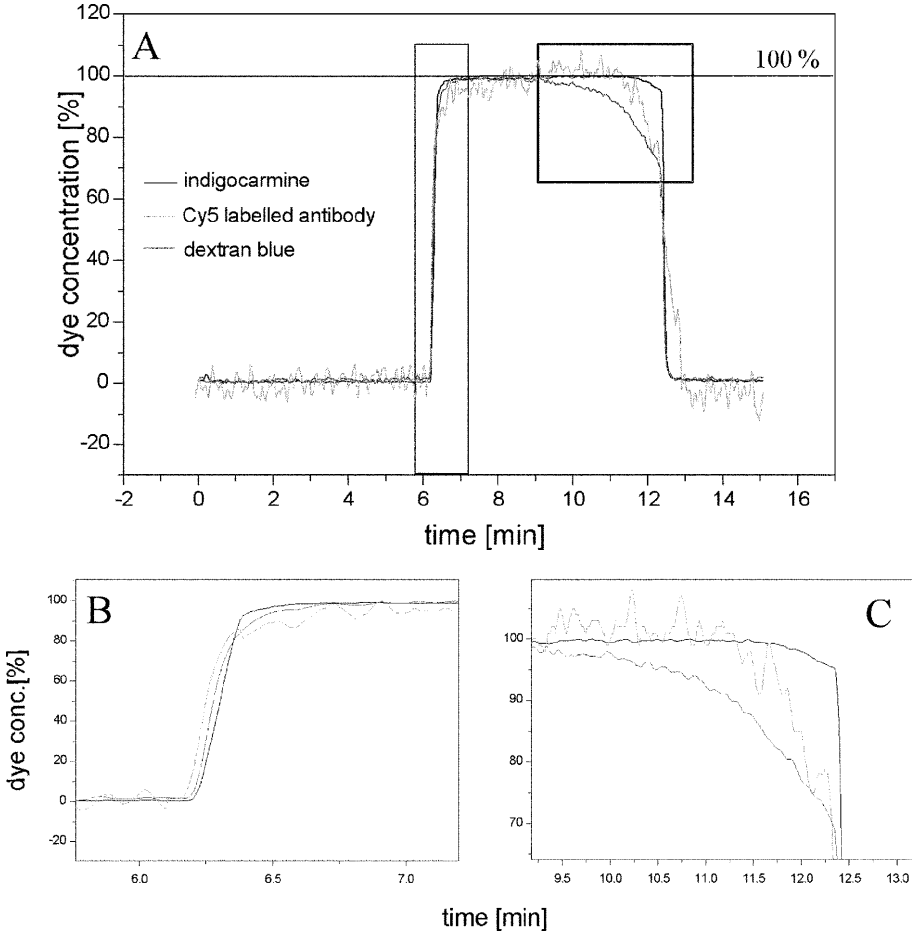

Fig. 2a–c Determination of evaluable region using the RIfS system limited by distribution of the FIA peak within the carrier liquid. (The noise of the cy5-labelled antibody solution is caused by the very low concentration of the compound within the solution). **a** Concentration profiles of the RIfS fluidic system for determination of the sample concentration by injecting dye solutions (overlay of three different molecular weight dyes). **b** Enlarged rise region of the injection, limitation is caused by the construction of the fluidic system such as dead volume. **c** Enlarged fall region of the injection, distribution depending on the size of the molecule

mum sample concentration. Concentration profiles were measured with the RIfS system to register the peak of the sample during the flow injection and specify the distribution. To this purpose, the reflected intensity was monitored while dye solutions were injected under the same conditions as the thrombine samples. Due to the different diffusion dyes with different molecular weights used, namely indigocarmin (MW 466 g/mol), dextranblue (MW 1 million g/mol) and Cy5-labelled antibody (150,000 g/mol). Thrombine has a molecular weight of 39,000 g/mol. Concentration profiles are given in Fig. 2. Maximum concentration was set to 100% in this plot.

Using this plot, the interesting evaluable region can be defined. Maximum concentration is reached at nearly the same time for each dye. Within 10 s up to 90% and within 20 s maximum concentration is reached using peristaltic pumps. Removing the sample from the flow chamber strongly depends on the molecular weight of the dissolved dye. When the sample plug is directly flanked by carrier liquid, diffusion takes place. Smaller molecules diffuse faster than bigger molecules. Fast radial diffusion inhibits dispersion of the sample plug caused by axial convection, which means samples of small molecules do not mix with the carrier stream as fast as larger molecules [28].

