

Covalent attachment of functionalized cardiolipin on a biosensor gold surface allows repetitive measurements of anticardiolipin antibodies in serum

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Abstract Antiphospholipid antibodies (aPL) are a relevant serological indicator of antiphospholipid syndrome (APS). A solid-state surface with covalently bound ω -amine-functionalized cardiolipin was established and the binding of β 2-glycoprotein I (β 2-GPI) was investigated either by use of surface plasmon resonance (SPR) biosensor, by electrically switchable DNA interfaces (switchSENSE) and by scanning tunneling microscopy (STM). STM could clearly visualize the attachment of β 2-GPI to the cardiolipin surface. Using the switchSENSE sensor, β 2-GPI as specific ligand could be identified by increased hydrodynamic friction. The binding of anti-cardiolipin antibodies (aCL) was detected against the ω -amine-functionalized cardiolipin-

modified SPR biosensor (aCL biosensor) using sera from healthy donors, APS patients and syphilis patients. Our results showed that the aCL biosensor is a much more sensitive diagnostic device for APS patients compared to previous methods. The specificity between β 2-GPI-dependent autoimmune- and β 2-GPI-independent infection-associated types of aPLs was also studied and they can be distinguished by the different binding kinetics and patterns.

Keywords Antiphospholipid syndrome · Antiphospholipid antibody · Cardiolipin · β 2-glycoprotein I · Phospholipid · Surface plasmon resonance

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Abbreviations

aCL	Anticardiolipin antibody
aPL	Antiphospholipid antibody
APS	Antiphospholipid syndrome
AUC	Area under the curve
a β 2-GPI	Anti- β 2-GPI antibodies
BB	Blocking buffer
BFP	Biological false positive
FC	Flow cell
FCS	Fetal calf serum
HBS	HEPES-buffered saline
HRP	Horseradish peroxidase
LA	Lupus anticoagulant
mAb	Monoclonal antibody
MW	Molecular weight
PBS	Phosphate-buffered saline
PL	Phospholipid
ROC	Receiver operating characteristic
RU	Resonance units
SAM	Self-assembled monolayer
SD	Standard deviation
SLE	Systemic lupus erythematosus

SPR	Surface plasmon resonance
STM	Scanning tunneling microscopy
TMB	3,3',5,5'-tetramethylbenzidine
VDRL	Venereal disease research laboratory
WB	Washing buffer
β 2-GPI	β 2-glycoprotein I

Introduction

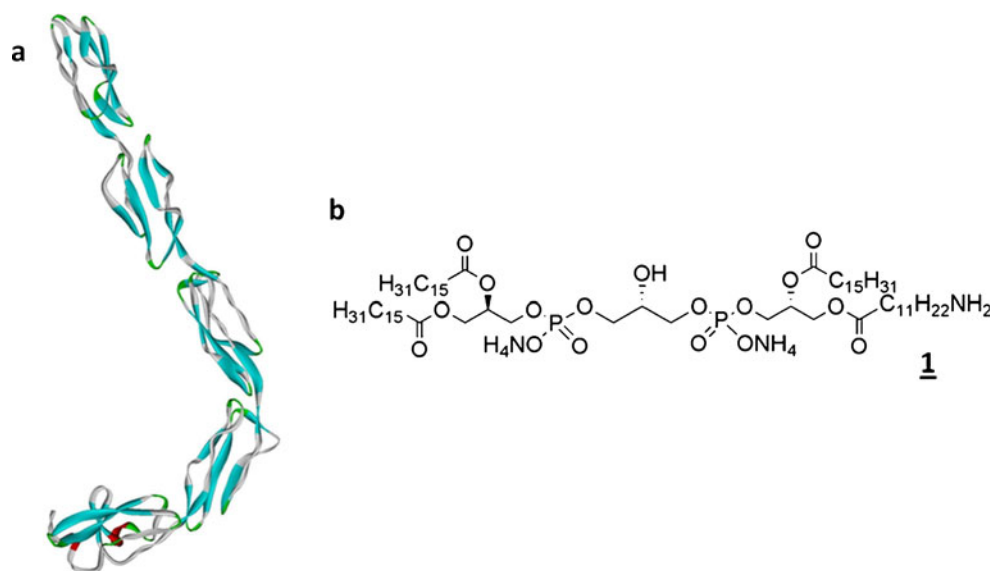
Although parainfectious anti-cardiolipin antibodies (aCL) were first described in 1906, when developing a test for syphilis [1], it took 35 years until cardiolipin was identified as the main antigenic structure [2]. This phospholipid (PL) consists of two phosphatidyl glycerols connected via a central glycerol (1,3-bis(*sn*-3'-phosphatidyl)-*sn*-glycerol). Both lateral glycerols are acylated with fatty acids via the primary and secondary hydroxyl groups. The venereal disease research laboratory (VDRL) developed a serological test using cardiolipin for mass screening to control syphilis. The application of this test identified groups of people who had positive results for syphilis without physical evidence of syphilis infection, the so-called biological false positive (BFP) VDRL reactions [3]. Further, one third of patients with BFP serological tests were found to have lupus anticoagulant (LA) [4]. Today, it is known that the reactivity of antibodies responsible for LA and BFP serological tests depend on protein targets that bind anionic PL such as cardiolipin, phosphatidylcholin or phosphatidylserin. Thus, the aPL from syphilis patients which directly bind to phospholipid [5, 6] are different from those for LA and BFP. The allocation of patterns of clinical symptoms to the laboratory finding of persistently elevated titers of aPL led to the description of the antiphospholipid syndrome (APS) [7], often also called Hughes syndrome. This autoimmune disease is characterized

by a humoral hypercoagulability caused by aPL. As a result, thrombosis in both arteries and veins as well as placenta-related obstetrical complications occur. APS can develop as primary disease without association with other autoimmunities, whereas a secondary form of APS is found frequently in patients suffering from systemic lupus erythematosus (SLE) or other autoimmune disorders. A minor fraction of patients with primary APS later develop SLE. The Sydney classification criteria for APS [8] demand at least one clinical and one laboratory criterion. The latter one comprise the detection of either LA or anti-cardiolipin (aCL) or anti- β 2-glycoprotein I (a β 2-GPI) antibodies and has to be confirmed by a second determination performed after 12 weeks.

As mentioned above, aCLs in association with APS require the presence of the PL binding serum protein β 2-GPI (Fig. 1a), presumably due to the involvement of β 2-GPI in immune complex formation [9–11]. This 54-kDa single-chain serum glycoprotein binds to negatively charged PLs with high affinity [12]. During the binding event to cardiolipin, β 2-GPI undergoes a conformational change and converts from a circular to a J-shaped structure [13, 14]. This event presumably increases the binding capability for aPL. Nevertheless, the role of cardiolipin for the analysis of aPLs in APS patients remains a topic of controversial discussion. This is due to the observation that binding of aPLs to β 2-GPI can also be demonstrated in the absence of cardiolipin [15–17].

