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An integrated system for optical biomolecular interaction analysis

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Abstract: Since the introduction of a commercial system for monitoring affinity reactions by BIACORE AB, biomolecular interaction analysis (BIA) has become a valuable tool for many areas in fundamental and applied research. In addition to the BIAcore system, two other optically-based systems are commercially available, all of which are dedicated instruments, developed for the specific requirements of BIA. We introduced reflectometric interference spectroscopy (RIfS) as a transducer in BIA some years ago. Here we present an approach to an integrated RIfS system based on a modified commercially available bench-top spectrometer. We have used the Zeiss SPEKOL 1100 photometer containing the ZEISS MMS integrated optical spectrometer device. Only minor changes of the spectrometer optics were required to set up the RIfS detector. A root mean square baseline noise of 0·5 pm optical thickness was achieved. A change of approximately 1 nm in optical thickness was observed for 1 ng protein bound per square millimetre of transducer surface. Hence, the minimum change in protein mass coverage detectable with the RIfS set-up investigated is below 2 pg protein/mm^2. The set-up of a BIA system based on a non-dedicated hardware platform may lead to significant reduction of the entry level price of BIA systems. This could make BIA affordable for routine research laboratories and might broaden the range of applications. We will discuss the ultimate performance expected for the set-up investigated. Advantages and basic limitations of the RIfS approach will be discussed. ©1997 Elsevier Science Limited

Keywords: biomolecular interaction analysis, optical transducer, reflectometric interference spectroscopy

INTRODUCTION

At the present time at least three systems for label-free biomolecular interaction analysis (BIA) are commercially available. The most prominent system is BIAcore, developed by Pharmacia Biosensor (now BIACORE). The transducer used in this system is based on surface plasmon resonance (Liedberg *et al.*, 1983; Löfas *et al.*, 1991) and detects binding of matter at a liquid–solid interface as changes in local refractive index. IASys, distributed by Fisons Affinity Sensors, uses a prism coupler (resonant mirror transducer, Cush *et al.*, 1993), while BIOS-1, developed by ASI, is based on a grating coupler (Nellen *et al.*, 1988). Both systems also detect changes in the local refractive index in the vicinity of the transducer surface. The detection limits of these systems are 10 pg/mm^2 for IASsys and BIOS-1, whereas the limit for BIAcore 2000 is below 1 pg/mm^2. The relatively high prices for all these systems (US$70 000–200 000) are prohibitive for broad application of the technology. Affordable

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systems with good performance could enter a new market. In this paper we present an approach realized with commercially available components. This may lead a way to more affordable technology for BIA applications.

Thin film interference can serve as a transducer for label-free binding assays (Langmuir & Schaefer, 1937). We introduced a set-up for reflectometric interference spectroscopy (RIfS) for BIA in 1993 (Gauglitz *et al.*, 1993). White light interference is used to monitor the optical thickness of a thin transparent film. A schematic drawing of the light path is given in Fig. 1. White light illuminating an interface between two media of different refractive index is partially reflected. Therefore thin films can cause multiple reflected beams. The reflected partial beams can superimpose and result in an interference pattern, depending on the angle of incidence of radiation,