BIAcore 2000 has a very low dead volume and ensures a highly precise exchange of sample in the flow cavity by using a micro-flow cartridge. Because of successively filling the chamber, the different spots are reached one after the other. The marginal resulting offset can be nearly eliminated by software.

IAsys plus uses stirred liquid in a cuvette with two cells. Limitations in this case could be not having a total change of liquid while pipetting into the cuvettes, not filling both chambers simultaneously and evaporation during long lasting equilibrium measurements because there is no lid to cover the chambers.

By changing liquid three times, total exchange of liquid is ensured. Nevertheless, a slight offset between reference and measuring chamber caused by a time shift is unavoidable; a correction by software is possible. Temperature control and short measurement times keep evaporation low.

The comparison of the fluid handling systems shows that RIfS and IAsys have to manage without a sophisticated fluidic system. This could be a disadvantage in the reproducibility of data and should be considered throughout evaluation.

Comparison of performance criteria

The performance of a method first of all depends on general parameters such as noise or drift. So first of all, noise or drift obtained during the measurements should be compared. For determination of LOD, signal calibration has to be known. Measurements with radioactive labelled proteins give the signal level for each method [29, 30] as shown in Table 1.

The noise of the signal was derived from linear regression of recorded baselines as standard deviations of the signal after subtraction of linear drift. It was taken from 5 min pre-run baseline measurements with every instrument using the surfaces with the immobilized TI.

Table 1 Comparison of general parameters such as noise and drift derived from baseline measurements with reflectometric interference spectroscopy (RIfS), BIAcore 2000 and IAsys plus. Data were taken during investigations of interaction between thrombine and immobilized thrombine inhibitor (TI). For a better comparison the results of the signal calibration by using radioactive labelled compounds is shown. Signal calibration: 1 ng protein/mm^2=1600 pm optical thickness (RIfS), 163 arcsec (IAsys), 1000 RU (BIAcore)

Instrument	rms-noise	Limit of detection (3×rms)/sensitivity	Drift	Drift (in units of concentration)
RIfS	0.80 pm	1.50 pg/mm^2	10 pm/h	6 pg/h mm^2
BIAcore	0.26 RU	0.78 pg/mm^2	6 RU/h	6 pg/h mm^2
IAsys	0.40 arcsec	7.36 pg/mm^2	4 arcsec/h	24.5 pg/h mm^2

Another limitation is the drift, which should not exceed the signal. Detection of slow interactions is limited. The drift is obtained from linear regression of this baseline runs. Results are shown in Table 1.

Comparable data resulting from signal noise are the LODs received from each instrument (3×rms/sensitivity). The lowest values were obtained from BIAcore 2000 in this study. LOD of RIfS measurements is twice as high as BIAcore data, and noise data achieved with IAsys plus in this measurements gives an LOD more than 9 times higher than the BIAcore data. All achieved values are within one order of magnitude. Binding of proteins where masses are in a range of several kilo Daltons can be easily resolved and monitored.

Evaluation of rate constants

For the evaluation of rate constants several concentrations of thrombine binding to the TI-modified surface were monitored. Each sample concentration was measured three times. For SPR and RM, referencing of the signal was necessary. Signals obtained from the reference cell were subtracted from that received from the measuring cell. RIfS measurements were carried out without referencing. Binding curves for RIfS, referenced signals obtained with BIAcore and also referenced binding curves for IAsys are presented in Fig. 3.

Reproducibility of the binding curves

Considering the binding curves, the reproducibility of the binding of thrombine with a certain concentration can be estimated. Triple experiments have been carried out and the standard deviation of the k_{obs} values, which has been percentally referred to the k_{obs} values has been evaluated. The average of the percental standard deviation is shown in Table 2.

