Biosensors and Bioelectronics 25 (2009) 198–203

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Optical biosensor-based characterization of anti-double-stranded DNA monoclonal antibodies as possible new standards for laboratory tests

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ARTICLE INFO

Article history:
Received 18 May 2009
Received in revised form 24 June 2009
Accepted 25 June 2009
Available online 3 July 2009

Keywords:
Surface plasmon resonance
Optical biosensor
Bioconjugation
Double-stranded DNA
Autoantibodies
Systemic lupus erythematosus

ABSTRACT

The serum determination of circulating anti-double-stranded (ds)DNA autoantibodies is a routine measure for the laboratory diagnosis of systemic lupus erythematosus. Since available assays differ substantially and no feasible calibrator is available, the aim of this study was to evaluate a recently introduced surface plasmon resonance (SPR) biosensor chip for binding studies between dsDNA and anti-dsDNA autoantibodies and to demonstrate its usefulness for the characterization of new monoclonal antibody (mAb) standards and standardization of assays.

We characterized two human and one murine monoclonal anti-dsDNA antibodies by measuring the kinetic on- and off-rates using the biosensor and calculating functional affinity (avidity) as the ratio of these. Obtained equilibrium dissociation constants were verified by an independent method and inhibition experiments were performed to determine reactivities to DNA of various length and composition.

While all mAbs exhibited comparable avidities, which could be confirmed by gel shift experiments, one of them proved to have slower association and dissociation kinetics. This was the only mAb providing positive results in the Farr RIA. In inhibition experiments with ss- and ds-oligonucleotides 10, 24 and 42 bp in length, the mAbs acted substantially different.

The study demonstrates how putative standards for the anti-dsDNA determination can be characterized using SPR biosensor technology. Our results suggest that kinetic rate constants seem to be decisive in explaining the behaviour of mAbs. Different reactivities to various DNA species should be taken into account with respect to varying DNA sources in commonly used laboratory assays.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder, potentially causing damage to virtually any organ of the human body. In the case of major organs being affected, e.g., when developing lupus nephritis, the disease can be lethal (Kotzin, 1996; D'Cruz et al., 2007). Due to its systemic nature, clinical features vary widely, making correct and early diagnosis difficult. Since therapeutical options are most helpful at an early stage, however, there is a strong need for an early diagnosis. The ACR classification criteria therefore contain, amongst clinical features, laboratory tests such as the detection of antinuclear antibodies and findings of "antibodies to native DNA in abnormal titer" (Tan et al., 1982; Hochberg, 1997). The latter are often present years before the onset of clinical symptoms (Arbuckle et al., 2003) and thus of high diagnostic significance. Anti-double-stranded (ds)DNA autoantibodies are, due to their high specificity for SLE, especially helpful in terms of guiding the diagnosis in the right direction (Isenberg, 2004). They are also feasible for follow-up, since rising titers of high-avidity IgG predict disease flares (Riboldi et al., 2005; Bootsma et al., 1997).

Considering the importance of correct anti-dsDNA detection for the rheumatologist, it is disappointing how ineffectually the matter of assay calibration has been solved by the developers. Back in 1988, an international standard for anti-dsDNA, Wo/80, was presented (Feltkamp et al., 1988). Wo/80, prepared by recalcification of 21 of plasma from one single SLE patient, followed by clotting and centrifugation, became a WHO reference preparation and was distributed by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service CLB (now Sanquin) with the arbitrary specification of containing 100 International Units (IU) anti-dsDNA per 500 μl reconstituted solution. All commercial assays were sub-

Abbreviations: Anti-dsDNA, double-stranded DNA autoantibodies; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; IU, International Units; mAb, monoclonal antibody; ODN, oligodeoxynucleotide; RIA, radioimmunoassay; SA, streptavidin; SLE, systemic lupus erythematosus; SPR, surface plasmon resonance; ssDNA, single-stranded DNA.

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doi:10.1016/j.bios.2009.06.037

sequently calibrated against Wo/80. Obviously, the amount was limited and the standard was recently withdrawn without replacement. In the original paper, Feltkamp et al. (1988) proposed the establishment of national, regional or local standards using Wo/80. However, since different assays measure different subgroups of anti-dsDNA and quantify them differently (Riboldi et al., 2005), it is evident that a secondary standard calibrated with one analytic system will not be suitable for another. It has become apparent for the anti-dsDNA autoantibody that not only binding affinity and avidity, but also the concentration and selectivity are closely related to its specificity as a marker of SLE. Thus, antibodies reacting with single-stranded (ss)DNA are less disease specific compared with antibodies reacting with dsDNA (Reveille, 2004). In this context, mAb standards were suggested to be useful (Simon et al., 2001), since they are homogeneous reagents with constant binding properties. To provide sufficient clarity about the standards' assay behaviour and their suitability as calibrators, it is necessary to characterize them extensively regarding their binding characteristics within the commonly used assay design with surface-attached dsDNA.