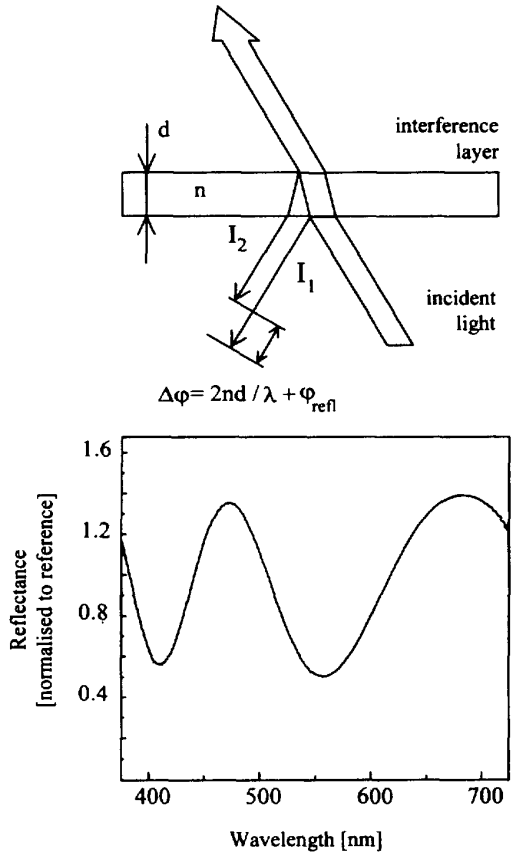

Fig. 1. Schematic of the light path in the RIfS transducer. The reflected partial beams I_1 and I_2 create a spectral pattern due to the phase difference $\Delta\varphi$.

the wavelength, the physical thickness of the film, and its refractive index. For perpendicular incidence, a non-absorbing layer, and low reflectances one finds for the reflectance R:

$$R = R_1 + R_2 + 2\sqrt{R_1 R_2}\cos(4\pi nd/\lambda) \quad (1)$$

where R_1 and R_2 denote the Fresnel reflectance at the two interfaces, d the physical thickness of the film, n its refractive index and λ the wavelength of incident light. A typical interference pattern showing the modulation of reflectance with $\cos(1/\lambda)$ is given in Fig. 1.

The optical thickness $n{\cdot}d$ can be determined from the position of an extremum with a given order value m from

$$n{\cdot}d = \frac{m\lambda}{2} \quad (2)$$

Illumination of the interference layer with white light and acquisition of the reflected light is performed by fibre optics. Reflected light is acquired by a simultaneous spectrometer which detects a complete spectrum at a time. This in turn allows one to monitor the optical thickness of the layer in real time. For biomolecular interaction analysis glass chips with an interference layer of SiO_2 are used as transducer. Molecules binding at the surface of this layer increase the optical thickness of the interference film which can be determined as mentioned above (Gauglitz *et al.*, 1993). Since RIfS detects any organic matter binding at the transducer surface, the interference layer must be modified to reduce non-specific binding. Modification is done by silanization with aminobutyl-dimethylmethoxysilane and covalent coupling of a hydrophilic polymer (e.g. dextran, polethylenglycol) by carbodiimide chemistry (Piehler *et al.*, 1996a).

This short description of a RIfS device shows the four major components of the system:

- light source;
- diode array spectrometer;
- fibre optics;
- transducer element (glass chip with interference layer).

Tungsten light sources are relatively noise-free and are an adequate choice for the application requirements. Diode array spectrometers are routinely used in analytical instrumentation and in colour measurement systems. Cost-effective fibre optical components are available as spin-off from telecommunications. Transducer elements for

RIfS can be produced by standard vacuum techniques on common float glass substrates. We used these commercially available components to set up an integrated system for affinity measurements. A bench-top cuvette photometer with a diode array system was modified with a polymer fibre optics and a lamp with integrated reflector to set up a RIfS device. The aim of this paper is to show an approach to a low-cost system with performance sufficient for affinity interaction studies. The price for such a system under commercial conditions can be estimated to be below US$6000 for the optical part and about US$10 000 for the fluidics. The latter could be reduced by using a more cost-effective miniaturized flow system (Schmitt *et al.*, 1996). The performance of the integrated system is compared to a standard laboratory set-up (Piehler *et al.*, 1996a).

EXPERIMENTAL

The standard laboratory set-up for RIfS (Fig. 2) consists of a 20 W tungsten halogen lamp with fibre incoupling optics (front surface spherical mirror, collimating lens, infrared absorption filter), a polymer fibre coupler (1 mm PMMA fibre, 50/50 coupling ratio, Microparts, Dortmund, Germany) and a diode array spectrometer MCS 410 (Carl Zeiss GmbH, Jena, Germany). A gap