In a previous work, it was shown that surface plasmon resonance (SPR)-based assays are auspicious tools for autoimmune diagnostics [18, 19]. The advantage of the SPR biosensor is the real-time gathering of data concerning the association as well as the dissociation phases of antigen/antibody interactions. This enables the monitoring of the reactivity of autoantibodies in patient sera to a complementary surface-immobilized antigen.

Fig. 1 Structures of the two main antigens used for the SPR assays: **a** β 2-glycoprotein I; **b** ω -amine-functionalized cardiolipin **1** [21]



To gain deeper insights into the role of surface-immobilized cardiolipin for the determination of aCL in APS patients, a new SPR-based assay was developed. For preparation of phospholipid surfaces two main approaches have been described in the literature, the supported bilayers and immobilized bilayers or liposomes [20]. In contrast to these well-described techniques where PLs are immobilized by adsorption, in this work, a ω -amine cardiolipin analogue **1** (Fig. 1b) was immobilized covalently to a gold surface modified with self-assembled monolayer (SAM) to generate a stable and regenerative assay enabling multiple measurements.

The aims of this study were not only the development and evaluation of the aCL biosensor and its comparison with the existing a β 2-GPI SPR assay [18], but also the application of two alternative sensor techniques to investigate the specific interaction of β 2-GPI and cardiolipin which determines the performances of the both SPR assays.

Materials and methods

Biochemicals

Gold-coated glass slides (SIA Kit Au), the BIAcore amine coupling kit and ethanolamine-HCl was purchased from GE Healthcare (Freiburg, Germany). 11-mercaptopundecanoic acid, 11-mercaptopundecan-1-ol, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 3,3',5,5'-tetramethylbenzidine (TMB) and transferrin were obtained from Sigma-Aldrich (Taufkirchen, Germany). Potassium chloride, sodium chloride, potassium phosphate monobasic, sodium phosphate dibasic dihydrate and ethanol were purchased from Merck (Darmstadt, Germany). β 2-GPI was from Scipac (Sittingbourne, UK). Fetal calf serum (FCS) was purchased from PAA Laboratories GmbH (Pasching, Austria).

The ω -amine cardiolipin analogue **1** was synthesized by the group of Andrew B. Holmes, as previously described [21].

Patients

We studied 21 patients with APS, 21 healthy controls, and 10 patients with positive results in the VDRL test. All APS patients satisfied the Sydney classification criteria for APS [8]. The serological parameters aCL and a β 2-GPI were determined by use of the Alegria system from Orgentec (Mainz, Germany). LA was analyzed with the BCS XP from Siemens Healthcare Diagnostics (Eschborn, Germany). APS patients and controls were age-matched. The latter were assumed to be free of any acute or chronic disease on the basis of a medical and clinical chemistry examination. Each blood sample was collected in 10 mL tubes without anticoagulant. After clotting, the samples were centrifuged at 1,500 $\times$ g for 15 min. Sera were collected and stored at -80°C .

The study was approved by the institutional ethics committee of the Klinikum rechts der Isar, Technische Universität München. All participants gave written informed consent. No financial compensation was provided.

ELISA

ELISA plates (Nunc PolySorp 96, Thermo Electron LED, Langenselbold, Germany) were coated with either cardiolipin or the ω -amine-functionalized cardiolipin (50 $\mu\text{g/mL}$ diluted in ethanol, 30 $\mu\text{L/well}$) overnight at 4°C . After incubation, wells were washed three times with washing buffer (WB: phosphate-buffered saline (PBS); 2.6 mM KCl, 138 mM NaCl, 10 mM $\text{HNa}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.8 mM H_2KPO_4 , pH 7.2; 100 $\mu\text{L/well}$), blocked for 1 h with 200 $\mu\text{L/well}$ blocking buffer (BB: PBS, 10 % fetal calf serum), and washed once again with WB. Serum samples were diluted 1:100 in BB and 100 μL of each sample was added to wells at each dilution in duplicates and incubated for 30 min at room temperature. The wells were washed three times with WB, before 100 μL of horseradish peroxidase (HRP) labeled anti-human IgG (or IgM) (Dako, Glostrup, Denmark) were added at a 1:5000 dilution in BB, followed by incubation for 1 h. After three times washing with WB, TMB as substrate solution was added at 200 $\mu\text{L/well}$ and color was allowed to develop for 30 min. The reaction was stopped with H_2SO_4 (5 %) and plates were read for absorbance at 450 nm in an ELISA plate reader (BioTek Germany, Bad Friedrichshall, Germany).

SPR measurements

SPR measurements were performed using a BIAcore X (GE Healthcare) device at a working temperature of 25°C . All measurements were performed on SIA Kit Au sensor chips (GE Healthcare). The gold surfaces were modified with a SAM by incubating the surface with a 10 mM solution of 11-mercaptopundecanoic acid and 11-mercaptopundecan-1-ol (6/4 molar ratio) in ethanol. The surface was activated by using the BIAcore amine coupling kit and HBS (HEPES-buffered saline; 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 500 mM NaCl, pH 7.5) as running buffer. After reducing the flow to 1 $\mu\text{L/min}$, 100 μL of a 74 nM solution of (–)-1-*O*-(1'-*O*-[12"-Aminododecanoyl]-2'-*O*-hexadecanoyl-*sn*-glycer-3'-yl-*O*-phosphoryl)-3-*O*-(1',2'-di-*O*-hexadecanoyl-*sn*-glycer-3'-yl-*O*-phosphoryl)-*sn*-glycerol diammonium salt (**1**) (MW, 1,357 g/mol) in HBS were injected over the specific flow cell (FC 2). The reference cell (FC 1) was prepared under the same conditions by injecting 100 μL of a 166 nM solution of human transferrin in HBS as non-APS-antigenic protein. This concentration of transferrin was chosen to ensure a complete surface coverage by the protein in order to decrease unspecific binding. Subsequently,

1.0 M ethanolamine-HCl, pH 8.5 (10 min) was added to each flow cell to block the remaining activated carboxylic groups.

Signal differences between the two flow cells were monitored as a function of time (SPR sensorgrams), expressed in arbitrary resonance units (RU). For most macromolecules, one RU is equivalent to a change in concentration of $\sim 1 \text{ pg/mm}^2$ on the sensor surface [22]. The binding of different concentrations of $\beta 2$ -GPI (1, 0.5, 0.25, 0.125, 0.0625 μM) was investigated at a continuous flow (1 $\mu\text{L/min}$) with HBS as running buffer. The surface was regenerated by a 5 s pulse of regeneration solution (50 mM NaOH, 1 M NaCl). For measuring the biomolecular interactions between the sera and the immobilized cardiolipin, a PBS-buffer (2.6 mM KCl, 138 mM NaCl, 10 mM $\text{HNa}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.8 mM H_2KPO_4 , pH 7.6) with 5 % of FCS was used as running buffer. The measurements were performed at a continuous flow rate of 1 $\mu\text{L/min}$ conducting 45 μL of diluted sera (1:10 with running buffer) over the surface to allow association, followed by a 300 s period of buffer injection to monitor the dissociation. After each measurement, the biosensor surface was regenerated with a 5-s pulse of regeneration solution (50 mM NaOH, 1 M NaCl).