We have recently introduced a biosensor device for the detection of anti-dsDNA in serum on the basis of surface plasmon resonance (SPR) technology, which had a superior discrimination power between sera from SLE patients and controls (Buhl et al., 2007). The system allows the label-free monitoring of dsDNA and anti-dsDNA interactions in real time and under homogeneous conditions, meaning that no buffer changes or washing steps are performed during the actual analysis. The change of mass concentration at the interface caused by specific binding of the autoantibodies to surface-immobilized dsDNA is detected as changes in the refractive index via the SPR effect (Jönsson and Malmqvist, 1992; Van Regenmortel et al., 1998). The established biosensor surface meets the requirements for multiple measurements of serum samples under routine conditions by using two coupling chemistries at the same time (Buhl et al., 2007): Biotin/streptavidin (SA) for an efficient capturing of ligand onto a SA-coated chip surface, followed by covalent amine coupling for the final stabilization and rigid surface presentation of dsDNA. Starting reagent was a synthetic ss-oligodeoxynucleotide (ODN) which was covalently coupled to biotinylated transferrin. After hybridization with the complementary antistrand and ligation with a human recombinant dsDNA fragment of 233 bp in length, this antigenic compound was finally immobilized to the biosensor surface, consisting of a carboxymethyldextran-coating on the gold surface with pre-immobilized SA.

The aim of this study was to apply this SPR biosensor for the characterization of putative anti-dsDNA mAb standards. Kinetic rate and equilibrium constants were evaluated for three available mAbs, two of human origin and one of mouse origin. The validity of the determined equilibrium constants was then confirmed by an independent method. Furthermore, the impact of different kinetic properties and differences in the reactivity to ds- or ssDNA and to DNA of variable length were demonstrated.

2. Materials and methods

2.1. Antibodies

The human anti-dsDNA mAbs dsDNA-mAb32 and dsDNA-mAb33 were purchased from DIARECT AG (Freiburg, Germany). The manufacturer specifies their activities, determined in a not further denominated ELISA system relative to Wo/80: 1.4×10^8 IU l^{-1} for dsDNA-mAb32 at a protein concentration of 0.43 g l^{-1} and 2.2×10^8 IU l^{-1} for dsDNA-mAb33 at 0.075 g l^{-1}. The monoclonal mouse IgG2a HYB331-01 (Heegaard et al., 1996) was developed from a NZW × NZB F_1 mouse strain by Statens Serum Institut (Copenhagen, Denmark).

2.2. Chemicals and oligonucleotides

All ODNs were synthesized by MWG Biotech (Ebersberg, Germany) using standard phosphoramidite chemistry. When ssODNs are mentioned, we used 5′-ACG GTG TAT C-3′, 5′-TCA CGG GCT CAC TGA CGG TGT ATC-3′ and 5′-TCC TGT ATC CTG GTA TTA TCA CGG GCT CAC TGA CGG TGT ATC-3′. DsODNs were prepared by mixing equimolar amounts of the aforementioned ODNs and their complements in HEPES buffered saline (HBS, containing 0.01 M HEPES pH 7.4, 0.15 M NaCl) and denaturing at 95 °C for 5 min followed by slow cooling to room temperature at a rate of 1 °C/min. All other chemicals of analytical grade were used without further purification.

2.3. Instrumentation

Biosensor measurements were accomplished using a BIAcoreX (GE Healthcare, Freiburg, Germany) device at a working temperature of 25 °C. All measurements were performed on sensor chips SA (GE Healthcare), consisting of a carboxymethyldextran-coating on the gold surface, pre-immobilized with SA. Preparation and immobilization of dsDNA antigen were performed as described previously (Buhl et al., 2007). In brief, an ODN was covalently coupled to biotinylated transferrin, hybridized with the antistrand and ligated with a recombinant human dsDNA fragment. This conjugate was then captured on the SA sensor chip via biotin/SA interaction and covalently fixed by catalyzed amide bond formation.

2.4. Biosensor measurements

Signal differences between the two flow cells were monitored as a function of time (SPR sensorgrams), expressed in arbitrary resonance units (RU). For most proteins, one RU is equivalent to a change in concentration of about 1 pg/mm^2 on the sensor surface (Stenberg et al., 1991). For the measurement of dsDNA/anti-dsDNA interactions we applied 0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.1% albumin from bovine serum, 0.005% octyl β-D-glucopyranoside as running buffer maintaining a continuous flow rate of 30 μl min^{-1}. For kinetic measurements, antibody stock solutions were serially diluted using the running buffer and 90 μl were conducted over the surface to allow association, followed by a 300 s period of buffer injection to monitor the dissociation of immune complexes. For competition measurements, a fixed concentration of antibody and varying concentrations of competitive ODNs were mixed in running buffer and 90 μl were conducted over the surface without monitoring the dissociation phase. Thereafter, the biosensor surface was regenerated with a 60 s pulse of regeneration solution (50 mM NaOH, 1 M NaCl).