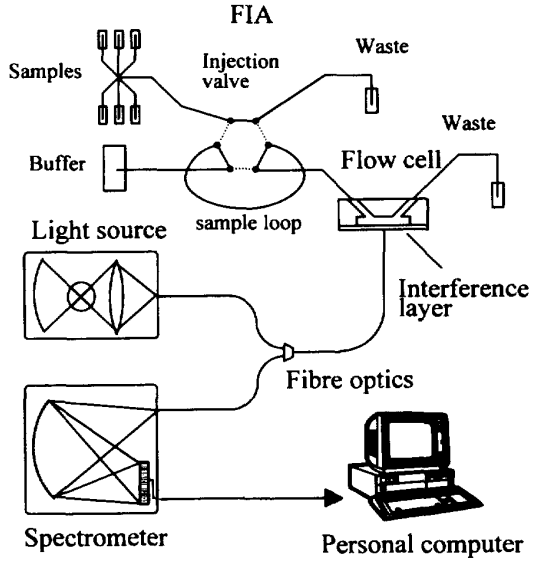

Fig. 2. Standard set-up for RIfS measurements.

(approximately 100 μm) between transducer chip and the fibre output is filled with glycerol (80%) for refractive index matching. Interference layers on float glass (20 nm Ta_2O_5/500 nm SiO_2) were obtained from Schott (Mainz, Germany). Transducer chips were mounted to a flow cell (Perspex™, varnished outside with black paint, 50 μm × 2 mm, < 200 nl). A FIA system (ASIA, Ismatec/Zurich/CH) was used for sample handling. Data acquisition and control of the FIA system are performed by self-written software (MEASURE) running under Windows on a personal computer. The raw spectra are corrected for the dark current of the spectrometer by subtraction and normalized to the reflectance spectrum from a glass chip without an interference layer. The position of an interference extremum at 550 nm is determined by a parabolic fit to one halfwave of the interference spectrum. Optical thickness is calculated according to Eq. (2). The evaluation of optical thickness is performed online by a self-written program (IFZ).

A compact RIfS device was developed around a bench-top cuvette photometer as a core unit (SPEKOL 1100 developed by Carl Zeiss Jena GmbH, distributed by Analytik Jena GmbH, Germany). The SPEKOL is a single-beam photometer. The signals from the spectrometer unit are read out by a 14 bit AD electronics interface on a single board PC. A whole spectrum can be measured, evaluated and plotted on the integrated LCD device with the SPEKOL within 1 s. Programs for additional applications are available on memory cards. The system contains a diode array spectrometer module (MMS, Zeiss), a tungsten halogen lamp and some optics. In Fig. 3(a) a schematic of the original light path of the SPEKOL is drawn.

The MMS module consists of a diode array (S 3904-256Q HAMAMATSU), a concave grating as monochromator and a fibre cross-section converter, which are all fixed to a glass ceramic body (see Fig. 4).

The spectrometer module shows a high mechanical and thermal stability since no moving parts were used.

For RIfS measurements a PMMA fibre optic coupler as in the standard set-up (see above) was connected to the MMS module by a SMA connector. The light source of the SPEKOL was replaced by a 5 V/10 W tungsten halogen lamp with integrated parabolic reflector (Welch Allyn, New York, USA) and a collecting lens for

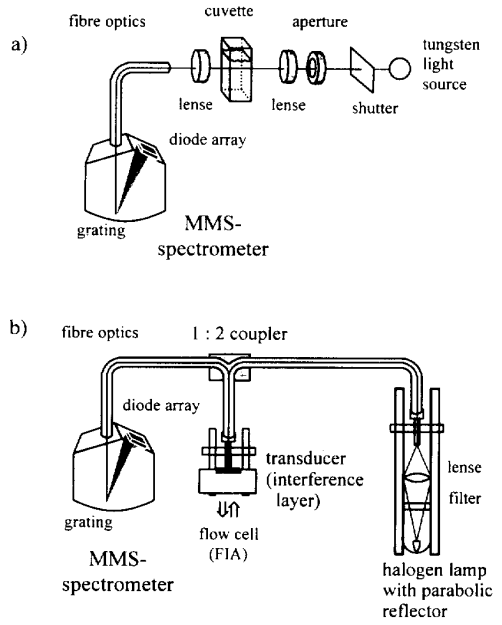

Fig. 3. Schematic of the SPEKOL: (a) original set-up; (b) modified SPEKOL for RIfS measurements.

