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RESEARCH PAPER

Development of a new parallelized, optical biosensor platform for label-free detection of autoimmunity-related antibodies

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Abstract Autoimmune diseases are characterized by the presence of autoantibodies in serum of affected patients. The heterogeneity of autoimmune relevant antigens creates a variety of different antibodies, which requires a simultaneous detection mode. For this reason, we developed a tool for parallelized, label-free, optical detection that accomplishes the characterization of multiple antigen–antibody interactions within a single measurement on a timescale of minutes. Using 11-aminoundecyltrimethoxysilane, we were able to immobilize proteinogenic antigens as well as an amino-functionalized cardiolipin on a glass surface. Assay conditions were optimized for serum measurements with a single spot antigen chip on a single spot 1-λ detection system. Minimized background signal allows a differentiation between patients and healthy controls with a good sensitivity and specificity. Applying polarized imaging reflectometric interference spectroscopy, we evaluated samples from three APS patients and three control subjects for this proof-of-principle and already obtained good results for β2-glycoprotein I and cardiolipin.

Keywords Label-free detection · Optical sensors · Antiphospholipid syndrome · β2-Glycoprotein I · Reflectometric interference spectroscopy · Microarray

Abbreviations

aa	Amino acids
anti-CL	Cardiolipin antibody
anti-PL	Phospholipid antibody
anti-β2-GPI	β2-GPI antibodies
anti-BSA	Bovine serum albumin antibodies
anti-PT	Prothrombin antibody
AMD	Aminodextrane
APS	Antiphospholipid syndrome
BSA	Bovine serum albumin
CCD	Charge-coupled device
CL	Cardiolipin
ELISA	Enzyme-linked immunosorbent assay
IgG	Immunglobulin G
IgM	Immunglobulin M
LED	Light-emitting diode
LA	Lupus anticoagulant
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pi-RIfS	Polarized imaging reflectometric interference spectroscopy
PL	Phospholipid
PS	Phosphatidyl serine
PT	Prothrombin
RIfS	Reflectometric interference spectroscopy
RT	Room temperature
SD	Standard deviation
11-AUTMS	11-Aminoundecyltrimethoxysilane
β2-GPI	β2-Glycoprotein I

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Introduction

Parallelized screening of multiple analytes within one measurement is of growing importance in numerous fields of biomolecular interaction analysis. Applications are given in the fields of pharmaceutical screening [1] and epitope mapping [2] of antigens and medical diagnostics [3]. In these areas, the combination of a short analysis time and low sample consumption is particularly important and means a step forward compared to classical analytics.

We describe here a newly developed technical setup based on reflectometric interference spectroscopy (RIfS) [4–6] that allows for label-free, multiplexed screening of up to several hundreds of biomolecular interactions. As it is the case in 1-λ-reflectometry [5], the signal on the parallelized platform is generated via interference of reflected light at thin layers and the resulting intensity shifts at a specific wavelength. The setup creates a two-dimensional picture of a glass-transducer and uses a quasi-monochromatic LED light source in combination with a polarization filter—therefore, it will be referred to as "polarized imaging reflectometric interference spectroscopy" (pi-RIfS). Using this label-free technique, it is possible to record time-resolved antigen–antibody binding events, which opens up possibilities to evaluate kinetic constants of the interaction [7]. Compared to evanescent field techniques, such as surface plasmon resonance imaging (SPRi) [8], grating couplers [9] or resonant mirrors [10], pi-RIfS surrenders temperature control, as the detected optical thickness is nearly independent from temperature changes. This is due to the fact that refractive index and physical thickness are of opposing trends when temperature changes, thus the optical thickness remains unaffected.

In this work, we present the parallelized detection of a variety of antigen–antibody interactions on the pi-RIfS platform, relevant for diagnosis of the antiphospholipid syndrome (APS). APS is an autoimmune disease with a prevalence of 2–5 %, where patients suffer from thrombophilia and recurrent fetal loss. Antiphospholipid antibodies (anti-PLs) target not only anionic phospholipids (PLs) but also phospholipid-binding plasma proteins and phospholipid-protein complexes [11]. Main antigens are cardiolipin (CL) and β2-glycoprotein I (β2-GPI). Lower specificity for APS diagnosis have prothrombin (PT) and phosphatidylserine (PS) [12]. Still, they seem to be important as anti-PT is a more reliable marker for possible thrombotic events than anti-CL and anti-β2-GPI [13]. Other possible self-antigens are Annexin V, Protein C, and Protein S.

β_2-GPI is a 326 amino acids (aa) plasma protein [14]. It comprises five short consensus repeat domains with about 60 aa. The fifth domain exhibits an additional 6-residue insertion forming a hydrophobic loop as well as a 19 aa long C-terminal extension. These features are responsible for the binding of β_2-GPI to anionic PLs like CL [15, 16]. It circulates as a globular protein with a concentration of about 0.2 mg/mL in human plasma, but its physiological role is not yet completely elucidated [17]. Upon binding to a negatively charged surface, it changes into a J-shaped conformation, exposing the epitope in domain 1 [18]. When the concentration of PL-bound β_2-GPI exceeds a certain level, one antibody can bind two antigens. This leads to stabilized β_2-GPI dimers with an increased affinity for anionic PLs. The role of CL as diagnostic parameter for APS remains controversial due to the observation that anti-PLs also bind to β2-GPI in the absence of the PL.