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

2.5. Electrophoretic mobility shift assay (EMSA)

The *Bss*SI digested 255 bp PCR product for chip fabrication (Buhl et al., 2007) was used as a probe and labeled by primer extension with the Klenow fragment of DNA polymerase I (Roche, Mannheim, Germany) in the presence of [α-^{32}P]dCTP (>3000 Ci/mmol; PerkinElmer, Brussels, Belgium) and deoxynucleoside triphosphates (Roche). MAbs at varying concentrations were incubated with different amounts of radiolabeled DNA probes for 30 min at 25 °C in 20 μl of binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol). Samples were run in 0.5× TBE buffer (10× TBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.0) on non-denaturing 4% polyacrylamide gels. Gels were dried and analyzed by autoradiography.

2.6. Farr assay

We used the anti-dsDNA RIA reagent set from Trinity Biotech (Bray, Ireland) according to the manufacturer's instructions. Values >7.0 × 10^3 IU l^{-1} are regarded as indicative of SLE.

2.7. Evaluation of inhibition curves

Each data point of inhibition experiments is shown in the plots. Curves were obtained by fitting calculated averages of the data points (n = 3) to the logistic four-parameter function using SigmaPlot 2000 (v 6.00, SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Farr RIA

The well-established Farr assay (Farr, 1958), a RIA adapted to use with anti-dsDNA (Wold et al., 1968), is believed to detect only high-avidity anti-dsDNA (Isenberg and Smeenk, 2002; Egner, 2000). The assay uses radiolabeled dsDNA from the bacteriophage PM2 (6 × 10^6 Da) as antigen to detect the specific antibodies in serum. The dsDNA reacts with the anti-dsDNA and is precipitated using a 50% ammonium sulphate solution. The bound labeled immunoprecipitate is then quantified by measuring the I-125 radioactivity. It is often regarded as a gold-standard for anti-dsDNA measurement since it is so far the most specific assay for a positive diagnosis of SLE (Rouquette and Desgruelles, 2006). The mAb dsDNA-mAb32 is described as a calibrator by the manufacturer, showing strong signals in the Farr assay, while for dsDNA-mAb33, a calibrator for anti-dsDNA ELISAs, as well as for HYB331-01, no corresponding information is available. However, dsDNA-mAb32 and dsDNA-mAb33 are delivered with activity specification (for details see Section 2). The three mAbs were diluted in control serum (tested negative for anti-dsDNA in various assays) and measured in triplicate with the Farr method. According to the manufacturer's specifications, the spiked serum samples should then contain 1400 × 10^3, 140 × 10^3 IU l^{-1} (4.3 and 0.43 μg ml^{-1} dsDNA-mAb32), 2200 × 10^3 and 220 × 10^3 IU l^{-1} (0.75 and 0.075 μg ml^{-1} dsDNA-mAb33) anti-dsDNA, respectively. HYB331-01 was diluted to comparable concentrations prior to analysis (5 and 0.5 μg ml^{-1}). Only dsDNA-mAb32 yielded a positive result (39.6 × 10^3 IU l^{-1} with a SD of 0.63 × 10^3 IU l^{-1} at 4.3 μg ml^{-1} and 11.1 × 10^3 IU l^{-1} with a SD of 0.39 × 10^3 IU l^{-1} at 0.43 μg ml^{-1}) which was below the activity declaration based on ELISA data. While a certain deviation was to be expected due to the known differences between assays, the reason for a complete lack of binding of two mAbs was hitherto inexplicable.

3.2. General considerations for kinetics and functional affinity studies

In general our intent was not a thorough kinetic and thermodynamic analysis of anti-dsDNA antibodies. Our main goal was the characterization of monoclonal anti-dsDNA antibodies as possible new standards for the determination of SLE-characteristic autoantibodies in human serum. A valid interaction analysis would demand to avoid multiple attachment of a single analyte molecule to multiple surface sites. This would mean to turn the system up-side-down: immobilization of the respective mAb, which is typically recommended in order to avoid avidity effects and allows for a straightforward affinity analysis using the 1:1 Langmuir model. This is, however, impossible due to the fact that the dsDNA molecule as ligand has more than one binding site for the antibody.

Thus, due to its exceptional antigenic properties, dsDNA substantially hampers the kinetic characterization of anti-dsDNA. It is a multiepitopic repetitive molecule, which may be bound simultaneously by more than one antibody, being of the same clonality or not. Furthermore, for most anti-dsDNA the epitope is spatially not strictly limited: since the phosphodiester backbone is bound by ionic interactions (Radic and Weigert, 1994), it may be recognized at different, non-predictable positions (Fig. 1). Due to this high "intrinsic" epitope density, a profound rebinding effect was to be expected in our biosensor measurements. To minimize the respective deviations, only the initial dissociation phases were analyzed.