All buffers for the biosensor measurements were sterile-filtered using 0.2 μm FP CA-S filters (Whatman, Dassel, Germany) and carefully degassed with helium before use.

Scanning tunneling microscopy measurements

Scanning tunneling microscopy (STM) images were obtained either by an in-house Veeco/MI EC-STM consisting of a Nanoscope IIIA controller (Bruker AXS, Mannheim, Germany, former by Veeco Instruments) and a PicoSPM STM base (Agilent Technologies, Böblingen, Germany, former Molecular Imaging) together with an EC-Tec bipotentiostat/galvanostat BP600 and an EC-Tec bi-scangenerator SG600 or an electrochemical Veeco Multimode system with the universal bipotentiostat, a combined STM/SECPM head, a Nanoscope 3D Controller and the Nanoscope 5.31r2 software. All STM images were taken under in situ conditions in HBS in constant current mode ($U_{\text{Bias}}=100\text{--}300 \text{ mV}$, $I_{\text{T}}=0.2\text{--}1 \text{ nA}$) using etched Au tips insulated with Apiezon wax. All experiments were performed in a PTFE cell with an electrolyte volume of 100–200 μL and an exposed surface area of 0.13 cm^2 . The PTFE cell was cleaned with Caro's acid (95–98 % H_2SO_4 and 33 % H_2O_2 , volume ration of 1:1, both Merck, pro analysis) and then thoroughly rinsed with deionized water prior to each experiment. Image processing was performed with WSxM 4.0 [23] and ImageJ [24].

The gold surface was modified with a SAM as described above. The activation of the carboxyl groups was achieved by incubation of the surface with a solution of EDC/NHS (4:1) in deionized water for 1 h at room temperature. After washing the surface three times with deionized water it was covered with a solution of **1** (1 mg/ml) in HBS for 12 h at 4 °C. The surface

was then washed again three times with deionized water before being covered with a solution of $\beta 2$ -GPI (1 mg/ml) in HBS for 2 h at room temperature. Prior to the STM image measurements the surface was washed three times with HBS.

SwitchSENSE sensor

Oligonucleotides obtained from IBA (Göttingen, Germany) with a mixed, non-self complementary 48 bp sequence [25] were used as switchable DNA probes. Circular gold work electrodes of 120 μm diameter and adjacent platinum reference electrodes were deposited on glass substrates using standard metallization techniques. Double-stranded DNA layers were immobilized on the gold electrodes according to a previously described protocol, which is described together with the electro-optical measurement setup in references [25, 26]. After DNA layer preparation, the double-stranded DNA layer was dehybridized by rinsing 10 mM NaOH pH 11 for 3 min, and hybridized with 5' **1**-modified complementary sequences. Thus, the cardiolipin moiety was located at the surface-distal end of the DNA molecules. Carboxyl-DNA was activated by NHS/EDC and modified with the cardiolipin derivative **1**. Reaction products were purified by RP-HPLC (C18 Hydrosphere). The incubation of $\beta 2$ -GPI (1 μM) was performed in 150 mM NaCl, HEPES 20 mM, pH 7.3, in stopped flow for 30 min at room temperature. The frequency responses of DNA switching were compared in 50 mM NaCl, HEPES (20 mM, pH 7.3).

Statistics

The Analyse-it™ (Analyse-it Software, Leeds, UK) add-in program for Windows Excel was used for statistical analysis. Receiver operating characteristic (ROC) curves were plotted and areas under the curves (AUC) as a measure of the differentiation ability ill vs. healthy were determined; $p_{0.5}$ was calculated to check whether the individual AUCs are significantly different from 0.5 [27] and p_c whether they are significantly different from each other [28]. For ROC analysis, all 21 patients were considered to be ill even if negative in the serological analyses of aCL and a $\beta 2$ -GPI. Optimal cutoff values were set to yield a maximum Youden Index ($J = \text{sensitivity} + \text{specificity} - 1$). For comparison of the ELISA tests, Spearman's rank correlation coefficient r_s was determined.

Results

ELISA

Development of a reusable biosensor for the detection of aCL requires the covalent attachment of cardiolipin to a functionalized surface. Therefore a cardiolipin derivative had

to be synthesized with an amine group at the end of one of the four fatty acid side chains (Fig. 1) [21]. To ensure that this synthetic derivative still retains its antigenicity, ELISA plates were coated with either cardiolipin or the ω -amine-functionalized derivative **1** by physisorption. Using these assays for determination of aCL levels (IgG/IgM) in APS patients as well as in healthy controls, a comparable performance of the two compounds was observed. For the IgG-ELISAs, AUC for cardiolipin was 0.837 ($p_{0.5} < 0.0001$) and 0.805 ($p_{0.5} < 0.0001$) for **1**. IgM-ELISAs yielded lower AUCs (cardiolipin, 0.697, $p_{0.5} = 0.0127$; **1**: 0.658, $p_{0.5} = 0.0433$) than the respective IgG-ELISAs. AUCs of IgG- and IgM-ELISAs were not significantly different from each other (IgG: $p_c = 0.5914$; IgM: $p_c = 0.5955$). The Spearman's rank correlation coefficient was $r_s = 0.78$ for the IgG-ELISAs and $r_s = 0.67$ for the IgM-ELISAs (ELISA data shown in the [Electronic supplementary material \(ESM\)](#), Table S1/S2).

Immobilization of cardiolipin

Several publications deal with carboxymethyl dextran hydrogel, modified with lipophilic *n*-alkyl chain anchors (L1 chip, Biacore) being used to capture PL vesicles in order to analyze the binding of proteins to lipid surface by surface plasmon resonance [29, 30]. The drawback of this non-covalent immobilization strategy is the cleavage of the antigen from the surface when regenerating the chip. As a consequence, prior to every measurement the PL has to be immobilized again, leading to longer analysis time and to a lack of reproducibility of the active surface. To overcome these problems we established planar carboxyl-terminated SAM surfaces, allowing the covalent coupling of the ω -amine-functionalized cardiolipin **1** (Fig. 2). Modification of the gold surface was visualized by STM (Fig. 3). Figure 3b shows a typical STM image of the SAM-modified gold

