

Immunosensing by a Synthetic Ligand-Gated Ion Channel**

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Cell signaling is often mediated by pore- and channel-forming membrane proteins, whose function can be controlled either by changing the transmembrane electrical potential or by the binding of ligands. Although the structure of most native membrane channels is unknown at a molecular level, it is rather attractive to copy this principle of biological signaling by incorporating synthetic ligand-gated ion channels (SLICs) into an electrically insulating lipid bilayer tethered chemically to a gold electrode. The newly designed SLICs should change their transmembrane ion conductance by selectively binding an analyte.^[1] By this intrinsic amplification of a binding reaction it should be possible to develop highly sensitive biosensing devices. Recent studies have shown that molecular reactions of proteins in tethered lipid membranes can be measured with high sensitivity, either optically or electrically.^[2]

Figure 1 shows an example of a new class of SLIC molecules made of branched polypeptides. Conceptually, a SLIC comprises a ligand-binding and a channel-forming region. As a ligand-binding region we have chosen a peptide, the repetitive NANP sequence,^[3] which is recognized by a specific monoclonal antibody. The NANP sequence is the major B-cell epitope of the circumsporozoite protein of *Plasmodium falciparum*, the cause of human malaria.^[4] The channel-forming part is built by four melittin peptide segments that are chemically attached to the branched spacer. The melittin sequence was chosen because of its well-known channel-forming properties.^[5] The SLICs were directly attached to a sensing gold electrode through a sulfur-bearing peptide spacer in order to create a robust device. The direct chemical tethering of the protein has the advantage of fixing its orientation in the supported lipid bilayer. A synthetic thiolipid^[2f, 6] containing a sulfur-terminated spacer of similar length as that of the SLIC and the phospholipid POPC were