RIfS, 1-λ-reflectometry and pi-RIfS, utilizes glass carrier chips as transducer that allow for a series of bioconjugate chemistries. For general evaluation purposes of the technical arrangements, the transducers have been modified with diaminopoly(ethylene)glycol (DAPEG) or aminodextrane (AMD) which are well known in this context [19]. Using 11-aminoundecyltrimethoxysilane, we found the surface being suitable for the immobilization of proteins as well as an amino-functionalized CL. Thus, all antigens could be applied to the chip with the same chemical approach. Early detection of autoantibodies can be hard to realize but indispensable for a successful treatment of the patient. The challenge is the sensitive detection of specific antibodies, directed against a variety of substances produced by the own body. From a microarray-based assay format, we expect a more detailed diagnosis of APS. We are able to detect a broader antibody pattern of each patient within one single measurement that is less time-consuming than standard ELISA procedures and provides for a result in less than 20 min depending on the measurement protocol.

Materials and methods

Biochemicals

Transducers are made of a 1-mm BK7-glass substrate with a layer of 45 nm Ta_2O_5 covered by a 20-nm layer of SiO_2.

N,*N*-dimethylformamide (DMF), acetone, *N*-hydroxsuccinimide (NHS), *N*,*N*′-diisopropyl-carodiimide (DIC), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), guanidine hydrochloride, Tween 20 and human serum albumin (HSA) are purchased from Sigma Aldrich (Steinheim, Germany). Glutaric anhydride (GA), hydrogen peroxide 30 %, sulfuric acid 95–97 % and sodium chloride are obtained from Merck (Darmstadt, Germany), 11-aminoundecyltrimethoxysilane (11-AUTMS) from Sikemia (Clapiers, France). AMD is purchased from Innovent e.V. (Jena, Germany) and DAPEG from Rapp Polymere (Tübingen, Germany). 3-Glycidyl(oxypropyl)trimethoxysilane (GOPTS) is obtained from Fluka (Neu-Ulm, Germany).

Human β2-GPI is purchased from Scipac (Sittingbourne, UK), a monoclonal aβ2-GPI from USBiological (Swampscott, MA, USA) and a polyclonal anti-β2-GPI from Bethyl Labratories (Montgomery, TX, USA). Human prothrombin is purchased from Haematologic Technologies Inc. (Essex

Junction, VT, USA) and a polyclonal anti-prothrombin (anti-PT) from Thermo Fisher Scientific (Rockford, IL, USA). Polyclonal anti-BSA antibody is ordered from Acris Antibodies GmbH (Herford, Germany). Polyclonal anti-cardiolipin antibody (anti-CL) is purchased from LifeSpan Biosciences, Inc. (Seattle, WA, USA) and a monoclonal anti-transferrin from Dako (Glastrup, Denmark).

The ω-amine cardiolipin analogue is synthesized as previously described [20].

Distilled water is taken from an in-house Millipore (Cork, Ireland) device.

For running buffer, we use 20 mM HEPES with 150 mM NaCl, 0.2 % Tween 20 and 0.05 % HSA with a pH of 7.4.

Regeneration is accomplished with 6 M guanidine hydrochloride at a pH of 2.

Patients

For the 1-λ-reflectometry measurements we investigate five sera of APS patients according to the Sydney classification criteria [12] and five healthy controls. For the pi-RIfS analysis, three positive patients and three healthy controls are tested. The serological parameters anti-CL and anti-β2-GPI are routinely measured with the Alegria system from Orgentec (Mainz, Germany). LA is determined by using the BCS XP from Siemens Healthcare Diagnostics (Eschborn, Germany). Serum aliquots are stored at −80 °C. The study is approved by the institutional ethics committee of the Klinikum rechts der Isar, Technische Universität München. All participants gave written informed consent. No financial compensation is paid.

Purification of human antibodies

Antibody purification is carried out as described by the product information of the supplier with a recombinant A/G fusion protein covalently immobilized onto sepharose beads, purchased from Thermo Fisher Scientific (Waltham, MA, USA). All eluted fractions of one patient sample are pooled in centrifugal filter devices with a cut-off of 30 kD from Millipore and buffer is exchanged into HEPES running buffer at 14,000×*g* for 5 min at RT. Final volume of each sample is adjusted to 250 μL. The protein content is verified with Bradford [21] solution from Bio-Rad (Hercules, CA, USA).

Surface chemistry

Glass chips are cleaned and activated in freshly prepared H_2SO_4/H_2O_2 (2:1) for 15 min in an ultrasonic bath (Bandelin electronic, Berlin, Germany). Afterwards, they are rinsed with Millipore water and dried in a nitrogen stream.

Subsequently, they are silanized with 11-AUTMS diluted 1:10 with DMF. To this end, 100 μL of the silanization solution is pipetted on one transducer which is then covered with a second transducer, forming a sandwich. They are kept in a dry atmosphere at RT for 1 h, rinsed with acetone and dried again in a nitrogen stream. The terminal amino group is changed into a carboxyl function using 100 μL of 2 mg/μL GA in DMF over night. Again, two chips are put together as sandwich and kept under DMF atmosphere for at least 2 h. After washing with Millipore water and drying with nitrogen, the transducers are activated with 100 μL 1 M NHS/1.5 M DIC in DMF for 2 h. Finally, the transducers are rinsed with acetone, dried and placed into the microarray spotter. The steps of surface modifications are illustrated in Figure S1 of the Electronic Supplementary Material.

