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REVIEW

Biosensor analyses of serum autoantibodies: application to antiphospholipid syndrome and systemic lupus erythematosus

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Abstract Autoimmune disorders are rare human diseases characterized by the presence of circulating autoantibodies that bind the body's own structural compounds as target antigens. The detection of autoantibodies is important for the diagnostic process. Immunofluorescence and immunoassay methods do not allow a reliable characterization of binding characteristics. Therefore, novel analytical techniques should be considered. This review describes the application of surface plasmon resonance biosensor systems for the diagnosis of autoimmune disorders. The covalent attachment of native antigens to the sensor chip is a suitable method for obtaining highly reproducible analyses of autoantibodies, allowing the evaluation of kinetic rate and affinity constants, and it may enable the identification of disease-relevant autoantibodies linked to disease progression. The autoantibody microarray is another future-oriented technique. Patterns of differential antigen recognition should allow early diagnosis. This is due to the fact that a broad range of autoreactive B cell responses in autoimmune disorders can only be mirrored by including a sufficient number of antigens in a microarray format.

Keywords Antiphospholipid syndrome · β2-glycoprotein I · Cardiolipin · Double-stranded DNA · Systemic lupus erythematosus · Surface plasmon resonance

Abbreviations

ACR	American College of Rheumatology
anti-β2-GPI	anti-β2-glycoprotein I autoantibodies
anti-dsDNA	antibodies directed towards double-stranded DNA
aPL	antiphospholipid antibodies
APS	antiphospholipid syndrome
β2-GPI	β2-glycoprotein I
CCP	cyclic citrullinated peptide
CL	cardiolipin
CLIF	*Crithidia luciliae* immunofluorescence test
CMD	carboxymethyldextran
ds	double-stranded
EDC	*N*-ethyl-*N*′-[(dimethyl-amino)propyl]-carbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
FC1	flow cell 1
FC2	flow cell 2
FCS	fetal calf serum
IC_{50}	half-maximal inhibitory concentration
IIF	indirect immunofluorescence
IU	international unit
K_D	equilibrium dissociation constant
n.d.	not determined
NHS	*N*-hydroxysuccinimide
pelCL	1,1′,2,2′-tetra-9-oxopelargonoyl cardiolipin

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PL	phospholipid
RI	refractive index
RU	resonance unit
SAM	self-assembling monolayer
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
Tf	human transferrin
TTG	tissue transglutaminase

Introduction: autoimmunity and autoantibodies

Autoimmune disorders are rare human diseases; their prevalence in the Caucasian population is 1.5%. These diseases are classified as systemic (e.g., rheumatoid arthritis, antiphospholipid syndrome or systemic lupus erythematosus) or organ-specific (e.g., Graves' disease or primary biliary cirrhosis) [1]. Autoimmune diseases are characterized by the presence of circulating autoantibodies which bind specific proteins, peptides or other structural compounds of the human body as target antigens [2]. The autoantigens are not clearly defined or are even unknown for a series of disease entities. Others depict autoantibodies which are closely linked to characterized antigenic compounds [3, 4]: rheumatoid arthritis, cyclic citrullinated peptide (CCP); pemphigus vulgaris, desmoglein 1 and 3; myasthenia gravis, acetylcholine receptor; autoimmune adrenal insufficiency, steroid-21-OH-hydroxylase; Hashimoto's thyroiditis, thyroid peroxidase and thyroglobulin; Graves' disease, thyrotrophin receptor; celiac disease, tissue transglutaminase (TTG). Some, but not all, autoantibodies are associated with typical clinical signs and symptoms. During the natural courses of these diseases, organ destructions are mediated by T-cell cytoxicity and/or a direct autoantibody-related injury [5].

The measurement of autoantibodies in sera of affected patients is an important guide to diagnosis and clinical decision-making. The set of tests, however, is complex and often leads to confusion [6, 7]. When used appropriately, the determination of autoantibodies can be a valuable adjunct to diagnosis. This is due to the fact that many serum autoantibodies may be detected a long time before clinical onset as well as during the course of organ-specific autoimmune diseases [8, 9]. Performing follow-up for the levels of autoantibodies may occasionally help to assess disease activity [10].

Several different methods with varying specificities and sensitivities are used to detect autoantibodies. The most widely used methods are indirect immunofluorescence (IIF) microscopy and heterogeneous immunoassays, such as enzyme-linked immunosorbent assay (ELISA) methods. Both techniques, however, have analytical limitations [11]. The analytical quality of IIF microscopy largely depends on the experience of the investigator. Overall, IIF shows poor diagnostic sensitivity. On the other hand, immunoassay methods depend on adequate antigen presentation, which may deteriorate the respective specificity data. Another major drawback of both techniques is that they do not allow a reliable differentiation of binding characteristics. Associations between the affinities/avidities of autoantibodies and their pathogenic potentials as well as the clinical signs and courses of the respective diseases have been shown for several autoimmune disorders [12–15]. Hence the determination of the binding characteristics of autoantibodies may help to ameliorate the diagnosis and management of autoimmune diseases. Moreover, a unique analytical issue concerning autoantibody determination has to be considered: many measuring systems determine the levels of autoantibodies in serum only semiquantitatively. No quantification in terms of absolute concentrations of the specific autoantibody isotypes can be made. According to FDA terminology, the use of the term "semiquantitative" is allowed for assays that compare to a cut-off calibrator, and for those that use a standard curve [16]. In most cases, international reference preparations for autoantibodies in terms of certified calibrators are not available. Thus, many assay systems are calibrated in relative and arbitrary units and are related to internationally recognized reference sera. These sera test positive for the respective individual autoantibody. IIF microscopy methods face the same problem: results are presented as threshold titers.

Novel analytical approaches should improve the diagnostic armamentarium. Biosensor technology, in particular, offers the possibility of label-free determination of autoantibody concentrations in serum. Additionally, optical biosensors may allow the real-time monitoring of the association and dissociation phases of the biospecific interaction between autoantibodies and the respective autoantigens. Detailed information on the reactivities of these autoantibodies could be beneficial for diagnosis.

Biosensor techniques

In the last two decades, the successful development of *immunoassay* technology has had a great impact on the clinical laboratory due to the outstanding lower detection limits afforded by this technology, which allows the detection of analytes at levels that can even reach down to the attomolar range. Further development and automation are still expanding the potential of immunoassay analysis in the clinical sciences [17, 18].

A major future-oriented development in the immunochemical field, the *immunosensor* is still rarely used in clinical diagnostics [19, 20]. The label-free immunosensor

is conceived as a continuously working heterogeneous assay system. It represents a specialized biosensor system that is solely concerned with monitoring antibody–antigen interactions, with either the antibody or antigen immobilized on the sensor surface [20, 21].