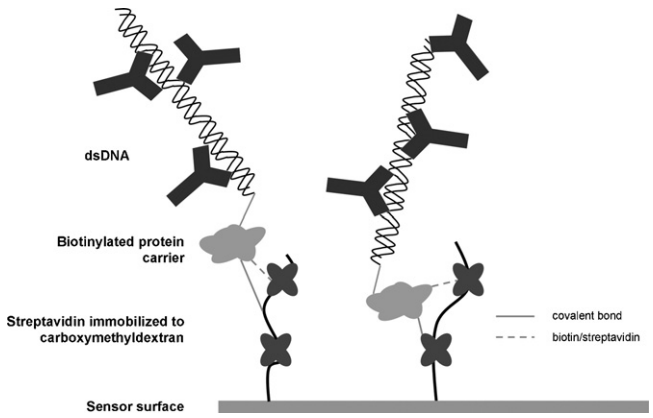

Fig. 1. Chip design as described elsewhere (Buhl et al., 2007) and supposed binding mode. Every dsDNA strand may be bound by more than one antibody at variable locations, hampering the exact determination of kinetic rate and affinity constants.

A further analytical pitfall of the immunologic interaction is the fact that it is meaningless to mention molar concentrations of DNA. It is obvious that DNA strands differing in length do not provide the same amount of potential binding sites per mole. Respective concentrations are therefore given in g/l.

3.3. Kinetic SPR studies

SPR biosensor technology allows the monitoring of biological interactions, thus enabling the measurement of kinetic rate and equilibrium constants. We investigated the formation of immune complex AY from immobilized dsDNA antigen A and dissolved mAb Y. One major constraint has to be considered: IgG antibodies are bivalent molecules and cooperative binding effects almost certainly occur since DNA of any noteworthy length will allow bivalent binding. Therefore the biosensor yields binding data representing the observed functional affinity, describing the combined strength of multiple bond interactions. This equates the term observed avidity. However, according to the argumentation of Schuck et al., who explicitly emphasize the weakness of such methods (Schuck et al., 2004), we did not use any complex fitting models like those including bivalent binding. In pilot evaluations with the BIAevaluation program we applied the bivalent model and found the results to be untrustworthy. The reason is that in each sensorgram the starting points for the association and dissociation phases have to be selected manually before the automated fitting procedure starts. In dependence of the choice of starting points the calculated kinetic data of the bivalent model vary considerably. With the Langmuir fitting procedure, however, the resulting data set was found to be more robust. Thus, we exclusively present kinetic rate constants based on a curve fitting with the simple 1:1 monovalent Langmuir model. However, it must be kept in mind that the mAb are bivalently binding and avidity effects can be anticipated. Therefore the 1:1 Langmuir binding model can only be an approximation and the derived data should only be used for a semiquantitative ranking of various antibodies.