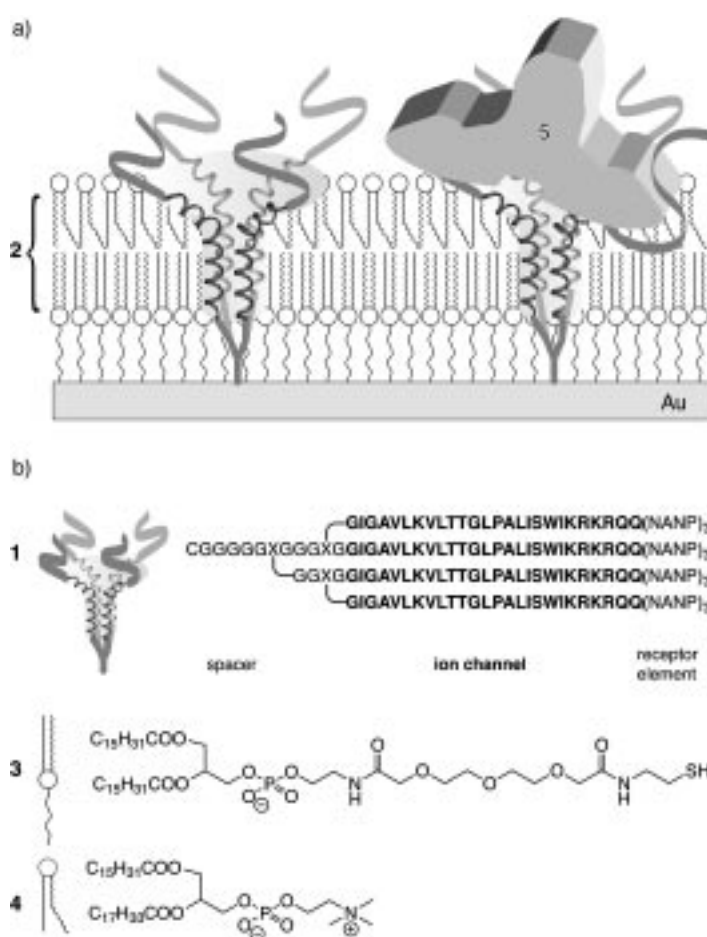


Figure 1. a) Schematic view of a synthetic ligand-gated ion channel (SLIC) in a tethered lipid bilayer (2). The SLICs and the thiolipids were covalently attached to the gold surface through terminal SH groups. The gating of an ion channel by the binding of an antibody molecule (5) is schematically illustrated. b) Amino acid sequence of the SLIC (1) in one-letter-code. X is used for the amino acid ornithine, which allows the synthesis of branched polypeptides. The SLIC is shown to traverse the bilayer as a four-helix bundle formed by the hydrophobic melittin parts (bold regions). Chemical formulas of the synthetic thiolipid (3) and of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; 4) are also depicted.

added sequentially to build a lipid bilayer (Figure 1). The supported molecular layer on the gold surface was characterized at every stage of formation by surface-sensitive techniques: 1) infrared (IR) spectroscopy provided details about the structural organization of the layers, 2) surface plasmon resonance (SPR) was used to measure the surface concentration of each component of the supported molecular layer, and 3) impedance spectroscopy (IS) showed that an initial tethered monolayer of thiolipids increased the electrical insulation of the supported bilayer substantially.

Reference IR spectra were first recorded from bulk SLIC samples on CaF₂ plates (Figure 2a). Deconvolution of the amide I and II bands of the SLIC on CaF₂ reveals there is a large content of α -helix that compares well with pure melittin, both in frequency and relative intensity.^[7] Hence the α -helical structure of melittin is preserved in the SLIC.

Reflectance absorbance (RA) spectra (Figure 2, spectra (b)–(d)) of the tethered thiolipid/SLIC layers on gold proved that the SLICs were not replaced by the thiolipid

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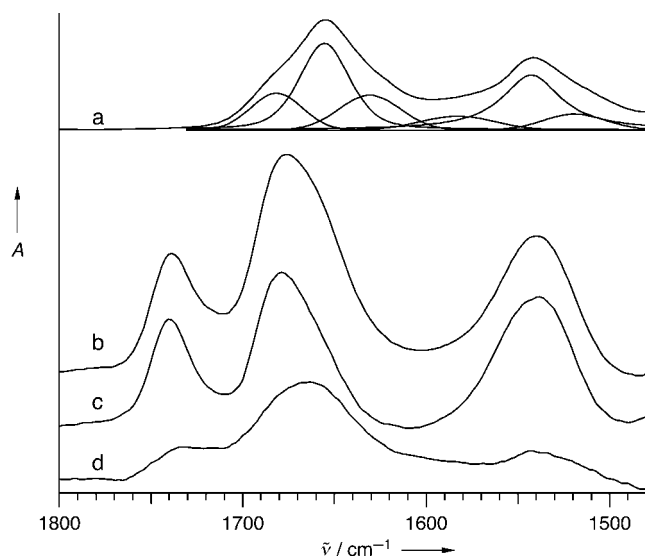


Figure 2. IR spectroscopy of the SLIC. Transmission spectrum of bulk SLICs on CaF_2 (a). The maximum of the amide I band is located at 1655 cm^{-1} . Curve fitting shows that this component accounts for 57% of the total amide I absorbance. In the RA spectrum of the mixed thiolipid/SLIC layer on gold (b) the amide I and II modes of the peptide are obscured by the strong amide I and II modes of the thiolipid (c). In the difference spectrum (d) the spectral contribution of the thiolipid has been subtracted from the spectrum of the mixed layer. For the subtraction, the thiolipid spectrum was weighted by a factor of 0.85 to account for the lower lipid content in the mixed layer. This factor was derived from the estimated 10 to 20% surface coverage determined by SPR. The subtraction leads to an almost complete extinction of the lipid ester band at 1738 cm^{-1} .

during layer formation. The orientation of the melittin subunits can be deduced from the ratio of the intensities of the amide I:II bands of the SLICs. This is possible because the major components of amide I and II transition moments of α -helical peptides are orthogonal, and on metal surfaces only p-polarized light can be absorbed.^[8] Intensity ratios of 0.1:1 or 5.7:1 have been previously reported for helix orientations parallel or perpendicular to the surface, respectively.^[8] An intensity ratio of amide I:II of greater than 3:1 was measured for the α -helical moieties of the SLIC (Figure 2, spectrum (d)). This value confirms the preferentially perpendicular orientation of the melittin moieties with respect to the surface.