In general, a *biosensor* is a self-contained integrated analytical device that combines both a biological element on a solid-state surface, enabling a reversible biospecific interaction with the analyte, and a signal transducer. In other words, a biosensor is an analytical tool that combines a biochemical recognition component with a physical transducer. The label-free biosensor is able to detect the physical changes during the biospecific interaction between ligand (in general the analyte) and a surface-immobilized binding structure (receptor, antibody, etc.). There are three major types of biosensors: electrochemical, acoustic and optical biosensors.

In electrochemical sensors, the biochemical recognition element is provided by an enzyme-coated electrode that selectively catalyzes the reaction of a substrate to produce an electroactive product, which subsequently is detected ampero- or potentiometrically [22–24]. Acoustic biosensors obtain information about biomolecular interactions via changes in the resonant properties of a quartz crystal [25–27]. In optical biosensors, biomolecular interactions are exploited by recording any changes that occur in the intrinsic optical properties of the biomolecule-coated surface as a result of its interaction with the target analyte. Such changes can occur in the absorbance, emission, polarization, or the luminescence decay time of an immobilized ligand. On metal surfaces, differences in reflected light intensity can be measured near the resonance response; these are due to differences in film thickness or refractive index [28–32]. Optical biosensor devices have been available on the market since the late 1980s. Besides the IAsys resonant mirror sensor from NeoSensors Ltd. (Sedgefield, UK) [33], the Biacore systems from GE Healthcare (Freiburg, Germany) are the commercially available devices that are most commonly used in research laboratories due to their sophisticated apparatus and user-friendly control software; the latter use the optical phenomenon of surface plasmon resonance.

Surface plasmon resonance

Surface plasmon resonance (SPR) is a quantum optical phenomenon that can be utilized for monitoring biospecific binding events on solid-state surfaces. In most SPR biosensors, a wedge-shaped beam of plane-polarized monochromatic light is directed from a layer with a high refractive index (RI) (glass prism) towards a layer with a low RI (buffer). At the interface between the two different media, a thin (approximately 50 nm) gold film (with suitable free electrons) is interposed. The biological sample is attached to the top of the gold film. The light propagates into the high-RI medium and undergoes total internal reflection at the interface with the low-RI medium. This does not result in a loss of energy. As a result, the electric field vector of the light beam penetrates into the buffer medium, and is thus called the "evanescent field" because the amplitude decreases exponentially with distance [34, 35]. This may reach up to 300 nm. At a specific incident angle, this field wave causes a resonant absorption of energy by electromagnetic surface plasmon waves within the gold film. This results in a sharp drop in light intensity at the respective angle of reflection. Since this so-called resonance angle is highly sensitive to RI changes near the metal surface, which in turn are directly proportional to changes in the biomolecule concentration at the surface, the position of the angle depends on the number of surface-bound molecules.

The monitoring of the resonance angle as a function of time results in a "sensorgram," which is used for the real-time direct visual observation or software-controlled quantitative evaluation of association and dissociation events at the sensor surface. The signal intensity is expressed in arbitrary resonance units (RU), where 1 RU corresponds to a bound mass of 1 pg/mm^2 [36].

The most prevalent applications are related to protein–protein interactions, especially receptor–ligand and antibody–antigen, although DNA–protein and DNA–DNA interactions are also under investigation. Here, the interacting partner in solution is delivered to the flow cell using a microfluidic flow system that allows contact with the solid-state surface on which the second binding partner is immobilized. The combination of a miniaturized flow system and efficient sample and buffer delivery to the sensor surfaces allows the label-free investigation of specific interactions of biomolecules via continuous monitoring of the association and dissociation phases of the analyte from the immobilized ligand as well as the quantitative determination of kinetic rate constants [34, 37].

The quality of an organized biomolecular substrate on a sensor surface is the pivotal factor in the stable binding of a ligand of interest. Commonly, functionalized chip layers such as planar *self-assembling monolayers (SAMs)* of long-chain *n*-alkylthiol compounds, *hydrogels* composed of carboxymethyldextran (CMD), or various polyethylene glycols are the starting point for covalently immobilizing ligands, typically peptides/proteins or DNA, to the sensor surface by amine, thiol or aldehyde bioconjugation chemistries. This is advantageous, as all noncovalent immobilization strategies are prone to analytical inadequacies due to sensor surface instabilities.

Analysis of autoantibodies using immunosensor and microarray techniques

In the detection of autoantibodies via commonly used immunoassay techniques, i.e., ELISA methods, and the emerging immunosensor analytical techniques, endogenous antibodies from the patient serum are the analytes to be determined. Usually antigens are immobilized onto a microtiter plate or a solid-state immunosensor surface as binding structures for these autoantibodies. The subsequently performed detection step constitutes the principal difference between an immunoassay and an immunosensor: whereas the former uses labeled secondary antibodies directed against the human autoantibodies, the latter system uses a signal transducer for the direct (label-free) or indirect detection of physical changes during immune complex formation. Hence, the detection mode is the fundamental differerence between the systems. Thus, in contrast to immunoassays, immunosensors are able to monitor the process of immune complex formation in real time and to collect kinetic binding data.

The routine determination of autoantibodies is currently generally performed using IIF and/or immunoassays [38]. The latter have become more important due to the availability of the highly purified or recombinant autoantigens used in these methods [39]. The application of immunosensor techniques for autoantibody detection, however, is still in its infancy.

Two major advantages of optical immunosensors should be outlined. First, compared to immunoassays such as ELISAs, they allow the reliable characterization of the affinity and avidity of antibody binding. This is of particular interest in the identification of immunoglobulin subtypes that contribute differently to clinical signs, e.g., of antiphospholipid syndrome [13, 40, 41] or of systemic lupus erythematosus [12, 42]. Therefore, the applicability of immunosensor techniques goes beyond determining the antibody serum titer. In fact, real-time resolution allows the association and dissociation phases of the antigen/antibody interaction to be surveyed, thereby providing insights into the binding characteristics of the respective autoantibody [43]. Detailed information on the binding activities of autoantibodies could be beneficial for diagnosis. The second aspect is the great interest in multiplexed assays for clinical applications. Biosensors and accordingly immunosensors are very well suited to multiplexed analysis: the sensing surface can easily be extended to house multiple antigen spots, and many immunosensor systems allow a label-free readout of the assay [44].

