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An electrochemical biosensor for rapid detection of anti-dsDNA antibodies in absolute scale†

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Autoimmune diseases are chronic inflammatory pathologies that are characterized by the presence of antibodies against the body's own epitopes in serum (autoantibodies). Systemic lupus erythematosus (SLE) is a common autoimmune pathology characterized by the presence of antinuclear antibodies (ANAs). These include anti-dsDNA (α-dsDNA) antibodies, which are widely used for diagnosis and disease monitoring. Their determination is carried out using traditional techniques such as Indirect Immunofluorescence (IFI) or Enzyme-Linked Immunosorbent Assay (ELISA), which are time consuming, require qualified technicians, and are not compatible with decentralized analysis outside a laboratory facility. Here, we show a sandwich-format electrochemical biosensor-based method for α-dsDNA determination in a rapid and simple manner. The total assay time is only 30 minutes, and the sensor is capable of detecting 16 ng (8 μg mL^{-1}) of α-dsDNA antibodies. Using the current derived from the detection limit of the method as a cut-off, we could discriminate positive from negative serum samples with 90% sensitivity and 100% specificity. Using monoclonal antibodies for calibration curves, our results are presented in absolute scale (*i.e.*, concentration instead of serum titer) which will help us to perform comparisons between methods and carry out further improvements of this protocol. In an effort to render the sensor compatible with automation, we minimized the manipulation steps without compromising the analytical performance, even in complex samples such as serum.

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Introduction

Autoimmune disorders are chronic inflammatory pathologies that affect over 5–8% of the world's population.[1] In these disorders, the immune response of the individual is directed against his/her own components causing tissue- or organ-specific damage, generating local or systemic responses. Common autoimmune diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, systemic sclerosis, type 1 diabetes, inflammatory bowel disease, and antiphospholipid syndrome. Diagnosis of these diseases is challenging for professionals, since their symptoms can vary between individuals and overlap with other rare pathologies.[2,3] Moreover, their origin is still difficult to understand because of the genetic and environmental factors involved in their appearance.[4] However, they share some common features, such as the presence of antibodies directed against the body's own epitopes (autoantibodies).

SLE, considered as a model autoimmune disease, has been studied in depth and more than a hundred autoantibodies have been reported. These involve reactivity against nuclear, cytoplasmic and membrane components. Antinuclear antibodies (ANAs) are reactive against single- and double-stranded DNA, histones, nucleosomes and chromatin, as well as other nuclear antigens (Ro, La and Sm ribonucleoproteins).[5] Specific antibodies are associated with distinct clinical features. For example, anti-dsDNA antibodies (α-dsDNA) are associated with the development of lupus nephritis. Total ANAs, α-dsDNA and α-Sm antibodies are considered as the hallmarks of this pathology and are included in the serological American College of Rheumatology (ACR) criteria for diagnosis.[6–10] SLE is also characterized by flares and remission steps.[8,11] Thus, determination of the aforementioned antibodies in serum samples is relevant not only for diagnosis but also for classification, determination of the state of the disease, as well as for therapeutic evaluation and drug adjustment.[3,12]

The gold standard technique for ANA determination is *Crithidia luciliae* indirect immunofluorescence (CLIF). In this assay, fluorescence intensity is used for titer determination,

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and, in addition, the specific autoantibodies contained in the sample can be defined as a consequence of the different staining patterns. Although several commercial kits have been developed with different cellular substrates, these require complex instrumentation and experienced staff, being unsuitable for point-of-care applications.[13–15] Recently, Enzyme Linked Immunosorbent Assay (ELISA) kits have been developed and commercialized. These kits use single or multiple antigen-coated wells (purified or synthetic), such as Ro, La, and dsDNA. However, diversity in antigen and adsorption strategies can affect comparability of the results between labs using kits from different suppliers. Line immuno-semi-quantitative assays (variation of immuno-blots) are easy to use and facilitate detection of multiple autoantibodies in one strip. Nevertheless, they generally offer low sensitivity and specificity for certain antibodies.[16]

All the assays described above are either time-consuming or can only be developed in a centralized laboratory with certified equipment and technicians, being unable to provide immediate results for flare prediction and drug adjustment therapy. Biosensors emerge as suitable platforms for quick point-of-care tests. A biosensor can be described as an analytical device that includes a biological component as a sensor, which works in association with a physicochemical transducer.[17,18] Most biosensors oriented to the detection of autoantibodies employ a dsDNA-coated surface (or other auto-antigen) that is used to capture the autoantibodies in the sample. These can be detected directly (label-free) or indirectly (*e.g.*, with the help of an enzyme-coupled secondary antibody). Some label-free approaches include Surface Plasmon Resonance (SPR) chips or Quartz Crystal Microbalance (QCM) measurements.[13,19–21] However, electrochemical label-based biosensors are very promising for point-of-care tests because of the low costs associated with their automatization and miniaturization.[22] In this regard, Konstantinov, Rubin and coworkers have developed an electrochemical sandwich-type immunoassay where nuclear antibodies are detected through current measurements (using redox enzyme-coupled secondary antibodies).[23–25]

