Biosensors and Bioelectronics 51 (2014) 177–183

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Electrochemical biosensor for quantitation of anti-DNA autoantibodies in human serum

Robert L. Rubin [a,*], David Wall [b], Konstantin N. Konstantinov [c]

[a] Department of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA
[b] Voss Scientific, Albuquerque, NM, USA
[c] Department of Internal Medicine, Division of Rheumatology, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA

ARTICLE INFO

Article history:
Received 3 May 2013
Received in revised form
12 July 2013
Accepted 21 July 2013
Available online 1 August 2013

Keywords:
Electrochemical flow-through biosensor
Anti-DNA autoantibody
Human serum

ABSTRACT

Measurement of serum autoantibody is a critical tool in the diagnosis and management of autoimmune diseases. However, rapid and convenient methods at the point-of care have not been achieved in large part because any one antibody species is a heterogeneous and miniscule fraction of the total serum immunoglobulin displaying identical properties other than its antigen-binding specificity. The present system addresses these challenges by vacuum-mediated transport of diluted serum through an antigen-coated porous membrane. To measure anti-DNA autoantibodies, native DNA was immobilized into a poly (vinylidene fluoride) membrane pre-coated with a synthetic phenylalanine/lysine co-polymer. Flow-through of primary and peroxidase-conjugated secondary antibodies over the course of 3 min enhanced productive antibody–antigen interactions by bringing the reactants into close mutual proximity. Signal was quantified electrochemically during the enzymatic conversion of the tetramethylbenzidine substrate to a charge-transfer complex. The electrochemical signals generated by sera from patients with systemic lupus erythematosus using this device showed good quantitative correlation with a standard enzyme-linked immunosorbent assay and displayed similar detection limits. Inter- and intra-assay variability and electrode uniformity were favorable as was a two-month test of the stability of the DNA-coated membrane. While refining the fluidics requirements of this biosensor will be needed, its capacity to quantify over the course of 30 min anti-DNA antibodies in fresh human serum without background reactivity of normal serum makes this a promising technology as a point-of care device of clinical utility.

1. Introduction

Anti-native (double-stranded, ds) DNA autoantibodies are the serological hallmark of the autoimmune disease systemic lupus erythematosus (SLE) (Isenberg et al., 1997) and as such are included in its classification criteria defined by the American College of Rheumatology (Tan et al., 1982). The diagnostic and prognostic importance of IgG anti-dsDNA antibodies has led to the development of numerous detection techniques based on diverse physical principles and/or sources of critical reactants including immunoprecipitation of radiolabelled DNA, indirect immunofluorescence of mitochondrial DNA in kinetoplasts of *Crithidia luciliae*, and immunoassays involving immobilized DNA in which the antibody is detected either fluorescently using bead-based flow cytometry or optically via enzyme-mediated amplification (ELISA) (Hahn, 1998). These methods have different sensitivity, specificity, selectivity and reproducibility, and they require centralized clinical or research laboratories for needed equipment and expertise. The disconnection between the clinical laboratory and the physician has resulted in increased turn-around time for critical laboratory results and misunderstandings by laboratory personnel concerning which test(s) the physician actually needs (Wiik and Bizzaro, 2012). Also, misinterpretation of results by the physician can be related to unfamiliarity with the technical or performance characteristics of a particular clinical laboratory assay.

Point-of-care (POC) testing has the potential to overcome these problems and is showing increasing use in aiding clinical diagnosis; recent trends in its application may be especially relevant to prioritizing patients who are severely ill or in need of therapy adjustment due to active disease. Biosensor technology offers the promise to streamline diagnostic laboratory testing, thereby improving productivity of health care systems by minimizing costs, time and errors. If available, reliable POC testing for anti-dsDNA and other autoantibodies associated with rheumatic diseases would deliver more timely, unambiguous results that could improve diagnostics in primary or urgent/emergency care clinics

* Correspondence to: Department of Molecular Genetics and Microbiology, MSC08-4660, University of New Mexico Health Sciences Center, 1 University of New Mexico Albuquerque, NM 87131, USA. Tel.: +1 505 272 4640; fax: +1 505 272 9550.
E-mail address: rlrubin@salud.unm.edu (R.L. Rubin).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.07.041

and enhance medical intervention for patients (Konstantinov et al., 2013).

We describe the development and application of an electrochemical biosensor for quantitation of anti-dsDNA antibodies with performance characteristics comparable to ELISA. This prototype has promise as a POC device for measuring autoantibodies in fresh human sera in a rheumatology setting by virtue of its simple and flexible design and use of inexpensive materials, reagents and electronics.

2. Material and methods

2.1. Human serum samples

Blood was obtained from donors under auspices of a human subjects protocol approved by the institutional human research review committee. After clotting, serum was removed by centrifugation and stored at 5 °C in the presence of 0.05% sodium azide for <2 weeks prior to use. Blood from patients diagnosed with SLE based on accepted criteria (Tan et al., 1982; Hochberg, 1997) was used for anti-DNA positive samples. Donors of normal blood were anonymous employees of the university.