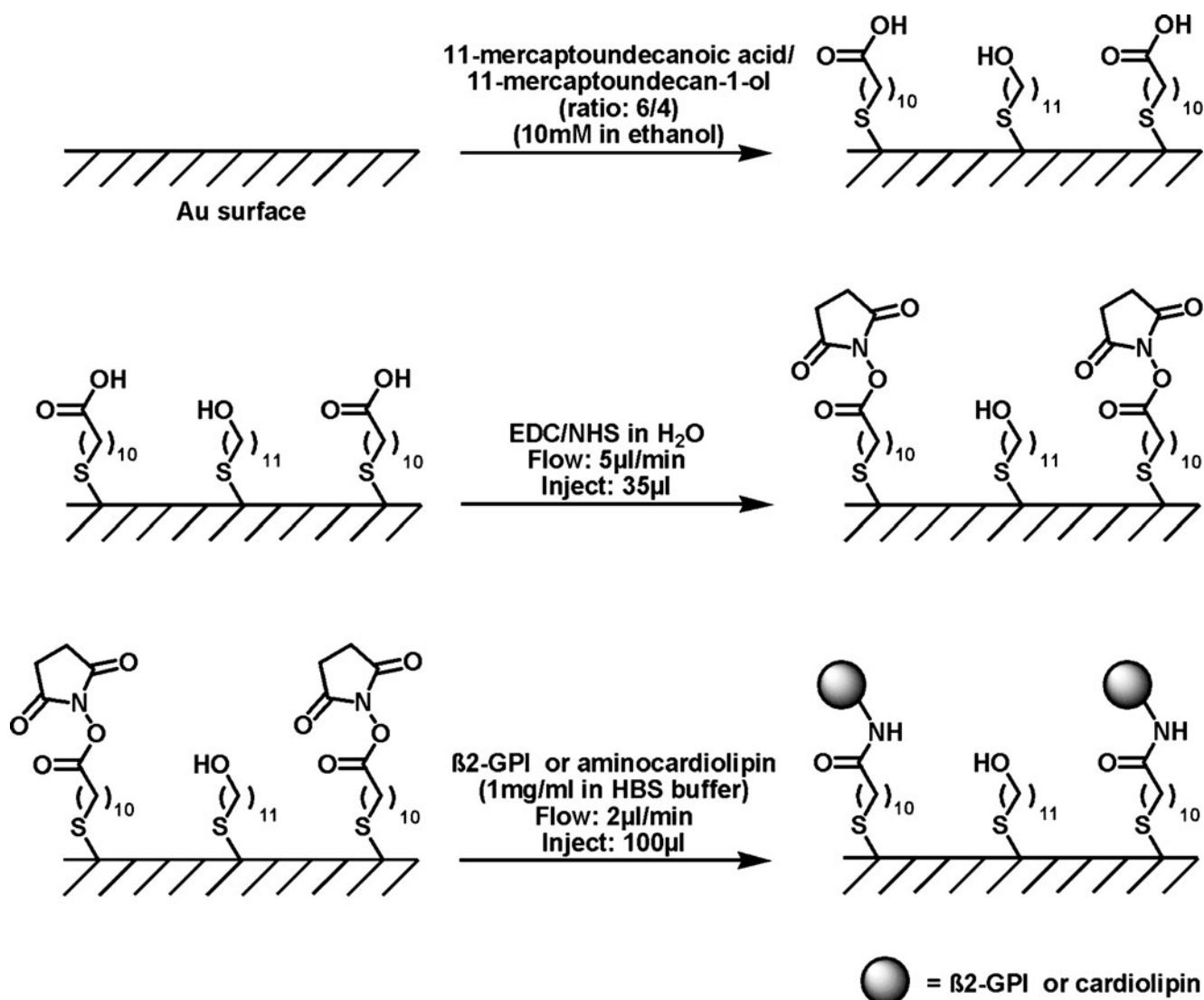
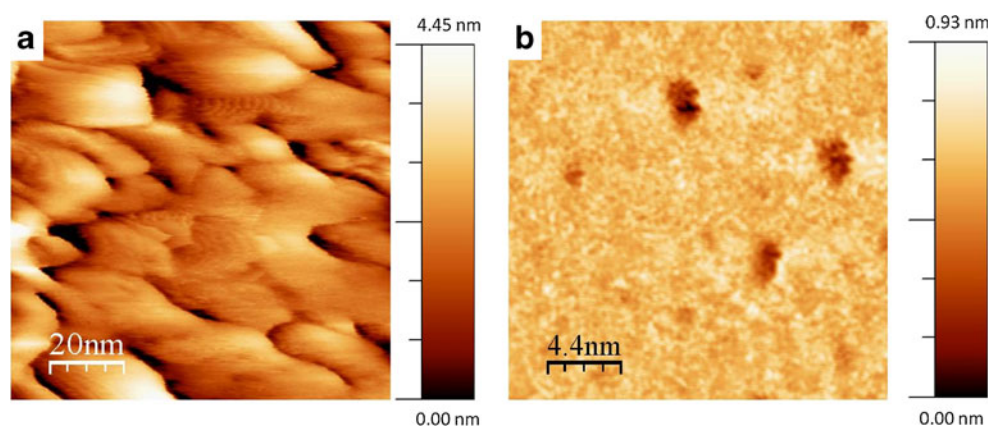


Fig. 2 Covalent immobilization of antigens on a SAM-modified gold surface

Fig. 3 Successful modification of the gold surface with a self-assembled monolayer imaged by STM. **a** Blank gold surface; **b** gold surface after 12 h incubation with a 10 mM solution of 11-mercaptoundecanoic acid and 11-mercaptoundecan-1-ol (ratio, 6/4) in ethanol



surface decorated by pit-like defects, so-called vacancy islands. These pit defects were first observed by Häussling et al. [31]. Later, it could be shown that the pits are defects in the Au surface layer, not defects in the *n*-alkanethiol layer [32].

Binding of β 2-GPI to immobilized cardiolipin

We first tested the functionality of the **1**-coated surface by measuring the binding of β 2-GPI. SPR sensorgrams for different β 2-GPI concentrations (1–0.0625 μ M) showed clear concentration-dependent signals (Fig. 4a), whereas no binding events were observed to transferrin at the surface of the reference cell. For visualization, the SAM-modified gold surface with immobilized **1** was incubated for 2 h with a solution of β 2-GPI (1 mg/ml) in HBS and then examined by STM (Fig. 4b). The surface image exhibits a dense packing of the proteins, which indicates that the surface is functionally structured, with the antigenic sites of the protein being sterically accessible for potential autoantibodies. The average size of the single proteins imaged in the STM is $100 \times 50 \times 20$ Å with a standard deviation (SD) of 10 %, which is in good accordance with the overall dimensions

of the β 2-GPI molecule of $132 \times 72 \times 20$ Å [33, 34]. The measured size is slightly smaller than the crystallographic. However, one has to keep in mind that STM images always show a convolution of the surface topography and its electronic properties, such as conductivity, as well as the surface topography of the tip, especially in scanning direction. Therefore, in STM experiments, the electron transfer pathway is a crucial point for mapping the topography of protein structures. If only the amide skeleton is involved in the tunneling process and not all the sidechains, the enzyme appears smaller in the STM image than it is in reality [35]. Another point that might be responsible for the mismatch of apparent morphology and crystallographic data is the fact that STMs are normally calibrated against a monoatomically high Au(111) step in *z*-direction and against the atomic structure of HOPG in *x*–*y*-directions, not against a protein calibration standard which would be more suitable due to the different conductivity. However, such calibration standards are not available. Therefore, absolute values should not be overrated. Measured dimensions only describe the apparent topography, which is a convolution of electronic and