Recently reported electrochemical biosensors as well as commercial kits use a WHO-standardized serum as a reference to measure autoantibody concentrations. Thus, concentrations are reported either in arbitrary units or in titer units (maximum serum dilution to obtain a signal with a suitable signal-to-noise ratio), which are compared against the WHO reference or other previously calibrated positive samples. This is suitable for most clinical applications, but we argue that results expressed in absolute scale (*i.e.*, molarity or grams per liter) are convenient for both basic research and applications where the inclusion of a previously calibrated control is not always possible (*e.g.*, self-monitoring). Furthermore, the WHO-standardized reference serum is not available any longer.[2]

The previous reasons pushed us to develop an amperometric biosensor capable of detecting α-dsDNA antibodies, which could serve as a diagnostic and disease tracing tool in the near future. We also propose a methodology to obtain absolute α-dsDNA antibody concentrations (in terms of mass per volume), without the need for positive and previously calibrated human sera.

Materials and methods

Reagents and solutions

Lyophilized genomic salmon sperm DNA ("dsDNA", Sigma-Aldrich, Cat. No. D1626) was employed for the ELISA assays and in all electrode modification procedures. Genomic DNA ("gDNA") was extracted from cow liver using a Quick-gDNA MiniPrep kit from Zymo Research. Plasmid DNA ("pDNA") was purified from *Escherichia coli* cells transformed with the pGEM-T Easy Vector (Promega) with conventional plasmid purification based on alkaline lysis, potassium acetate precipitation of genomic DNA, and ethanol precipitation of plasmid DNA. A PCR product ("PCR-DNA") obtained using actin gene-specific primers to amplify a 200 bp fragment from porcine DNA was purified using a QIAquick PCR Purification Kit (Qiagen) and was also tested using ELISA. Poly-L-lysine and 3,3′,5,5′ tetramethylbenzidine (TMB, ≥98%) were purchased from Sigma-Aldrich. Freeze-dried bovine serum albumin (BSA ≥96%) was obtained from Spectrum Chemical Mfg Corp. (USA), and 1% fresh solutions were prepared in phosphate buffered saline ("PBS": 10 mM sodium phosphate buffer, 150 mM NaCl, pH = 7.4). Mouse monoclonal anti-dsDNA antibodies (α-dsDNA: sc-58749), normal mouse immunoglobulins (m-IgG: sc-2025) and mouse monoclonal anti-human CD9 (α-CD9: sc-13118) and anti-Cy3 (sc-166894) antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit HRP-conjugated anti-mouse IgG (α-m-IgG/HRP, ab6728) was purchased from Abcam. Fetal bovine serum (FBS) was supplied by Gibco. Adult mouse serum was obtained from a 12-week-old BALB/c mouse. Stock DNA concentrations were estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Screen-printed three or four (bi-electrode) electrode strips (working: carbon; counter: carbon; pseudoreference: silver) were supplied by DropSens (Oviedo, Spain). All electrochemical measurements were carried out with a CHI760D workstation (CH Instruments).

ELISA assay

The ELISA assay was carried out as previously described.[26] A 96-well ELISA plate (CELLSTAR, Cat.-No. 655 180) was pretreated with 100 μL of 50 μg mL^{-1} poly-L-lysine solution (in water) for 30 min at room temperature (RT) and washed three times with a Tris-borate saline buffer ("TBS", 10 mM, 150 mM NaCl, pH = 7.5). Then, 100 μL of dsDNA solution (4 μg mL^{-1} in TBS) was incubated for 60 min at RT and washed in the same way as in the previous step. After this, the plate was blocked overnight with 100 μL of 1% BSA solution at RT and washed vigorously three times with a PBS–0.1% Tween-20 buffer. Then, 100 μL of PBS dilution containing either α-dsDNA or mouse (m) IgG (specific and unspecific antibodies, respectively) was incubated for 60 min at 37 °C and washed with

PBS–0.1% Tween as previously described. 100 μL of α-m-IgG/HRP (1/2000 dilution, plus 1% BSA) was dispensed and incubated for 60 min at 37 °C. The plate was washed and finally incubated for 30 min with 100 μL of TMB–H_2O_2 solution (2 mM TMB, 1 mM H_2O_2, diluted in 50 mM acetic acid/sodium acetate buffer, pH = 5). The reaction was stopped with 50 μL of 5 M HCl, and the absorbance at 450 nm was measured in a microplate reader (Thermo Scientific, Multiskan EX).