2.2. DNA-coated membrane for immunosensor

A sheet of polyvinylidene fluoride (PVDF) membrane (0.45 μm pore size, Pall Life Sciences Corp., Port Washington, NY, USA) was cut into 1.0 cm × a8.25 cm strips. Just before coating, each strip was placed in an individual 13 × 100 mm glass test tube and hydrated by sequential immersion in methanol, 25% methanol, water and phosphate buffered saline ("PBS", 0.14 M NaCl, 0.01 M Na phosphate, pH 7.4, 0.01% thimerosol). A synthetic polypeptide co-polymer of lysine and phenylalanine at a 1:1 ratio and molecular weight of 20–50 kDa (Sigma-Aldrich, St. Louis, MO, USA) stored frozen at 10 mg/ml in 0.01 M phosphate buffer, pH 7.4 was diluted just before use to 8 μg/ml in PBS, and 9 ml was added to each membrane. After gentle continuous tube inversion at room temperature for 1–2 h, the membrane was washed 2 × with PBS. DNA (Calbiochem EMD Biosciences), pre-treated with S1 nuclease (Invitrogen Life Technologies, Carlsbad, CA) as previously described (Burlingame and Rubin, 2002), was diluted to 10 μg/ml in PBS, and 9 ml was transferred to the membrane. After continuous tube inversion for 0.5 h, the membrane was incubated overnight at 5 °C, rinsed with PBS and immersed in a solution consisting of 0.1% bovine serum albumin (BSA, Sigma), 1.0% Tween-20 (polyoxyethylene sorbitan monolaurate, stabilized and peroxide/aldehyde-free, Sigma) in PBS for 2 h at room temperature. This post-coating stage was followed by rinsing the membranes in PBS and replacing it with membrane "wash solution" consisting of 0.005% NP-40 (Sigma-Aldrich) in PBS. Membranes were either used the same day or stored at 5 °C in wash solution. Based on uptake analysis, 0.8 μg DNA was bound to 0.5 cm^2 well-exposed membrane.

2.3. Immunosensor design

An 8-well manifold was machined in two parts from stabilized acrylic as shown in Fig. 1. Each well had a diameter 0.8 cm, exposing 0.5 cm^2 of the underlying membrane. Below the membrane was placed another acrylic block with 8 cavities aligned with those in the top piece. A gasket was inserted at the interface between the membrane and the top piece, which was sealed to the bottom piece by wing nuts tighten onto two threaded posts separated by 8.25 cm. Perpendicular to the walls of the wells in the bottom piece was drilled a 1 mm diameter channel, exiting through a stainless-steel hollow rod to which a vacuum can be applied. During the immunoassay after the final wash step (see Section 2.4), the apparatus was disassembled and a plastic strip containing 8 independently-addressable electrode sets (Alderon Biosciences, Beaufort, NC, USA) was aligned with the cavities in the bottom piece with the aid of alignment rods inserted into holes drilled through the plastic strip containing the electrode sets. The immunoreactant-exposed membrane was then aligned with the electrode and the top piece was sealed to the bottom piece as described above. Each electrode set consisted of a central working carbon graphite electrode surrounded by carbon graphite counter electrode and a silver chloride reference electrode within the 0.8 cm diameter well and at a 0.9 cm center-to-center separation from the adjacent set(s). The electrode terminals were connected through a cable to an amperometric reader (Alderon Biosciences), whose parameter settings were controlled and output monitored by interface with a computer using the manufacturer's software.

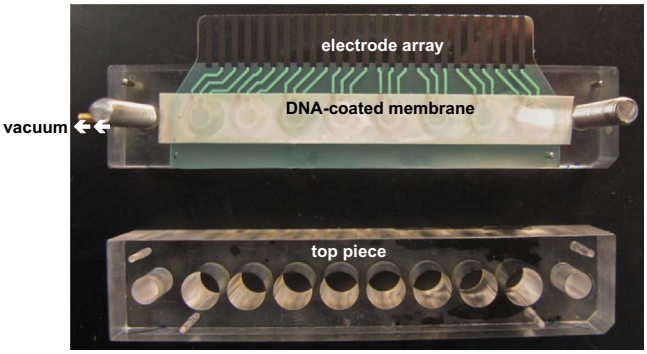

Fig. 1. Flow-through device for measuring anti-DNA antibodies in 8 samples. The fully assembled apparatus, in which the top piece is placed over the DNA-coated membrane, is sealed with a gasket and wing nuts on the threaded posts. The electrode array would be inserted under the membrane after the primary and secondary antibodies are drawn and washed through the membrane using a weak vacuum applied below the membrane to the lucite bottom piece.

