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# A synthetic membrane protein in tethered lipid bilayers for immunosensing in whole blood

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ABSTRACT

Tethered lipid bilayers, containing a transmembrane synthetic ligand-gated ion channel (SLIC), have been formed on gold surfaces. The SLIC was designed as a highly selective receptor and reporter protein to detect antibodies in whole blood, which are of importance in malaria diagnosis. The specific binding of the antibody to the sensor surface was monitored on-line with label-free surface-sensitive techniques either optically by surface plasmon resonance in whole blood or electrically by measuring the channel activity of SLIC in blood serum. We demonstrate the feasibility of a highly sensitive and easily applicable whole blood biosensor on the basis of simple commercially available components. The sensor might find applications in the field of infectious diseases such as point-of-care diagnostics of malaria, high content quality control of blood samples of donors, or monitoring the efficacy of vaccination.



## 1. Introduction

Immunosensing in whole blood is classically performed by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay techniques, which require time-consuming preparation steps including the use of fluorescent-, radioactive- or enzyme-labeled antibodies. Direct immunoassays in whole blood without sample preparation would improve speed and reliability of point-of-care diagnostics (Warsinke et al., 2000; Toner and Irimia, 2005; El-Ali et al., 2006; Lee, 2008; Skottrup et al., 2008; Yager et al., 2008; Chan et al., 2008). However, the high interference of blood, as a complex and concentrated solution of blood cells, proteins and lipids poses a still largely unmet challenge. Specific protein sensing has been demonstrated in diluted or undiluted blood serum only recently with fluorescence assays (Moreno-Bondi et al., 2006), quartz crystal microbalance (Hirst et al., 2008), surface plasmon resonance (SPR) (Masson et al., 2007; Phillips et al., 2007; de Boer et al., 2008; Nagel et al., 2008; Vaisocherová et al., 2008), aptamer-based electrochemistry (Lai et al., 2007), magnetic relaxation of nanoparticles (Fornara et al., 2008). Serum preparation requires removal of blood cells by filtration or centrifugation and may substantially affect the analysis by missing the antibodies bound to blood cells. To date, direct protein sensing has been achieved in diluted whole blood samples with gold nanoshells (Hirsch et al. 2003; Wang et al, 2008), porous silicon sensors (Bonanno and DeLouise, 2007), and fluorescent antibodies in surface plasmon-coupled emission (Matveeva et al., 2005).

Here, we report on an optimized biosensor based on label-free and surface-sensitive techniques that allows the detection of antibodies in whole blood with SPR and in blood serum with impedance spectroscopy. Such simple and robust biosensors are urgently needed to detect infectious diseases in the population of for example developing countries, during vaccine development to monitor the development of antibodies in the blood of vaccinated probands, or for high content quality control of blood samples from donors. Surface-sensitive techniques provide a few substantial advantages in this context: (i) The analyte on the sensor surface is sensed distinguishingly from the bulk and without any separation step. (ii) The possibility to work label free avoids further additional preparation procedures. (iii) On the basis of these sensing techniques a number of remarkably reliable bioanalytical devices have evolved. They range from high-tech instruments suitable for research and clinical applications to extremely simple and cheap devices suitable for home- and in-field point-of-care diagnostics: examples are SPR (Rich and Myszka, 2008), planar wave guide technology (Weissenstein et al., 2006), quartz crystal microbalance (Cooper and Singleton, 2007), electrophysiology (Schulz et al., 2008).

Fig. 1 depicts the concept of our generic biosensor: it is based on a synthetic ligand-gated ion channel (SLIC) integrated in a lipid bilayer, both tethered to a gold surface (Terrettaz et al., 2001). This