For immobilization of DAPEG and AMD 10 μL GOPTS is pipetted on the transducers surface and incubated as a sandwich for 1 h at RT. Afterwards, they are rinsed with acetone and dried in a nitrogen stream. Dextrane chips are prepared by a solution of 100 mg/mL AMD in Millipore water, which is incubated on the transducers surface for at least 6 h at RT. Chips are thoroughly rinsed with Millipore water and dried in a nitrogen stream. For preparation of PEG chips, the cleaned and activated transducers are modified with GOPTS as described above. Subsequently, a solution of 4 mg/mL DAPEG in DCM is pipetted on the surface, without forming a sandwich. For incubation the chips are placed at 70 °C in an oven for at least 6 h. Modified DAPEG transducers are also rinsed with Millipore water and dried in a nitrogen stream. Amino functions for both AMD and PEG surfaces are changed into carboxyl functions by incubation with GA as described above.

Antigen spotting

Antigen microarrays are produced via contact printing, using the BioOdyssey Calligrapher MiniArrayer (Bio-Rad Laboratories GmbH, Munich, Germany). Twenty microliters of a 1 mg/mL protein solution is prepared in PBS, containing 0.01 % (*v*/*v*) Tween 20. CL is prepared similar to the proteinogenic antigens but without adding Tween 20. Each antigen solution is placed in a single cavity of a 96-well microtiter-plate. PBS containing 0.01 % (*v*/*v*) Tween 20 and Millipore water is used for cleaning of the pin within the spotting run. The spotting procedure is carried out on the activated glass transducers by use of a Bio-Rad software program, resulting in antigen spots of about 170 μm in diameter with gaps of 800 μm in between. The microarrays spotted with the BioOdyssey Calligrapher consist of seven replicate spots for each antigen aligned in rows with BSA spotted between every two antigens.

For a simplified spotting procedure, microarrays are also being produced by direct spotting of droplets via an Eppendorf pipette (reference series). Therefore, a single droplet containing 0.5 μL of the described spotting solution is placed on the activated transducers. Microarrays spotted by hand consist of three replicate spots for each antigen with a diameter of about 1 mm each, gaps in between the spots vary due to the spotting

procedure. Buffer measurements have been performed with microarrays produced with the BioOdyssey Calligrapher, serum measurements with microarrays spotted by hand.

After completion of the spotting procedure the glass transducers are incubated under water atmosphere at RT over night and rinsed thoroughly with Millipore water. Chips are always used the next day and not stored at all. This should guarantee for reproducible sensor surface conditions.

Reflectometric interference spectroscopy

1-λ-reflectometry setup

The 1-λ-reflectometry setup has already been described by Ewald et al. [22]. The flow cell is in this case connected to a tubing pump (ISMATEC, IDEX Health & Science, Wertheim, Germany) that allows for manual control of the fluids and sample injection speed.

Direct assay for the 1-λ-reflectometry measurements

To determine chip validity 2.5 μg of monoclonal anti-β2-GPI, 2.5 μg of polyclonal anti-PT and 2.5 μL of polyclonal anti-CL are diluted in running buffer to a final volume of 100 μL and injected into the flow cell with a flow rate of 10 μL/min. The amount of anti-CL is given in microliters due to the fact, that the product is prepared from human serum and not quantified by the supplier. Two hundred microliters of running buffer are used for the dissociation phase (10 μL/min) followed by 100 μL of regeneration solution (50 μL/min). For serum measurements, 10 μL of patient or control serum are filled up to 100 μL with running buffer. Measurements are taken as described in the "1-λ-reflectometry setup" section.

pi-RIfS setup

Figure 1 illustrates the general arrangement of the pi-RIfS setup. The basic parts of the technical setup include a telecentric objective with coaxial LED light source for illumination, a PDMS flow cell in a mounting frame, a system of syringes and valves to control the fluids (not shown in the scheme) and a CCD camera for detection purposes. The 14bit CCD camera "pco.1600" from PCO AG (Kelheim, Germany) is controlled by the PCO "Camware" software version 3.03. A telecentric objective "S5LPJ2060" from Sill Optics (Wendelstein, Germany) is mounted to the camera. The PDMS flow cell is provided by Biametrics GmbH (Tübingen, Germany) and affixed to the transducer using a magnet-based framework (a scheme of this arrangement can be found in the Electronic Supplementary Material, Figure S2). For illumination purpose, a second telecentric objective (S5LPL2060, Sill Optics, Kelheim, Germany) is used in combination with a coaxial LED (high power, 470 nm wavelength) light source. The fluidic control system is purchased from NMI (Reutlingen, Germany) and includes two Tecan XLP6000 syringe pumps and two Tecan 9-way valves. It allows pump rates between 6.25 and 7,500 μL/min.

