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Diagnostic performance study of an antigen microarray for the detection of antiphospholipid antibodies in human serum

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Abstract

Background: The parallelization of clinically relevant antigens in a microarray format is of growing importance due to the ability to measure multiple antigen-antibody interactions. With the development of a microarray for the detection of antiphospholipid antibodies we focussed on one important autoimmune disease that is still diagnostically challenging. Reasons are the heterogeneity of the autoantibodies and the unspecific clinical symptoms.
Methods: For the covalent immobilization of antigenic structures, glass transducers were coated with 11-aminoundecyltrimethoxysilane (11-AUTMS). In total 35 antiphospholipid syndrome (APS) patients, six patients with lupus erythematosus and 24 healthy controls were investigated on a microarray format using polarized imaging reflectometric interference spectroscopy.
Results: The novel surface modification based on the short derivative 11-AUTMS resulted in a selective biosensor allowing a clear differentiation of patient and control samples. It combined proteinogenic as well as phospholipid-derived antigens, namely β_2-glycoprotein I (β_2-GPI), prothrombin, cardiolipin (CL) and a β_2GPI/CL complex. With optimized regeneration conditions, up to 20 consecutive measurements could be performed on one chip. Sensitivity was determined to be 0.800–0.929, specificity was between 0.733 and 0.969, depending on the respective antigen.
Conclusions: Multiplexed determination of serological parameters has a great potential. We have shown that our biosensor is capable of detecting four different APS relevant antibodies in parallel exhibiting a sensitivity and specificity comparable to existing ELISA methods.

Keywords: antigen microarray; antiphospholipid syndrome; autoantibodies; label-free biosensor; reflectometric interference spectroscopy (RiFS).

Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmunopathy associated with peculiar clinical manifestations, such as vascular thrombosis and recurrent fetal loss or prematurity.

Antiphospholipid antibodies (aPL) are a heterogenous group of autoantibodies that provoke this prothrombotic disorder. The predominant reactivity of aPL is against serum proteins, mainly β_2-glycoprotein I (β_2-GPI), that bind to negatively charged phospholipids like cardiolipin (CL). Other aPL targets include phosphatidylcholine, phosphatidylinositol, phosphatidylserine, prothrombin (PT), annexin A5, annexin A2, protein C, protein S and tissue plasminogen activator [1, 2].

β_2-GPI is a 326 amino acid (aa), highly glycosylated plasma protein, that belongs to the so-called complement control protein (CCP) superfamily [3]. It consists of five homologous CCP domains: four of them with the regular, conserved sequence, and a fifth, exhibiting an additional 6-residue insertion forming a hydrophobic loop and a 19 aa long C-terminal extension. These extra aa include several positively charged lysine residues responsible for β_2-GPI binding to anionic phospholipids [4–6]. It has been shown, that plasma-derived β_2-GPI is in a circular conformation caused by interaction of domains I and V. As a result, the known epitope for aPL binding to domain I

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is not accessible. Therefore, no circulating immune complexes between β_2-GPI and anti-β_2-GPI can be observed [4]. Upon binding to negatively charged surfaces, β_2-GPI changes from a globular to a fishhook conformation. This exposes the epitope located in domain I [7].

According to the Sydney classification [8], one of the clinical criteria, namely vascular thrombosis and pregnancy morbidity, as well as one laboratory finding is necessary for a definite diagnosis of APS. Latter are lupus anticoagulant (LA), measured with activated partial thromboplastin time or dilute Russell's viper venom time, anti-CL IgG or IgM and anti-β_2-GPI IgG or IgM, both determined with a standardized ELISA. Laboratory criteria have to be tested positive two or more times at least 12 weeks apart. Additional laboratory testings could be incorporated in the Sydney classification providing a better differentiation of the patients' antibody status. These could include determination of anti-CL and anti-β_2-GPI IgA, anti-PT or anti-phosphatidylserine-PT complex antibodies, to give just some examples [2].