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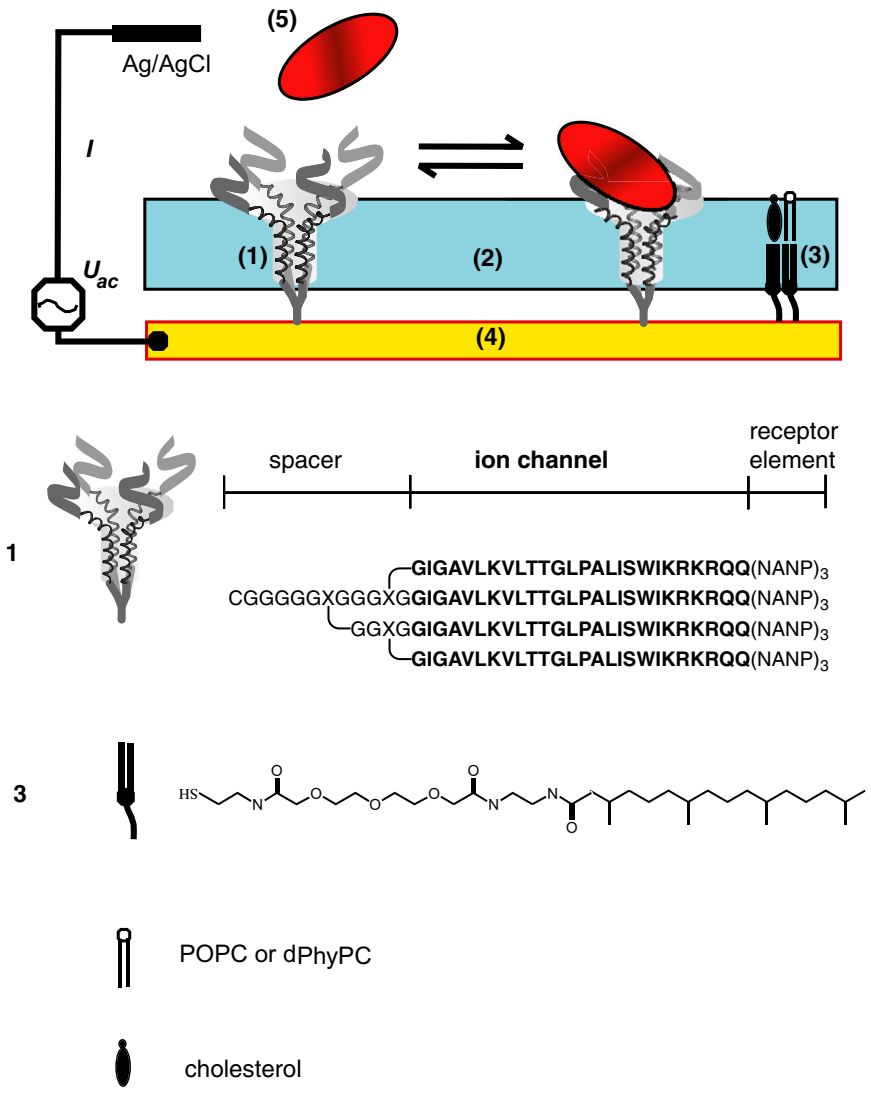


**Fig. 1.** Scheme of a synthetic ligand-gated ion channel SLIC (1) in a lipid bilayer (2) tethered via a thiolipid (3) to a gold surface (4). The binding of an antibody (5) from the bulk solution to the SLIC receptor can be measured either optically by SPR or electrically by impedance spectroscopy as the modulated ion channel activity or by a combination of both techniques. The amino acid sequence of SLIC 1 in one-letter-code and the chemical structure of thiolipid 3 are shown. The lipid bilayer comprises gold tethered thiolipids and untethered phospholipids such as POPC or DphyPC for SPR measurements, and a mixture of DphyPC and cholesterol (9/1) for electrical measurements.

configuration combines the high resistance of phosphatidylcholine lipid bilayers to unspecific protein adsorption (Chapman, 1993; Terrettaz et al., 1993; Glasmästar et al., 2002; Svedhem et al., 2003; Phillips and Cheng, 2005) and the intrinsic amplification of ligand binding provided by ion channels (Lang et al., 1993; Terrettaz et al., 1993; Cornell et al., 1997; Stora et al., 1999; Bayley and Cremer, 2001; Terrettaz et al., 2001; Janshoff and Steinem, 2006; Uram et al., 2006; Matile et al., 2007; Mayer et al., 2008). Long term mechanical stability allowing measurements over several days has been obtained using lipid bilayers, which are tethered to gold surfaces via hydrophilic spacers, reported the first time by us (Lang et al., 1992, 1994) and others (Spinke et al., 1992; Naumann et al., 1995). In this configuration the lipid bilayer is decoupled from the solid support and allows the integration of bilayer-traversing proteins (Naumann et al., 1995; Heyse et al., 1998; Bieri et al., 1999; Stora et al., 1999; Terrettaz et al., 2001). These original concepts have since then be used by many groups to design various configurations of tethered lipid bilayers in pure form, or with integrated polypeptides or membrane proteins, as reviewed elsewhere (Danelon et al., 2008).