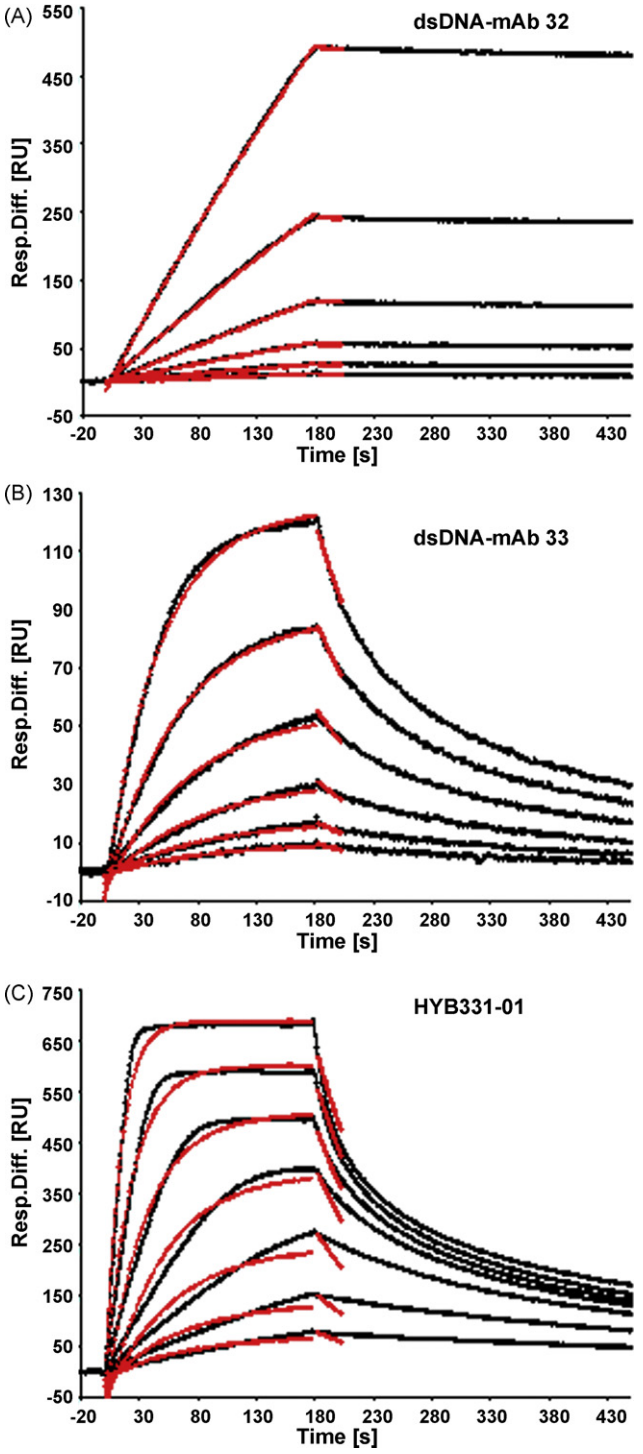

Fig. 2. Representative sensorgrams of the three mAbs. Concentration series (serial 1:1 dilutions) (A) of dsDNA-mAb32 ranging from 14.4 to 0.45 nM; (B) of dsDNA-mAb33 from 2.5 nM to 0.078 nM and (C) of HYB331-01 from 16.4 nM to 0.26 nM. *Remark*: The measured sensorgrams are given as black lines, whereas the red lines represent the fitted curves.

To determine the affinity of the three mAbs dsDNA-mAb32, dsDNA-mAb33 and HYB331-01 from their kinetic properties, a series of at least six concentrations on three different biosensor chips was used. Typical binding curves are shown in Fig. 2. For data evaluation, the curves for all concentrations of one certain antibody on one certain chip were fitted by use of the BIAevaluation software applying the Langmuir binding model. This was done separately

Table 1
Observed kinetic rate constants and equilibrium dissociation constants for anti-dsDNA antibodies determined with the SPR biosensor. *Remark*: The intra-chip SD's were negligible. Therefore only the global SD's are given.

	Chip1	Chip2	Chip3	Average ± 1 SD
dsDNA-mAb32				
$(k_a)_{obs}$ [1/Ms × 10^6]	0.0843	0.479	0.265	0.276 ± 0.198
$(k_d)_{obs}$ [1/s × 10^{-2}]	0.0295	0.0949	0.0358	0.0534 ± 0.0361
$(K_D)_{obs}$ [M × 10^{-9}]	3.50	1.98	1.35	2.28 ± 1.11
dsDNA-mAb33				
$(k_a)_{obs}$ [1/Ms × 10^6]	4.39	2.26	2.69	3.11 ± 1.13
$(k_d)_{obs}$ [1/s × 10^{-2}]	1.12	0.505	0.319	0.648 ± 0.419
$(K_D)_{obs}$ [M × 10^{-9}]	2.55	2.23	1.19	1.99 ± 0.711
HYB331-01				
$(k_a)_{obs}$ [1/Ms × 10^6]	3.90	10.2	1.94	5.35 ± 4.32
$(k_d)_{obs}$ [1/s × 10^{-2}]	1.35	1.56	0.584	1.16 ± 0.514
$(K_D)_{obs}$ [M × 10^{-9}]	3.46	1.53	3.01	2.67 ± 1.01

for the rate constant of dissociation, $(k_d)_{obs}$ using the data from the first 20 s of dissociation and for the association rate constant, $(k_a)_{obs}$, using the complete 180 s of association and the previously established $(k_d)_{obs}$. The equilibrium dissociation constant $(K_D)_{obs}$ was then calculated as the ratio of these. Since in many injections steady state levels were not reached (compare Fig. 2), Langmuir analyses (denoted as "steady state affinity" in BIAevaluation) could not be carried out.

As already mentioned, the 20 s analysis of $(k_d)_{obs}$ was chosen because due to the high epitope density of dsDNA rebinding effects were to be expected. The only way to minimize the respective deviations was to analyze the initial 20 s of the dissociation phase. Our approach is supported by the thesis of Nieba et al. that rebinding depends on the available antigens on the surface and therefore on the coating density as well as the total amount of bound antibody. Consequently, the surface empties with time during the dissociation phase and rebinding becomes more frequent (Nieba et al., 1996). Additionally, the increase of the flow rate from 30 to 40 and 50 μl min^{-1}, which should be also useful to suppress rebinding of the antibody, did not alter the kinetic data.