The binding of the Sp3E9 antibody to a pure layer of SLICs was monitored by SPR (Figure 3 (a)). As a consequence of efficient rebinding at the surface, only a small amount of antibody could be desorbed by washing with buffer (b).^[9] A rapid dissociation was however induced by the competitive binding of the free antigen C(NANP)₆Y (c). Although, as seen previously,^[10] not all surface-bound antibodies could be displaced under these conditions, the true nonspecific adsorption measured using bovine IgG was negligible. The amount of bound Sp3E9 at saturation scaled with the SLIC content in mixed layers of lipids and SLIC. The binding of the antibody Sp3E9 did not induce any remarkable change in the electrical impedance spectra of tethered lipid bilayers with molar thiolipid:protein ratios smaller than 200:1 (data not shown). At these high surface concentrations of SLICs the tethered lipid bilayers are not sufficiently electrically blocking. There-

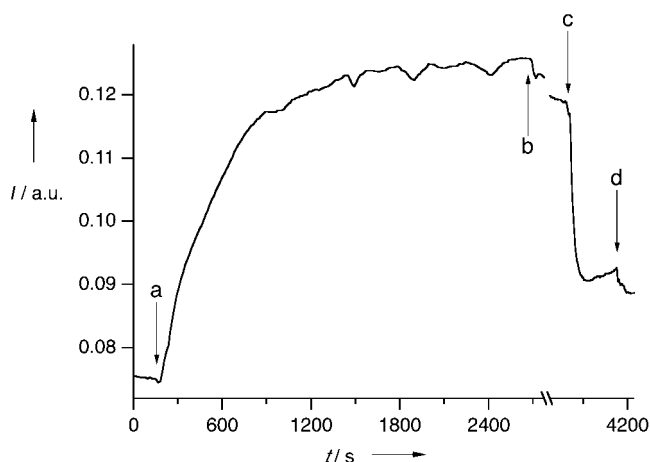


Figure 3. Change of the SPR signal of a gold film covered by a SLIC monolayer after adding 10^{-7} M antibody Sp3E9 to the aqueous phase (a), washing with buffer (b), adding of 10^{-3} M competitive ligand C(NANP)₆Y (c), washing with buffer (d). The change of reflectivity at (b) originates from a shift of the SPR resonance angle of 0.72° as a result of bound antibodies at a surface concentration of 1 antibody per 81 nm^2 .

fore, the charging current of the large interfacial (double-layer) capacitance dominates the impedance spectra at all frequencies.

Increasing the thiolipid:protein ratio to approximately 2200:1 increased the membrane resistance R_M sufficiently so that the charging current of the membrane dominated the intermediate frequency range. The resistances of the solution, of the membrane, and of the interface (charge transfer) were then well separated in the impedance spectra (Figure 4). This spectral behavior is typical of supported lipid bilayers on electrodes.^[2c-e] Accurate values of R_M were calculated with the