The SLIC comprises two functional elements: (i) An extramembraneous site, which serves as a receptor to selectively recognize and strongly bind the analyte, here an antibody. (ii) A transmembrane amphipathic four-helix bundle forming an ion channel whose activity is gated by the binding of the analyte to the receptor part of SLIC. This design allows the dose-dependent detection and quantification of the analyte either optically (SPR), or electrically with potential single molecule sensitivity, exploiting the SLIC ion channel activity as an intrinsic electrical amplification as shown elsewhere (Terrettaz et al., 2001, 2003; Danelon et al., 2008). The modular architecture of the SLIC allows us to design our biosensing molecule to be precisely adapted to the analyte to be detected by choosing the proper receptor part of SLIC. Here, the ligand-binding region of SLIC is the repetitive NANP sequence, which is recognized by a specific monoclonal antibody. The NANP sequence was chosen, because it is the major B-cell epitope of the circumsporozoite protein of *Plasmodium falciparum*, the cause of human malaria (Miller et al., 1986), and therefore is of interest in malaria diagnosis. The channel-forming part of the SLIC is built by four melittin peptide segments that are chemically attached to a branched

spacer to form a lipid bilayer traversing four-helix bundle. The melittin sequence was chosen because of its well-known bilayer-traversing, channel-forming properties in lipid bilayers (Tosteson and Tosteson, 1981; Vogel, 1981, 1987; Pawlak et al., 1994).

## 2. Experimental

### 2.1. Chemicals

SLIC 1 and 1-phytanoylamide 2-[14′-mercapto-1′-11′-dioxo-3′,6′,9′-trioxa-12′-azatetradecylamide]ethane (thiolipid 3), (Fig. 1) were synthesized as described elsewhere (Terrettaz et al., 2001, 2003). 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DphyPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were from Avanti Polar Lipids (Alabaster, AL, USA). *n*-Octyl-β-glucopyranoside (OG) was from Bachem (Switzerland). The monoclonal antibody SP3E9, raised against the synthetic peptide $(NANP)_{40}$ was a gift from H. Matile (Roche, Switzerland). Other chemicals were from Fluka and were of the highest purity available. The buffer comprised 5 mM sodium phosphate (pH 7.4) and 0.1 M KCl in deionized water.

Human serum was from Sigma and comprised more than 50 mg/mL protein. Blood samples were obtained from rats. 5 mL of blood were stabilized with 9 mg $Na_2EDTA$ to prevent coagulation and used without further treatment. When stored at 4 °C, blood samples could be used for up to one week with no detectable influence on our measurements.

### 2.2. Formation of biosensing surface

For SPR measurements, SF10 glass slides with a refractive index of $n = 1.723$ (Schott-Guinchard SA, Switzerland) were used as supports. They were cleaned by ultrasonication, first in a 2% solution of the detergent Hellmanex (Hellma, Germany) and then in pure water. A 1-nm thick chromium and then a 45-nm thick gold layer was deposited sequentially on a cleaned glass slide by evaporation at a pressure of $5 \times 10^{-6}$ mbar. After being cooled to room temperature in the vacuum chamber, the gold-coated glass slide was mounted in the measuring cell and used immediately. Alternatively, a gold-coated glass slide was stored overnight in a solution of 1 mM β-mercaptoethanol in ethanol and used the following day after intensively washing with ethanol. Gold surfaces were incubated in a solution of 0.1 μM of SLIC in buffer for 800 s leading to a surface coverage of 56 ng/cm$^2$. The surface-immobilized SLIC was integrated in a tethered lipid bilayer by forming first the thiolipid monolayer from an OG solution. The lipid bilayer was then completed by an additional monolayer of either POPC or DphyPC formed by diluting the corresponding phospholipid solution in OG below the critical micellar concentration of the detergent (Terrettaz et al., 1993).