Microarrays The main feature of microarray technology is its potential to simultaneously detect multiple analytes in one sample by an affinity-binding event at a solid-state surface interface. In general, the specific affinity (hybridization) reactions of nucleic acids or antibodies with antigens provide the bioanalytical principle for generating multiplexed quantitative results. There are already many reports of the use of antigen microarrays in microfluidic platforms [45, 46]. State-of-the-art arrays consist of up to 200 peptides and proteins representing candidate autoantigens for the respective autoimmune disease [47]. Thus, it is evident that proteomic analyses of autoantibody reactivities may provide diagnostic information and may allow the stratification of patients suffering from autoimmune diseases into clinically relevant disease subsets. Despite recent advances, a substantial improvement in antigen microarray technology is still needed in relation to technical issues (e.g., printing and probing, normalization strategies, lack of reference samples for standardization) as well as biochemical problems (e.g., the simultaneous measurement of low- and high-abundance proteins) [48].

Some examples of applications of immunosensor and microarray techniques in the field of autoimmune disease and autoantibody characterization are as follows. Rarok [49] applied a SPR device for the determination of epitope shifts of antineutrophil cytoplasm autoantibodies specific for proteinase 3 during the course of the form of autoimmune vasculitis known as “Wegener’s granulomatosis.” More recently, Lokate [50] facilitated the simultaneous detection of various anti-CCP antibodies using a scanning SPR microarray imaging platform. Screen-printed gold electrodes used as support for an electrochemical immunosensor were applied by Balkenhohl and Lisdat [51] for the detection of anti-TTG autoantibodies in patients with celiac disease. Salama [52] developed a chemiluminescent optical immunosensor setup which allowed the detection of autoantibodies to ovarian- and breast cancer-associated antigens. Various microarray platforms are in the development phase [53]. Graham [54] successfully demonstrated the application of antigen microarrays in the characterization of autoantibody profiles and in multiplexed isotype analysis. Similarly, Balboni [55] and Kattah [56] highlighted improvements in the analysis of antibody subtype classes using several fluorescently labeled secondary antibodies in multiplexed protein arrays. Nevertheless, the authors acknowledge the limitations of this technology concerning structural changes of the antigen during solid-state immobilization. Other applications include the identification of clinically relevant subtypes of autoantigens in rheumatoid arthritis [47, 57], as well as the detection of autoantibody clusters that predict lupus nephritis disease activity using glomerular proteome arrays [58]. Recently, McBride et al. [59] presented a presumably low-cost modular system for the multiplex analysis of autoantibodies: the so-called nanodot array luminometric immunoassay. The bases of the wells of a conventional microtiter

plate consist of a nitrocellulose/cellulose acetate membrane; the antigen array is spotted on the underside of this membrane.

Antiphospholipid syndrome (APS)

Antiphospholipid syndrome (APS) is an autoimmune disease that was initially described by Graham Hughes in 1983 [60]. When APS presents without an underlying disease, it is termed "primary" APS, whereas it is called "secondary" APS in cases where a concomitant rheumatological disease can be identified. The most common disease associated with secondary APS is systemic lupus erythematosus (SLE) [61]. The main clinical manifestations of APS are recurrent thromboses, primarily appearing as venous thromboses of the legs with danger of pulmonary embolism. Arterial thromboses may also occur, with brain and heart being the most frequently affected organs, resulting in stroke and myocardial infarction. In women, APS is frequently associated with obstetric complications encompassing pregnancy losses and premature delivery [62]. In view of these main clinical manifestations, the Sydney classification for the diagnosis of APS was developed [63]. Accordingly, the definite diagnosis of APS requires at least one clinical and one laboratory criterion. The *clinical criteria* comprise vascular thrombosis and defined states of pregnancy morbidity. Detection of lupus anticoagulant and IgG- or IgM-anti-cardiolipin (CL) and anti-β2-glycoprotein I (β2-GPI) antibodies constitute the possible *laboratory criteria* which must be confirmed by a second determination performed at least 12 weeks after the first measurement.

Autoantibodies in APS As CL and β2-GPI have been identified as cardinal antigenic factors, the abovementioned anti-CL and anti-β2-GPI antibodies form the serological correlate of APS. These two autoantibodies are members of the heterogeneous group of antiphospholipid antibodies (aPL), which were discovered early last century [64]. The positive predictive value of the aPL titer for the occurrence of thrombotic events in patients with systemic lupus erythematosus and other autoimmune diseases, however, was not discovered until 1983 [60, 65–67]. The common feature of aPL is their ability to interact with anionic or neutral phospholipids (PL), complexes of PL and proteins, or with PL-binding proteins alone [68]. Originating from the inner mitochondrial membrane, anionic phospholipid CL was initially thought to be the major antigen for aPL in APS. It was demonstrated, however, that anti-CL antibodies bind more strongly to their antigen in the presence of β2-GPI, presumably due to the involvement of β2-GPI in immune complex formation [69–71]. Additionally, if β2-GPI is immobilized on solid supports at a high density, the interaction with aPL is even detectable in the absence of CL [72].

β2-GPI is a 54-kDa single-chain serum glycoprotein that binds with high affinity to negatively charged PLs [38]. The crystal structure has been reported [73], and shows four complement control protein modules in a β-barrel shape and a distinctly folding fifth C-terminal domain which are arranged like beads on a string to form an elongated J-shaped molecule. The C-terminal loop region carries a positive charge and a distinct sequence motif, and therefore is an excellent counterpart for interactions with anionic PL. During the binding event to PL, β2-GPI can perform a certain conformational change in the secondary structure. In case of high ionic strength, the bound J-shaped protein coupled by the fifth C-terminal domain changes the conformation of the four coiled-up (so-called "Sushi") domains, creating a globular structure [74, 75]. This step seems to increase the binding capability for aPL.

Further studies have revealed that anti-β2-GPI are associated with clinical manifestations of APS [76], and in rare cases are the sole antibodies detectable in patients presenting with clinical signs of APS [77]. In view of reports that show significant correlations between aPL titers measured by anti-CL-ELISAs and anti-β2-GPI-ELISAs, with a higher specificity observed for the latter [78], anti-β2-GPI themselves have been proposed to be helpful for APS diagnosis. The aforementioned data illustrate that the relevance of each different autoantigen responsible for the generation of aPL is still a matter of discussion. SPR is an elegant method for assessing how single PL, PL-binding proteins and their respective complexes build up an antigenic potential, leading to the binding of aPL and to the deleterious effects associated with this.