2.4. Amperometric immunoassay

All steps were performed at room temperature. The test sera were diluted 1:50 in "serum diluent" consisting of 2.5 mg/ml bovine gamma globulin (BGG, Calbiochem EMD Biosciences) and 3.5 mg/ml BSA in "wash solution". Serum diluent was ultrafiltered through a 0.2 μm pore-size membrane and stored at 5 °C. Sera were unfiltered and used within a few hours after dilution. After assembly of the apparatus with the DNA-coated membrane, each of the 8 wells was loaded with 200 μl diluted serum, and the samples were simultaneously drawn through the membrane over the course of ~3 min at ~70 μl/min using a weak vacuum applied to the lower chamber by a peristaltic pump (W-M Alitea AB, Stockholm, Sweden). Each well was then rinsed 3 × with ~0.4 ml wash solution at a flow rate of 0.7 ml/min, requiring a total wash time of ~3 min. Then 0.2 ml peroxidase-conjugated goat anti-human IgG (SouthernBiotech, Birmingham, AL) diluted 1:20,000 in "serum diluent" was added to each well, drawn through the membrane at ~70 μl/min and washed as described above. After re-assembling the apparatus with the electrode array in place (see section 2.3), 0.15 ml peroxidase substrate solution consisting of commercially-stabilized hydrogen peroxide (H_2O_2)+2 mM 3,3′,5,5′ tetramethylbenzidine ("TMB liquid substrate system", Sigma) was added to each well. Beginning at 1 min, readings were taken by intermittent pulse amperometry at 2 min intervals at a potential of −100 mV using 20, 5 ms voltage pulses over the course of 4 s, and the current at the last 15 pulses was averaged to produce the recorded signal at the selected timepoint. Product accumulation

was monitored continuously for up to 20 min, and the 13 min results are reported in some figures. Total elapsed time from addition of serum to addition of substrate was less than 20 min. Electrode arrays were re-used after rinsing with soap and soaking sequentially in 0.5 M NaCl, 0.01 N HCl and 0.1% H_2O_2 for 10 min each.

2.5. Limit of detection determination

Human IgG (Sigma) was dissolved in 0.15 M NaCl and its concentration determined by absorption spectroscopy based on $E_{280\ m\mu}=1.4$ mg/ml. Serial dilutions down to 1 ng/ml were made in PBS with 2 μg/ml BSA as a carrier, and 0.2 ml was slowly drawn through individual wells of a virgin membrane. After disassembly, the membranes were post-coated as described in Section 2.2 and subjected to the standard immunoassay (Section 2.4) starting with the peroxidase-conjugated goat anti-human IgG step.

2.6. Determination of anti-DNA antibody activity by ELISA

Anti-DNA antibody measurements were performed by a standard ELISA as previously described (Burlingame and Rubin, 1990,2002) except for substitution of the several materials in order to maximize congruence with the electrochemical method. Solutions were prepared in PBS (Section 2.2). Immulon 2HB 96-well microtiter plate wells (Thermo Scientific, Rochester, NY) were pre-coated overnight at 5 °C with 0.2 ml lysine/phenylalanine co-polymer (described above) at 2.0 μg/ml. After rinsing with PBS, 0.2 ml of DNA at 5 μg/ml was incubated in each well for 6 h at 5 °C and replaced with 1 mg/ml gelatin (type B, Baker, Phillipsburg, NJ) blocking solution overnight at 5 °C. Plates were rinsed the next day with 0.05% Tween-20, and 200 μl serum diluted 1:200 in 0.75 mg/ml BGG, 1 mg/ml BSA, 0.05% Tween-20 was added in duplicate and incubated for 2 h with agitation at room temperature. Plates were rinsed with 0.05% Tween-20 and agitated for 1.5 h with peroxidase-conjugated goat anti-human IgG diluted 1:20,000 in 1 mg/ml BGG, 5 mg/ml BSA. After rinsing, colored product was developed over the course of 1 h after addition of 0.2 ml 0.005% H_2O_2+1 mg/ml 2,2′ azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, MP Biomedicals, Santa Ana, CA) in McIlvaine's buffer, pH 4.6. Optical density (O.D.) at 410 mμ was measured using a spectrophotometric plate reader (Thermo Labsystems, Fisher) at various times over the course of 1 h. Values greater than 3.5 O.D. were extrapolated from values at time-points earlier than 1 h as previously described (Burlingame and Rubin, 2002) so that all antibody activities were expressed as O.D. at 1 h.

2.7. Preparation of TMB product

The products of the catalytic action of horseradish peroxidase on TMB+H_2O_2 substrate solution were prepared using solid phase peroxidase in order to control the extent of product formation without chemically altering the solution. Human gamma globulin was enriched from serum by 50% saturated ammonium sulfate precipitation and adsorbed overnight at 25 μg/ml to wetted PVDF membrane. The membrane was blocked and incubated with peroxidase-conjugated goat anti-human IgG (Section 2.4) by continuous tube inversion (Section 2.2) for 1 h at RT. After extensive washing, the membrane was floated in TMB/H_2O_2 substrate solution at 37 °C with agitation and removed when 10-fold diluted product accumulated to ~2.2 O.D. at 370 mμ (12–16 min). Once removed from the peroxidase-coated membrane, the solution showed no further change in $O.D._{370\ m\mu}$ over the subsequent 30 min, indicating absence of peroxidase leakage into the liquid phase. The product was stored up to 1 h at 0 °C and ultrafiltered just before use to remove small amounts of precipitate that accumulated. For measurements of light absorption and electrochemical activity, the product was serially diluted in TMB substrate solution. The concentration of the TMB semiquinone (charge-transfer complex) was based on $E_{652m\mu}=39$ (mM/cm) and of TMB benzidiimine on $E_{450\ m\mu}=59$ (mM/cm) (Josephy et al., 1982).