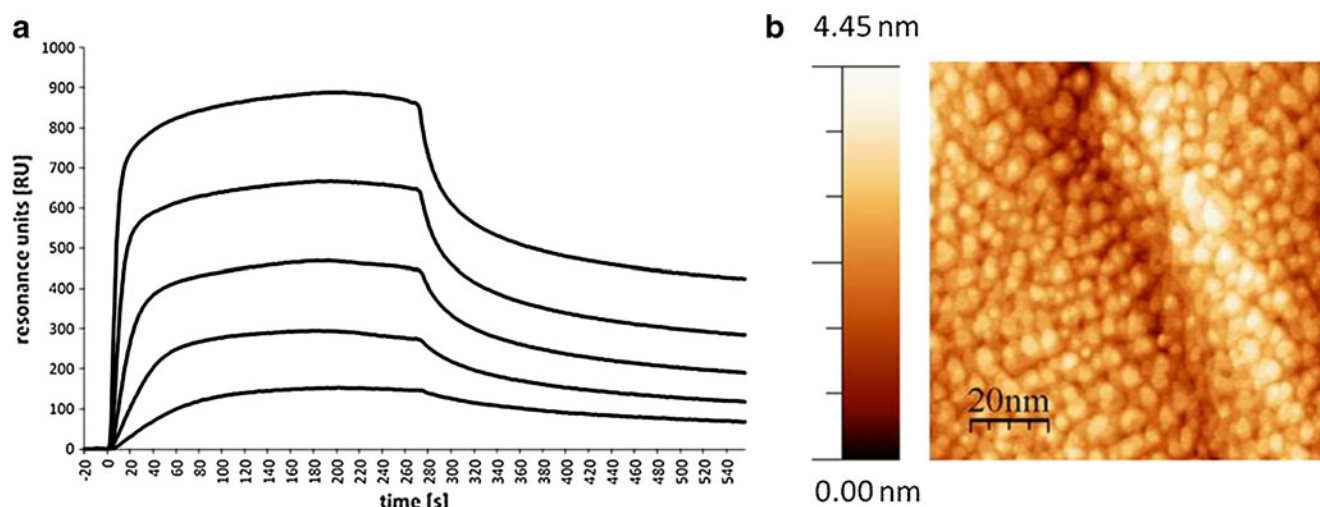


Fig. 4 **a** Sensorgrams of the aCL SPR biosensor for different concentrations of β 2-GPI (1 μ M, 0.5 μ M, 0.25 μ M, 0.125 μ M, 0.0625 μ M); **b** STM imaging of the SAM/1 surface after incubation with β 2-GPI

morphological properties. By contrast, no β 2-GPI molecules could be detected in the STM image when incubating only the SAM-modified surface with β 2-GPI under the same conditions (data shown in the [ESM](#), Fig. S1).

For further characterization of the biospecific interaction between β 2-GPI and **1**, a new label-free technology based on electrically switchable DNA layers was applied. The advantage of the switchSENSE sensor is the possibility not only to detect the binding event but also to estimate the molecular size of bound ligand molecules. The probes are short (48 bp) DNA molecules which are tethered at one end to gold microelectrodes on a chip. The DNAs' top ends are modified with receptors, in this case cardiolipin. By applying alternating electric voltages to the electrodes, the charged DNA molecules are dynamically switched between an upright and a reclined conformation, as indicated schematically in Fig. 5a. This conformational switching process is monitored in real-time mode by the fluorescence signals emitted from dyes attached to the DNA top ends (Fig. 5b). The DNA switching can be driven at frequencies exceeding 100 kHz, however DNA velocity is limited by hydrodynamic friction damping the oscillation amplitude ΔF (Fig. 5b) at high frequencies. The maximum achievable DNA velocity can be characterized by the cutoff frequency f_c being defined as the frequency value for which the switching amplitude ΔF has decreased to 50 % of its low-frequency value [25]. The cutoff frequency of a DNA layer modified with **1** as receptor is $f_c = 172$ kHz. After incubation with β 2-GPI (1 μ M) as ligand for cardiolipin in HBS, a pronounced decrease of f_c by -17 kHz can be observed which corresponds to a slowdown of the switching dynamics by approximately 10 %. By contrast, the cardiolipin-negative control layer does not show any response. The 17 kHz frequency shift can be attributed to an increase in hydrodynamic friction due to the binding of β 2-GPI to **1**-modified DNA. By comparison with previous measurements in which the frequency shift was experimentally determined for proteins of different molecular weight [25], the added molecular weight at the **1**-modified DNA end can be estimated to be ~ 50 kDa. This is in agreement with the expected MW of β 2-GPI. Hence, the results obtained by the switchSENSE method confirm the binding of β 2-GPI to the ω -amine-functionalized cardiolipin **1**.

SPR biosensor analysis of APS patient sera

Healthy donors showed no aCL-specific activity under optimized SPR sensor conditions (see above). The maximum binding signals after injection of diluted sera (1:10) of healthy donors with negative syphilis serology under appropriate experimental conditions (PBS with 5 % FCS) was as small as 126 RU, whereas sera of aCL ELISA-positive APS patients showed values in the broad range of 266–1748 RU

(Fig. 6a). It is known that the titers of aCL positive APS patient extend over a broad range [18]. By setting a cutoff value of 126 RU (Youden Index=1) [36], we were able to detect aCL in patient sera with confirmed APS with 100 % diagnostic specificity and 100 % sensitivity. Analyzing the same set of sera in a routine aCL IgG/IgM ELISA (Orgentec, Germany), the APS patients were detected with 100 % specificity at a sensitivity of 85.7 %. Hence, the developed biosensor is more sensitive in comparison with the standard ELISA tests used in routine diagnostics. Reproducibility was assessed on three separate biosensor chips by performing multiple injections of a single APS serum with CL IgG ELISA titers of >120 U/ml in the routine ELISA. Each sensor surface was stable for 15 measurements with a SD of 3.6 % for the maximum binding measured at the end of the association. Chip-to-chip reproducibility has a SD of 2.6 % (SPR data are shown in the [ESM](#), Fig. S2/S3).