Application of SPR for the diagnosis of APS

Chip design In order to prepare a robust, regenerative, reproducible and functionalized SAM surface for the aPL biosensor [79], gold slides were immersed in an ethanolic solution of 11-aminoundecanethiol, 11-mercaptoundecanol and 11-mercaptoundecanoic acid in a defined molar ratio (1:3:6) for several hours at room temperature. The molecules were linked to the surface via gold–sulfur binding, while the aliphatic chains were oriented coordinately via hydrophobic interactions. After rinsing with 96% ethanol and drying under a stream of argon, the SAM-coated gold surfaces were assembled onto a chip carrier and inserted into the biosensor flow system. Crosslinkers such as *N*-ethyl-*N*′-[(dimethylamino)propyl]-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) allowed the covalent coupling of ligands to the exposed

functional groups on top of the aliphatic moieties in the biosensor device itself. As three functional chemical groups are present on the surface, combinations of different antigens could be loaded onto the sensor chip by different coupling chemistries. SAM layers were used to immobilize an oxidized CL derivative and/or the protein cofactor β2-GPI. As considerable controversy exists regarding the antigenic potential of APS antigens, this experimental setup should provide new insights in the recognition of CL, β2-GPI and their combination by aPL.

The SPR biosensor device used two flow cells, FC1 and FC2, within the same sensor chip. This assembly permits the parallel monitoring of the binding of the same sample to each one of the flow cells at all measuring times. FC2 is usually coated with the antigen of interest and FC1 with an irrelevant antigen. This means that FC2 constitutes the measuring cell and FC1 the reference cell. The net sensorgrams (Figs. 1, 3, 4 and 5) depicted in the following sections are calculated as difference curves for the RU values, FC2–FC1. This approach attenuates refractive bulk effects and also many unspecific binding phenomena from the serum matrix. It also allows baseline correction.

SPR analyses As described in the introduction to APS, aPL occur not only in APS but also in several other autoimmune diseases and in infections, e.g., those by *Treponema pallidum* or parvovirus B19. Infection-associated anti-β2-GPI differ from anti-β2-GPI in primary APS in their avidity [80, 81], and are usually not correlated with thrombosis. Nevertheless, they are detected by conventional anti-β2-GPI ELISAs [82, 83], leading to a poor positive predictive value for diagnosis of primary APS [84]. In order to differentiate disease-relevant from infection-associated anti-β2-GPI, we immobilized β2-GPI in high densities using EDC/NHS chemistry on a SAM surface consisting of just 11-mercaptoundecanol and 11-mercaptoundecanoic acid [79]. The reference cell FC1 was coated with human transferrin (Tf) by again using covalent EDC/NHS chemistry to immobilize the amino groups of the protein onto the carboxylic functions of the SAM. As Tf is a physiologic human serum component, no antibody reactivity against this iron transporter should be present in patient sera. Tf is therefore suitable for use as a reference protein. We analyzed a series of sera from patients suffering from primary APS, SLE, syphilis, parvovirus B19 infections, and sera from healthy subjects as controls. All of the sera from SLE patients without secondary APS and from patients suffering from syphilis or parvovirus B19 infection lacked the β2–GPI-reactive response, unlike the APS patient samples, which exhibited highly specific anti-β2–GPI binding responses. Hence, the assay design enabled us to clearly discriminate between pathogenic and infection-associated anti-β2-GPI, and it therefore offers pivotal diagnostic advantages. Furthermore, the biosensor system showed near-perfect discrimination between APS patients and healthy controls (Fig. 1).

β2–GPI aside, CL represents the second major autoantigen in APS. We therefore subsequently investigated the role of CL using the oxidized CL-variant 1,1′,2,2′-tetra-9-oxopelargonoyl-cardiolipin (pelCL), which contains alkanal moieties that can be easily immobilized as binding partners on the sensor surface. It has been shown that oxidized CL derivatives and/or adducts of oxidized CL and β2-GPI are suitable epitopes for aPL [85, 86]. Therefore, this approach is reasonable.

PelCL was synthesized from 1,1′,2,2′-tetraoleyl-cardiolipin (Avanti Polar Lipids Inc., Alabaster, AL, USA) by ozonolysis and subsequently performed reductive workup procedures using dimethylsulfide (Fig. 2). The resulting end-standing aldehyde functions are suitable for covalent coupling with hydrazine to the carboxylic acid function of the 11-mercaptoundecanoic acid in the SAM. It should be noted that approximately 30–50% of the four aldehyde groups undergo acetalation with methanol during synthesis.

In a deviation from the SAM surface used by Metzger [79], this SAM additionally contained 11-aminoundecanethiol. The amino group of 11-aminoundecanethiol is able to covalently react with oleic acid on the surface of the reference cell FC1 via the EDC/NHS chemistry described above. As oleic acid is a common component of triglycerides, serum autoantibody reactivity against this fatty acid is unlikely. Therefore, oleic acid is suitable for use as a reference compound for FC1.

Figure 3 shows the sensorgrams obtained when sera from patients with definite diagnoses of APS and those from healthy controls were injected. There was no difference in the binding behavior of the sera between the healthy and ill subjects; regardless of the study group, all sera bound to the pelCL to various degrees, indicating

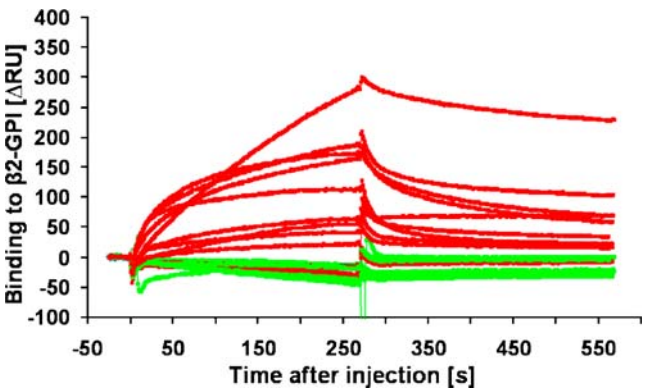

Fig. 1 Binding curves obtained by injecting 1:100-diluted sera from patients with APS (*red lines*) and healthy controls (*green lines*) onto a biosensor surface coated with 1161 RU β2-GPI