3. Results

3.1. Anti-DNA antibody detection in human serum by electrochemical immunoassay

The electrochemical immunoassay (EC) immunoassay platform employed poly(vinylidene fluoride) membranes coated with a synthetic polypeptide consisting of co-polymer of phenylalanine for hydrophobic binding to the membrane and lysine for ionic binding to DNA. Membranes immobilized 1.6 μg DNA/cm^2 of strictly native DNA conformation to preclude detection of antibodies to denatured (single-stranded) DNA that is common at low levels in human serum (Schwartz and Stollar, 1985). Porous membranes facilitated productive interaction between the primary antibody solution and solid phase antigen by transporting diluted serum through the antigen-coated membrane using weak negative pressure over the course of 3 min. A similar procedure facilitated binding of the secondary antibody, peroxidase-conjugated anti-human IgG Fc, to bound primary antibody. Both antibody binding steps were followed by membrane rinses at a 10-fold higher flow rate. An array consisting of eight independently addressable electrodes was then inserted under the membrane followed by addition of the peroxidase substrate solution. Continuous production of peroxidase-generated TMB product was monitored every 2 min by electrochemical reduction at the working electrode.

The device is shown in Fig. 1, and typical results with seven sera containing anti-DNA antibodies from patients with SLE are shown in Fig. 2A. After a lag time of ~3 min, largely due to time for diffusion of the peroxidase substrates into the membrane (data not shown), each sample showed a time-dependent and non-overlapping increase in current due to voltagenic reduction of an intermediate TMB product (see Supplement), which continued for about 10 min. The parallel nature of the response curves and their similar plateau time suggested a quantitative relationship with the amount of primary and secondary antibody bound to antigen on the membrane. This view was supported by the data in Fig. 2B in which the same human sera produced negligible reactivity above that of the secondary antibody background on a blank membrane devoid of antigen.

As shown in Fig. 3, sera from normal individuals failed to display detectable reactivity in the EC immunoassay on either DNA-coated (Fig. 3A) or blank (Fig. 3B) membranes. The small reactivity on blank membrane at late time points can be accounted for by low-level reactivity of the secondary antibody in this experiment. The positive control in Fig. 3B and some samples in Fig. 1B did display reactivity several fold above background on the blank membrane; this is the result of a serum storage artifact (see discussion).

Fig. 4 compares the anti-DNA reactivity of a set of SLE sera measured by the EC assay versus an ELISA, the standard immunoassay for measuring anti-DNA antibodies. EC data was obtained from three independent assays on separate days. Regression analysis between the two assays showed good correlation ($r=0.92$) and a similar limit of detection. Intra-assay variability (RSD) based on 4 replicates was 8% with high level reactors and 11% for moderate level reactors; inter-assay reproducibility (RSD) based on 3 independent assays was 14%, 16% and 23% for high, moderate, and low level anti-DNA reactors, respectively.

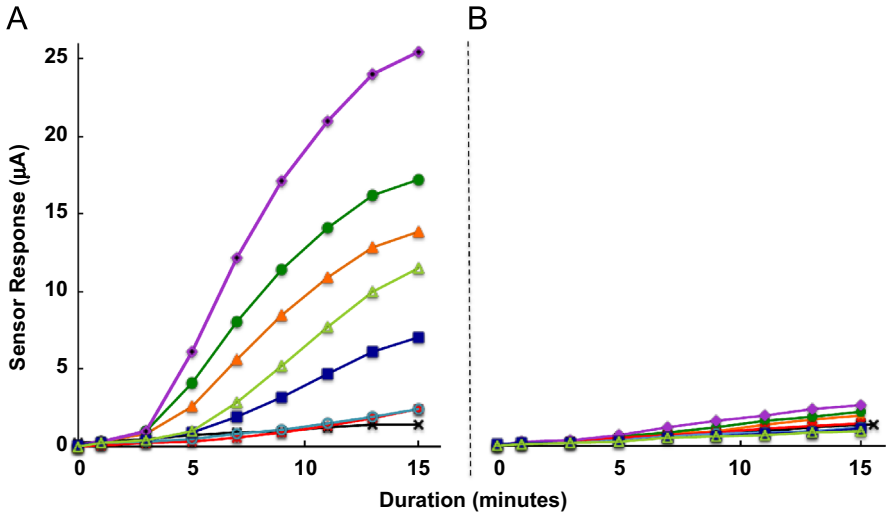

Fig. 2. Electrochemical response of antibody in sera from 7 patients with SLE to a DNA-coated membrane (A) or blank membrane (B). Sample marked as x—x is the negative control without primary antibody.