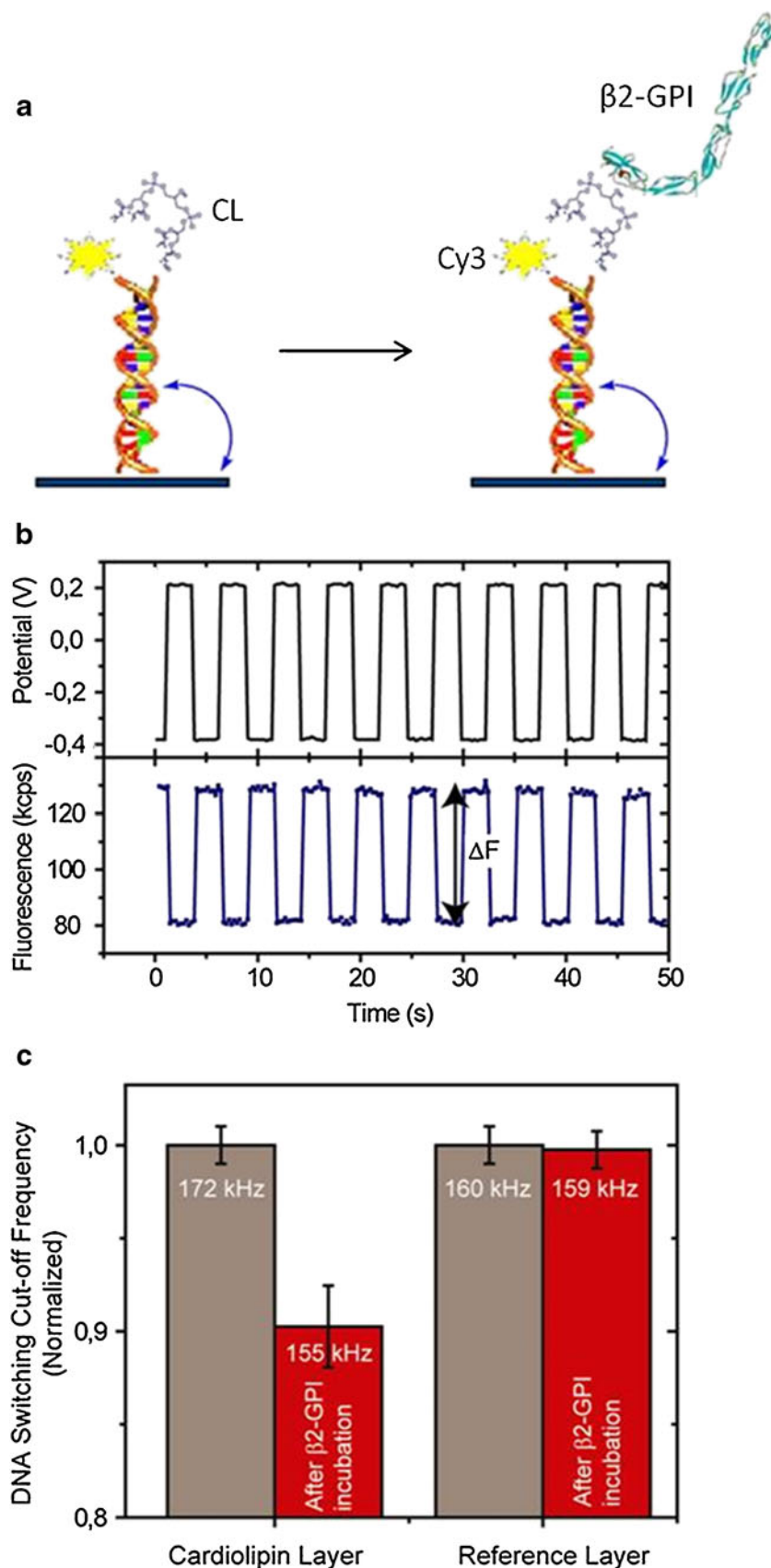
A primary problem using cardiolipin as the antigen in immunoassays for APS is the presence of parainfectious aCL. Therefore, we also tested sera from syphilis patients with positive VDRL test in the SPR biosensor and, as it was observed in the ELISA, a significant binding to cardiolipin could be detected (Fig. 6b).

In contrast to the ELISA which does not allow reliable differentiation of affinity and avidity of antibody binding, the SPR biosensor device allows resolution of association and dissociation phases to monitor the reactivity of antibodies in patient sera to surface-immobilized cardiolipin. We could show that it may be possible to distinguish patients with APS and syphilis by assessing the profile of the binding curve (Fig. 6): The aCL found in APS patient sera showed a higher association rate, whereas the ascending dissociation curves of the aCL from VDRL positive sera point to rebinding phenomena often seen in low affine IgM antibodies. Comparing the ratios of the resonance units at the beginning of dissociation (285 s) and after a dissociation phase of 100 s (385 s), the values for APS patients are lower than 100 %, whereas for patients with syphilis the values are higher than 100 % (data shown in the [ESM](#), Fig. S4). This points to the predominance of a highly refined IgG autoantibody type in APS.

aCL biosensor vs. a β 2-GPI biosensor

Previously, we reported the development of a SPR biosensor for the detection of a β 2-GPIs in APS patient serum [18]. Although we were able to detect the respective autoantibodies against immobilized β 2-GPI, further experiments showed that the assay performance strongly depends on the β 2-GPI preparation used [37]. The same set of patient and healthy control sera tested in the aCL SPR system presented herein were also investigated with the a β 2-GPI assay. As in the aCL SPR assay, sera of healthy controls

Fig. 5 Detection of β 2-GPI by the switchSENSE sensor. **a** Schematic illustration of electrically switchable DNA molecules, oscillating in an applied ac field. The DNA top end is modified with a fluorescence dye (Cy3) for detection, and with CL receptors for β 2-GPI binding. **b** Electric potential (top panel) applied to gold microelectrodes and Cy3 fluorescence emission (bottom panel) from the switching DNA layer. The fluorescence is high when the DNA stands and low when the DNA lies on the surface, because the Cy3 emission is quenched in proximity of the gold. **c** Maximally attainable switching frequency (cutoff frequency f_c) for CL-modified DNA layers and reference DNA layers without CL before and after the incubation with β 2-GPI. Column heights are normalized to $f_{c, \text{ before}}$ values, numbers denote the measured f_c values