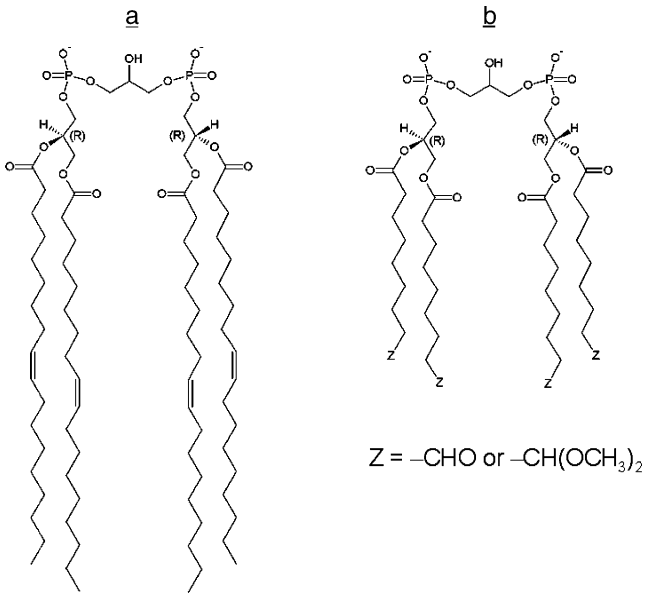

Fig. 2 1,1′,2,2′-Tetraoleoyl-cardiolipin (CL, *a*) and its oxidized derivate 1,1′,2,2′-tetra-9-oxo-pelargonoyl-cardiolipin (pelCL, *b*)

strong unspecific binding to the immobilized pelCL. This was a surprising result, as CL is often considered to be the main antigenic structure in APS. To further evaluate this observation, we examined the effects of the co-immobilization of pelCL and β2-GPI. A SAM chip with both amino and carboxylic acid moieties was established as follows. In a first step, pelCL and oleic acid were coupled covalently to FC2 and FC1, respectively. In a subsequently performed second immobilization step, β2-GPI as well as Tf were covalently attached to FC2 and FC1. For this elaborate coupling procedure, the same immobilization strategies and identical concentrations as used for the sole immobilizations of these components were used. When the same sera from the APS patients and from the healthy controls as used before were injected onto a surface coated with pelCL and β2–GPI simultaneously, it became evident that this combichip provides significantly better discrimination of patients vs. controls than the pelCL chip. A comparison of the sensorgrams of Fig. 4 with those of Fig. 3 back up this observation.

When compared, however, to the sensor chip onto which β2-GPI had (solely) been immobilized in high densities (see Fig. 1), no beneficial effects were observed, regardless of the SAM used for attachment (with or without the amine function). As a consequence, it should be stated that the good diagnostic efficiency of our biosensor system seems to be caused by the immobilization of β2-GPI in high densities, and not by CL. This is in agreement with the abovementioned hypothesis of Arvieux et al. [72], that the interaction with aPL is detectable even in the absence of CL if β2-GPI is immobilized on a solid support in high

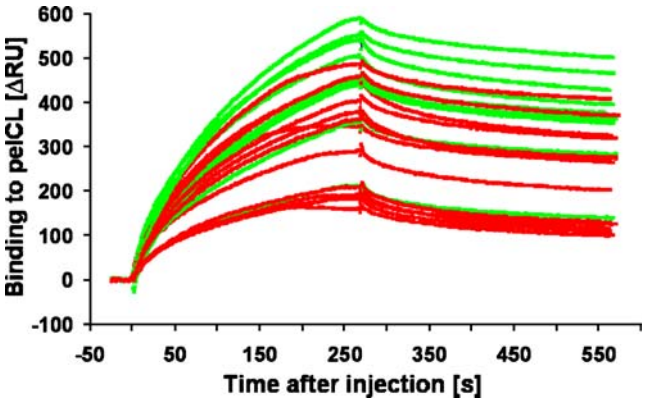

Fig. 3 Binding curves obtained by injecting 1:100-diluted sera from patients with APS (*red lines*) and healthy controls (*green lines*) onto a biosensor surface coated with 745 RU pelCL

densities, resulting in the previously described globular conformation.

Synopsis Our data indicate that β2-GPI is the principal antigenic structure for the aPL in APS. In our SPR biosensor system, the known conformational changes of β2–GPI [74, 75], even those induced by CL, do not significantly amplify its antigenicity and are not necessary for aPL-binding. This observation could shed light on the design that is commonly used in commercially available anti-CL-ELISAs. For a standard anti-CL-ELISA, CL is noncovalently adsorbed onto the wells of a polystyrene microtiter plate [87]. A blocking step is subsequently performed with a solution of human or nonhuman β2–GPI, essentially to obtain sufficient diagnostic sensitivity and specificity. Historically, adult bovine or fetal calf serum (FCS) was used for this purpose. Serum or plasma samples are then added to the wells at dilutions of 1:50 to 1:100. After washing, enzyme conjugates of anti-human-IgG or -

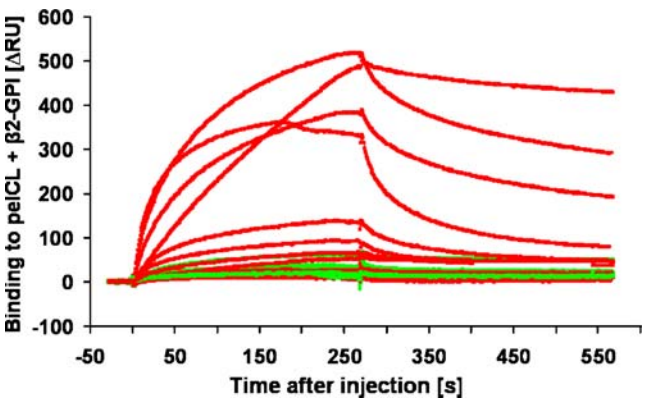

Fig. 4 Binding curves obtained by injecting 1:100-diluted sera from patients with APS (*red lines*) and healthy controls (*green lines*) onto a biosensor surface coated with both 745 RU pelCL and 2052 RU β2-GPI

IgM are added. After incubation and additional washing, the chromogenic reaction is started [68].

In view of the considerable quantities of β2–GPI found in adult bovine serum and FCS, and its known high affinity for CL, it is conceivable that β2–GPI binds to the immobilized CL when blocking the ELISA microtiter plate, and subsequently remains attached to the plate. The improved performance of the described ELISA design compared to a non-blocking approach could then be explained by our biosensor results, which showed comparably high immunogenicities of β2-GPI and its complex with pelCL, and where there was no differentiation of sera from ill and healthy subjects by sensor chips with only pelCL immobilized on the surface. In fact, two separate groups have shown that, during the coating of the ELISA microtiter plate, a substantial amount of the immobilized CL is oxidized [85, 88]. This oxidation affects the four unsaturated fatty acid groups. Hence, pelCL may be a suitable antigen for biosensor measurements.