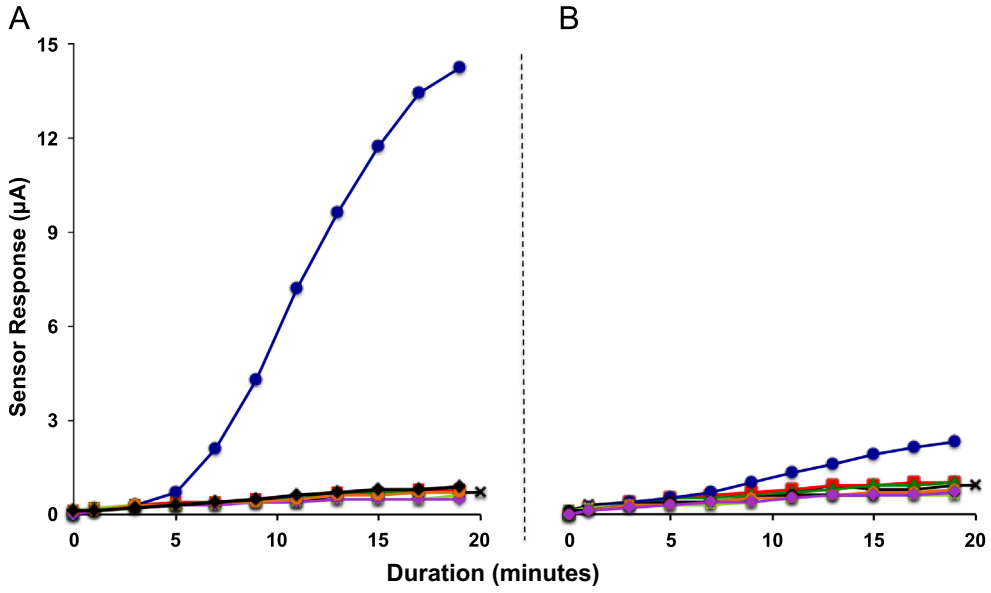

Fig. 3. Normal serum reactivity to the DNA-coated membrane (A) or a blank membrane (B). Sample marked as x—x is the negative control without primary antibody. The positive control from an SLE patient is indicated as ●—●.

The EC assay and the ELISA displayed similar sensitivity and capacity to discriminate among differences in anti-DNA antibody levels based on the samples employed in Fig. 4, although many more samples would be needed to fully demonstrate this. Specificity for measuring only antibodies to native DNA is determined by the quality of the DNA preparation, which was the same in both assays. We increased the dynamic range of the ELISA by use of an early type-point extrapolation method as described (Burlingame and Rubin, 1990); without this method, the ELISA would be limited to the maximum measurable optical density, usually <3.0 O.D., while the EC assay does not have this limitation. In addition the ELISA requires at least 4 h, while the EC assay is complete in $\sim$30 min.

The detection limit for human IgG was also assessed by sensor response to various amounts of purified IgG directly adsorbed to the membrane and then measured in the same manner as with specific antibody bound to the membrane via DNA antigen. In this system 1 μg IgG/well generated a response of 8 μA at 13 min; the amperometric response fitted to a power regression analysis using the log of the input IgG concentration produced an $r^2=0.96$ (data not shown). Based on a background signal of 1.4 ± 0.2 μA (S.D.) at 13 min, the absolute limit of detection was conservatively estimated as 0.04 μg IgG, which produced a signal of 3.3 μA at 13 min.

3.2. Electrode performance

The EC device we constructed has 8 separated sample chambers juxtaposed against an 8-component independently addressable electrode array (Fig. 1). The absolute responsiveness of each position was compared within an array and between three electrodes. This is shown in Fig. 5A in which the same amount of preformed TMB oxidation product was added to each well. The average responsiveness of the 8 positions for each of the 3 electrodes was 7.7 μA, 6.9 μA and 6.6 μA, the decrease due mainly to decay in the TMB charge-transfer complex (see Supplement) over the course of this experiment (data not shown). Intra-electrode variability was 7.1%, 6.9%, and 14.6% (SEM) among the 8 positions for electrodes 7, 8, and 9, respectively. Fig. 5B shows electrode

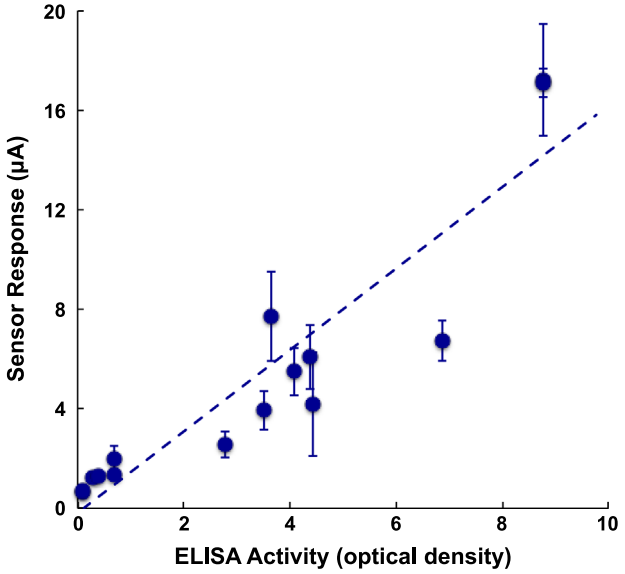

Fig. 4. Comparison between a standard ELISA method and the electrochemical method for detection of anti-DNA antibodies in 13 SLE patients. Error bars are S.D. derived from 3 independent EC assays.