Construction of dsDNA-modified electrodes

100 μL of an acetic acid/sodium acetate buffer (200 mM, pH = 5) was placed in order to cover the three or four screen-printed electrodes in each strip, and a potential of +1.7 V was applied for 120 s as electrode pretreatment, followed by three washes with the same buffer. A dsDNA solution (0.5 μg μL^{-1} in an acetic/acetate buffer) was prepared and vortexed for 30 s and then 50 μL were placed on the electrode system. A constant potential of +0.5 V was applied for 300 s for dsDNA immobilization. After washing three times with PBS, 2 μL of 1% BSA were deposited (on the working electrode only) and the electrodes were incubated for 30 min at 37 °C in a wet chamber with 100% humidity.

The dsDNA immobilization was confirmed using cyclic voltammetry (CV) after successive washing steps. Briefly, 50 μL of an acetic acid buffer were placed on the three electrode system and CV was carried out between −0.2 V and +1.0 V at a scan rate of 0.05 V s^{-1} in order to observe the guanine oxidation signal.

Detection of specific antibodies

Different dilutions of α-dsDNA and m-IgG were prepared in PBS. Two microliters of these solutions were placed on the working electrode and the working electrode was incubated for 20 min at 37 °C in a wet chamber. For the bi-electrode strips, each antibody was placed in a different working electrode. After this, the electrodes were washed three times with PBS, and 2 μL of α-m-IgG/HRP antibody (1/2000, 1% BSA) was added and the electrodes were incubated under the same experimental conditions. The electrodes were then washed with PBS, and 50 μL of a TMB/H_2O_2 solution was added. Immediately, the working electrode was placed at a constant potential of −0.1 V, and the TMB reduction current was registered. For optical measurements, only monoelectrodes were employed. After antibody incubations, the TMB/H_2O_2 solution was incubated for 5 min, followed by the addition of 5 μL of 5 M HCl. Two microliters of the mixture were measured at 450 nm using a NanoDrop spectrophotometer.

Performance in serum samples

As an approximation to the analysis of real samples, assays were performed in the bioelectrode strips by spiking α-dsDNA or irrelevant antibodies directly in 1/80 FBS. Here, two different strategies were tested. One strategy (which we called the "two-step" method) consisted of incubating α-dsDNA-containing FBS on the working electrode, performing two washing steps with PBS and then incubating (20 min at 37 °C) the electrodes with 2 μL of the α-m-IgG/HRP antibody (1/2000 dilution, plus 1% BSA), followed by a new washing step and addition of the TMB/H_2O_2 solution. In contrast, the "one-step" method consisted of preincubating α-dsDNA (or irrelevant antibodies) with α-m-IgG/HRP in FBS. Briefly, 5 μL of α-dsDNA (in 1/40 FBS) was mixed with 5 μL of α-m-IgG/HRP (1/1000) and incubated for 5 min at 37 °C. Then, 2 μL of the mixture was placed on the working electrode, and the procedure continued the same way as described above. As a consequence, one single incubation with the sample and HRP-conjugated antibodies was needed, avoiding the washing step in between, reducing the assay time approximately two-fold.

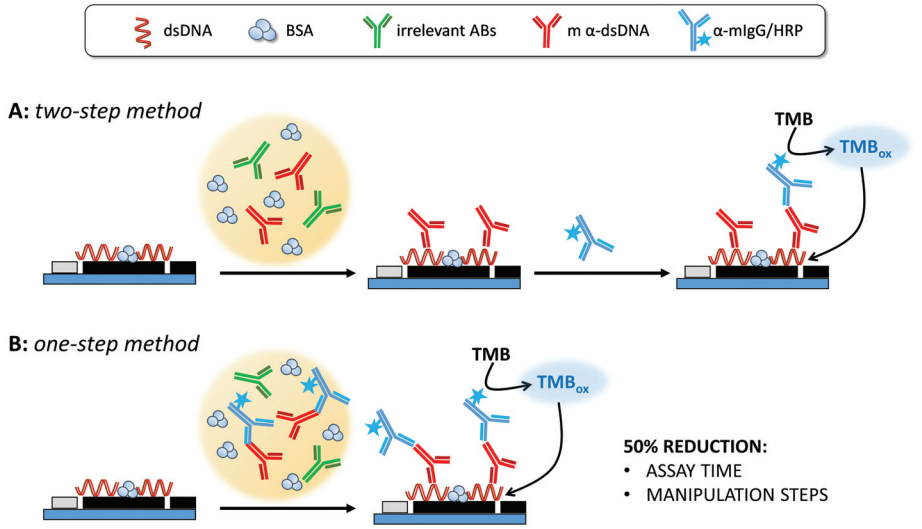
Samples and informed consents from SLE patients were obtained in the Autoimmune Disease Unit of the Hospital de Clínicas (Faculty of Medicine, Universidad de la República, Uruguay). All procedures involving the acquiring, handling and use of human samples were performed under the guidelines of the Universidad de la República (Uruguay) and approved by the Bioethics Committee of the Hospital de Clínicas (Faculty of Medicine, Universidad de la República, Uruguay). Adult mouse serum was obtained in accordance with the Guidelines for Care and Use of Laboratory Animals of the Institut Pasteur de Montevideo (Uruguay) and approved by the Animal Ethics Committee of the Institut Pasteur de Montevideo.