With regard to the basic pathophysiological processes, it is doubtful that the "β2–GPI-dependent anti-CL ELISA" really measures anti-CL antibodies or in fact solely anti-β2–GPI antibodies. However, the biosensor measurements are not readily transferable to ELISAs. The data obtained for the biosensor device may have resulted from the fact that the attachment of β2–GPI in the specific SAM environment in high densities allows the protein to adopt its most immunogenic conformation, independently of any PL cofactors. Presumably CL or other PL are required on ELISA plates solely to transform a sufficient number of β2–GPI molecules to the abovementioned globular conformation with amplified immunogenicity.

Systemic lupus erythematosus (SLE)

SLE is often referred to as the prototypic systemic autoimmune disease due to its complex clinical and molecular manifestations [89]. Patients develop a wide variety of clinical and serological features, making correct and early diagnosis cumbersome. This is illustrated by a delay of ~2 years between the onset of symptoms and final diagnosis [90], depriving patients from effective and potentially life-saving therapy. The plethora of presentations range from rash and arthritis through to thrombocytopenia and anemia as well as serositis, nephritis, and psychotic symptoms [91]. According to the SLE classification criteria of the American College of Rheumatology (ACR) [92, 93], at least 4 out of 11 criteria must be met for correct disease diagnosis. These criteria include many unspecific symptoms that are not restricted to SLE. These are: cutaneous involvement (malar rash, discoid rash, photosensitivity), oral ulcers, arthritis and serositis. However, renal, neurological and hematological disorders also occur. Besides the detection of anti-nuclear antibodies using IIF microscopy, antibodies directed towards native double-stranded DNA (anti-dsDNA), ACR criterion 10b, are of high diagnostic significance, especially since they provide clues regarding the correct diagnosis.

Assays for anti-dsDNA A series of different assay types are available for determining anti-dsDNA; the most widely used are IIF microscopy (*Crithidia luciliae* IIF test, CLIF), the Farr assay and ELISAs [94, 95]. They differ substantially with respect to isotype specificity, DNA antigen source, steric DNA presentation, and exact reaction conditions. For this reason, different subgroups of circulating anti-dsDNA are determined. While all isotypes but only antibodies with high avidity are detected with the Farr assay, most ELISA methods display only IgG, but also those with a lower affinity. CLIF only shows IgG too, but it does not enable real quantification. Thus, the same serum sample can give utterly different values when measured with different methods [96]. Any comparison between them is challenging, and analytic results must be interpreted carefully in light of the assay's characteristics and the patient's condition [97, 98]. The development of a reference method is therefore strongly desirable.

Application of SPR for the diagnosis of SLE

In the following, a novel concept is presented: SPR is used to untangle the disparate results obtained with the abovementioned methods. To our knowledge, no assay method is currently able to provide results that come even close to an overall picture of a patient's anti-dsDNA status. Therefore, we demonstrate the general possibilities of the SPR system with some illustrative measurements.

Chip design Buhl et al. established a SPR biosensor based on the covalent immobilization of human recombinant dsDNA via a carrier protein (Tf), enabling the visualization of the dsDNA/anti-dsDNA interaction in real time [99]. Briefly, a 5′-aldehyde-modified 24-mer oligodeoxynucleotide is covalently attached to Tf, whereupon the resulting protein–DNA conjugate is ligated to a digested PCR product with a well-defined length and sequence. The Tf can then be utilized as an immobilization anchor, allowing fast, efficient and stable immobilization of DNA antigen on a CMD sensor chip. The SPR biosensor established permitted the specific detection of anti-dsDNA. Sera from SLE patients with proven diagnoses could be distinguished from healthy control sera. The specificity of the biosensor was also confirmed by competition experiments using salmon sperm dsDNA spiked into positively tested sera,

which blocked the surface-binding of anti-dsDNA in a concentration-dependent manner [99].

SPR analyses We initially performed CLIF (from Euroimmun Laboratory Diagnostics, Lübeck, Germany) and the FARRZYME™ High Avidity anti dsDNA ELISA (The Binding Site, Birmingham, UK) with SLE sera, for which SPR-binding data and Farr results were published in [99]. Three of these samples (a-1, c-1 and k-1 in the original publication), as well as an additional one from a patient suffering from mixed connective tissue disease (referred to in the following as serum n) were then chosen as examples in order to perform a more detailed examination of the binding characteristics. All were strongly positive in the SPR biosensor assay (the cut-off was 25 RU), but performed quite differently in the other assays (Table 1).

Since the immunoglobulin isotypes IgG, IgM and IgA are reported to be present in parallel in sera from SLE patients [100], it seemed to be necessary to clarify the role of their distribution. A protocol similar to that reported in [79] was used to specifically remove respective isotypes, but in this case we depleted either no antibodies (positive control), IgG and IgM (IgA response), IgA and IgM (IgG response), IgG and IgA (IgM response) or all antibodies (negative control). The results (Fig. 5) clearly indicate that samples a-1 and k-1 are mainly IgM-dominated, while samples c-1 and n contain anti-dsDNA IgGs. This explains why a-1, in spite of the positive outcome in the Farr assay, shows a relatively weak response in the CLIF and is negative in the ELISA. Both methods utilize an IgG-specific secondary antibody and will not recognize anti-dsDNA IgMs. The high association value in our biosensor system also fits with these findings. IgMs have an about 5 times higher molecular weight than IgGs and generate a higher signal on the mass-sensitive biosensor surface. The Farr assay, however, does not discriminate between the isotypes. Accordingly, samples c-1 and n both show good binding in the CLIF and high values in the ELISA. The extreme response of serum n in all assays could either result from a very high concentrations of anti-dsDNA IgG or superb affinity, leading to more stable binding.

Table 1 Anti-dsDNA concentrations in sera from SLE patients, as determined using different methods[a]

SLE serum no.	Farr assay ($\times 10^3$ IU L^{-1})	CLIF titer	Farrzyme ELISA ($\times 10^3$ IU L^{-1})	SPR association level (RU)
a-1	100.9	1:50	Negative	181.6
c-1	75.1	1:640	172.15	140.8
k-1	42.1	n.d.	n.d.	412.7
n	550.0	1:1280	> Max	325.0

[a] *IU*, international units (calibrated against the WHO WO/80 reference preparation); *RU*, arbitrary resonance units; *n.d.*, not determined