A collective of APS patients and healthy controls has formerly been investigated by our group, using a surface plasmon resonance (SPR) biosensor [9]. In this work we describe the development and the performance of a microarray test format for the detection of several APS relevant antibodies, established with polarized imaging reflectometric interference spectroscopy (pi-RIfS) [10–12].

RIfS is a label-free technique based on the interference of partially reflected light beams at the interfaces of a chip-ligand-analyte system. Upon binding of the analyte, the optical thickness of the sensor surface changes. This results in a different interference spectrum and a change in the intensity of the reflected light. A two-dimensional picture of the biosensor platform is generated by including a CCD-camera in the instrumental setup. Thereby the intensity of the reflected light is monitored time-resolved and independently for every spot on the transducer. This enables kinetic measurements [13], as well as parallelized screening of several antigen-antibody interactions without the necessity of a labelled analyte or secondary antibody.

RIfS utilizes glass carrier chips, compatible with a series of bioconjugate chemistries. The transducers are coated with a thin layer of tantalum pentoxide (Ta_2O_5), an optically transparent metal oxide with a high refractive index [14]. This Ta_2O_5 layer leads to improved signal dynamics of the label-free biosensor.

The main focus of our work was the establishment of a microarray system for detecting four different APS relevant antibodies in parallel. Since a proof of principle of the underlying detection method has already been published by our group in Bleher et al. [15], we now focussed on the validation of the microarray to measure larger patient collectives. We further characterized the array in terms of sensitivity and specificity in comparison to standard APS antibody detection performed with ELISA systems.

Materials and methods

Biochemicals

Transducers were 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. Glass chips for the 1-λ-reflectometry were 9×9 mm and for the pi-RIfS system 75×25 mm due to the different size of the flow-cells of the two systems.

Standard reagents were purchased from Sigma Aldrich (Steinheim, Germany) and of analytical purity, unless otherwise indicated. Glutaric anhydride, hydrogen peroxide 30%, sulphuric acid 95%–97% and sodium chloride were obtained from Merck (Darmstadt, Germany). 11-Aminoundecyltrimethoxysilane (11-AUTMS) was from Sikemia (Clapiers, France).

Human β_2-GPI was purchased from Scipac (Sittingbourne, UK) and a polyclonal anti-β_2-GPI from either US Biological (Swampscott, MA, USA) for 1-λ-reflectometry measurements or from Bethyl Laboratories (Montgomery, TX, USA) for pi-RIfS measurements. Human PT and a monoclonal anti-PT was ordered from Haematologic Technologies Inc. (Essex Junction, VT, USA), a polyclonal anti-PT from Thermo Fisher Scientific (Rockford, IL, USA). Polyclonal anti-BSA was purchased from Acris Antibodies GmbH (Herford, Germany), a polyclonal anti-CL from LifeSpan Biosciences, Inc. (Seattle, WA, USA) and a monoclonal anti-transferrin from Dako (Glastrup, Denmark).

The ω-amine CL analogue was synthesized as previously described [16]. As running buffer 20 mM HEPES with 150 mM NaCl, 0.2% Tween 20 and 0.05% HSA with a pH of 7.4 was used. Regeneration was accomplished with 6 M guanidine hydrochloride at a pH of 2.

Patients

In total 35 subjects, diagnosed as APS patients, according to the laboratory and clinical features defined by the Sydney classification criteria [8], six disease controls suffering from systemic lupus erythematosus (SLE, referred to as SLE controls) and 24 negative controls were investigated with the 1-λ-reflectometry as well as with the pi-RIfS system. The patient samples of the collective were randomly chosen in terms of autoantibody concentrations. Samples of 11 APS patients and all SLE patients and healthy donors were collected at the Klinikum rechts der Isar (n=41). The latter were found to be free of any acute or chronic disease by clinical examination and clinical chemistry testing. A total of 24 APS positive serum samples were obtained from university hospital Würzburg. Whole blood was collected in 10 mL tubes without anticoagulant. Clotted blood samples were centrifuged at 1500×*g* for 15 min. Anti-CL and anti-β2-GPI were measured with the Alegria system from Orgentec (Mainz, Germany).