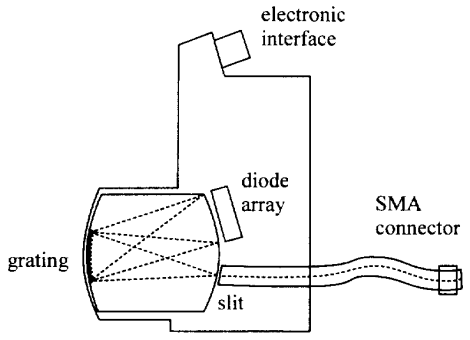

Fig. 4. Schematic of the MMS module.

launching light to the fibre. Fig. 3(b) shows the modifications to the original set-up of SPEKOL. Original Zeiss software SFREE for the SPEKOL was modified to provide an interface for serial communication with a host PC. Two modes were built in. One mode provides only spectra acquisition and transfer to the host PC where evaluation of thickness is carried out by software of the standard laboratory set-up. In the second mode the program performs the data evaluation on the single board PC and only optical thickness values are transferred to the host. For this mode a control program for the host PC was written under Testpoint (Keithley Instruments GmbH, Germering, Germany) which also provides FIA control.

The system's performance was determined with the following measurements. Thickness resolution is the primary performance parameter of the device. The noise of repeated determinations of optical thickness was observed by recording baselines for preconditioned transducer chips under buffer flow. Potential noise sources from the optoelectronics system were characterized by measurements of drift and fluctuation of the dark current of the diode array and the stability of the light source. Dark current measurements were done by repeated scanning of the diode array without illumination. The stability of the light source was characterized with the set-up as described above, but the transducer was removed and the fibre end face was exposed to air to ensure stable reflectance conditions.

The performance of the system in binding assays was investigated with thrombin and a thrombin inhibitor immobilized at the transducer surface according to following protocol: the inhibitor (a derivate of the 'Thrombstop' thrombin inhibitor group with a reactive amino group, kindly supplied by the BASF/Ludwigshafen, Germany) was immobilized at a transducer modified with carboxymethyldextran (CMD) by diisopropylcarbidiimide (DIC) activation. A CMD modified transducer was covered with 2 mg of inhibitor and 2 μl DIC in 8 μl DMF for 12 h in a DMF saturated chamber. After rinsing, the transducer was mounted into the flow cell and thrombin was injected in various concentrations with an auto sampler (Ismatec, Zurich). Thrombin (0–10 μg/ml) in PBS (phosphate-buffered saline, pH 7·4) was loaded into a 500 μl sample loop and injected retrograde. Regeneration was carried out by a short pulse of 20 mM HCl.

RESULTS AND DISCUSSION

The cuvette bench-top spectrometer SPEKOL was used as platform for an integrated system. Only a few modifications were required to set up the RIfS device. All added parts are commercially available at low prices since they are products from mass production applications. Several measurements were performed to characterize the integrated system. Fig. 5 shows a spectrum measured with the RIfS device (solid line). This spec-

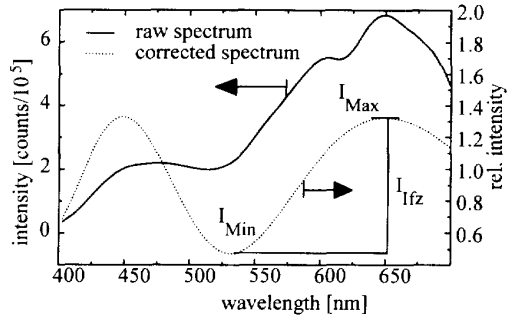

Fig. 5. Raw signal (left ordinate) and interference pattern (right ordinate) of the RIfS device.

trum contains the radiation characteristics of the light source, the transmission characteristics of the optics (lenses, fibre optics, monochromator), the spectral sensitivity distribution of the detector element, and the interference pattern of the transducer chip. The decrease of intensity in the range 650–700 nm is mainly due to the PMMA fibre optics. The corrected interference spectrum I_{cor} of the transducer is derived by normalizing the measured spectrum to the intensity distribution I_r measured with a glass chip without interference layer according to

$$I_{cor} = \frac{I_m - I_d}{I_r - I_d} \tag{3}$$

where I_m denotes the intensity measured with an interference transducer and I_d the dark signal of the detector.