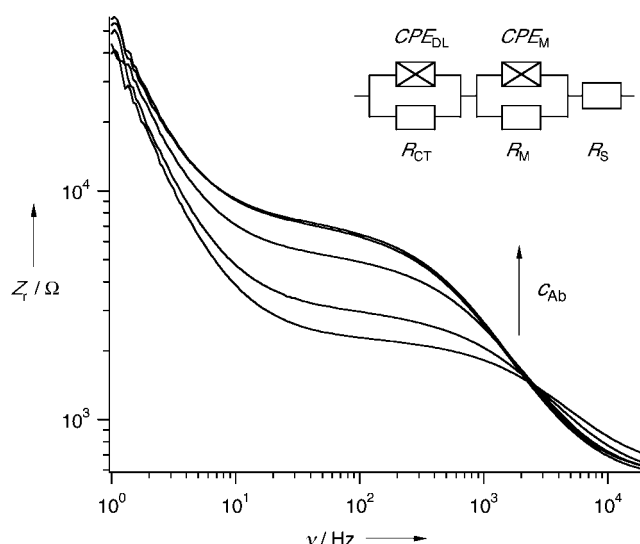


Figure 4. Real part of the impedance spectra of a lipid bilayer containing SLICs at various concentrations of antibody (from bottom to top): 0 , 10^{-9} , 3×10^{-9} , 10^{-8} , and $3 \times 10^{-8}\text{ M}$. The resistances of the interface (charge-transfer, R_{CT}), the membrane (R_M), and the electrolytic solution (R_S) are spectrally well resolved; distributed elements (CPE_{DL} and CPE_M) are used for a very accurate fit of the dielectric properties of the interface and of the membrane, respectively. The increase of R_M as a function of the antibody concentration in the bulk solution is seen at frequencies between 10 Hz and 1 kHz.

equivalent circuit of Figure 4. Here, the important finding is the increase in the value of R_M in the presence of nanomolar concentrations of the antibody Sp3E9 in the bulk solution. Figure 5 shows the time course of the corresponding admit-

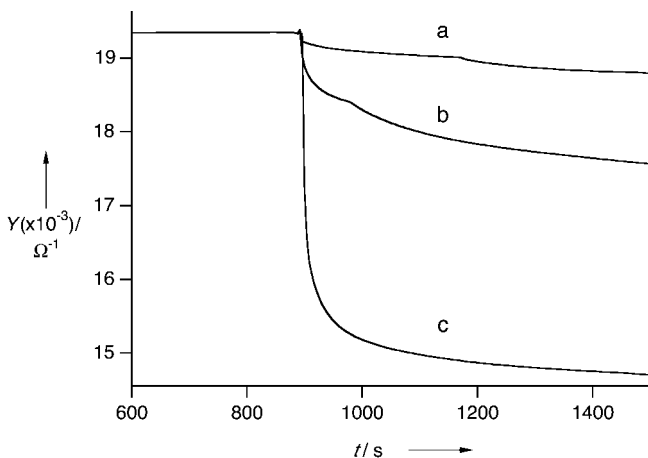


Figure 5. Electrical admittance (measured at 180 Hz) of a tethered lipid bilayer containing SLICs after the addition of buffer containing 3×10^{-7} M IgG (a), 3×10^{-9} M Sp3E9 (b), and 3×10^{-8} M Sp3E9 (c).

tance at various antibody concentrations. Unspecific adsorption of IgG at even a hundredfold higher concentration than used for Sp3E9 (Figure 5, spectrum (a)) was much smaller than the specific binding of Sp3E9 (Figure 5, spectra (b) and (c)). The major part of the response could be reversed by washing with buffer, since the fraction of rebound antibodies Sp3E9 was very small at this low density of surface-bound antigens.^[9]

The modulation of the R_M value with the antibody concentration in the electrolyte is plotted in Figure 6. The binding curve indicates some cooperativity and is best fitted by a Hill function with a dissociation constant of 2.4×10^{-9} M and a Hill coefficient of 2.1. From a comparison of the values of R_M measured before and after a buffer wash the reversibility of the response is shown to be always larger than 50%. The reported relative effect of antibody binding is large: the