Data show that RIfS provides the lowest standard deviations throughout the measurement. Despite the use of

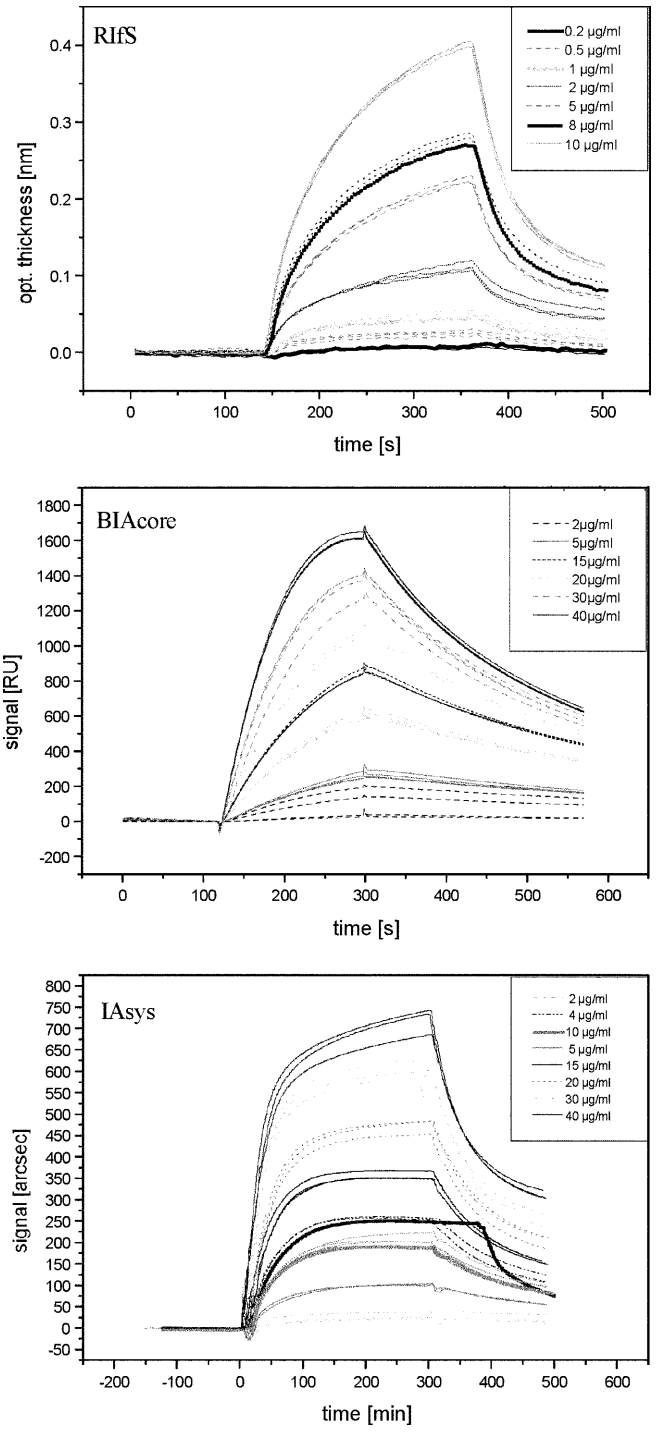

Fig. 3 Binding curves of thrombine to immobilized TI received with the *RIfS* set-up, with BIAcore 2000 and IAsys plus. Each concentration measured with an instrument is given within one plot to show the reproducibility between repeated measurements

reference cells the reproducibility of the commercial instruments is not as good as the data obtained with RIfS. The standard deviations are twice as high as received from the RIfS-set-up.

Table 2 Comparison of the average of the percental standard deviationof k_{obs} measured with RIfS, BIAcore 2000 and IAsys plus

Instrument	Mean value of the standard deviation of k_{obs} in percent referred to k_{obs} [%]
RIfS	8
BIAcore 2000	15
IAsys plus	15

Association rate constants

The kinetic of association of an analyte A binding to the immobilized partner B to form the complex AB on the surface is a bimolecular binding process. The rate of the complex formation depends on the free concentration of A and B components and on the stability of the formed complex.

$$d[AB]/dt = k_a[A][B] - k_d[AB] \quad \text{(a)}$$

k_a is the association rate constant ($M^{-1}s^{-1}$) and k_d the dissociation rate constant (s^{-1}). [*A*] is in this case concentration of thrombine and [*B*] concentration of immobilized TI. Second order kinetics and, if one component is constantly replenished by laminar flow or stirred solution, resulting in pseudo first order kinetic for binding reactions to a surface are well described in literature [2, 31] and [23]. According to these considerations, the change of signal *R* during time can be expressed as:

$$dR/dt = const. - (k_a A_0 + k_d)R \quad (1)$$

Solving the differential Eq. 1, the signal measured at a time can be described as:

$$R_t = R_{eq}[1 - e^{-k_{obs}t}] \quad (2)$$

where

$$k_{obs} = k_a C + k_d \quad (3)$$

whereas R_t denotes the response at time *t* and R_{eq} the response at equilibrium. A_0 gives the initial molar concentration of the component binding to the surface.