For electrical impedance measurements, 50 μm disk gold electrodes were produced by photolithography on glass slides (Danelon et al., 2008). They were cleaned by hot chromic acid (saturated potassium dichromate in concentrated sulfuric acid), then rinsed thoroughly with water, dried in a stream of nitrogen and sealed at the bottom of the electrochemical cell by means of an O-ring. The electrodes were then left for 15 min in ethanol in order to remove the labile gold oxide and washed again in water. Larger electrodes of 500 and 2000 μm diameter were prepared by vapor deposition of 50 nm thick gold films at $5 \times 10^{-6}$ mbar through a metallic mask on glass slides and transferred immediately in the electrochemical cell. The gold electrodes were then incubated for 5–30 min in a $10^{-9}$ to $10^{-7}$ M solution of SLIC in buffer. Highly insulating membranes were obtained by overnight incubation with thiolipid and the subsequent deposition of a mixed monolayer of DphyPC and cholesterol formed by the dilution of a detergent solution of DphyPC/cholesterol (9/1) (Terrettaz et al., 2003).

### 2.3. Surface plasmon resonance (SPR)

SPR measurements were performed on a home-built apparatus (Terrettaz et al., 1993). The intensity of the reflected light was measured as a function of the angle of incidence, using a photodiode with a chopper/lock-in amplifier technique. Organic molecules adsorbed at the gold surface led to a shift of the resonance angle, which was used to characterize the molecular layers, while the kinetics of adsorption were monitored at an angle slightly lower than the resonance angle at the steepest part of the resonance curve. Assuming a refractive index of $n = 1.45$, an experimental shift of 1° corresponded to a layer thickness of 6.4 nm in buffer. The mean molecular area of adsorbed proteins was calculated with a refractive index increment of 0.18 mL/g as previously described (Boncheva and Vogel, 1997). The onset of total reflection of the SPR curve lead to refractive indices of 1.33 and 1.34–1.35 for buffer and blood samples, respectively.

The adsorption of blood protein was quantified from the angle shift obtained from measurements in buffer before and after the exposure to blood samples. The reflectivity curves measured in human blood serum or whole blood were shifted to higher angles compared to the corresponding measurements in buffer due to a higher refractive index of the bulk medium but retained a very well-defined resonance angle (Fig. 2). On line measurements in whole blood were performed at a fixed angle slightly below the resonance angle measured in blood. A stable baseline was first acquired before adding the antibody in whole blood. Adsorption was monitored *in situ* for approximately 1 h until a plateau was reached. A blood wash was then performed to remove unbound material and to avoid any small refractive indices mismatches. Specifically bound antibodies were desorbed in buffer in the presence of 0.1 mM $(NANP)_6$ as an excess antigen. The desorption kinetics were fitted by a double exponential. The time constant of the rapidly decaying exponential is taken for calculating the rate constant of dissociation of the antibody–antigen complex. The slowly decaying exponential is taken as a fitting element that reflects the retardation through re-adsorption of antibodies. (Duschl et al., 1996).