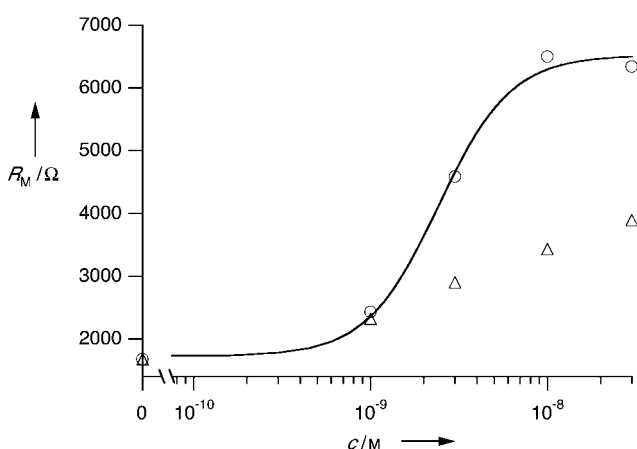


Figure 6. Electrical resistance R_M of the tethered lipid bilayer containing SLICs after antibody binding at different bulk concentrations c of Sp3E9 before (○) and after (△) washing with buffer.

membrane resistance increases by almost a factor of four at saturation. The absolute increase of approximately 4500Ω is however limited by the number of defects in the lipid layer. The values of R_M obtained after complete antibody binding are indeed very close to the limiting values measured for the pure lipid bilayers. The complete blocking of 1% of the surface-bound SLIC is sufficient to account for the reported effect.^[11] Enhanced sensitivity is therefore expected with membranes having higher electrical resistances. Measurements on smaller electrodes would be useful if smaller changes in conductance need to be detected.

In conclusion, the gating of an artificial receptor was monitored for the first time by impedance spectroscopy in tethered lipid bilayers. Subnanomolar concentrations of antibody were detected by modulation of the channel activity. The principle of gating by an antibody is particularly attractive because of its general character, and thus opens new possibilities for immunosensing.^[12]

Experimental Section

The branched polypeptides (SLICs) and $C(NANP)_6Y$ were assembled by linear solid-phase peptide synthesis on Wang resin following the Fmoc/Bu strategy (Fmoc = 9-fluorenylmethoxycarbonyl). The polypeptides were cleaved from the resin by treatment with a mixture of trifluoroacetic acid (88%), water (2%), and triisopropylsilane (10%). The crude products were prepurified by size-exclusion chromatography on Sephadex G50 Superfine using 50% acetic acid as the eluant. The fractions containing the target products were pooled and purified by RP-HPLC on a Vydac C_{18} column. The molecular weights were controlled by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MW: 17155 Da for the SLIC). The thiolipid N -(14'-sulfanyl-1',11'-dioxo-3',6',9'-trioxa-12'-azatetradecyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine^[2f] and the competing antigen $C(NANP)_6Y$ ^[10] were synthesized as described earlier. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was obtained from Avanti Polar Lipids (Alabaster, AL), and the bovine IgG from Sigma (St. Louis, MO). The monoclonal antibody Sp3E9 was a generous gift from H. Matile (Hoffmann – La Roche, Switzerland); it was raised in mice against the synthetic peptide $(NANP)_{40}$ as described elsewhere.^[13]

A 45-nm thick film of gold was prepared by evaporation through a metallic mask on a glass slide silanized with (3-trimethoxysilyl)propylthiol. Sulfur-bearing molecules were self-assembled on gold directly in the measurement cells and the layer formation was monitored in situ by SPR and IS. In order to prepare layers with a thiolipid:protein ratio smaller than 200:1, SLIC molecules were adsorbed by spreading small unilamellar vesicles composed of POPC and SLIC followed by a detergent or methanol wash. SLIC monolayers with a thiolipid:protein ratio of 2200:1 were produced by prior self-assembly of SLIC at a low concentration (10^{-8} M) in buffer. The surface-immobilized SLICs were first integrated into a tethered lipid monolayer by adding thiolipids from a detergent solution. The lipid bilayer was then completed by an external POPC monolayer by using a dilution method from detergent^[2a] or a methanol^[14] solution. Antibody binding to the sensor surface was measured under constant antibody concentration in the bulk as described earlier.^[10]