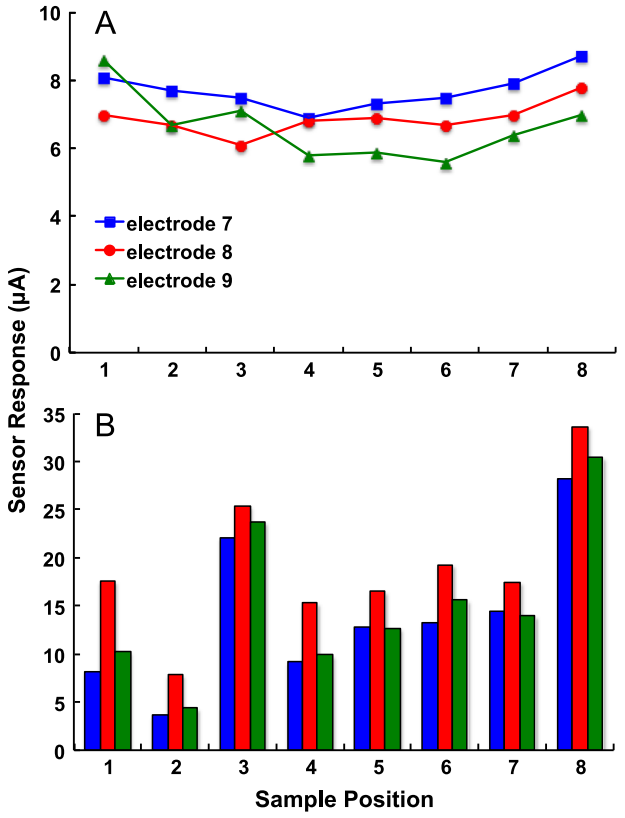

Fig. 5. Electrode performance. In panel (A) pre-formed TMB product was added to each well of the assembled apparatus without a membrane, and the response of electrode #7 was recorded at 3 min. This was promptly repeated for two other electrodes. In panel (B) the same 3 electrodes were used in a complete anti-DNA assay of 8 SLE sera in which de novo-produced TMB product at 13 min is shown.

responsiveness in an anti-DNA assay in which a different SLE serum was assayed in the same well position using three different electrodes. While electrode #8 produced substantially higher values than the other two electrodes, especially with the low-level reactors, paired comparisons between electrodes were highly correlated ($r=0.96$ for electrode 7 vs. 8, $r=0.99$ for electrode 7 vs. 9, and $r=0.98$ for electrode 8 vs. 9), indicating that minor differences in absolute electrode sensitivity did not affect relative autoantibody activity.

3.3. Stability of DNA-coated membranes

We assessed the device performance after storing the antigen-coated membrane in wash solution at 5 °C up to two months by comparison to membranes prepared the same day they were used. Surprisingly, reactivity of membranes stored for one or two months displayed an average ($\pm$S.D.) of 1.66 ± 0.21-fold and 1.70 ± 0.39-fold, respectively, greater antigenicity than freshly prepared membranes without any substantial increase in background binding of the secondary antibody ($n=5$ serum samples, data not shown). This suggests that enhanced membrane antigenicity upon storage was essentially complete by 1 month with no loss in performance for at least one more month.

4. Discussion

The flow-through design of this biosensor enhanced antigen-antibody interaction by slowly delivering the reactants into close mutual proximity. The concentrating effect produced by solution transport is particularly important for measuring spontaneously arising antibodies because their heterogeneous affinities and concentrations can result in assay-dependent selection of only a portion of the molecular population displaying particular properties. In the current system, autoantibodies from the equivalent of 4 μl serum were exposed over the course of the 3 minute transport process to 0.8 μg DNA embedded in a 0.15 mm thick membrane, producing a similar magnitude signal to that in ELISA format but which necessitated 40-fold more time just for the primary antibody incubation step. Based on comparison to IgG directly bound to the membrane, we conservatively estimate that the limit of specific antibody detection in serum in this system is 10 μg/ml, which is normally ~0.1% of total serum IgG. The 3-dimensional structure of the membrane required a substantial wash-out process to completely remove trapped immunoglobulin; this was also facilitated by the design of the apparatus in which the membrane was continuously exposed to virgin wash solution at a high flow rate.