Serum aliquots were stored at −80 °C for biosensor measurements and anti-PT determination with an ELISA system from Orgentec. The study is approved by the institutional Ethics Committee of

the Klinikum rechts der Isar, TU München. All participants gave written informed consent. No financial compensation was paid.

Purification of human antibodies

Antibody purification was carried out with a Protein A/G column kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's prescription. For each measurement 50 µL of the respective serum was prepared as described in Bleher et al. [15].

Surface chemistry

The SiO_2 surface was modified as previously described in detail by Bleher et al. [15].

In the last step, 10 µL of β_2-GPI solution (1 mg/mL) were incubated over night between two 1-λ-reflectometry chips forming a sandwich. In case of the pi-RIfS system, antigens were spotted on the chip surface using a pipette. Therefore, three replicate spots using 0.5 µL of the desired antigen solution were placed on the activated transducers. For the spotting procedure β_2-GPI and PT were mixed with HBS buffer to a final concentration of 1 mg/mL. Approximately 0.5 mg/mL of CL were solubilized in HBS buffer and spotted alone or mixed 1:1 with 1 mg/mL of β_2-GPI solution prior to the immobilization.

Reflectometric interference spectroscopy

1-λ-reflectometry The 1-λ-reflectometry setup was described by Ewald et al. [17]. In our system, the flow cell was connected to a tubing pump (ISMATEC, IDEX Health & Science, Wertheim, Germany) that allows for manual control of the injection speed. Immobilization was verified by measurements with 25 µg/mL polyclonal anti-β_2-GPI and monoclonal anti-PT as positive and with 25 µg/mL monoclonal anti-transferrin as negative control, respectively. One hundred microliters of antibody solution was injected with a flow rate of 10 µL/min. The dissociation phase was recorded by injection of 200 µL running buffer at a flow rate of 10 µL/min. The chip surface could be completely restored and was ready for a subsequent measurement using 100 µL of regeneration solution (50 µL/min). Serum measurements were performed analogous with 10 µL of patient or control serum filled up to 100 µL with running buffer (dilution factor is 1:10).

pi-RIfS Our biosensor setup, as well as the measurement performance, was described in detail by Bleher et al. [15]. A summary of the technical features, including data processing, is given in the Supplemental Material. A technical setup is depicted in Figure 1.

Statistical analysis

For statistical analysis, SPSS (software version 20.0, IBM, Armonk, NY, USA) or Origin (software version 8.5, OriginLab, Northampton, MA, USA) was used. For every sample slope values from three measurements were averaged and depicted as box plot using Origin. Area under the curve (AUC) was calculated with SPSS. As gold standard, the respective ELISA system was taken and samples were grouped into 'positive' for patients exhibiting the respective antibody and 'negative' for patients without the respective antibody and the control samples. Cut-off values were determined by calculating the maximum Youden Index (J=sensitivity+specificity–1).

Results

Immobilization of β_2-GPI on 11-AUTMS modified glass carriers

For the development of a complex microarray combining several APS-relevant antigens, we started with a simplified system that could only detect one antibody