The next step was therefore to determine the relative binding affinities of the samples. In order to do so, the binding of 1:400 dilutions of the individual sera was inhibited prior to the SPR sensor measurements by incubating the serum with various concentrations of a double-stranded 42-mer oligodeoxynucleotide. Measurements were done in triplicate and the residual responses were then plotted as percentages of the uninhibited sample against the oligomer concentration. The resulting log (inhibitor) vs. response curves had roughly symmetrical sigmoidal shapes. The midpoint, representing the oligomer concentration where half of the binding activity is inhibited, can be deduced from the plot. This half-maximal inhibitory concentration (IC_{50}) is equivalent to the equilibrium dissociation constant (K_D) of the autoantibody/inhibitor interaction and can thus be interpreted as a measure of affinity. Affinity is inversely correlated to IC_{50}. This technique has been used with ELISA methods for a long time [101], and was also shown to be valid for BIAcore systems [102]. Values are given in g L^{-1} since the dsDNA is a multiepitopic antigen, and so values in mol L^{-1} would simulate absolute affinity constants that cannot be deduced solely from the presented experiment. As shown in Fig. 6, the autoantibodies present in our samples behaved very differently in that experiment. Those in serum n could be inhibited by very small amounts of oligodeoxynucleotide ($K_D = 4 \times 10^{-5} gL^{-1}$), indicating a very high strength of binding. That finding explains the excessive responses seen in all other assays, as discussed above. The second affine serum, serum c-1 ($K_D = 1.5 \times 10^{-3} gL^{-1}$), shows an affinity which is nearly two orders of magnitude lower than n. The autoantibodies in this serum are, however, strong enough for stable binding in all assays. It should be mentioned that these two samples both contain mainly IgG antibodies, which are known to generally show more affinity than the respective IgMs. Samples a-1 ($K_D = 5 \times 10^{-3} gL^{-1}$) and k-1 ($K_D = 5 \times 10^{-2} gL^{-1}$) in particular, are both IgM-dominated, yet bind with even less affinity to the oligodeoxynucleotide. The fact that they are found to be positive in the Farr assay, which is known to require a certain minimum antibody affinity, seemingly contradicts our results, but only at first glance. The DNA strand used in the Farr assay is isolated from calf thymus and is obviously long enough to allow cooperative binding effects for the ten IgM valences, while this is not possible for steric reasons with the short 42-mer oligodeoxynucleotide used in our experiment.

Synopsis These results show that the SPR biosensor is a very versatile system that allows different experimental

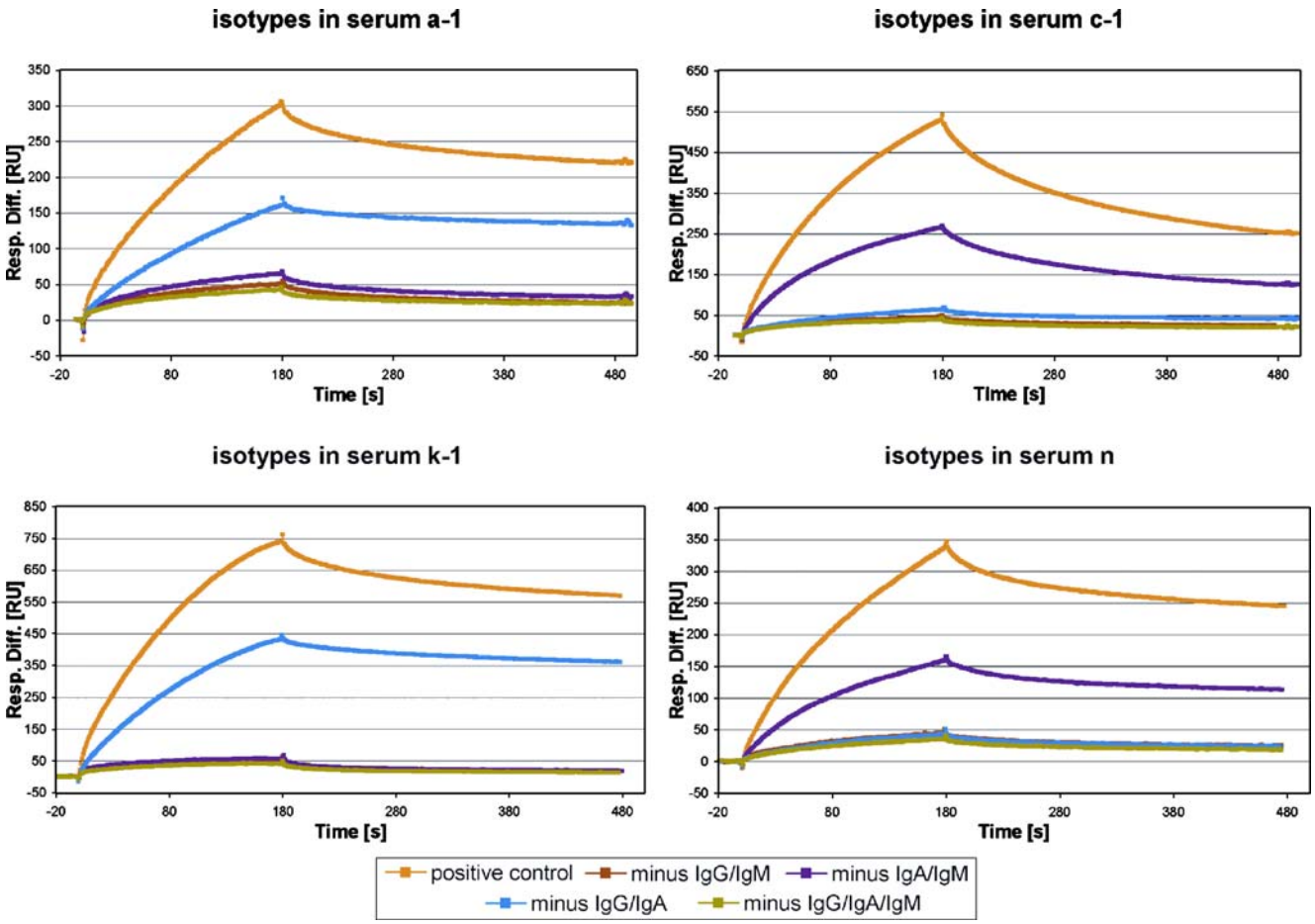

Fig. 5 Isotype distributions in the four selected sera. Shown are the binding curves of 1:100 dilutions of the respective samples after specific removal of individual isotypes

setups that are valuable for autoimmune diagnostics. Compared to other analytical systems, the biosensor offers some pivotal advantages. First of all, the interaction process itself can be monitored in real time, allowing the extraction of kinetic rate and affinity constants solely from the binding curves. Since the binding reaction itself is performed under homogeneous conditions without any buffer changes or washing steps, interactions with slow kinetics or low affinities can also be detected. Furthermore, due to the time-resolved nature of the binding curves, there is no need to reach the equilibrium state, so that measurements can be performed within a few minutes. By using more sophisticated analyzers, automation (e.g., BIAcore T100, BIAcore A100) or high parallelization (e.g., BIAcore Flexchip) can even be realized. Finally, the flow-through system provides a virtually constant analyte concentration at the chip surface, facilitating the more complex and error-prone determination of kinetic rate and affinity constants of autoantibodies of interest.