Results

We propose the construction of an amperometric biosensor capable of detecting anti-double stranded DNA antibodies, which offers diagnostic and prognostic value in several autoimmune diseases. This biosensor is based on the specific binding of α-dsDNA antibodies present in a test sample to dsDNA molecules immobilized on the surface of a disposable screen-printed carbon graphite electrode, and the subsequent binding of anti-mouse IgG antibodies conjugated to the electroactive enzyme HRP (conjugated ABs). The electrochemical reduction of the oxidized TMB generated by the catalysis of HRP is measured,[27] which is dependent on the amount of α-dsDNA antibodies located in the surface of the sensor. In an attempt to render this biosensor compatible with automatization, the conjugated ABs were directly introduced in the test sample, greatly simplifying the detection procedure (one-step method, Scheme 1).

Specificity of the α-dsDNA antibody

The specificity of our α-dsDNA antibody was studied using ELISA (Fig. 1). Wells sensitized with either vortexed or intact dsDNA showed a characteristic binding response when incubated with increasing concentrations of mouse monoclonal α-dsDNA antibody, with a linear response below 0.07 μg mL^{-1}. In contrast, incubation with normal mouse immunoglobulins (m-IgG) showed basal absorbance independently of the concentration used, confirming the specificity of the assay. Secondly, we assayed the α-dsDNA antibody against genomic



Scheme 1 Detection strategy. Positive or negative serum samples (containing 1/80 fetal bovine serum plus either murine monoclonal anti-dsDNA [red] or irrelevant [green] antibodies) were incubated with dsDNA-modified carbon electrodes, washed, and later incubated with anti-mouse IgG antibodies conjugated to the HRP enzyme [blue] (A). In a more time-efficient and automatization-compatible strategy, conjugated antibodies were mixed with the serum samples before incubation (one-step, B).

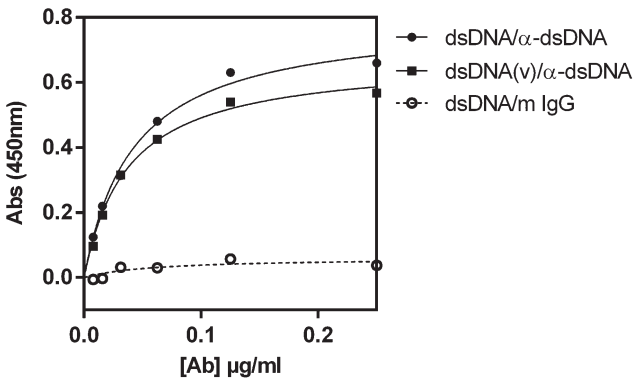


Fig. 1 Specificity of the α-dsDNA antibody using the enzyme-linked immunosorbent assay. Plates were sensitized with vortexed (squares) or intact (circles) salmon sperm DNA and incubated with different concentrations of α-dsDNA antibody (black) or mouse IgGs as a control (white).

DNA, plasmid DNA and a purified PCR product, confirming that it is the dsDNA molecule itself (rather than co-purified nucleosomes present in certain genomic DNA preparations) that is being recognized by the antibody used throughout this study (ESI, Fig. 1†).

Electrode modification

dsDNA was deposited on the working electrode at a constant potential as indicated in the Materials and methods section. We asked whether that immobilization method would be suitable for keeping the dsDNA attached to the electrode surface during the whole procedure. To test this, we subjected dsDNA-modified BSA-blocked electrodes to a number of washes in PBS, which was equivalent to the number of washes in our longest assay (three washing cycles, three washes per cycle). The presence of DNA in the electrodes was then analyzed by measuring the irreversible guanine oxidation signal[28] at +0.83 V using cyclic voltammetry, and comparing this signal with those of naked (Fig. 2A) or BSA-only electrodes (data not shown).