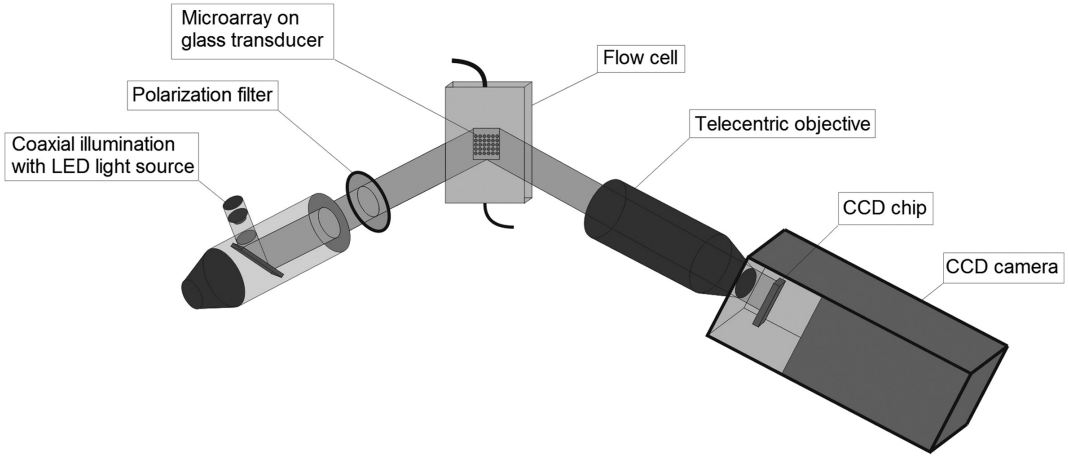

Figure 1 Schematic overview of pi-RIfS setup, comprising a telecentric objective including an LED for illumination purpose (left), flow cell with sensor chip (middle) and another telecentric objective with CCD camera for signal detection (right).
Modified from [15]. Copyright Springer-Verlag, 2014.

at a time. On an 11-AUTMS modified surface, β_2-GPI was immobilized via amine coupling reaction. The short silane 11-AUTMS forms a two-dimensional surface resulting in low antigen density but reduced unspecific binding of the serum matrix. Antigeneity of the covalently bound protein was confirmed by binding of a monoclonal anti-β_2-GPI. Similar measurements with a monoclonal anti-transferrin gave almost no signal and therefore verify the specificity of the system (described in [15]). A titration experiment with anti-β_2-GPI (5.0–40.0 µg/mL) is additionally shown in Supplemental Material Figure 1.

First screening of APS patients and healthy controls on the 1-λ-biosensor

As single-spot biosensor, the 1-λ-reflectometry allowed the detection of anti-β_2-GPI in diluted serum. In total 35 APS patients, six SLE-controls and 24 healthy controls were investigated.

Stability of the surface and regeneration with guanidine hydrochloride was tested with repeated injections of one serum sample. After 10 consecutive measurements on one chip a loss of 8.5% of binding signal was detected. After 20 measurements the signal intensity still was 83.1% of the initial binding signal. Further injections of the sample revealed strong decrease in signal intensity (73.1% of initial binding signal after 30 injections, see also Supplemental Material, Figure 2). Therefore, with this biosensor surface and using guanidine hydrochloride as regeneration solution, not more than 20 consecutive measurements on one chip should be performed.

Levels of anti-β_2-GPI were determined in triplicate with calculated variation coefficients between 0.02% and 0.68%. Figure 2 shows a box plot of all samples. Thereby, all antibody positive (Ab positive) APS patients were depicted in one box as well as the SLE controls. Healthy controls and Ab negative patients were combined in one box (for further explanation see Material and methods, 'Statistical analysis'). A list of the results is given in Table 1, Supplemental Material.

At a cut-off of 3.85 mV, the ROC analysis revealed a good sensitivity of 0.969 with a specificity of 0.970 (Table 1).

Detection of further antibodies required purification of serum samples

The above-mentioned surface chemistry was used to couple other antigens, namely PT and the ω-amine-CL, to the glass transducer. The CL-derivative was synthesized with an amino-group at the end of one carboxyl chain [16]. This allowed an immobilization with the head group, the recognition element, facing towards the biosensor flow cell. The assay conditions established for the β_2-GPI biosensor could not be easily transferred to the other antigenic surfaces. Especially measurements in diluted serum were not possible due to high background binding to the hydrophobic CL surface as well as to the immobilized PT. Latter might be caused by inactive coagulation factors that have not been removed by clotting and centrifugation of the collected blood sample. Identical assay conditions are an indispensable prerequisite for microarray measurements with the pi-RIfS system due to the fact that the technical setup consists of only one flow channel. As a solution, the whole IgG fraction was isolated with recombinant protein A/G coated sepharose spin columns prior to the measurement.