Anti-DNA is an important biomarker for diagnosis of SLE (Hahn, 1998). However, the reliability of the assay is strictly dependent on the presentation of native, double-stranded DNA structure, because IgG binding to denatured (single-stranded, ss) DNA occurs in normal individuals (Schwartz and Stollar, 1985), although anti-ssDNA antibodies are substantially elevated in SLE and 10–15% of patients with other rheumatic diseases (Teodorescu, 2002). Sources for native DNA can be closed circular plasmid DNA or preparations of double-stranded DNA treated with the single-strand-specific enzyme S1 nuclease. However, because native DNA is hydrophilic, it will not stably bind to hydrophobic surfaces such as PVDF membranes or polystyrene microtiter plates. The current system employed a synthetic polypeptide pre-coating agent that binds to the PVDF solid support through its hydrophobic tryptophan side-chains and to native DNA by electrostatic interactions between the polypeptide lysine residues and DNA phosphates. Membranes coated with DNA in this way were stable for at least two months when stored in aqueous solution.

There has been explosive progress in the development of biosensors involving antibodies, although the vast majority of these devises employed immobilized antibodies to capture their cognate antigen in biological fluids. Measurement of antibodies in blood or serum to immobilized antigens is technically more challenging because the specific antibody of interest is usually a

miniscule fraction of the total sample immunoglobulin that displays similar immunochemical properties. In addition some sera stored frozen or at 5 °C for as little as one month produced background reactivity on a blank membrane, precluding their use in this system but not in ELISA (data not shown). The current device dealt with these problems by the use of solution carrier agents that minimized non-specific binding to the antigen-supporting membrane, by the extensive washing steps, and by the use of relatively fresh serum samples. Lack of non-specific binding phenomena was demonstrated by the minimum reactivity of anti-DNA positive samples on blank membranes and that of normal serum reactivity in this system.

Despite the wide use of horseradish peroxidase+TMB secondary substrate in electrochemical detectors, there is little information on the basis for the signal generation. Fig. 6 is a proposed mechanism underlying autoantibody-mediated signal generation in this system based on properties of the semi-stable intermediates generated during catalysis (Supplementary materials). We propose that TMB is co-oxidized with H_2O_2 to a free radical intermediate which forms a unstable charge-transfer complex with unreacted TMB. A portion of this product is reduced back to TMB by intermittent voltage application at the working electrode, resulting in current flow from the counter electrode. The molecular complex undergoes another oxidation event producing the yellow-colored diimine product. As substrate is consumed, the rate of charge-transfer complex formation decreases. Because of its instability, the charge-transfer complex must be measured de novo during the catalytic process to maximize signal intensity.

Reports of devices for measuring antibodies, including autoantibodies, more rapidly than traditional techniques are increasing (reviewed in Konstantinov et al., 2013). Surface plasmon resonance (SPR)-based technology applied to measurement of autoantibodies to DNA (Buhl et al., 2007), cyclic citrullinated peptide (Lokate et al., 2007), β2-glycoprotein 1 (Real-Fernandez et al., 2012) and cardiolipin (Schlichtiger et al., 2013) can produce results in just 5 min, but the difficulty of distinguishing among clinically-relevant antibody isotypes and the need for sophisticated instrumentation limit applicability in clinical settings. Nevertheless, the rapidity of SPR methodology and its capacity to measure immune complexes without the need for secondary antibodies could be favorable when combined with pulse amperometry (German et al., 2013). Techniques that show particular promise for measuring (auto)antibodies in a POC setting include lateral flow chromatography (Renger et al., 2010), bead-based luciferase luminescence (Burbelo et al., 2009), reflectometric interference spectroscopy (Hilbig et al., 2012) and other enzyme amplified electrochemical techniques (Neves et al., 2012; Purvis et al., 2003; Rosales-Rivera et al., 2011). A non-flow-through device for measuring immunoglobulin class-specific anti-gliadin antibodies (Rosales-Rivera et al., 2011) showed performance characteristics similar to the present device although required considerably longer assay time.

A potential limitation of the present device is the requirement for a controlled vacuum applied to the sealed assembly in order to draw through antibodies followed by a substantial volume of wash solution. In addition, because the immunological reactions occur on a porous membrane, the electrode must be inserted under the membrane prior to application of the enzyme substrate. We believe these requirements could be readily overcome. Because of its superior performance based on assay speed, negligible background, stability, and good correlation with an established method to measure disease-relevant autoantibodies, this device has considerable promise for POC applications.

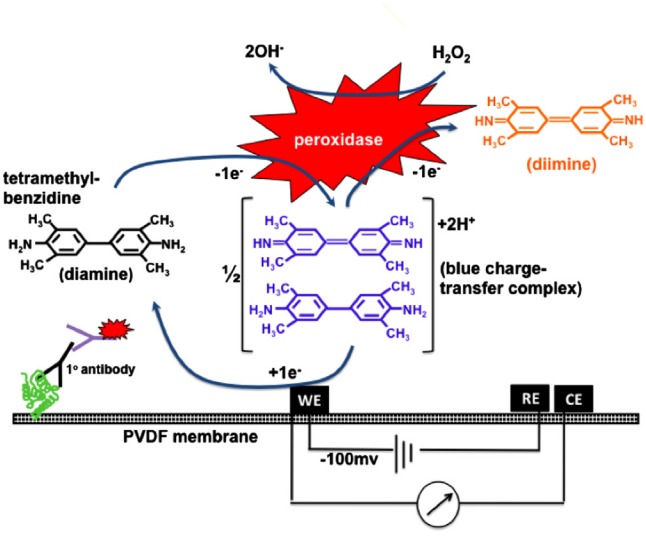

Fig. 6. Immunochemical, enzymatic, and electrochemical phenomena underlying the autoantibody biosensor. The objects at the lower left depict membrane-immobilized DNA antigen to which serum antibody is bound followed by peroxidase conjugated anti-human IgG. After the substrates H_2O_2 and TMB are added, de novo product formation occurs, which is detected by electochemical reduction of the TMB charge-transfer complex at the working electrode (WE) due to negative potential produced through the reference electrode (RE), causing current to flow from the counter electrode (CE).