Each monitored binding curve derived from several concentrations of analyte were exponential fitted using Eq. 2 in order to get k_{obs} which is shown as an example for one of all monitored binding curves in Fig. 4. For evaluation of each dataset Origin 5.0 was used.

For each binding curve a value for k_{obs} were obtained. According to Eq. 3 a linear fit was accomplished from plot k_{obs} versus initial concentration A_0 for each dataset. The slope results gives directly the association rate constant k_a as is shown in Fig. 5 for datasets monitored by each method. Theoretically the dissociation rate constant is also achieved from the intercept of the *y* axis. However this intercept cannot be determined exactly when k_d is low.

Limitations of the pseudo-first-order analysis are given in [23]. In order to make sure of detecting the binding rate instead of mass transport, derivative of the signal d*R*/d*t* is plotted versus signal *R*. According to Eq. 1 the plotted curve should be linear where mono-exponential behaviour

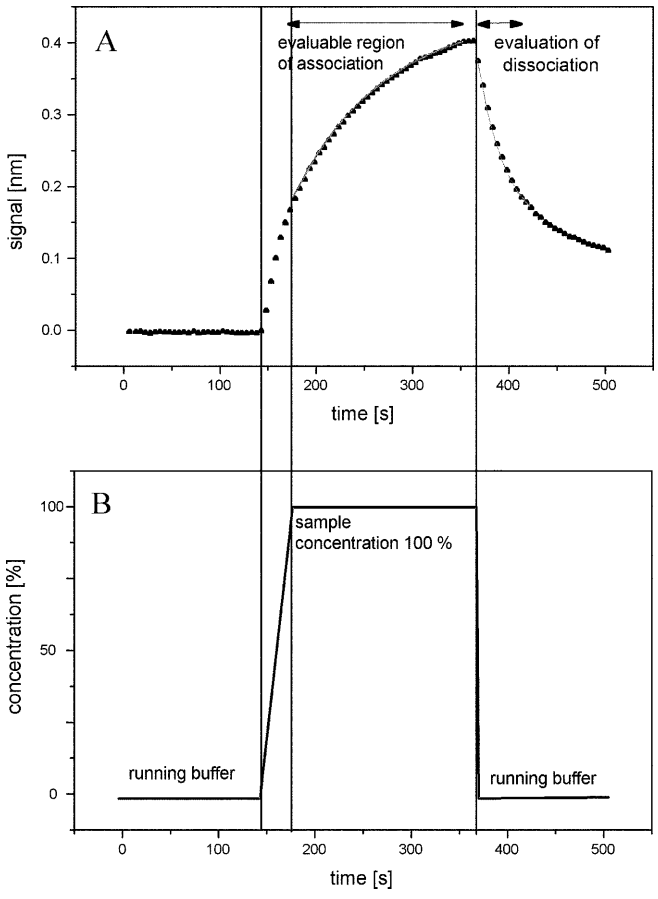

Fig. 4 **a** Binding curve with evaluable region limited by mass transport. Pseudo first order kinetic is true only within the marked section of the association, which is shown with a binding curve of the RIfS measurement as an example. **b** Schematic drawing of the sample concentration within the flow cell during determination of dissociation. In order to reduce back binding effects k_d is evaluated from a binding curve received from a high sample concentration because the lack of free binding sites