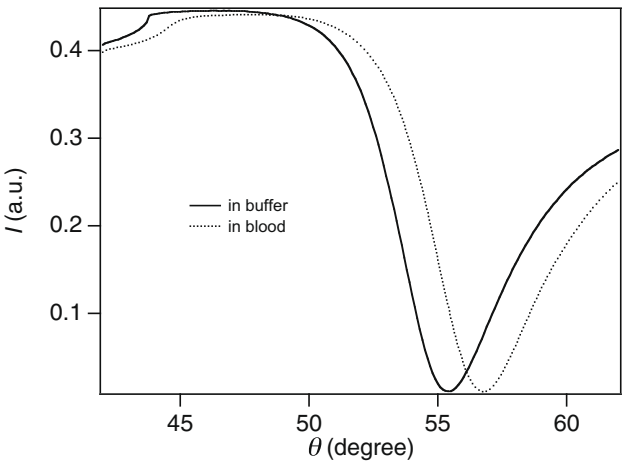


**Fig. 2.** SPR $I(\theta)$ sensorgram (intensity $I$ of reflected light versus angle $\theta$ of incidence) of a gold surface coated with a tethered SLIC/lipid bilayer in buffer or in whole blood. $I(\theta)$ sensorgram in whole blood has the same shape as in buffer but is shifted to higher angles due to a larger bulk refractive index of blood. SPR measurements could thus be performed directly in whole blood at higher angles of incidence.

### 2.4. Impedance spectroscopy (IS)

IS was performed in an electrochemical cell comprising a lipid membrane-covered gold electrode and a counter Ag/AgCl electrode directly in human blood serum or in whole blood. A sinusoidal voltage of 10 mV was applied to the cell at successive frequencies between 0.1 Hz and 20 kHz. The resulting current was recorded via a phase-sensitive lock-in amplifier to calculate the complex impedance. The data were analyzed using the same electrical equivalent circuit as in previous work (Terrettaz et al., 2003). The same model fitted the data equally well the biosensor in blood serum and whole blood as in buffer.

## 3. Results and discussion

The sensor surface was composed of a lipid bilayer and bilayer-spanning SLIC, both tethered to a planar gold surface (Fig. 1). SPR was used first to optimize each step of the formation of the sensing surface and then to detect the interaction between the antigens on the tethered membrane and the antibody in whole blood. Details are given in Fig. S1 of Supplementary material. Due to the surface-confined evanescent light, SPR is ideally suited to measure specific interactions on the sensor surface even in highly concentrated lipid/protein media such as blood (Fig. 2). In order to test the performance of our tethered membrane biosensor, we incubated it for 1 h in whole blood. After washing with buffer we did not measure unspecific protein adsorption within the detection limit of our SPR instrument (<0.03°). On the other hand a pure tethered SLIC monolayer on gold (in the absence of a tethered lipid bilayer) showed substantial unspecific adsorption in whole blood (>1°, corresponding to >430 ng/cm$^2$). This demonstrated that the surface of a densely packed self-assembled phosphatidylcholine lipid membrane was of central importance for suppressing undesired unspecific binding events on the sensor surface in whole blood.

Once the optimal composition and conditions for formation of the biosensor surface for suppressing nonspecific binding were found, we investigated the specific interaction of the antibody to the SLIC receptor molecule comprising four antigenic $(NANP)_3$ peptides (Terrettaz et al., 2001; Duschl et al., 1996). Fig. 3 depicts the time course of the binding of the specific antibody to the biosensor surface in whole blood. The intensity versus time sensorgrams were recorded at an angle slightly below the resonance angle. After about 1 h of incubation at this antibody concentration the SPR signal reached a constant value. Due to mass transfer limitations the antibodies were not displaced by buffer and blood washes (Terrettaz et al., 2001). Also, the addition of 0.3 μM unspecific antibody IgG did not result in any measurable protein adsorption (Fig. 3). The specificity of the antibody–antigen interaction was further probed by a competitive assay adding an excess of free $(NANP)_6$ peptides to the bulk solution (Fig. 4). Under these conditions, more than 93% of antibodies were displaced from the sensor surface. Antibodies adsorbed either from buffer or from whole blood were displaced in buffer to allow a direct comparison of the kinetics (Fig. 4). The obtained $k_{off}$ values were identical for both cases demonstrating that antibodies were also bound identically to the SLIC receptor in buffer and in whole blood. Fig. 5 depicts dose–response curves for the antibody binding to SLIC in buffer and in whole blood. The detection limit ($5 \times 10^{-8}$ M) of our SPR instrument allowed measurements of clinically relevant antibody concentrations. It is remarkable that reliable dose–response curves could be obtained in blood samples of different origin and different storage conditions. All the results obtained from SPR measurements in whole blood were in total agreement with corresponding measurements in buffer (Terrettaz et al., 2001) showing that there was no degradation of the sensor performance in blood.