Theoretical calculations indicate that obtained signal intensities with the pi-RIfS setup are a function of the incident angle of light onto the transducers surface (data not shown). This is due to the fact that reflection coefficients referring to the Fresnel equations depend on the angle of incidence but also on the polarization of light according to the plane of incidence. The correlation between binding signal intensity, angle, and polarization of the incident light has been evaluated for this setup. Using a reference system of BSA as ligand and anti-BSA as analyte, we applied different angles and polarizations, while keeping all other factors constant (fluidic program, antibody concentration, pixel saturation). Hence, an angle of 35° to the lead of the transducers backside is chosen in combination with a TE polarization of the incident light (Figure S3, Electronic Supplementary Material). The resulting binding signals are of negative values, due to the thin-layer system of the transducer and the resulting intensity shifts by changing the optical thickness of the sensitive layer.

Direct assay for the pi-RIfS measurements

For monoclonal antibody detection, anti-BSA, anti-PT, anti-β2-GPI, or anti-CL are diluted in running buffer to a final

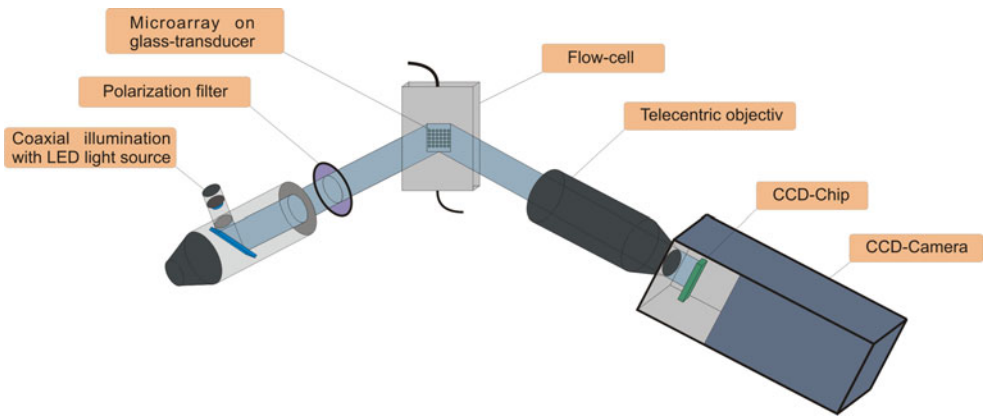

Fig. 1 Scheme of the technical alignment of the pi-RIfS setup. The *left part* includes a telecentric objective with coaxial LED illumination and a polarization filter. This part is for illumination of the transducers backside. *Right side* is a CCD camera with a second telecentric objective for detection purpose

concentration of 50 mg/L, 12 mg/L, 12 mg/L, 40 mL/L, respectively. For the detection of patients' antibodies, 50 μL of purified serum is diluted with running buffer to a final sample volume of 250 μL. All measurements are carried out with the same fluidic program: after a 200 s baseline, a sample volume of 250 μL is injected automatically with a flow rate of 50 μL/min followed by a dissociation phase of 200 s with a flow rate of 200 μL/min. Complete regeneration is achieved with 500 μL of regeneration solution and a flow rate of 200 μL/min. Twenty consecutive measurement cycles could be performed using this regeneration procedure on a single chip with the real patient samples. All measurements are carried out without controlling the temperature. Therefore all parts should be at normal RT.

Data processing

Data from 1-λ-reflectometry is given in mV from the photodiode, which reflects the change in reflection coefficient during the detected binding event. The curve progression is negative caused by the same reflectivity behavior as for the pi-RIfS setup (for further details, see the "pi-RIfS setup" section). All obtained values of one binding event are divided by the first data point. This results in relative signal intensities which are then plotted as a function of time (in seconds).

Data from pi-RIfS measurements are received as pixel values (counts) from the CCD detector and further processed using ImageJ (Version 1.46e). Mean pixel values of each spot are calculated and referenced to mean pixel values of a reference spot on the transducer where no specific binding occurs. To eliminate discrepancies in general illumination levels for different spots, these referenced values are normalized to the starting values of the particular spot. To this end, the first intensity where binding occurs is subtracted from all other referenced values. Equation 1 illustrates this procedure, with I_j being the referenced and normalized signal intensity of each spot j, s is the count value of each pixel within the spot, r refers to the count value of each pixel within the reference spot, and Z is the referenced intensity of the zero point where the sample injection starts.

$$I_j = \frac{1}{n}\sum_{i=1}^{n} s_i \Big/ \frac{1}{n}\sum_{i=1}^{n} r_i - Z_j \qquad (1)$$

The resulting signals are plotted as a function of time (in seconds). These real-time binding curves are further evaluated by applying a linear fit to the onset of the binding event. This region covers at least 20 data points (equates to 20 s of measurement time) and is selected due to the diffusion controlled linear behavior common for every binding curve at that point. Further discussions of the results are based on the slope of this linear fit.

Results and discussion

The key challenge within this work was to find suitable conditions for the parallelized immobilization of chemically different antigens and the detection of all the relevant APS antibodies in one single measurement.

Surface development for antibody detection in human sera

With 11-AUTMS, a short linker was attached to the glass chip likewise a self-assembling monolayer. It allows the immobilization of a series of proteins as well as amino-functionalized PLs. Even very small molecules can be presented on the surface and recognized by their antibodies. Compared to the short 11-AUTMS, larger polymers like PEG or AMD provide a more homogenous surface that is essential for some optical methods. But especially the small CL could not be detected by use of these polymers. This might be due to steric reasons but has not been further evaluated.