Specificity of the electrochemical biosensor

The electrochemical behavior of TMB on our screen-printed dsDNA-modified carbon electrodes was studied using cyclic voltammetry (ESI, Fig. 2†) in order to determine the working potential, where electrochemical TMB oxidation was virtually zero, and to also register the electrochemical reduction of oxidized TMB. By doing so, we defined −0.1 V as a suitable applied potential for subsequent constant potential assays.

As a proof-of-principle, intensity *vs.* time (*I vs. t*) assays at −0.1 V are shown in Fig. 2B. Incubation of dsDNA-modified electrodes with the α-dsDNA monoclonal antibody (followed by detector ABs and TMB/H_2O_2 solution) showed the expected response,[27] with a negative (*i.e.*, reduction) signal decreasing in absolute value with time and approaching a concentration-dependent plateau as predicted by the Cottrell equation.[29] Here, the total TMB concentration is constant, but the concentration of oxidized TMB depends on the number of HRP molecules present at the surface of electrodes. In contrast, the electrodes incubated with normal mouse IgG displayed a negligible signal, comparable to the electrodes only exposed to the conjugated ABs. This finding demonstrates that unspecific binding of conjugated ABs to our electrodes is negligible in the absence of an analyte (*i.e.*, α-dsDNA antibodies).

Optical and electrochemical response

Based on the fact that our electrochemical signal depends on the concentration of enzyme-oxidized TMB, which also absorbs visible light with a maximum at 450 nm after acidifi-

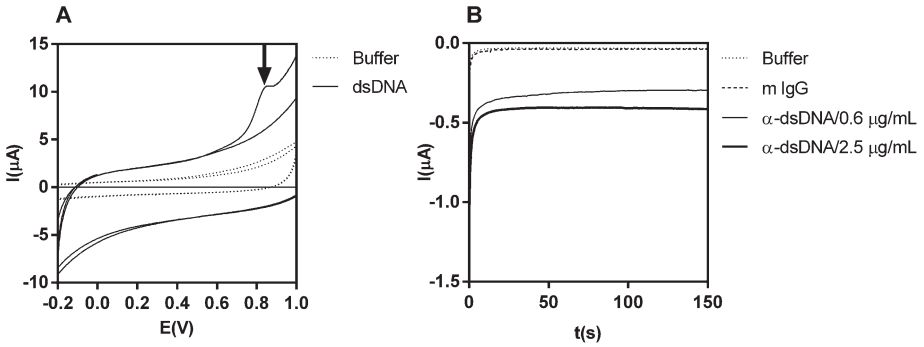


Fig. 2 Electrode modification with dsDNA and electrochemical sensing of α-dsDNA antibodies. (A) Cyclic voltammograms showing guanine oxidation (arrow) in dsDNA-modified electrodes (solid line) *vs.* buffer-treated (dashed line) screen-printed carbon electrodes. (B) Intensity *vs.* time curves for TMB reduction on the electrode surface at −0.1 V. dsDNA-modified BSA-blocked electrodes were incubated with α-dsDNA antibodies at two different concentrations (solid lines), normal mouse IgG at 2.5 μg mL^{-1} (thick dashed line), or buffer (thin dashed line), and then incubated with α-mouse IgG/HRP conjugates, followed by addition of a TMB/H_2O_2 solution.

cation of the medium, we compared the analytical performance of the sensor with both optical and electrochemical readouts. Measuring oxidized TMB absorbance in droplets deposited on top of modified carbon electrodes also served to validate antibody targeting to DNA-modified electrodes using a previously validated and electrochemistry-independent technique. Similar to what was previously observed in ELISA plates (Fig. 1), the absorbance of oxidized TMB was saturated above 0.05 μg mL^{-1} of α-dsDNA antibodies in carbon/dsDNA/BSA electrodes (Fig. 3A), with no signs of unspecific binding of antibodies to the electrode surface. In contrast, the electrochemical response (measured as the electrical current in *I vs. t* plots at exactly t = 100 s) was linear up to approximately 0.5 μg mL^{-1}, showing a 10-fold increase in the dynamic range of the method when compared to absorbance measurements under the same conditions. It is important to mention that these assays were performed in bi-electrodes, where both working electrodes were modified simultaneously using the same dsDNA solution and incubated with either α-dsDNA antibodies or normal mouse IgGs at exactly the same concentration. Thus, batch effects (*i.e.*, disparities in electrode fabrication and dsDNA immobilization) are minimized due to the use of paired data in our assays.