As whole blood and blood serum are surface-active, highly insulating lipid membranes have been prepared for electrical measurements that are highly sensitive to defects. The formation of these highly insulating membranes required notably a prolonged incubation time of thiolipid and an optimized composition of the lipid outer monolayer (PhytPC/cholesterol 9/1) as described earlier (Terrettaz et al., 2003). The electrical parameters of the membranes used in this study and their resistance to blood destabilization are shown in Table S2 and Fig. S2 of Supplementary material. For highly insulating lipid bilayers the electrical properties remained unchanged after a prolonged (>1 h) incubation in whole blood. SLIC-containing membranes showed a decreased resistance of 50–70% after 1 h incubation in whole blood, while the membrane capacitance increased by less than 3%; this indicates that the global membrane integrity was well preserved. In human blood serum the decrease of resistance of SLIC-containing bilayers was less pronounced than in whole blood. In this case, after the destabilization

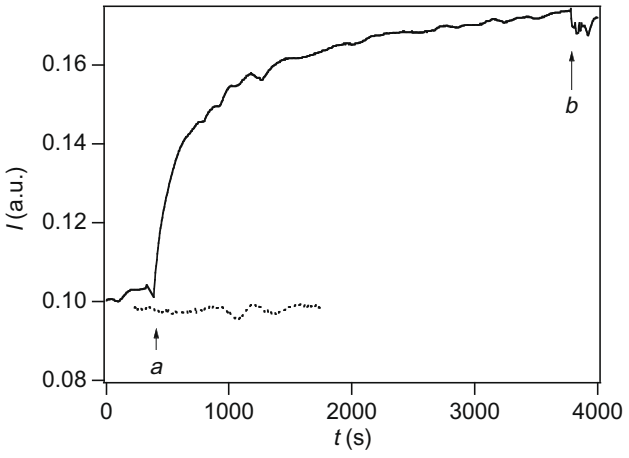


**Fig. 3.** *I(t)* (intensity of reflected light versus time) measured on the tethered SLIC/lipid bilayer biosensor in whole blood after adding 0.8 μM specific antibodies to SLIC (a), followed by purging the sensor surface with whole blood (b). The binding of antibodies as a function of time was monitored directly in whole blood. As a control experiment the addition of 0.3 μM unspecific antibody IgG is shown (dotted curve).

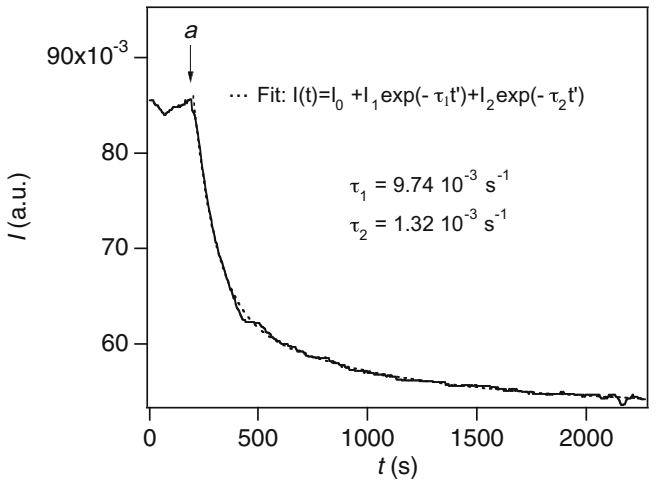


**Fig. 4.** Kinetics of dissociation of the specific SLIC antibodies, previously bound from whole blood, from the biosensor surface initiated by adding a $10^{-4}$ M competitive ligand $(NANP)_6$ in buffer (a). The experimental curve (solid line) was fitted with a double-exponential function (dotted line). $\tau_1$ represents the time constant of the dissociation of the antibody–antigen complex. $\tau_2$ considers antibody rebinding to the surface due to mass transfer limitations (Duschl et al., 1996).