Via amine coupling reaction, we were able to immobilize the PL ω-amine CL as well as the proteins β2-GPI and PT. The resulting binding curves indicate that the antigens on the surface kept their antigenicity to a sufficient degree during this coupling reaction, as specific binding of antibodies is still possible.

For the surface chemistry and assay development, the transducers were evaluated using the more simplified setup (compared to pi-RIfS) based on 1-λ-reflectometry which allows for transducers with one antigen at a time. The efficacy of every chip was determined by monoclonal antibodies (Fig. 2). Measurements with anti-transferrin show no binding to all immobilized antigens.

Five APS patient sera and 5 healthy controls were measured with the single spot β2-GPI biosensor. The antigen was covalently bound to 11-AUTMS, PEG, and AMD respectively. 11-AUTMS provides for a two-dimensional surface that has the

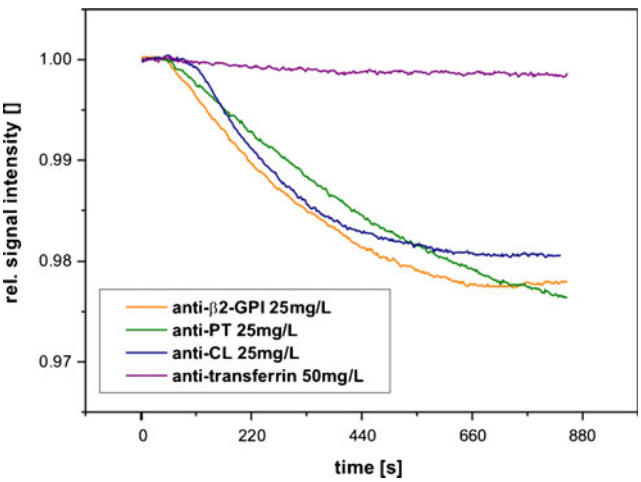

Fig. 2 Resulting binding curves on the 1-λ-reflectometry system for monoclonal anti-β2-GPI, polyclonal anti-PT and anti-CL antibodies in buffer. Anti-transferrin is measured to test for unspecific binding

advantage of low unspecific binding of the serum matrix. Although the density of the antigen is low on this surface, our β2-GPI biosensor can distinguish between patients' sera and healthy controls with a very good sensitivity and selectivity (Fig. 3). PEG shows a promising tendency, yet the overall performance is not convincing (Figure S4, Electronic Supplementary Material). The negative controls exhibit about the same binding behavior as on the 11-AUTMS, but not all positive sera would have been detected. The AMD surface is for this biosensor completely inappropriate (Figure S4, Electronic Supplementary Material). Both positive patients and negative controls bind very strong to the dextrane-modified surface making a differentiation impossible. Depending on their general state of health, samples from APS negative individuals can contain different amounts of antibodies not related to APS. But this cannot explain the intensity of false-positive binding signals from APS negative individuals to the sensor surface. Possible explanations are unspecific binding of matrix components to the immobilized antigens or the AMD surface itself. AMD tends to show more intensive unspecific binding due to its three-dimensional structure and high loading with antigen compared to a more two-dimensionally arranged aminosilane surface.

Prothrombin biosensor and antibody purification from human serum

Besides β2-GPI, PT plays an important role in establishing a differential APS diagnosis. For that reason further investigations have been performed to establish a reliable assay procedure for that additional antigen. After the successful immobilization of β2-GPI and a sensitive detection of anti-β2-GPI antibodies in patients' sera, PT was coupled to a transducer, using the same surface chemistry. The efficient immobilization was affirmed with a polyclonal anti-PT (Fig. 2). But measurements with serum samples gave strong binding curves for both, negative controls and APS patients. Since we had to find assay conditions suitable for β2-GPI and PT the optimization was limited to few alternatives. Overall, it was not possible to evaluate a buffer system that could reduce the unspecific interaction with the PT to an acceptable minimum. The reason might be the fact that PT plays the central role in the coagulation cascade. Thus, a series of coagulation factors and PLs bind to it. Although serum should not contain any clotting factors due to the coagulation process, it cannot be excluded that some remain in the sample and bind to the immobilized PT.

To eliminate the unspecific binding, the antibodies of serum samples were purified with A/G fusion protein bound to sepharose. After incubation of the serum with the A/G fusion protein, all other serum components are washed out and the antibody fraction can be eluted. It contains all subtypes of antibodies that have been in the serum sample [23, 24].

With the purification of the antibodies, a discrimination of APS patients and healthy controls was achieved. This is illustrated in the following sections.