Using the standard deviation of all electrodes incubated with mouse IgGs, we determined the limit of detection (LOD) of our sensor as the background signal (*i.e.*, the average of mouse IgG-incubated electrodes) plus three standard deviations. We obtained an LOD of 0.1 μg mL^{-1} of α-dsDNA antibodies, corresponding to a measured current of −0.1 μA. Currents (cathodic) higher than this could be taken into account to discriminate samples that contain α-dsDNA antibodies. It should be noted that, because of the variability in the background signal, the LOD obtained with the electrochemical readout was an order of magnitude higher than the absorbance measurements (Fig. 3A and B). However, the intrinsic compatibility of screen-printed electrodes with low-cost integration in portable electronic devices encouraged us to pursue electrochemical detection in real complex samples.

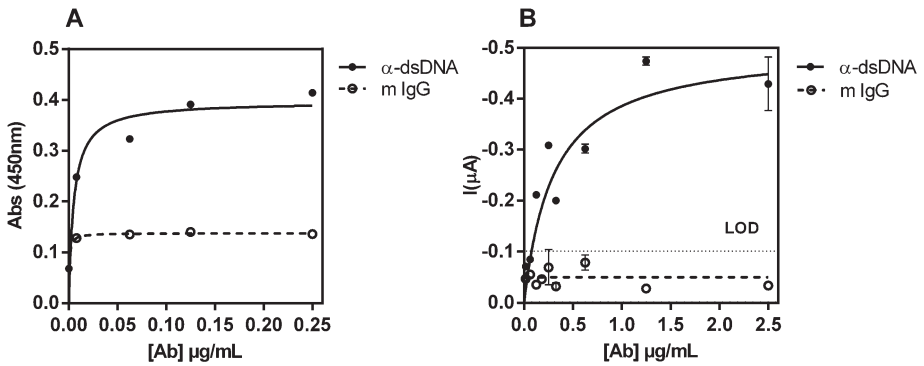


Fig. 3 Optical and electrochemical responses of the sensor. (A) Absorbance changes as a function of the concentration of m-α-dsDNA (black circles) or mouse IgGs (open circles). (B) Reduction current of TMB at 100 s as a function of the concentration of α-dsDNA (black circles) or mouse IgGs (open circles). LOD (limit of detection) was determined as the background signal plus three standard deviations. For visualization purposes, the current axis direction was inverted. Error bars correspond to the standard error of the mean of two independent replicates. Bi-electrodes containing specific and irrelevant antibodies at the same concentration were assayed in parallel.

Performance in serum samples

Once addressing the sensitivity and specificity of the sensor towards α-dsDNA antibody detection in buffer, we wondered whether this device would be able to detect these antibodies in a complex sample mixture such as serum. It is reported that many sensors fail to give a good response signal when exposed to real complex matrixes even if their performance indicates the recognition of specific analytes when using pure laboratory samples.[30] To test this, we performed standard additions of the monoclonal α-dsDNA antibody (or irrelevant antibodies) in 1/80 dilutions of fetal bovine serum. We decided to work in 1/80 serum as this is the dilution used to differentiate the positive samples from the negative samples in SLE.[14,15] Even though we could no longer detect a clear trend in the electrical current as a function of the specific antibody concentration, we did observe a statistically significant ($p < 0.0001$) higher reduction (*i.e.*, negative) current of TMB in electrodes incubated with α-dsDNA-containing FBS *vs.* mouse IgG-containing FBS (Fig. 4A). This also depended on the presence of dsDNA immobilized on the surface of the electrodes, as electrodes modified with BSA alone showed background currents even when incubated with α-dsDNA-containing FBS (Fig. 4A, diamonds). Assuming a cutoff value of 0.1 μA (which corresponds to the measured current at the LOD), the biosensor was 100% specific (no false positives) and 82% sensitive (3 false negatives out of 17). Similar results were obtained for current measurements at 50 s and for quantization of the electrical charge passed through the electrode over 100 s (ESI, Fig. 3†). However, the separation between the positive and the negative samples was much lower in these cases (though the difference was still statistically significant, the overlap prevented the establishment of a cut-off value). Thus, we empirically determined current measurements at 100 s as the most suitable readout for our system.