SPR measurements were performed on a home-made reflection apparatus.^[2a] Organic molecules adsorbed at the gold surface led to a shift of the resonance angle, which was used to calculate their optical thickness. Assuming a refractive index of $n = 1.45$, an experimental shift of 1° corresponded to a thickness of 6.4 nm. The mean molecular area of the adsorbed proteins was calculated with a refractive index increment of $0.18 \text{ g}^{-1} \text{ cm}^3$.^[2e]

Infrared spectra were recorded using a Bruker IFS28 spectrometer equipped with a HgCdTe detector. Spectra were background-corrected using the respective bare supports and presented at 4 cm^{-1} resolution with triangular apodization. Absorbance spectra of SLICs as bulk solid samples

were obtained in transmission mode by drying methanolic solutions of the SLICs on precleaned CaF₂ windows. Reflectance absorbance spectra were recorded at an angle of incidence of 85° using p-polarized light. The amide I and II bands were deconvoluted by applying a curve-fitting procedure based on the Levenberg–Marquardt algorithm using a combination of Gaussian and Lorentzian line shapes. Initial guesses for the curve fitting were determined from second-derivative spectra.

IS was done in an electrochemical cell comprising a membrane-covered gold electrode and a counter/reference Ag/AgCl electrode in 0.1M KCl/5 mm sodium phosphate buffer, pH 7.4. The surface area of the gold disk electrode was 3.34×10^{-2} cm². No direct current voltage was applied. A sinusoidal potential of 10 mV amplitude (root mean square) was applied to the cell at 199 successive frequencies equally spaced on a logarithmic scale from 1 to 20 KHz. The resulting current was recorded by a phase-sensitive lock-in amplifier to calculate the complex impedance and admittance. Details on the interpretation of IS spectra can be found in the Supporting Information.

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Super-“Amphiphobic” Aligned Carbon Nanotube Films**

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Carbon nanotubes (CNTs) are of tremendous interest for both fundamental and applied research^[1–6] since they show unique properties. Wettability is an important factor for a material. Ebbesen and co-workers have studied the wettability of CNTs in detail and found they could be wet and filled by different substances.^[7] In order to assess the properties of CNTs easily, it is highly desirable to prepare aligned carbon nanotube (ACNT) films in which the nanotubes are perpendicular to the substrate surface and densely packed with a fairly uniform length and diameter. To the best of our knowledge, no attention has been paid to the wettability of ACNT films.

In general, the wettability of solid surfaces is controlled by the chemical composition and the geometrical structures of the surfaces, and it is usually enhanced by surface roughness,^[8–12] especially by fractal structures.^[12] Recently, super-hydrophobic or super-lipophobic surfaces, that is, those with a contact angle for water or oil, respectively, that is higher than 150°,^[8, 12c] have attracted much interest for practical applications. These surfaces have been commonly prepared through the combination of surface roughening and the lowering of the surface energy. However, few reports have concerned super-“amphiphobic” surfaces,^[13] which have both super-hydrophobic and super-lipophobic properties. Öner and McCarthy reported that super-hydrophobic surfaces with a micrometer-scale post structure could be prepared by photolithography and made hydrophobic using silanization reagents.^[14] As the structure of ACNT films is similar to that of these super-hydrophobic surfaces, the films are expected to show special wettability features. Here we report that the nanostructured ACNT films show super-“amphiphobic” properties, namely, the contact angles for both water and oil are larger than 160°.

In a previous paper we demonstrated that honeycombl-like ACNTs could be prepared by pyrolysis of metal phthalocyanines.^[14] The approach used herein for the preparation of ACNT films is similar to that reported previously, namely ACNT films are prepared by pyrolysis of metal phthalocyanines.

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