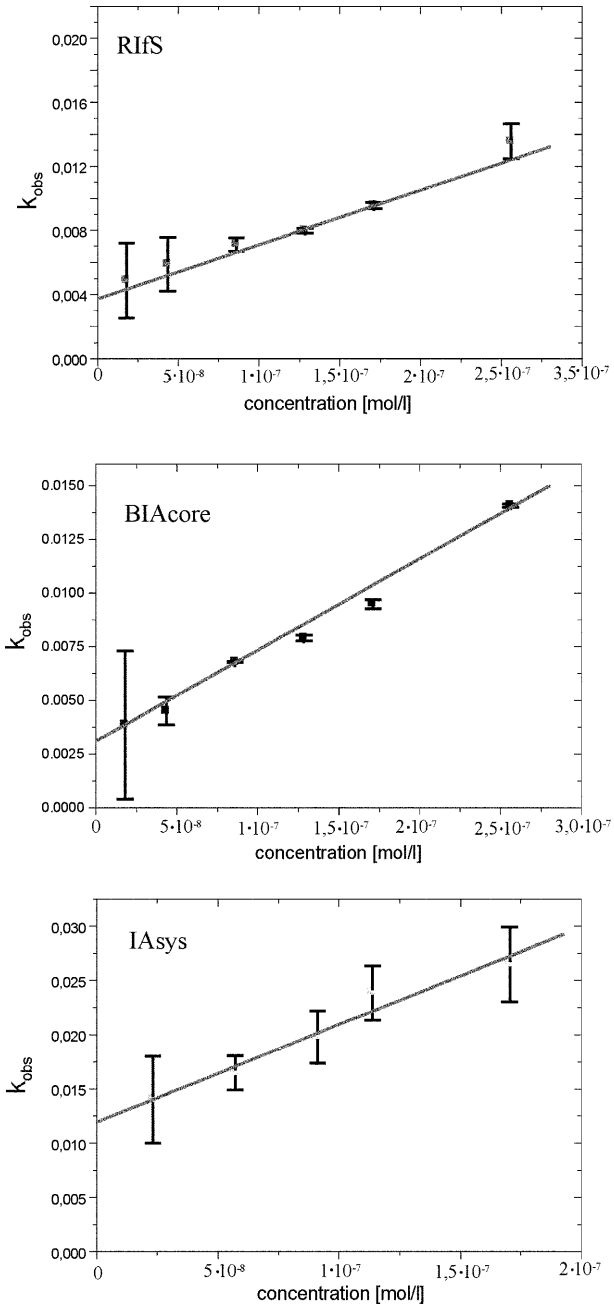

Fig. 5 Determination of association rate constants by linear regression using plots from k_{obs} vs. molar concentration for data achieved with RIfS, BIAcore and IAsys

of the curve is true [31]. Aware of other limitations, all data were evaluated in the same way to permit a comparison among each other.

Another method to determine association rate constants from binding curves is described in [31] using Eq. 1. Signal obtained from association dR/dt is plotted versus R as shown in Fig. 6. The slope value k_{obs} can be introduced into a new plot versus analyte concentration with Eq. 3 and the association constant is readily obtained from the slope of the plot. Data obtained in this way are also given in Table 3.

The results obtained with this method are one order of magnitude below the results achieved with the method described above. Linearization of an exponential relation holds a loss of information. However, data compared among each other demonstrate that association rate constants are within one order of magnitude. The lowest standard deviation among repeated measurements is achieved with the RIfS system; SD values obtained with IAsys plus were about 4 times higher and for BIAcore about 11 times higher. For evaluating association rate constants the first method is preferable.

Results achieved from association curves are within one order of magnitude for each instrument. Standard deviation of the fit for the dataset received with BIAcore gives the best results, 1/10 of the value of k_a. For both RIfS and IAsys, values for SD were 3 times higher.

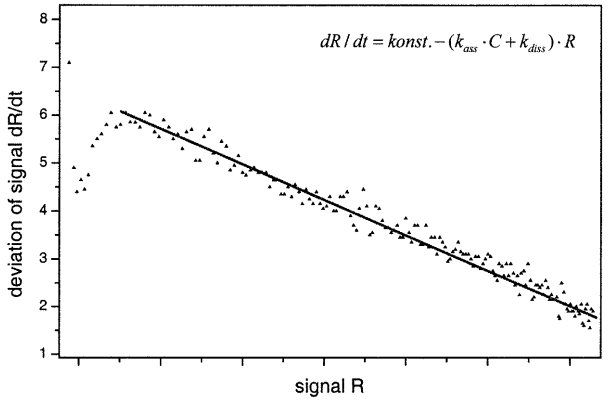

Fig. 6 Determination of evaluable section of the association curve and eliminating of the mass transport influenced regions by plotting derivative of the signal d*R*/d*t* vs. signal *R*. The plot can also be used to evaluate the association rate constant by the slope of the linear fit, which provides k_{obs}. The example has been taken from kinetic measurements with the RIfS set-up

Table 3 Comparison of the determined association and dissociation rate constants of the interaction between thrombine and immobilized TI measured with RIfS, BIAcore 2000 and IAsys plus

Instrument	k_a [10^4 $M^{-1}s^{-1}$] derived from k_{obs}-plot	k_a [10^3 $M^{-1}s^{-1}$] derived by linearisation	k_d [10^{-2} s^{-1}]
RIfS	2.1±0.9	5.2±0.2	3.00±0.04
BIAcore 2000	3.2±0.3	7.3±2.3	0.70±0.01
IAsys plus	9.0±1.0	9.2±0.2	2.00±0.10

Dissociation rate constants

More favourable for achieving dissociation rate constants then obtaining it from the *y* axis intercept is measuring dissociation of bound analyte in buffer flow.