Our next attempt was to reduce the assay time and manipulation steps in order to ease automatization of the sensor in the future. We hypothesized that by adding the conjugated ABs directly into α-dsDNA-containing FBS, we could then incubate both molecules on the electrode surface in a single step, without the need for a washing step in the middle. This method, called "one-step" in opposition to the "two-step" method, reduced the total assay time to 30 min (*vs.* 60 min in the "two-step" method) without compromising specificity (Fig. 4B), as evident from a comparison of ROC curves for both methods (Fig. 4C). In contrast, the current separation between the positive and the negative samples was even higher, with 100% specificity and 90% sensitivity when using the −0.1 μA cutoff. To discard that these results were not simply a consequence of using monoclonal antibodies instead of normal mouse IgG, we also analyzed other negative samples in which irrelevant monoclonal antibodies (*e.g.*, anti-human CD9) were added to 1/80 FBS at the same concentration as that of the positive samples (Fig. 4B, triangles). We also repeated these experiments in adult mouse serum instead of FBS to discard the non-specific binding of autologous immunoglobulins to the electrodes. Furthermore, adult mouse serum samples were also spiked with a new irrelevant monoclonal antibody at the same concentration as the α-dsDNA (ESI, Fig. 4†). Although electrical currents were generally lower in this case (implying that the cut-off value should be modified in a sample-dependent fashion), there was still complete and statistically significant ($p < 0.05$) separation between the positive and the negative samples. Overall, these results show that our biosensor is capable of discriminating between serum samples containing α-dsDNA antibodies and those which do not. This is promising for the future development of electrochemical biosensors for autoimmune disease-based applications.

Discussion

There is a need for rapid, simple and low-cost analytical devices capable of determining the presence and concentration of autoantibodies in blood specimens, to favor disease

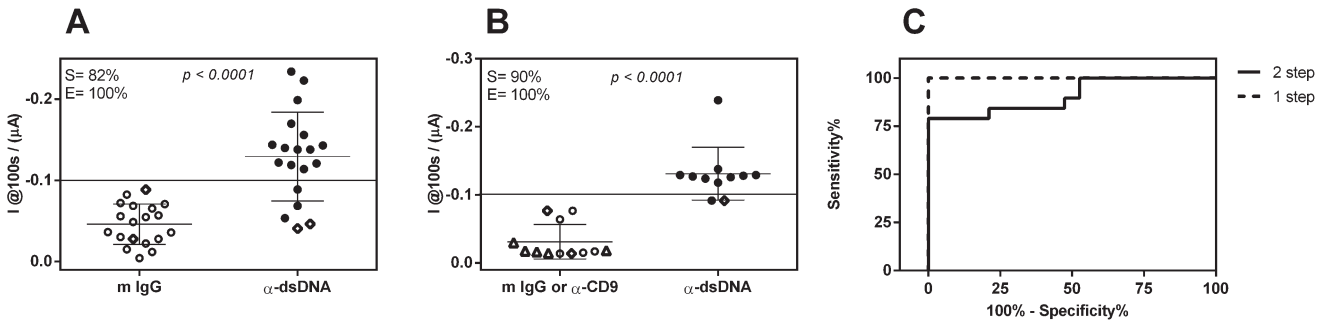


Fig. 4 "One-step" *vs.* "two-step" method. A reduction current recorder at 100 s after incubation with serum samples containing either relevant or irrelevant antibodies at the same concentration with the "two-step" (A) or "one-step" (B) method. To reduce batch effects, specific (black circles) and irrelevant (mouse IgG: open circles; monoclonal α-human CD9, open triangles) antibody-containing samples were incubated in parallel in bi-electrode strips. Diamonds correspond to carbon electrodes not modified with dsDNA (negative controls). Error bars correspond to the standard error of the mean. The Student's *t*-test (two-tailed) was carried out to test statistically significance in the difference between positives and negatives. The current corresponding to the detection limit of the method was used as a cut-off in order to establish assay specificity and sensitivity. Alternatively, a mobile cut-off was used in order to obtain ROC (receiving operating characteristic) curves for both methods (C).

diagnosis, treatment and monitoring.[12,31–33] Currently, these assays are carried out in clinical laboratories and demand time, costly equipment and specialized human resources. In contrast, electrochemical biosensors are intrinsically compatible with point-of-care testing,[22,27,34] and this makes them suitable for decentralized autoantibody determination. However, the number of reports describing electrochemical biosensors for anti-dsDNA autoantibodies (a hallmark of SLE and other autoimmune diseases) is rather low (Table 1).