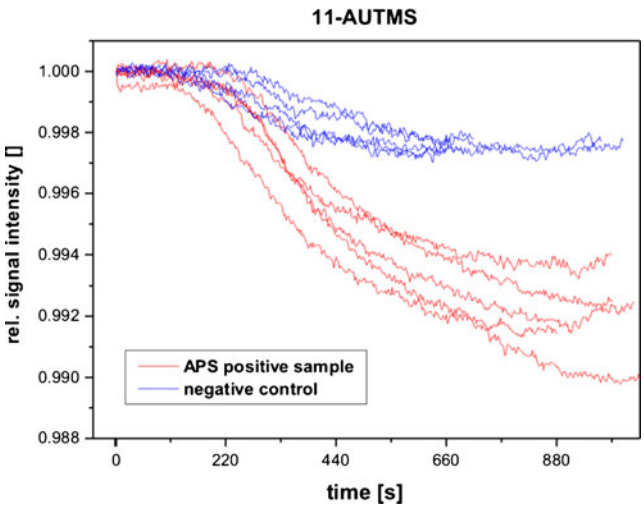

Fig. 3 Sensorgram of anti-β2-GPI interaction with β2-GPI covalently attached to 11-AUTMS. Samples diluted 1:10 in running buffer, flow rate 10 μL/min at RT. Patients ($n=5$) are depicted in *red* and healthy controls ($n=5$) in *blue*

Establishment of a microarray for the parallel detection of purified APS antibodies

For APS diagnostics, it is of high interest to evaluate each patients' antibody status in terms of incidence of different antibodies as well as kinetic behavior. Therefore, the aim was to transfer the knowledge we have gained from the single spot chips to a multiplexed measurement on the pi-RIfS setup, which allows us to monitor multiple biomolecular interactions within one measurement.

The overall functionality of the spotting procedure, the detection system and the microfluidic parts have been tested with a BSA / anti-BSA model-system. Figure 4 illustrates a resulting camera picture from this microarray.

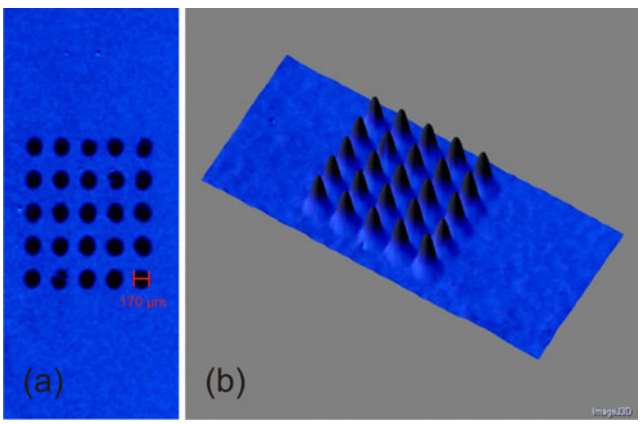

Fig. 4 **a** Camera picture of a 5×5 microarray on an AMD modified surface. Spots are BSA, detected with 50 mg/L anti-BSA antibody. **b** Three-dimensional view of the array after complete measurement illustrates the homogeneity of spot morphology and resulting signal intensity

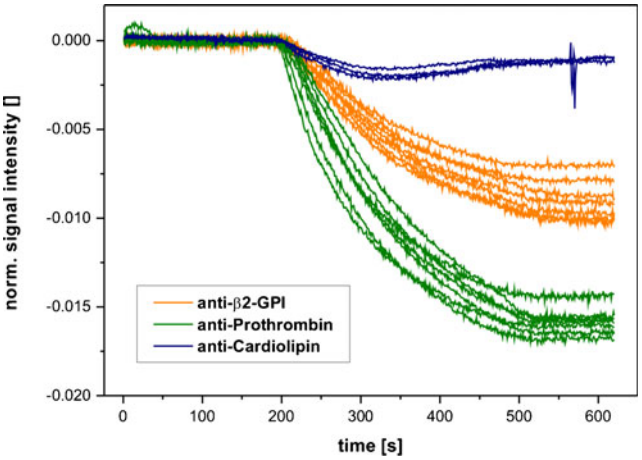

Fig. 5 Parallel detection of polyclonal anti-β2-GPI (*orange*), anti-PT (*green*) and anti-CL (*blue*) antibody on 11-AUTMS

The black spots indicate the binding event of anti-BSA antibody to the immobilized BSA protein. Spot morphology can be considered as homogeneous and well-defined, without spreading or so-called "donut" effects. The latter causes intense binding to the edge of the spot and almost none in the middle and can be observed in case of inappropriate spotting conditions. This proof-of-principle was followed by test measurements in buffer matrix with APS antigens immobilized on the transducer.

The successful immobilization of the two proteins β2-GPI and PT as well as the ω-amine CL on one chip was affirmed with polyclonal anti-β2-GPI, anti-CL and anti-PT antibodies (Fig. 5). To this end, the antibodies were mixed with running buffer to a final amount of 12 mg/L (anti-β2-GPI, anti-PT) and 40 mL/L (anti-CL) and injected. The anti-PT antibody gives the best signal, followed by the anti- β2-GPI. The anti-CL gives just very little signals. There are two possible reasons for that. First, its affinity might be reduced when CL is not in complex with its cofactor β2-GPI. Second, the real concentration of the antibody is not provided by the supplier. This means the weak binding compared to anti-PT and anti-β2-GPI might be due to differences in the absolute concentration of active antibody species in the sample. Absolute binding signal intensity should not be overvalued, as the polyclonal antibodies are just considered as a model for the autoantibodies we expect in human serum samples. Measurements with each antibody alone substantiate a selective binding of each antigen–antibody system without any cross reactivity (data not shown).