The observed kinetic rate constants and equilibrium dissociation constants (Table 1) indicate that dsDNA-mAb32 has both the lowest $(k_a)_{obs}$ and $(k_d)_{obs}$ value and thus a significantly different kinetic behaviour than the other two tested antibodies. This fact is also visible in Fig. 2, in particular for the slow dissociation rate. $(K_D)_{obs}$ values, however, are similar for the three mAbs and in the low nanomolar range. The unexpected deviant performance of the three mAbs in the Farr RIA cannot be explained by different avidities as commonly done in literature (Riboldi et al., 2005). We rather conjecture that the low $(k_d)_{obs}$ of dsDNA-mAb32 (Table 1, also visible as slower dissociation in Fig. 2), ensuring complex stability during washing or precipitation step, is crucial for significant activity using the Farr method. For the immunoprecipitation step in this assay a buffer solution with a high ionic strength is used. Only high-avidity autoantibodies are detected due to the fact that under these conditions anti-dsDNA with high dissociation rates uncouple easily from the immune complex. Thus, it is coherent that dsDNA-mAb32 was the only standard providing a positive signal in the Farr RIA.

3.4. Electrophoretic mobility shift assays (EMSA)

It is often criticized that affinity and avidity determinations with solid-phase methods are not valid due to a systematic difference to the situation in solution. It seemed therefore necessary for our SPR approach to confirm the observed functional affinities with an independent method, preferably working in solution. Since the antigen in the presented investigation is dsDNA, an electrophoretic mobility shift assay would be best applicable to characterize the mAbs.

Two concentrations of radiolabeled dsDNA were applied for the gel shift experiments, the higher one to visualize the multivalency of dsDNA as antigen and the lower one for avidity determinations. We found that all three mAbs form multiple immune complexes with the antigenic dsDNA showing different electrophoretic mobilities (Supplementary data, Fig. S1A–C). This is in accordance with the considerations given above. The effect is clearly concentration-dependent and inhibitable with unlabeled DNA. In contrast to publications of Khan et al. (Khan, 2006; Khan et al., 2007) who used pUC 18 plasmids and isolated human DNA from lymphocytes for similar experiments, the application of a well-defined, short (255 bp), radiolabeled PCR fragment allowed not only the detection of a general shift, but also to distinguish between various distinct immune complexes, indicating the complex binding stoichiometry.

As shown for a DNA binding peptide, EMSA can be utilized for the determination of K_D (Fairall et al., 1992). Shortly, it is deduced from the law of mass action

$$K_D = [A] \times [Y]/[AY] \quad (1)$$

with $[A]$ being the concentration of free antigen, $[Y]$ of free antibody and $[AY]$ of formed immune complex, that provided the total concentration of DNA is low enough, ensuring that the free antibody concentration $[Y]_{free}$ approximately equals the concentration of total (free and bound) antibody $[Y]_{total}$, then K_D equals $[Y]_{total}$ when 50% of the DNA is complexed. Strictly speaking, this is only valid for a 1:1 binding, which is obviously not the case with the examined dsDNA/anti-dsDNA interaction (see above). For higher stoichiometries, Eq. (1) needs to be generalized to

$$\beta_n = [A] \times [Y]^n/[AY_n] \quad (2)$$

with n being the number of antibodies bound to the DNA antigen and β_n the cumulative dissociation constant. When 50% of $[A]$ are bound, $[A]$ equals $[AY_n]$, meaning that

$$\beta_n = [Y]^n. \quad (3)$$

Since the *cumulative* dissociation constant β_n is defined as product of the *stepwise* dissociation constants for each individual binding antibody, and since each antibody has the same functional dissociation constant $(K_D)_{obs}$, it can be deduced that

$$(K_D)_{obs} = (\beta_n)^{1/n} = ([Y]^n)^{1/n}, \quad (4)$$

or

$$(K_D)_{obs} = [Y]. \quad (5)$$

This means that despite the definite deviation from a 1:1 binding reaction, $(K_D)_{obs}$ can be expressed as concentration of antibody that is needed to achieve a mobility shift of 50% of the DNA, given that the DNA concentration is very low.

The EMSA was performed by titrating the radiolabeled PCR product with a concentration of <70 pM with increasing amounts of mAbs. For dsDNA-mAb32, a clear shift can be seen between 1.4 and 14 nM, for dsDNA-mAb33 between 2.5 and 25 nM and for HYB331-01 between 5 and 20 nM (Supplementary data, Fig. S1D–F). According to the aforementioned theoretical considerations, these antibody concentration intervals correspond to the ranges of the respective $(K_D)_{obs}$ values. Taking into account that discrepancies of up to a factor of 500 have been reported between SPR biosensor measurements and values obtained in solution (Nieba et al., 1996), and antibody affinities may in principle vary over several orders of magnitude (approximately $K_D = 10^{-7}$ to 10^{-11} M), these value ranges are in good agreement with the biosensor data (Table 1), even more since affinity is a temperature-dependent constant and the good temperature control of the biosensor cannot be completely ensured in the EMSA experiments. Densitometric evaluations of the EMSA gels gave no better estimates of the apparent $(K_D)_{obs}$ values (data not shown).