The dashed line in Fig. 5 shows the corrected interference spectrum. The useful part of this signal is given by the relative depth of modulation F_{use} according to

$$F_{use} = \frac{I_{max} - I_{min}}{I_{max}} = \frac{I_{Ifz}}{I_{max}} \tag{4}$$

where I_{Ifz} denotes the difference between the intensity at the maximum I_{max} and at minimum I_{min} of the interference curve (Brecht & Gauglitz, 1994). For the integrated system the same value for F_{use} of 0·64 was determined as for the standard set-up since the same type of optic coupler and flow cell was used. This value of F_{use} is already close to the practical limits and indicates good performance and low backscatter of incident radiation. Acceptable signal quality is achieved for the interference minimum at 530 nm and for the interference maximum at 650 nm.

For interference measurements the integration time of the detector was adjusted to reach 90% of the detector saturation level (approximately 95 ms). Since for most affinity interaction measurements a sampling rate of 0·2 Hz is suitable, 48 spectra were recorded per data point. This leaves sufficient time for data transfer and control of the FIA. The noise of signal was derived from linear regression of recorded baselines as standard deviations of the optical thickness values after subtraction of linear drift. Noise values of about 0·5 pm r.m.s. were obtained. From measurements with labelled proteins (Brecht & Abuknesha, unpublished) it is known that an optical thickness of 1 pm corresponds to a surface coverage of 1 pg/mm^2. Therefore a minimum change of 2 pg/mm^2 protein can be detected within a confidence region of 99·7% (3 × SD). The baseline noise of standard laboratory set-up was found to be 0·5 pm (Piehler *et al.*, 1996b).

The thickness resolution and the drift stability of the system can be limited by the optoelectronic components or by the transducer element. The most critical parts besides the transducer element are the detector element and the light source. As the MMS spectrometer is monolithically integrated, wavelength drift is negligible. More important is the effective dynamics and the drift behaviour of the detection system. The effective dynamic of the detector is limited by the dark signal noise and was determined by repeated measurements of the dark current and calculation of the noise (standard deviations) for individual diodes. The mean standard deviation was 1·2 counts resulting in an effective dynamic range of 1:13 300 (≈ 13·7 bit). The true dynamic range of the spectrometer used in the standard laboratory set-up has been previously determined to be 13·8 bit (Brecht & Gauglitz, 1994).

Repeated dark measurements were performed for 24 h to characterize the long-term drift behaviour of the detector. The average dark signal varied only one to two counts over the whole 24 h period. No inhomogeneity was observed over the diode array, therefore the changes in dark current can be interpreted as a small and concerted shift of dark current for all diodes with negligible 'spectral' effects.

After ensuring constant operation of the detection system, the stability of the light source was investigated. Measurements were performed with the transducer removed and the common fibre end exposed to air for 24 h. To identify changes, irrespective of the signal of the individual diodes,

the dark current corrected spectra were normalized to the first measured spectrum. The intensity changes of the light source were estimated from the change in the mean of the normalized spectra. The mean values showed changes up to 10^{-4}. The fluctuations within 1 h were around 2 $\times$ 10^{-5} (peak to peak). As a typical binding experiment will take less than 1 h, this is relatively little drift. Theoretically, pure intensity shifts of the light source should not affect the thickness reading obtained with the RIfS device. Changes in the dark current should have only little effect, as long as the changes are small as compared to the light induced charge. This was confirmed by numerically and stepwise increasing the intensity values of measured spectra up to the levels observed. These simulated spectra were evaluated with the normal evaluation program (IFZ). Changes in optical thickness were found to be below 0·05 pm. This value is below the noise levels found above and indicates that neither the drift of dark current signals nor pure intensity variations of the light source will have significant impact on the system performance.