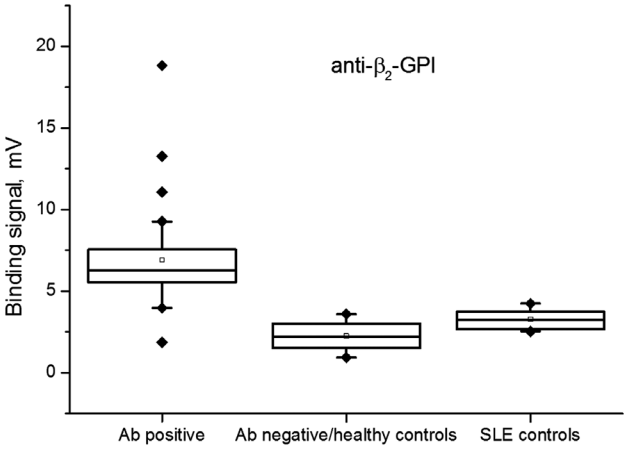

Figure 2 Box plot of antibody positive (Ab positive) APS patients, antibody negative (Ab negative) APS patients and healthy controls, as well as SLE controls investigated with 1-λ-reflectometry.

Antibody determination with the pi-RIfS microarray

β_2-GPI, PT, CL, a complex of β_2-GPI and CL, as well as BSA were combined to a microarray system (details are

Table 1 ROC analysis of anti-β_2-GPI determination performed with 1-λ-reflectometry. Cut-off was calculated with Youden Index.

n(positive)	33
n(negative)	32
AUC (CI)	0.974 (0.930–1.0)
Standard error	0.023
Cut-off, mV	3.85
Sensitivity	0.969
Specificity	0.970

described in Bleher et al. [15]). Transducer chips for the pi-RIfS system are in the dimension of a standard microscope slide (75×25 mm). The flow cell is of the same size with a flow channel of 30×8 mm and a depth of 100 µm. This might cause unequal flow over the sensor surface and therefore an uneven distribution of the injected analyte. Generally, a thinning of analyte concentration is possible, especially for high association constants [18, 19]. To assess the overall performance of the detection and microfluidic system, BSA/anti-BSA (50 µg/mL) was used as model system. This indicated an even distribution of analyte for our system. Furthermore, BSA served as negative control for the sample measurements.

Functionality of the different antigens after coating was tested with 12 µg/mL polyclonal anti-β_2-GPI and anti-PT, as well as 40 µL/mL anti-CL. Antibodies were measured alone and as mixture (detailed shown in [15]), indicating an efficient differentiation of the spotted antigens.

The same collective (35 APS patients, six SLE controls and 24 healthy controls) measured with the 1-λ-reflectometry was investigated with the pi-RIfS system (a representative example of an APS positive and a negative control is shown in Figure 3). Figure 4 illustrates all of the results collected with the microarray. A list of the results is given in Table 1, Supplemental Material.

Determination of the different antibodies was performed in parallel on one chip. Yet, binding signals are evaluated separately. For every serum sample three independent binding signals were generated per antigen and processed as described in Bleher et al. [15]. Variation coefficients (CV) were in over 95% of the measurements calculated to be <5%. A CV of more than 20% was only observed in about 1% of the measurements and restricted to negative controls. This can be explained by the extremely low binding intensities measured in general for these samples (10^{-6}–10^{-7} [normalized value]/sec, see Materials and methods). A summary of the statistical parameters of the pi-RIfS measurements is given in Table 2, Supplemental Material. Evaluation of the different antigen spots was performed separately and binding signals were depicted as box plot (Figure 4).