3.5. Competition SPR studies

To overcome certain analytical problems of SPR biosensors, the use of competition measurements was proposed (Nieba et al., 1996). This is in principle equivalent to the affinity determination with ELISAs (Friguet et al., 1985) with the advantages of a label-free detection and a flow-through system (constant analyte concentration in the liquid phase). The analytical configuration is virtually independent but uses the same technology. Fixed concentrations of the mAbs, resulting in sufficient signals in the biosensor, were mixed with varying concentrations of competitive ODNs and allowed to reach equilibrium. The resulting mixtures were then analyzed for residual binding. We found that antibody binding is inhibited with ss- and dsODNs of varying length (Supplementary data, Fig. S2). There is considerable heterogeneity regarding the reactivities of the three mAbs against 10, 24 and 42 bases/bp long ss- and dsODNs. While mAb32 is significantly inhibited only by dsODNs, mAb33 binds similarly to all of them with a certain preference for the 42-base ss one. HYB331-01 is inhibited stronger by ds and longer species.

Measurements with the respective two strongest inhibitors and with the 255 bp PCR product, used for chip immobilization, were repeated in triplicate and fitted to a four-parameter function (Supplementary data, Fig. S3). The midpoint values of the curves, representing the concentration of inhibitor needed for a 50% inhibition, are given in Table 2.

These data are valuable for the comparison of reactivities to different DNA species. Absolute binding constants, however, cannot be derived for several reasons. In contrast to the above-mentioned statement that $(K_D)_{obs}$ can be expressed as the concentration of *antibody* that is needed to achieve the binding of 50% of the DNA, $(K_D)_{obs}$ *cannot* be expressed as the concentration of *DNA* that is needed for the binding of 50% of the antibody when assuming a complex stoichiometry. This is due to the fact that it is impossible to solve Eq. (2) in a similar manner as shown above since the proportion of [Y] and $[AY_n]$ is unknown and certainly $\neq$ 1:1. The specificity profiles, however, have to be taken into account when choosing calibrators. For instance, mAbs with true specificity to dsDNA, such as mAb32, should be favoured over such with a high reactivity to ss species (e.g., mAb33), especially in assays where significant amounts of ssDNA contaminations could be present.

A further finding is that the midpoint of the 255 bp PCR product inhibition curve of mAb32 differs by more than one order of magnitude from those of mAb33 and HYB331-01 although all three antibodies show similar affinities in kinetic experiments and EMSA. This may be due to the fact that despite the high flow rate of 30 μl min^{-1} at a cell volume of 0.06 μl, implicating a surface contact time of at most 0.12 s, a substantial amount of the two antibodies with the faster kinetics, mAb33 and HYB331-01, can dissociate from the inhibitor and bind to the surface, pretending higher concentrations of free mAb and thus lower affinities to the inhibitor. If

Table 2
Binding specificities of monoclonal antibodies to different ODNs. *Remark*: The midpoint data are extracted from the experiments depicted in Supplementary data, Fig. S2.

DNA inhibitor	Midpoints of inhibition curves, given in $g l^{-1} \times 10^{-6}$		
	dsDNA-mAb32	dsDNA-mAb33	HYB331-01
255 bp PCR product	6.84	362	153
42 bp dsODN	3.40	95.2	173
24 bp dsODN	21.3	–	–
42-base ssODN	–	21.8	929

this dissociation occurs during a split second in the flow-through system, it will probably play an even greater role in similar experiments utilizing ELISA for the concentration measurement of free anti-dsDNA. Publications comparing anti-dsDNA on the basis of competition/inhibition measurements, like those for example performed by Spatz et al. (Spatz et al., 1997), may thus generally be criticized, provided that kinetic properties are not mentioned.

4. Conclusions

Our work presents the determination of kinetic rate constants and functional dissociation constants as a measure of antibody avidity for three anti-dsDNA mAbs. The applicability of our sensor has been demonstrated and the validity of $(K_D)_{obs}$ values was confirmed by EMSA experiments. Using the example of the Farr assay, we could show that the determination of kinetic properties seems to be important to explain the behaviour of mAbs in laboratory tests. Furthermore, it was demonstrated that antibody avidities vary with the choice of antigenic DNA, in the present work of different length and composition.