Some of the important parameters to study when describing new analytical technologies or methods are the sensitivity (often expressed as the limit of detection) and specificity of the assays, as well as the performance of the method in real samples. Previous reports, such as the seminal work performed by Rubin and Konstantinov, have used sandwich immunoassays in fluidic devices to detect anti-dsDNA, antinuclear or anti-chromatin autoantibodies with electrochemical biosensors based on a similar electrochemical readout to that used herein.[23–25] The authors compared the amount of autoantibodies in patient serum samples, and established the performance of their sensor against commercial ELISA kits. However, their output was based on the value of the measured electrochemical current; so, establishing comparisons of analytical performances with other methods was difficult, if not impossible. To overcome this problem, we performed calibration curves in artificial samples containing monoclonal anti-dsDNA antibodies, as suggested by Buhl *et al.* (2009).[2] We obtained a limit of detection [LOD] of 0.1 μg of α-dsDNA antibodies per mL and used the current in the LOD to define a cut-off in non-human serum samples, where known quantities of specific or irrelevant antibodies were spiked in. Through this procedure, we obtained 90% sensitivity and 100% specificity with the "one-step" method (*i.e.*, we could label 9 out of 10 positive samples as positive, and 10 out of 10 negative samples as negative). Considering the fact that we used 1/80 dilutions of serum, our real detection limit increases up to 8 μg mL^{-1}, which is slightly lower but comparable to those of previous reports.[23]

Since reported detection limits are usually based on the titer of autoantibodies (*i.e.*, 1/maximum dilution of a serum sample for which a positive signal is still detected) we wondered whether our detection limit of 8 μg mL^{-1} (16 ng total anti-dsDNA antibodies, since the sample volume is only 2 μL) was clinically relevant. To study this, we performed serial dilutions of FBS spiked-in with known amounts of α-dsDNA antibodies and looked for the amount that mimicked serial dilutions of an SLE patient serum using ELISA. By doing so, we estimated the α-dsDNA cargo in this patient sample to be precisely 8.8 μg mL^{-1}. Thus, the limit of detection of our sensor is in principle compatible with clinical applications. Further optimization will be needed to achieve this goal, as mammalian non-human antibodies and serum were used in this study.

The specificity of our sensor was considered high, as almost perfect separation between serum samples containing specific and non-specific antibodies was obtained. However, denaturation of dsDNA during immobilization can occur to some extent; so we cannot presently assess whether our method is suitable for distinguishing between α-dsDNA and α-ssDNA autoantibodies. Although this aspect deserves further study, the increase in α-ssDNA autoantibodies predicts forthcoming increases in α-dsDNA and SLE flares.[35] Thus, simultaneous detection of α-ssDNA and α-dsDNA antibodies might still be useful for the follow-up of SLE patients. However, α-ssDNA themselves have little diagnostic value as they are increased in a variety of autoimmune or inflammatory diseases.

One of the important aspects of our study is the minimization of assay time and manipulation steps, which is achieved with the thus called "one-step" method. If electrochemical biosensors are chosen for their compatibility with point-of-care tests, they should be compatible with automatization in order to avoid the need for specialized technicians. By decreasing the number of manipulation steps (including sample incubation, washing, and addition of reactants, among others) we facilitate the implementation of our sensing methodology in future analytical devices. By pre-incubating serum samples with the HRP-conjugated secondary antibodies, we have successfully decreased manipulation steps from 5 (sample incubation, washing, conjugate incubation, washing, TMB/H_2O_2 addition and electrochemical measurement) to 3 (sample incubation, washing, addition of a redox mediator and electrochemical measurement). Furthermore, this protocol reduced the total assay time from 60 to 30 minutes. Future work will be aimed at performing incubations at room temperature and reducing the need to introduce reactants with a micropipette, in order to make the method fully compatible with automatization.

Table 1 Assay time and limit of detection of different biosensors for analysis of autoantibodies

Biosensor/transductor	Target	Time assay (min)	Sample amount[a] (μL)	LOD	Ref.
QCM	α-TRIM21 (Ro52)/α-TROVE2 (Ro60)	>60	1000	NR	20
	α-dsDNA	NR	2000	NR	21
SPR	α-dsDNA	15	45	NR	13 and 19
Electrochemical	α-Chromatin	30	200	NR	25
	ANA	30	200	NR	24
	α-dsDNA	30	200	10 μg mL^{-1}	23
	α-dsDNA (two-step)	60	2	8 μg mL^{-1}	This work
	α-dsDNA (one-step)	30			

QCM: quartz crystal microbalance, SPR: surface plasmon resonance, ANA: total antinuclear antibody. NR: not reported. [a] Diluted serum samples.

Conclusion

We provide a simple protocol for the electrochemical sensing of α-dsDNA autoantibodies in serum samples, with excellent distinction between samples containing or not α-dsDNA antibodies in concentrations comparable to those present in the sera of autoimmune disease patients. The total assay time of 30 minutes and the few manipulation steps will aid in the automatization of this protocol in order to obtain portable sensors for their use outside of laboratory facilities.

Conflicts of interest

There are no conflicts of interest to declare.

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