More critical are spectral variations of the light source, as these will lead to an apparent shift of the spectral features observed in the interference spectrum. The spectral fluctuations of the light source were determined from the same 24 h measuring period used for estimation of pure intensity fluctuations. Spectral shifts of the light source can be due to a change in the drive current or due to changes in the local temperature of the tungsten filament. Such changes will lead to a wavelength-dependent decrease or increase of intensity levels. In consequence the normalized spectra will no longer be horizontal lines, but tilted or curved. As a simplified model we assumed that spectral fluctuations depend linearly on the wavelength. As a quantitative measurement of spectral fluctuations the slope of the linear regression for every normalized spectrum was calculated. Over 24 h the maximum slopes were found to be in the order of 10^{-6} nm^{-1}.

Model calculations were carried out to estimate the effect of spectral drifts of 10^{-6} nm^{-1} on the thickness readings. A measured spectrum was modified by multiplying each signal value with a wavelength-dependent factor. Slopes up to 10^{-6} nm^{-1} were applied. In Fig. 6 this approximation is shown schematically for raw spectra and for the resulting normalized curves. The slopes in the figure were set higher than in the

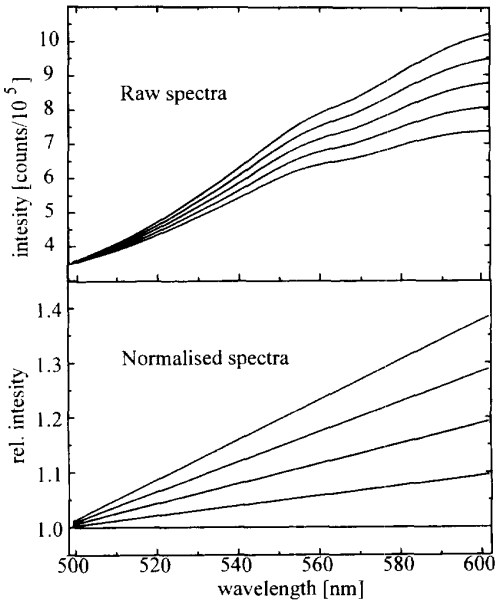

Fig. 6. Simulated spectra. Increasing red shift by linear increase of intensity with wavelength. Slope values are 0–4 $\times$ 10^{-2} nm^{-1} from the bottom curve to the top curve. Normalized spectra (bottom graph) were derived by dividing all curves by the first curve. Values shown exceed parameters actually used, for clarity.

calculations on purpose to show the effects more clearly. The effects of these variations on the corresponding interference spectra are shown in Fig. 7.

Simulations of spectra with factors corresponding to slopes of normalized spectra in the range of 10^{-7}–10^{-5} nm^{-1} resulted in changes of the optical thickness of more than 50 pm. For small changes, the shift in optical thickness is linear with the

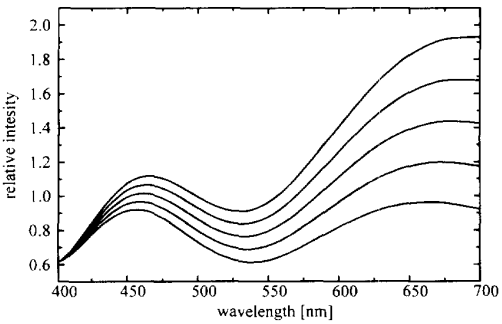

Fig. 7. Changes of interference spectrum with linear increase of intensity with wavelength. Slope is 0–4 $\times$ 10^{-2} nm^{-1} from the bottom curve to the top curve.

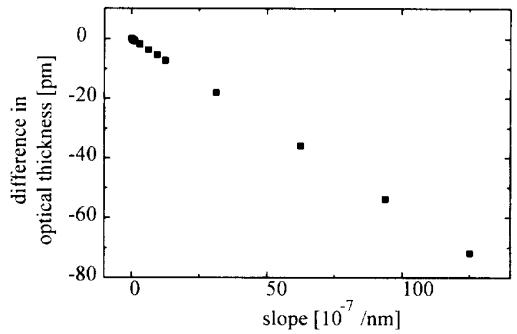

Fig. 8. Influence of linear increasing changes in a spectrum on the optical thickness. The changes are plotted versus the slope in the normalized spectrum.

slope of the spectral shift. These effects are shown in Fig. 8.