A ROC analysis is given in Table 2 with 'n(positive)' being the 'antibody positive' and 'n(negative)' the 'antibody negative' samples, evaluated with an ELISA. Therefore 'antibody negative' does not necessarily mean 'control serum', because not all APS patients have developed antibodies to every antigen tested. For instance, an APS patient without PT antibodies is thus classified as negative for the statistical evaluation of the anti-PT test.

Discussion

Antigen microarrays provide a unique tool to evaluate the immune response in autoimmune disorders, such as the APS [20].

Prerequisite for a successful establishment of an antigen microarray is a sophisticated surface bioconjugation of the antigenic structures. In the case of an APS microarray the simultaneous immobilization of proteins as well as phospholipids is mandatory. We have chosen a modification based on the short silane 11-AUTMS that forms a two-dimensional surface on the glass transducer. This only allows a low density of immobilized antigen but

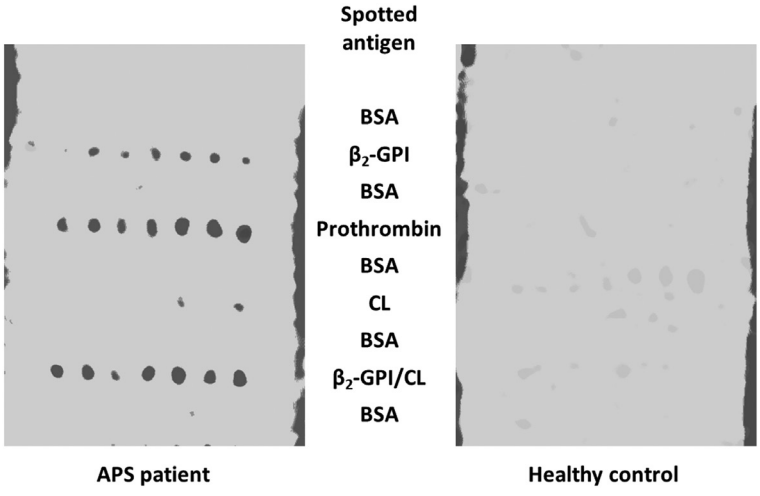

Figure 3 Example of microarray with seven replicate spots for each antigen.
A positive binding signal can be observed for the APS patient depicted on the left. A healthy control is shown on the right. Dark areas at the sides indicate chip border where refractive index is different from the rest due to air.