Overall, the biosensor can discriminate between different antigens displayed on one surface and gives reproducible binding curves. Differences in signal behavior between the 1-λ-system, that was used for the assay optimization, and the parallelized setup can be mainly explained by the differences in the microfluidic controls which is more sophisticated in case of the pi-RIfS system. As surface chemistry and immobilization techniques remain the same in both setups, the assay characteristics itself should remain unaffected while transferring the assays from the simpler 1-λ to the parallelized system.

Based on these promising results, the performance of the setup and the microarray was evaluated with antibodies purified from patient samples. Figure 6 illustrates the resulting binding curves of an APS patient positive for anti-β2-GPI (routinely measured at the Klinikum rechts der Isar, Technische Universität München) and a healthy control comprising three different APS antigens, which are β2-GPI, PT, and CL.

Both sets of binding curves show small drift effects within the first 50 s of the measurement. Small amounts of air in the microfluidic system produce artifact-signals at second 150 (positive sample) and 500 (negative sample). Clearly, the response especially for β2-GPI is much more intense in the case of the APS patient compared to the negative control. The slight binding to β2-GPI of the latter can be explained by unspecific interactions of the sample with the spotted antigen itself. The differences in absolute signal intensities for the

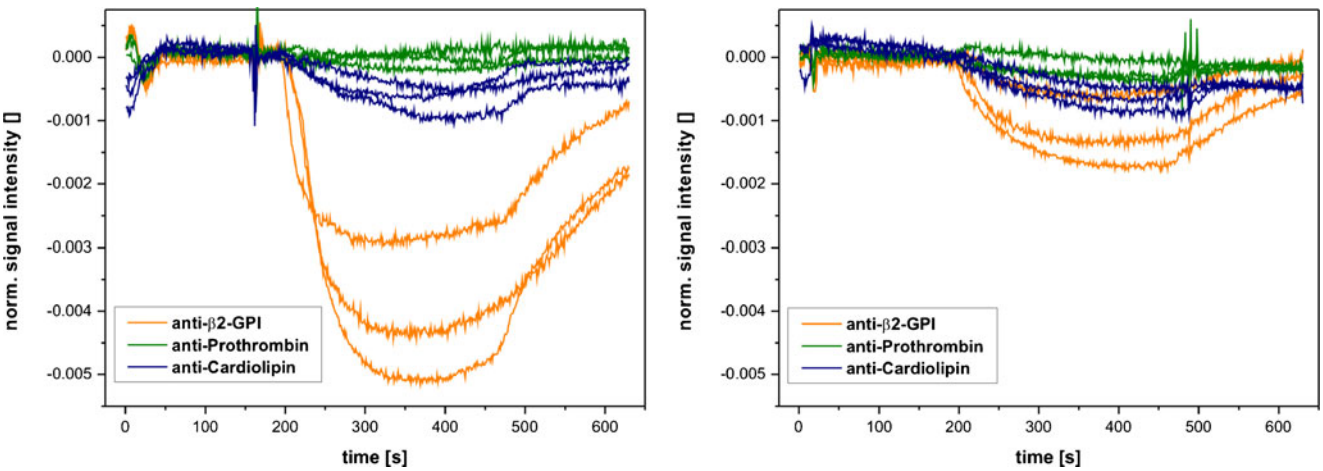

Fig. 6 Two separate measurements of a purified APS patients' sample (*left*) and a healthy control (*right*). Signals from three different spots per antigen are plotted. Immobilized antigens are β2-GPI (*orange*), PT (*green*), and CL (*blue*)

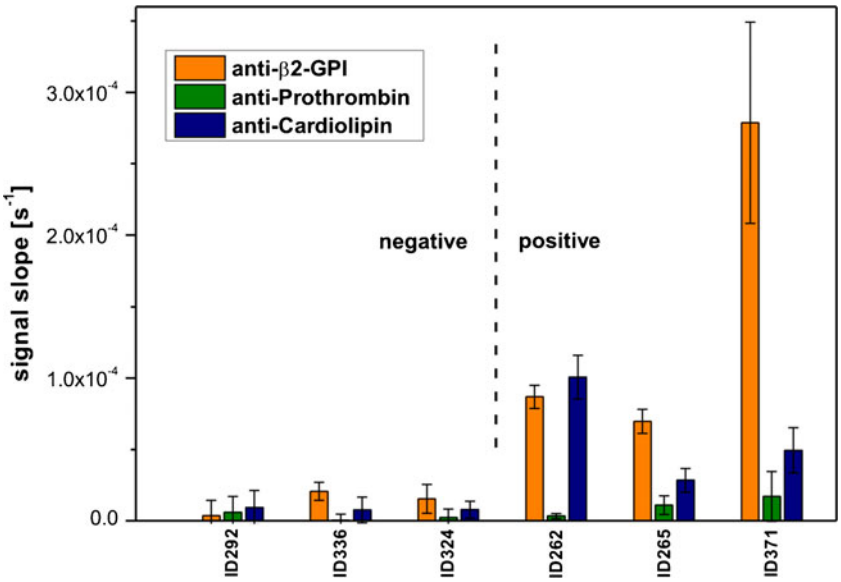

Fig. 7 Bar plot of three positive patient samples and 3 negative controls investigated on β2-GPI (*orange*), PT (*green*), and CL (*blue*). Measurements were performed in triplicate, SD is depicted as *error bars*

threefold determination of anti-β2-GPI antibodies within one measurement are due to spot inhomogeneities. Therefore, the spotting procedure still needs further optimization, although the evaluation via signal slope leads to acceptable standard deviations (illustrated in Fig. 7). Results are gained within approximately 10 min for all three antigens at once, which is far less time-consuming than a normal ELISA procedure with a commercially available kit that takes about 2–3 h for quantification of a single antibody type. Even if the antibody purification procedure, which takes about 30 min of time, is taken into account the gain of time is noteworthy. Investigating three APS patients and three healthy controls, we can substantiate a proof-of-principle for this microarray performance (Fig. 7).