The model of a linear change in a spectrum is a very primitive approximation to spectral fluctuations but shows that spectral variations of the magnitude observed have significant influence (approximately 5 pm) on the optical thickness observed. Therefore spectral stability of the light source is of high importance for the thickness resolution achievable.

The performance of the system in biochemical measurements was determined with a receptor–ligand model system as described above. Typical binding curves are given in Fig. 9. For concentrations low enough to allow the observation of diffusion-limited binding, a linear relation between concentration and slope of binding curve was found (see Fig. 10). The time resolution and resolution of optical thickness are adequate for binding experiments.

For simplified integration of the unit in other

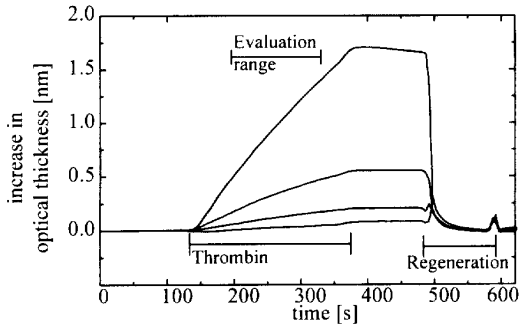

Fig. 9. Typical binding curves for various concentrations of thrombin: From bottom to top: 0·5, 1, 2 and 5 μg/ml.

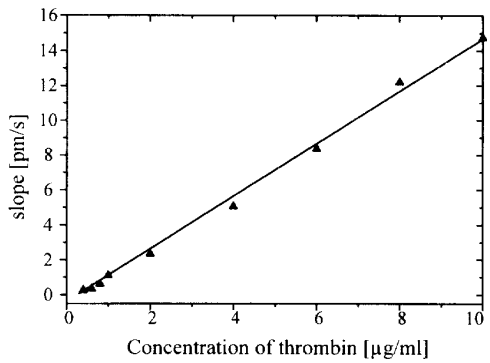

Fig. 10. Slope of binding curve versus concentration.

systems, the evaluation of optical thickness on the internal single board PC (8086 CPU) is best suited. This allows one to run the device as a stand-alone system with control *via* a serial port. The computational overhead for the evaluation of the optical thickness is about 1 s of CPU time. Owing to this fact, only 36 spectra with an integration time of 95 ms can be recorded in a 5 s interval. Therefore the noise of a baseline is increased by $\sqrt{(48/36)} = 1.15$. The time for evaluation could be reduced using a board with faster CPU.

SUMMARY AND CONCLUSION

A compact system for optical biomolecular interaction analysis was set up starting from a cuvette bench-top photometer. Only a few modifications with commercial components were required to set up the device. The compact system showed a performance comparable to a standard laboratory set-up. The baseline noise of 0·5 pg/mm^2 allows one to detect surface coverages of protein below 1‰ of a typical protein monolayer. The price of the system is low compared to existing commercial systems since only products from mass production are used. Owing to the basic principle of RIfS the signal of the transducer is only slightly affected by the bulk refractive index. Therefore in contrast to systems based on refractometric methods, no thermostatting is required.

Investigation of dark current and light source stability indicated that the system is mainly limited by spectral fluctuations of the light source. This was confirmed by evaluation of simulated spectra. An active control of the light source properties is possible but will be highly complex.

Therefore, further improvements may be achieved by referencing the light source with a second diode array detector. This should significantly reduce the influence of fluctuations of light intensity, but can not be easily achieved with the existing hardware platform. The performance for biomolecular interaction analysis of the system was demonstrated with a model ligand–receptor system. The performance of the device can be improved slightly by integration of a faster CPU for the embedded system control.

The sample handling system used for the device is a multiple-purpose FIA with corresponding price and size. We reported recently (Schmitt *et al.*, 1996) on a more cost-effective microconduit with a core unit that fits to the planar RIfS transducer. The miniaturized FIA system for hydrodynamic sample injection was realized based on printed circuit board technology. This core unit contains no active parts (actuators). The fluidics are completed by external valves and pumps. This approach may lead to a further integration of all parts of the affinity measurement device.

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