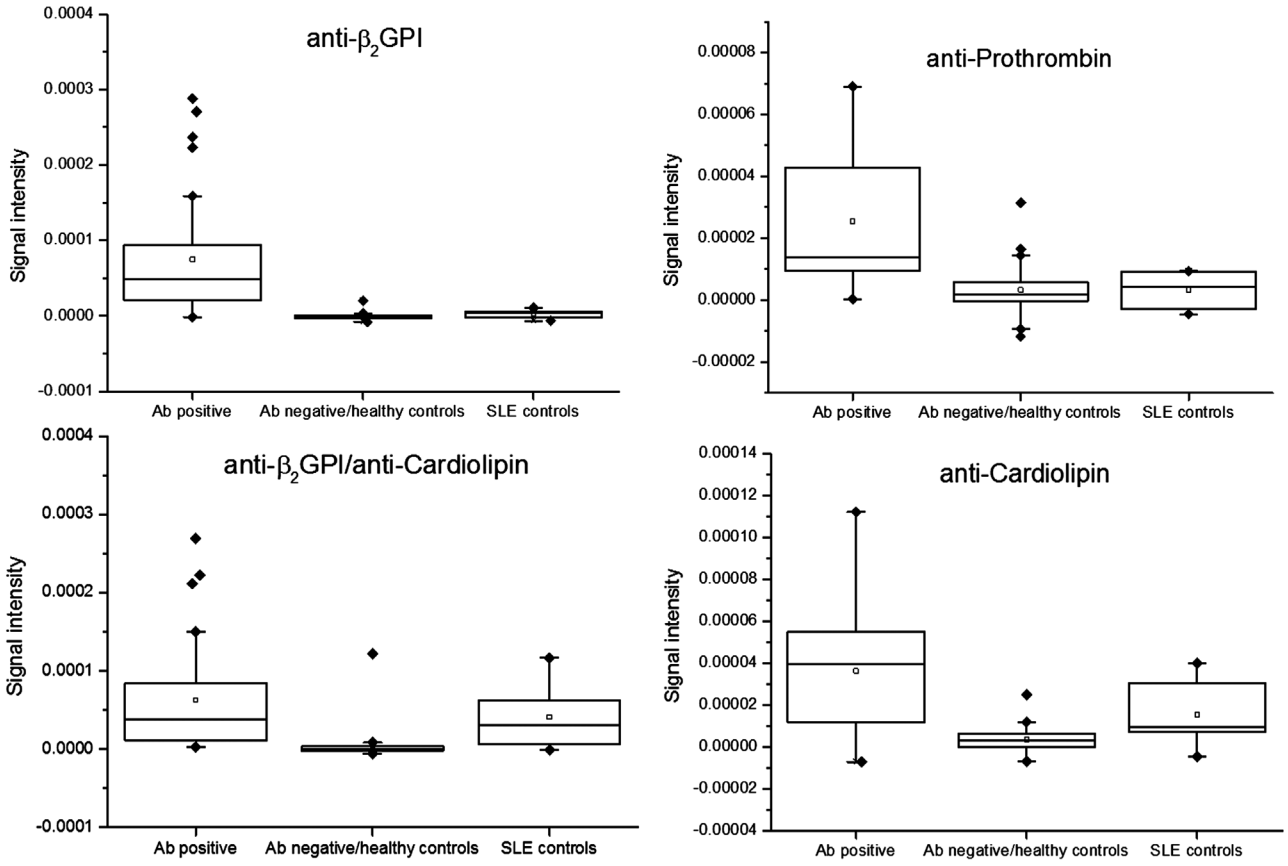

Figure 4 Box plots of the binding of serum samples to all four antigens investigated on the pi-RIfS microarray with antibody positive (Ab positive) APS patients, antibody negative (Ab negative) APS patients together with healthy controls, and SLE controls.

yields a sensitive biosensor surface with reduced unspecific binding of serum matrix. The advantages of short linkers have already been demonstrated for SPR measurements by previous works of our group [9, 21, 22]. Also for the 1-λ-reflectometry, this surface modification allowed a selective and sensitive discrimination of APS patients from healthy controls.

For the single spot β_2-GPI biosensor, measurements could be performed in diluted serum. Physiologically, β_2-GPI has been shown to interfere with several steps of the coagulation cascade. On one hand, it has anticoagulatory effects by inhibition of prothrombinase as well as tenase activity and factor XII activation. On the other hand, β_2-GPI also reduces activated protein C levels and is therefore procoagulant. Yasuda et al. [6] studied the effects of anti-β_2-GPI on fibrinolysis. They found that they significantly decrease the fibrinolytic activity in the extrinsic as well as intrinsic pathway. In case of the extrinsic fibrinolysis, they explain this finding by the observation that physiological concentrations of

Table 2 Summary of the statistical evaluation of the microarray-measurements referenced to the ELISA data of the respective samples.