The results depicted in Fig. 7 are compared to the reference values shown in Table 1, generated by the clinical chemistry laboratory (Klinikum rechts der Isar, Technische Universität München). The outcome of the microarray detection is overall in good agreement to the determined antibody activity given in units per milliliter (Table 1), although there are some discrepancies. According to the ELISA measurements, patient 265 is positive for anti-CL, but only small signals are detected in the pi-RIfS measurements. The same is the case for anti-PT antibodies of patients 265 and 371. Differences in anti-CL antibody evaluation might be due to the fact that during the ELISA procedure the β2-GPI is always immobilized together with the CL. That makes the results difficult to compare as in the routinely APS diagnostics; therefore, not only CL but also anti-β2-GPI and anti-β2-GPI/CL complex antibodies are visualized. With the approach of immobilizing both antigens separately we wanted to achieve a clearer separation of the detected antibodies. Binding signals for anti-PT antibodies of patient 265 and 371 are higher than for the negative controls, but the overall assay-performance is yet not satisfying. With the polyclonal antibodies from Thermo Fisher Scientific the PT spots gave quiet strong binding curves in the microarray format (Fig. 5), but for the human samples this assay still needs further improvement. β2-GPI spots from patient 371 resulted in the highest signal intensities compared to the other positive samples. This is not the case for the reference

Table 1 Antibody levels determined by ELISA for investigated APS patient sera and healthy controls. Test is assessed positive for anti-CL IgG/IgM >10 U/mL, anti-β2-GPI IgG/IgM ≥8 U/mL and anti-PT IgG/IgM ≥10 U/mL, respectively. Anti-PT IgG and IgM was not determined (n.d.) for negative controls

	Patient ID	anti-CL-IgG [GPL-U/mL]	anti-CL-IgM [MPL-U/mL]	anti-β2GPI IgG [U/mL]	anti-β2GPI IgM in [U/mL]	anti-PT IgG [U/mL]	anti-PT IgM [U/mL]
Negative	324	<0.1	1.2	2.5	1.8	n.d.	n.d.
	292	0.2	1.1	0.9	3.0	n.d.	n.d.
	336	<0.1	1.2	3.2	1.7	n.d.	n.d.
Positive	265	34.7	13.2	15.5	13.7	60.6	6.8
	371	>120	8.7	43.8	11.4	60.7	5.9
	262	>120	6.0	>100	3.0	4.6	0.7

experiments. One possible explanation is that low-affinity antibodies in the patients' serum are lost in the reference experiments due to repeated washing steps in the assay procedure, whereas in the pi-RIfS measurements these antibodies contribute to the signal. Nevertheless, comparisons of absolute binding signal intensities should not be overrated as the general assay procedures as well as surface modification differ a lot in pi-RIfS and ELISA system. Comparing these two systems in terms of reagent volumes and costs the microarray platform needs 5 μL of antigen solution (1 mg/mL) for the overall spotting process, whereas the amount of antigen used for surface modification of an in-house ELISA is 200 μL per well with a concentration of 5 μg/mL. Dependent on the commercially available ELISA system, at least 1 μL of patient serum (or plasma) is necessary for a single determination. Due to the sample preparation process, the pi-RIfS measurements currently require 50 μL but there remain a lot of possibilities for optimizing this value. This could be further improvement of the assay conditions and surface modifications towards better sensitivity of the sensor surface, as well as optimizations of the sample preparation step itself.

Conclusion

APS diagnostic is still a challenging field due to the heterogeneous group of antibodies developed by the patients. In this work we describe a new parallelized, label-free, optical biosensor setup that can evaluate different antigen–antibody interactions in a single measurement and opens up a wide range of applications. Different surface modifications were compared in terms of immobilization efficiency, unspecific binding and recognition by the antibodies. We present a surface chemistry suitable for the immobilization of proteins and an amino-functionalized CL. These different species can be coupled covalently to the transducer without compromising their antigenicity.

With the single spot β2-GPI biosensor, we optimized the assay conditions and established a detection system that allows for a sensitive and selective discrimination of APS patients and healthy controls. It works in serum matrix with a sample volume of 10 μL and is faster than the routinely performed ELISA.

Although the serum preparation for the microarray is more complex and time-consuming, the great advantage is the multiplexed detection with pi-RIfS. In pilot experiments, we can show a parallel determination of three different APS relevant antigens, namely β2-GPI, PT, and CL. Further investigations will evaluate concentration independent kinetic dissociation rate constants of the participating biomolecules. By introducing further antigens and optimized spotting conditions, this system can be even more efficient concerning time and sample volume. Also, a broader characterization of the patients' antibody spectrum can provide helpful information for a more individual treatment of this autoimmune disease.

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