5. Conclusions

A portable biosensor is described for measuring anti-DNA antibodies in the serum of patients with autoimmune disease. The read-out is derived from the electrochemical detection of the real-time oxidation of TMB substrate by peroxidase-conjugated secondary antibody. Negative control sera from normal people showed negligible reactivity in this system. Quantitative comparison with a standard ELISA was favorable as was reproducibility, uniformity, and reliability of the disposable reagents and materials. While its fluidics requirement for a vacuum source and ample wash solution remains a limitation, its capacity to produce reliable results in approximately 30 min argues for its promise as a POC device for measuring anti-DNA and other autoantibodies of utility in a rheumatology setting.

Acknowledgments

We thank Robert Henkens, Ph.D. (Alderon Biosciences) for providing the electrodes used in this work. There was no sponsor or specific funding source for this work.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.07.041.

References

Buhl, A., Metzger, J.H., Heegaard, N.H., von Landenberg, P., Fleck, M., Luppa, P.B., 2007. Clinical Chemistry 53, 334–341.
Burbelo, P.D., Ching, K.H., Issa, A.T., Loftus, C.M., Li, Y., Satoh, M., Reeves, W.H., Iadarola, M.J., 2009. Journal of Translational Medicine 7, 83.
Burlingame, R.W., Rubin, R.L., 1990. Journal of Immunological Methods 134, 187–199.
Burlingame, R.W., Rubin, R.L., 2002. In: Rose, N.R., Hamilton, R.G., Detric, B. (Eds.), Manual of Clinical Laboratory Immunology, 6th ed. American Society for Microbiology, Washington, D.C., pp. 951–960.
German, N., Kausaite-Minkstimiene, A., Kirlyte, J., Makaraviciute, A., Ramanavicius, A., Mikoliunaite, L., Ramanaviciene, A., 2013. Analyst 138, 1427–1433.

Hahn, B.H., 1998. New England Journal of Medicine 338, 1359–1368.
Hilbig, U., Bleher, O., Le Blanc, A., Gauglitz, G., 2012. Analytical and Bionalytical Chemistry 403, 713–717.
Hochberg, M.C., 1997. Arthritis and Rheumatism 40, 1725.
Isenberg, D.A., Ravirajan, C.T., Rahman, A., Kalsi, J., 1997. Lupus 6, 290–304.
Josephy, P.D., Eling, T., Mason, R.P., 1982. Journal of Biological Chemistry 257, 3669–3675.
Konstantinov, K.N., Tzamaloukas, A.H., Rubin, R.L., 2013. Autoimmunity Highlights 4, 55–61.
Lokate, A.M., Beusink, J.B., Besselink, G.A., Pruijn, G.J., Schasfoort, R.B., 2007. Journal of the American Chemical Society 129, 14013–14018.
Neves, M.M., Gonzalez-Garcia, M.B., Nouws, H.P., Costa-Garcia, A., 2012. Biosensors and Bioelectronics 31, 95–100.
Purvis, D., Leonardova, O., Farmakovsky, D., Cherkasov, V., 2003. Biosensors and Bioelectronics 18, 1385–1390.
Real-Fernandez, F., Passalacqua, I., Peroni, E., Chelli, M., Lolli, F., Papini, A.M., Rovero, P., 2012. Sensors (Basel), 12; , pp. 5596–5607.
Renger, F., Bang, H., Feist, E., Fredenhagen, G., Natusch, A., Backhaus, M., Burmester, G.R., Egerer, K., 2010. Arthritis Research and Therapy 12, R120.
Rosales-Rivera, L.C., Acero-Sanchez, J.L., Lozano-Sanchez, P., Katakis, I., O'Sullivan, C.K., 2011. Biosensors and Bioelectronics 26, 4471–4476.
Schlichtiger, A., Baier, C., Yin, M.X., Holmes, A.B., Maruyama, M., Strasser, R., Rant, U., Thaler, M., Luppa, P.B., 2013. Analytical Chemistry Journals 405, 275–285.
Schwartz, R.S., Stollar, B.D., 1985. Journal of Clinical Investigation 75, 321–327.
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 and Rheumatism 25, 1271–1277.
Teodorescu, M., 2002. Clinical and Applied Immunology Reviews 2, 115–128.
Wiik, A.S., Bizzaro, N., 2012. Autoimmunity Highlights 3, 15.