The rate equation is:

$$dR_t/dt = -k_d R_t \tag{4a}$$

and the dissociation rate constant can be obtained by an exponential fit of desorption data in terms of the integrated rate equation:

$$R_t = R_{eq} e^{-k_d t} \tag{4b}$$

as described also in the literature mentioned above. A typical fit taken from a RIfS-binding curve is shown in Fig. 4. It has to be taken into account that that the sample concentration should be maximal. And as explained above, in order to make sure of detecting the binding rate instead of mass transport, the plotted curve of derivative of the signal d*R*/d*t* plotted versus signal *R* should be linear where mono-exponential behaviour of the curve is true.

In order to avoid the influence of rebinding, data for evaluation of dissociation rate constants were taken from dissociation from highly loaded surface, i.e. after the binding of a highly concentrated sample. A comparison of dissociation rates of thrombine from immobilized TI obtained with RIfS, BIAcore 2000 and IAsys plus is given in Table 3.

The values are also matchable. The lowest standard deviation is achieved with BIAcore, RIfS is 4 times higher and IAsys 10 times higher.

Altogether the association and dissociation rate constants obtained with RIfS fit very well into the data received with the commercial instruments. The systems have been compared as a whole, including all advantages and disadvantages, to prove the feasibility of kinetic measurements with the RIfS-set-up.

BIAcore, with its highly sophisticated fluid handling, and IAsys own the ability to reference the signal and to reduce unspecific binding and drift. A temperature stabilization reduces signal increase caused by the change of refractive index with temperature. It also reduces the noise of the detector. The RIFS-set-up, as a cheap and robust device, has proved to obtain comparable data without a sophisticated flow system and an extensive temperature control.

To conclude, RIfS was compared with two other time-resolved and label-free optical detection methods, SPR and RM. Both were represented by commercially available instruments which are widely used. The RIfS set-up used in our laboratory was compared regarding its suitability for biomolecular interaction analysis with BIAcore 2000 and IAsys plus. Thus interaction between an immobilized TI and thrombine was measured under the same conditions with each instrument. Determination of affinity rate constants shows that RIfS is a comparable technique to the commercially available ones.

General parameters such as noise and drift are in good agreement with those obtained by BIAcore 2000 and IAsys plus. Regarding the LOD, the performance of RIfS is almost reaching BIAcore results and betters results achieved with IAsys instrument throughout these measurements.

Although referencing the signal is not required for the RIfS detection method, it could reduce drift caused by light source and upgrade the system in that way. Referencing has been tested in principle [32]. For reducing noise a cooled detector could be used.

Sample consumption with the RIfS set-up at this time is higher than consumption of the other devices. Improvements in sample handling such as using a micro fluidic system would decrease sample consumption. Using air gaps dividing the sample plug from the carrier stream would also reduce limitations given by sample handling.

Surface modifications suitable for various requirements as mentioned above is offered by the companies involved. Modifications of the RIfS transducer offer the same variety of surfaces and in addition a modification with polyethyleneglycol suitable for kinetic binding measurements.

The very low price of the RIfS set-up compared to the others is due to absence of temperature stabilization for detection and low costs of components. Altogether, RIfS is well comparable with other commercial available instruments.

Desirable for further development is a temperature controlled flow chamber in order to evaluate temperature-controlled binding, e.g. melting of DNA. Although the detection method RIfS needs no temperature stabilization

for steady and reproducible measurements, it would be favourable to evaluate temperature dependency of reactions at the surface.

Further, a miniaturization of the device is possible. Instead of using white light providing the whole spectra for the interference measurement, only four LEDs can be used, detecting intensity at only four different wavelengths with photodiodes instead of an diode array spectrometer, which has already been tested in our workgroup [33].

Calibration of the signal by specific binding of protein to the surface and solid phase detection of DNA-DNA interaction using radioactive labelled compounds has also been carried out.

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