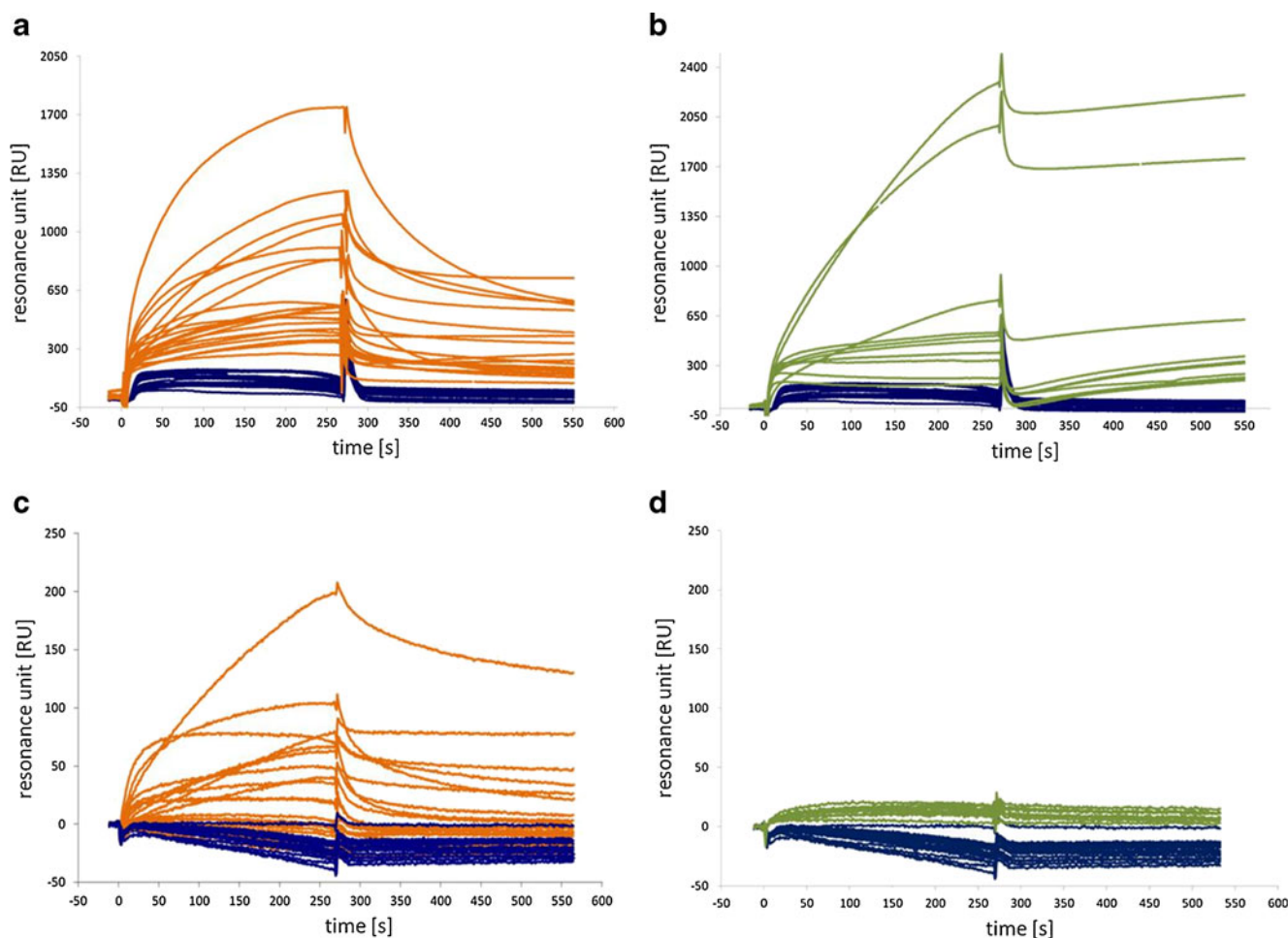


Fig. 6 SPR analysis. **a** aCL SPR assay of APS patients (*orange*) and healthy controls (*blue*); **b** aCL SPR assay of VDRL positive patients (*green*) and healthy controls (*blue*); **c** aβ2-GPI assay of APS patients

(*orange*) and healthy controls (*blue*); **d** aβ2-GPI assay of VDRL positive patients (*green*) and healthy controls (*blue*)

showed no binding to the surface-immobilized β2-GPI (Fig. 6c). When applying a cutoff value of 17 RU (Youden Index=0.49), the APS patients were detected with 96.8 % specificity at a sensitivity of 52.4 %. As shown in prior work, VDRL positive patients showed no binding to the aβ2-GPI biosensor [18].

Discussion

The specific interaction of β2-GPI with surface-immobilized ω-amine cardiolipin was investigated using two different biosensor approaches (SPR and a switchable DNA layer sensor) and visualized by STM. Additionally, the functionalized surface employed in a SPR biosensor system allowed the detection of aCL in APS-affected patient sera. The results were compared with routine ELISA tests as well as with the aβ2-GPI biosensor previously reported [18].

In contrast to established analytical methods, the label-free SPR technique allows monitoring of antigen–antibody

interactions in real time. The pivotal step in the development of regenerative solid-state biosensor surfaces is the covalent immobilization of the antigen (cardiolipin) on the gold support. As the native structure of cardiolipin contains no suitable functional group for covalent immobilization, it must be functionalized by chemical synthesis [21]. The amine group introduced at the end of one of the fatty acids (substance 1) allowed directed covalent immobilization on the carboxyl groups of the SAM-modified gold surface (Fig. 2). Changing the molecular structure of a substance, however, can affect biological characteristics. To ensure that the modification did not affect the biological activity of 1, the performance was tested in an aCL ELISA and compared with the ELISA using unmodified cardiolipin as the antigen. Statistical analysis revealed no significant difference in the diagnostic efficiency between the assays. This proved that the functionalization did not impair the biological function of the ω-amine cardiolipin analogue.

Besides, a potentially altered molecular structure, the covalent surface immobilization can also affect the antigenic

characteristics of the PL. STM and switchSENSE were used for characterization of the chip surface and for investigation of the binding of β 2-GPI to the immobilized cardiolipin. STM allows the imaging of surfaces down to a single-molecule level. The modification of the gold surface by SAM as well as the bound β 2-GPI on the covalently immobilized **1** could successfully be visualized (Figs. 3 and 4b). Furthermore the switchSENSE technology based on electrically switchable DNA layers was used not only to detect the binding event but also to estimate the molecule weight of the bound target ligand [25, 26]. The observed shift of the frequency signal after incubation with a solution of β 2-GPI corresponded to a mass change of $\sim$ 50 kDa, the MW of β 2-GPI (Fig. 5). Measuring the binding of β 2-GPI to cardiolipin at different concentrations with SPR, the sensorgrams showed a clear concentration dependency (Fig. 4a), whereas no binding was observed to transferrin on the reference cell. From these results, it can be concluded that the **1**-modified surface is suitable for detecting the specific binding of β 2-GPI in a concentration-dependent manner.

An important demand for the aCL SPR biosensor is the ability to differentiate sera from APS patients and healthy control subjects. The use of a cutoff value of 126 RU in the SPR biosensor assay offered high specificity (100 %) at equally high sensitivity. In comparison, the ELISA used in routine showed same specificity but was less sensitive (85.7 %). Additionally, the behavior of sera from syphilis patients in our assay was examined. As expected, we could observe positive results for all these sera. As the biosensor allows resolution of association and dissociation phases we speculate that it might be possible to discriminate between the different diseases on the basis of the shapes of the binding curves, which were caused by different binding kinetics. This phenomenon can be explained by the fact that aPLs in APS belong to β 2-GPI-dependent autoimmune-, whereas aPLs in syphilis belong to β 2-GPI-independent infection-associated types [38, 39].