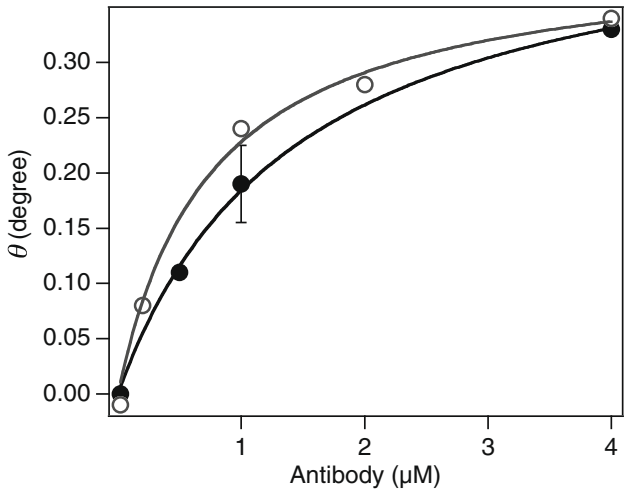


**Fig. 5.** Binding of specific SLIC antibodies measured in whole blood (●) and in buffer (○). The SLIC content of the tethered membrane was 56 ng/cm$^2$. The continuous line was calculated using the Langmuir adsorption equation. An angle shift of 0.1° corresponds to a surface coverage of antibody of 42.9 ng/cm$^2$ (1 molecule per 580 nm$^2$). A representative error bar of four different measurements is shown.

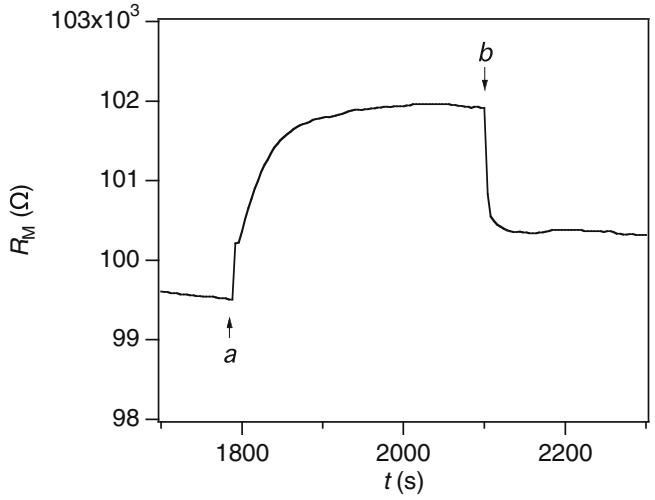


**Fig. 6.** Electrical resistance of the SLIC-containing tethered lipid bilayer in blood serum after the addition of 0.25 μM of SLIC antibodies (a) and a serum wash (b). The initial decrease of membrane resistance after incubation in blood serum was 30%.

phase, the membrane resistance increased by antibody binding (Fig. 6); this indicates that a SLIC-based immunosensor with electrical detection is feasible in human blood serum.

## 4. Conclusion

Tethered lipid bilayers have been used to detect molecular interactions in blood serum and whole blood. The excellent resistance to protein adsorption provided by standard phosphatidylcholine bilayers to the sensor allowed a direct specific detection and quantification of antibodies in whole blood using SPR. This is an important finding. It demonstrates that an easily applicable whole blood optical biosensor for the point-of-care diagnostics of infectious diseases or for evaluating vaccine development is feasible. The biosensor can be produced using presently available technologies and components, such as automated high-throughput instruments (VanWiggeren et al., 2007; de Boer et al., 2008) or miniaturized portable SPR devices as reported elsewhere (Stemmler et al., 1999; Whelan et al., 2002; Grunwald and Holst, 2004). We have also reported first experiments showing the feasibility of a biosensor based on electrical impedance measurements in human blood serum that has the potential of higher sensitivity, would be even simpler to use and cheaper to produce. We have shown that highly insulating pure tethered lipid bilayers (Terrettaz et al., 2003) remained exceptionally stable over hours upon exposure to whole blood, while the SLIC-containing tethered membranes appeared less stable in blood. Currently, we investigate the increase of robustness of tethered SLIC-containing bilayers based on membrane-spanning thiolipids inspired from bolalipids such as found in archaea (Febo-Ayala et al., 2006).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsb.2009.03.011.

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