The most important advantage of the presented system is, however, that the SPR anti-dsDNA assay not only determines a certain subpopulation of autoantibodies, but also provides more comprehensive results that help us to understand the patient's real anti-dsDNA status.

Main conclusion

Optical biosensor technology allows the label-free and real-time monitoring of the binding events associated with immune complex formation. Thus, the application of biosensor techniques for autoantibody detection represents a new analytical tool. The SPR biosensor analyses presented here demonstrate that attachment of β2–GPI or dsDNA through covalent linkages to a sensor chip is a suitable method for realizing aPL and anti-dsDNA measurements in patient sera. The novel biosensor-based analytical assay system will hopefully prove in future clinical studies to be diagnostically superior to conventional ELISA formats. This biosensor technique acquires information about the binding kinetics and affinities of the specific autoantibodies through its capacity to monitor

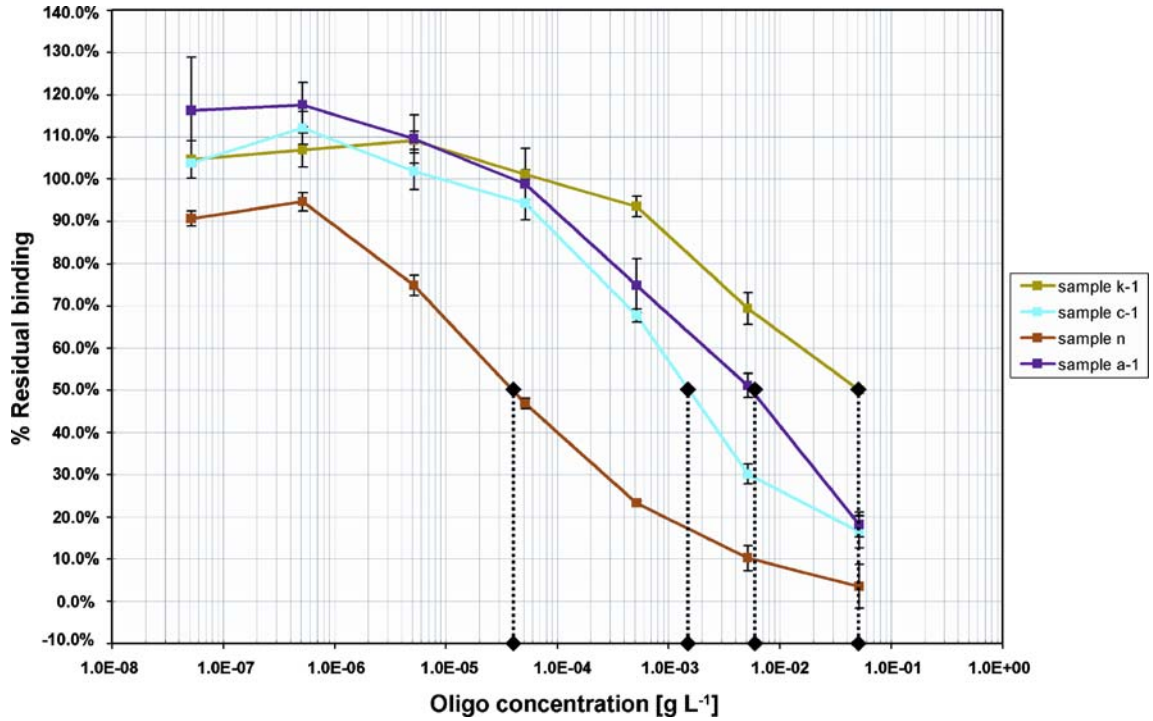

Fig. 6 Determination of relative binding affinities. The binding of 1:400 serum dilutions is inhibited with increasing concentrations of a double-stranded 42-mer oligodeoxynucleotide. Average response values (± standard deviation) of triplicate measurements are plotted as percentages of the uninhibited sample against the oligomer concentration on a logarithmic scale. The strength of antibody binding can then be determined as the oligomer concentration at the curve midpoint, indicated by the *dotted line*

biospecific interactions in real time. This technique cannot directly differentiate between concentrations and affinities/avidities of antibodies in polyclonal sera. The association concentration can, however, be determined. This parameter represents the degree of immune complex formation within a given time period, as defined by both concentration and affinity/avidity, and is a straightforward and accurate measure of antibody binding intensity.

Hence, the SPR biosensor shows diagnostic efficiencies that are at least comparable to those of established immunoassay and IIF microscopy techniques. As shown by Metzger [79] for APS patient sera, an association/dissociation plot of defined SPR binding parameter is able to prove that APS sera with dissociation values of 40%–80% are characterized by high β2-GPI-specific IgG titers, whereas those with 10%–30% dissociation contain high titers of the respective IgM isotypes. In other words, the position of an anti-β2-GPI-positive serum along the x-axis in the scatter diagram depends on the ratio of IgG and IgM autoantibodies.

SPR analyses are less time-consuming than conventional techniques and can easily be adapted to automated analyzer systems. The chip surfaces allow repetitive measurements—up to several hundreds of analyses. In conclusion, the SPR autoantibody biosensor approach analyzes the serum level of the respective autoantibody and additionally yields information about its binding characteristics. This may be of great diagnostic interest. Thus, the biosensor approach readily lends itself to adaptation to the serological evaluation of a wide spectrum of autoimmune diseases.

Future trends in the diagnosis and follow-up of patients with autoimmune diseases should address the key problems with today's laboratory evaluation: first the insufficient accuracy in the diagnosis of presymptomatic patients, and second the lack of adequate predictors of the individual disease severity [1]. With respect to the first analytical challenge, autoantibody microarrays with a high diagnostic sensitivity are clearly future-oriented. Patterns of differential antigen recognition should further facilitate an early diagnosis. This is due to the fact that a broad range of autoreactive B cell responses in many autoimmune diseases can only be mirrored by including a sufficient number of candidate antigens in a microarray format. Biosensor analyses with kinetic evaluations of kinetic rate and affinity constants of antibodies against cardinal autoantigens may be suitable for addressing the second analytical issue, identifying disease-relevant autoantibodies. The additional information from the sensorgram may help to identify autoantibody subsets which are linked to disease progression.

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