We previously developed a SPR biosensor for the detection of a β 2-GPI antibodies [18]. Testing the sera of the same patients (APS and syphilis) and healthy controls in this study, the a β 2-GPI assay was less sensitive (52.4 %) in comparison to the aCL SPR assay. In both assays β 2-GPI is the main antigenic substance, but binding to surface-immobilized **1** allows optimal orientation of β 2-GPI to be recognized by aPLs. The higher sensitivity of the aCL SPR assay is therefore conclusive. Cardiolipin, however, is not only involved as the antigenic structure in APS but also in many other diseases with parainfectious aPL (e.g., infection with parvovirus B19) [40, 41] making the aCL method less specific. Only the combination of the chips for a β 2-GPI and aCL achieves the clinically required sensitivity and specificity for the detection of aPL in APS patient sera.

Conclusion

In summary, switchSENSE and STM functionally revealed that ω -amine cardiolipin-modified surface was suitable for detecting the specific binding of β 2-GPI. It can also be demonstrated that the SPR biosensor with covalent attached functionalized cardiolipin was a suitable method for aPL measurements in patient sera. This biosensor-based analytical assay system offers relevant diagnostic advantages compared with conventional ELISA formats. It shows higher sensitivity and additionally allows analysis of binding kinetics and affinities. The latter data may enable differentiation between APS and other diseases where parainfectious aPLs are involved as well. This overcomes the drawback of lower specificity when using aCL in comparison to a β 2-GPI assays leading to a highly sensitive and specific diagnostic approach for APS.

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References

1. Wassermann A, Neisser A, Bruck C (1906) *Dtsch Med Wochenschr* 32:745–746
2. Pangborn MC (1941) *Proc Soc Exp Biol Med* 48:484–486
3. Moore JE, Mohr CF (1952) *J Am Med Assoc* 150:467–473
4. Johansson EA, Lassus A (1974) *Ann Clin Res* 6:105–108
5. Arnout J, Meijer P, Vermeylen J (1999) *Thromb Haemost* 81:929–934
6. Celli CM, Gharavi AE, Chaimovich H (1999) *Biochim Biophys Acta* 1416:225–238
7. Hughes GR (1983) *Br Med J (Clin Res Ed)* 287:1088–1089
8. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH PGDEG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis SA (2006) *J Thromb Haemost* 4:295–306
9. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA (1990) *Proc Natl Acad Sci U S A* 87:4120–4124
10. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ, Barbui T, Zwaal RF, Bevers EM (1990) *Lancet* 335:1544–1547
11. Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T (1990) *Lancet* 336:177–178
12. von Landenberg P, von Landenberg C, Schölmerich J, Lackner KJ (2001) *Med Klin (Munich)* 96:331–342
13. Wang SX, Cai G, Sui S (1999) *Biochemistry* 38:9477–9484
14. Wang SX, Sun YT, Sui SF (2000) *Biochem J* 348(Pt 1):103–106
15. Arvieux J, Roussel B, Jacob MC, Colomb MG (1991) *J Immunol Methods* 143:223–229
16. Cabral AR, Cabiedes J, Alarcon-Segovia D (1995) *J Rheumatol* 22:1894–1898
17. Keeling DM, Wilson AJ, Mackie IJ, Machin SJ, Isenberg DA (1992) *Br J Haematol* 82:571–574
18. Metzger J, von Landenberg P, Kehrel M, Buhl A, Lackner KJ, Luppa PB (2007) *Clin Chem* 53:1137–1143

19. Buhl A, Page S, Heegaard NH, von Landenberg P, Lippa PB (2009) *Biosens Bioelectron* 25:198–203
20. Beseničar M, Maček P, Lakey JH, Anderluh G (2006) *Chem Phys Lipids* 141:169–178
21. Johns MK, Yin MX, Conway SJ, Robinson DE, Wong LS, Bamert R, Wettenhall RE, Holmes AB (2009) *Org Biomol Chem* 7:3691–3697
22. Stenberg E, Persson B, Roos H, Urbaniczky C (1991) *J Colloid Interface Sci* 143:513–526
23. Horcas I, Fernandez R, Gomez-Rodriguez JM, Colchero J, Gomez-Herrero J, Baro AM (2007) *Rev Sci Instrum* 78:013705
24. Rasband WS, Image J (1997–2008) U.S. National Institutes of Health, Bethesda, Maryland, USA. <http://rsb.info.nih.gov/ij/> Accessed 28.06.2012
25. Rant U, Pringsheim E, Kaiser W, Arinaga K, Knezevic J, Tornow M, Fujita S, Yokoyama N, Abstreiter G (2009) *Nano Lett* 9:1290–1295
26. Rant U, Arinaga K, Scherer S, Pringsheim E, Fujita S, Yokoyama N, Tornow M, Abstreiter G (2007) *Proc Natl Acad Sci U S A* 104:17364–17369
27. Hanley JA, McNeil BJ (1982) *Radiology* 143:29–36
28. DeLong ER, DeLong DM, Clarke-Pearson DL (1988) *Biometrics* 44:837–845
29. Gamsjaeger R, Johs A, Gries A, Gruber HJ, Romanin C, Prassl R, Hinterdorfer P (2005) *Biochem J* 389:665–673
30. Krugmann S, Anderson KE, Ridley SH, Risso N, McGregor A, Coadwell J, Davidson K, Eguinoa A, Ellson CD, Lipp P, Manifava M, Ktistakis N, Painter G, Thuring JW, Cooper MA, Lim ZY, Holmes AB, Dove SK, Michell RH, Grewal A, Nazarian A, Erdjument-Bromage H, Tempst P, Stephens LR, Hawkins PT (2002) *Mol Cell* 9:95–108
31. Häussling L, Michel B, Ringsdorf H, Rohrer H (1991) *Angew Chem Int Ed Engl* 30:569–572
32. Edinger K, Goelzhaeuser A, Demota K, Woell C, Grunze M (1993) *Langmuir* 9:4–8
33. Bouma B, de Groot PG, van den Elsen JM, Ravelli RB, Schouten A, Simmelink MJ, Derksen RH, Kroon J, Gros P (1999) *EMBO J* 18:5166–5174
34. Schwarzenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P, Prassl R (1999) *EMBO J* 18:6228–6239
35. Albrecht T (2003) *Dynamik und Mechanismen der heterogenen Elektronentransferprozesse von synthetischen und natürlichen Haemproteinen*. Technische Universität Berlin, Berlin
36. Fluss R, Faraggi D, Reiser B (2005) *Biom J* 47:458–472
37. Müller C, Schlichtiger A, Balling G, Steigerwald U, Lippa PB, Thaler M (2010) *Thromb Res* 126:e102–109
38. Matsuda J, Saitoh N, Gohchi K, Gotoh M, Tsukamoto M (1993) *Br J Haematol* 85:799–802
39. Petrovas C, Vlachoyiannopoulos PG, Kordossis T, Moutsopoulos HM (1999) *J Autoimmun* 13:347–355
40. Chou TN, Hsu TC, Chen RM, Lin LI, Tsay GJ (2000) *Lupus* 9:551–554
41. Hsu TC, Tsay GJ (2001) *Rheumatology (Oxford)* 40:152–157