	β_2-GPI	Prothrombin	CL	β_2-GPI/CL complex
n(positive)	32	14	35	35
n(negative)	33	51	30	30
AUC (CI)	0.945 (0.882–1.0)	0.943 (0.889–0.996)	0.850 (0.750–0.949)	0.837 (0.731–0.943)
Standard error	0.032	0.028	0.051	0.054
Cut-off, mV	11.70×10^{-6}	8.84×10^{-6}	10.7×10^{-6}	4.06×10^{-6}
Sensitivity	0.818	0.929	0.800	0.914
Specificity	0.969	0.843	0.867	0.733

β_2-GPI can recover a suppression of fibrinolysis caused by PAI 1. Therefore, anti-β_2-GPI would negate this effect. Recently, Ninivaggi et al. investigated the influence of conformational changes of β_2-GPI on coagulation [23]. They found that only the open conformation of β_2-GPI is able to express inhibitory activity on the contact activation of blood coagulation. A possible reason might be the covered active site in the natural circular conformation. This hypothesis might explain the interference of anti-β_2-GPI with the anticoagulant properties of β_2-GPI and thereby causing thrombosis.

A multiplexed detection of APS relevant antibodies required, however, a purification of the whole IgG fraction from serum. Besides β_2-GPI, PT, CL and a β_2-GPI/CL complex were chosen as antigens for the microarray, investigated with the pi-RIfS system. The reason to immobilize these four antigens is the relevance of the respective antibodies detected. After anti-β_2-GPI and anti-CL, which are laboratory criteria for the diagnosis of APS, anti-PT are the most present antibodies, APS patients develop [8]. For the detection of anti-β_2-GPI and anti-PT, the pi-RIfS microarray gave excellent results, as demonstrated by excellent sensitivity and specificity (Table 2). The test for anti-CL and anti-β_2-GPI/CL-complex shows lower sensitivity as well as specificity. Reason might be the hydrophobicity of the CL which causes stronger background signals than a solely proteinogenic surface coating.

Comparing these pi-RIfS data with the respective ELISA assays, it can be concluded that both systems are in good agreement. Especially the detection of anti-β_2-GPI seems to be as valid as the ELISA (CI 0.882–1.0).

The collective investigated with the pi-RIfS microarray was also examined with the 1-λ-reflectometry. For the latter, an AUC of 0.974 (CI 0.930–1.0), a sensitivity of 0.969 and a specificity of 0.970 was calculated. The statistical analysis of the β_2-GPI spots on the pi-RIfS microarray gave an AUC of 0.945 (CI 0.882–1.0), a sensitivity of 0.818 at a specificity of 0.969. The lower sensitivity might be due to the conditions that were chosen for the microarray, the parallel detection of several biomolecular interactions as well as the sample preparation prior to the measurements.

Overall, the AUC of both systems is comparable to the β_2-GPI ELISA and microarray as well as single-spot biosensor exhibit good sensitivity and specificity. The other antigens show slightly lower AUC values, due to the fact, that the system was optimized for the β_2-GPI antigen. Still, the biosensor gives reliable results for all four antigens.

The single spot biosensor has the advantage of easy handling and no necessity of any purification steps prior to the measurement. In contrast, the microarray requires more preparation, but allows a multiplexed detection of several serological parameters. The parallel detection of multiple parameters is becoming more and more important, not only in the field of clinical diagnostics, but also in food and water analysis, detection of pesticides and toxins, biomarkers and drugs. Among a great variety of readout systems, real-time assays play a central role as they may provide kinetic data for analyte binding [24].

Conclusions

The multiplexed determination of several serological parameters is of great advantage compared to existing methods that measure just one type of antibody at a time such as ELISA methods. We have shown, that an appropriate surface modification allows the sensitive detection of different aPL in parallel. Although the running buffer had to be the same for every antigen-antibody interaction and could therefore not be optimized for each single pair, sensitivity as well as specificity is comparable to ELISA measurements, where every analysis has its own optimized reagents. The benefit of the microarray is the less time consuming procedure (all 4 parameters for one human sample were determined within 10 min). Compared to other multiplexed assays, the established RIfS microarray records the biomolecular interactions in real time and therefore allows the calculation of kinetic rate constants, affinity and avidity. This could provide deeper insights into the nature of the aPL-antigen binding.

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