We hence propose the use of the SPR biosensor for the characterization of anti-dsDNA mAbs in the context of establishing new standards for laboratory tests. Since at present there is no feasible calibrator available, and since a variety of completely different methodologies is used in common commercial assays, our approach readily lends itself to that purpose, not least due to the possibility to obtain kinetic binding information. A larger panel of dsDNA-mAbs should first be characterized, and can subsequently act as model systems to untangle the grossly disparate results achieved by the use of a series of non-comparable laboratory tests. Once the requirements of these tests, in terms of definite kinetic rate constants, concentration values and specificities/cross reactivities of mAbs are known, suitable antibodies can be chosen for calibration. This should improve reliability and comparability of anti-dsDNA determinations.

Acknowledgments

We gratefully acknowledge financial supports by the Kommission für Klinische Forschung des Klinikums rechts der Isar der TU München, and the Stiftung für Pathobiochemie und Molekulare Diagnostik der DGKL. We thank Ms. A. Schreiegg and Mrs. B. Miebach for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.06.037.

References

Arbuckle, M.R., McClain, M.T., Rubertone, M.V., Scofield, R.H., Dennis, G.J., James, J.A., Harley, J.B., 2003. N. Engl. J. Med. 349, 1526–1533.
Bootsma, H., Spronk, P.E., Ter Borg, E.J., Hummel, E.J., de Boer, G., Limburg, P.C., Kallenberg, C.G., 1997. Ann. Rheum. Dis. 56, 661–666.
Buhl, A., Metzger, J.H., Heegaard, N.H., von Landenberg, P., Fleck, M., Luppa, P.B., 2007. Clin. Chem. 53, 334–341.
D'Cruz, D.P., Khamashta, M.A., Hughes, G.R., 2007. Lancet 369, 587–596.
Egner, W., 2000. J. Clin. Pathol. 53, 424–432.
Fairall, L., Harrison, S.D., Travers, A.A., Rhodes, D., 1992. J. Mol. Biol. 226, 349–366.
Farr, R.S., 1958. J. Infect. Dis. 103, 239–262.
Feltkamp, T.E., Kirkwood, T.B., Maini, R.N., Aarden, L.A., 1988. Ann. Rheum. Dis. 47, 740–746.
Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L., Goldberg, M.E., 1985. J. Immunol. Methods 77, 305–319.
Heegaard, N.H., Olsen, D.T., Larsen, K.L., 1996. J. Chromatogr. A 744, 285–294.
Hochberg, M.C., 1997. Arthritis Rheum. 40, 1725.
Isenberg, D., 2004. Lupus 13, 881–885.
Isenberg, D., Smeenk, R., 2002. Lupus 11, 797–800.
Jönsson, U., Malmqvist, M., 1992. Real Time Biospecific Interaction Analysis—The Integration of Surface Plasmon Resonance Detection, General Biospecific Interface Chemistry, Microfluidics into One Analytical System. Elsevier Science & Technology, The Netherlands.
Khan, W.A., 2006. Scand. J. Immunol. 64, 677–683.
Khan, W.A., Alam, K., Moinuddin, 2007. Arch. Biochem. Biophys. 465, 293–300.
Kotzin, B.L., 1996. Cell 85, 303–306.
Nieba, L., Krebber, A., Plückthun, A., 1996. Anal. Biochem. 234, 155–165.
Radic, M.Z., Weigert, M., 1994. Annu. Rev. Immunol. 12, 487–520.
Reveille, J.D., 2004. Lupus 13, 290–297.
Riboldi, P., Gerosa, M., Moroni, G., Radice, A., Allegri, F., Sinico, A., Tincani, A., Meroni, P.L., 2005. Autoimmunity 38, 39–45.
Rouquette, A.M., Desgruelles, C., 2006. Lupus 15, 403–407.
Schuck, P., Boyd, L.F., Andersen, P.S., 2004. Curr. Protoc. Cell Biol. (Chapter 17) Unit 17.6.
Simon, T., Kneusel, R., Haubruck, H., Liedvogel, B., Diarect, A.G., 2001. Scand. J. Clin. Lab. Invest. Suppl. 235, 91–97.
Spatz, L., Saenko, V., Iliev, A., Jones, L., Geskin, L., Diamond, B., 1997. J. Exp. Med. 185, 1317–1326.
Stenberg, E., Persson, B., Roos, H., Urbaniczky, C., 1991. J. Colloid Interface Sci. 143, 513–526.
Tan, E.M., Cohen, A.S., Fries, J.F., Masi, A.T., McShane, D.J., Rothfield, N.F., Schaller, J.G., Talal, N., Winchester, R.J., 1982. Arthritis Rheum. 25, 1271–1277.
Van Regenmortel, M.H., Altschuh, D., Chatellier, J., Christensen, L., Rauffer-Bruyere, N., Richalet-Secordel, P., Witz, J., Zeder-Lutz, G., 1998. J. Mol. Recognit. 11, 163–167.
Wold, R.T., Young, F.E., Tan, E.M., Farr, R.S., 1968. Science 161, 806–807.