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Fast and sensitive diagnosis of autoimmune disorders through amperometric biosensing of serum anti-dsDNA autoantibodies

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ABSTRACT

This work reports the first amperometric biosensor involving the use of neutravidin-functionalized magnetic microbeads (NA-MBs) modified with a biotinylated-anti-dsDNA (b-dsDNA) as efficient magnetic microcarriers to selectively capture anti-dsDNA autoantibodies (IgG, IgA and IgM AAbs) present in the sera of patients with rheumatoid arthritis (RA). Subsequently, the attached anti-dsDNA AAbs are detected with a mixture of conventional HRP-labeled secondary antibodies (HRP-anti-human IgG/IgM/IgA mixture). The biorecognition event is monitored by amperometric transduction using the hydroquinone (HQ)/H_2O_2 system upon capturing the modified MBs on the surface of screen-printed carbon electrodes (SPCEs). The developed bioplatform exhibits a linear calibration plot ranging from 1 to 200 IU mL^{-1} with a LOD of 0.3 IU mL^{-1} for anti-dsDNA AAbs standards. In addition, the biosensor allows performing the determination of the anti-dsDNA AAbs levels directly in 100-times diluted serum samples from patients diagnosed with RA and in just 75 min. The obtained results are in agreement with those provided by an ELISA kit and allow discrimination between positive and negative samples.

1. Introduction

Autoimmune diseases are characterized by the presence of autoantibodies (AAbs) that react against own antigenic structures. Among AAbs, a prominent place is that of antinuclear antibodies (ANAs) that recognize autologous cellular components (nuclear and cytoplasmic). ANAs are reactive against histones, nucleosomes, single (ss) and double (ds) strand DNA, and other nuclear antigens and have been widely used for the diagnosis of autoimmune disease (Conigliaro et al., 2016; Bizzaro et al., 2018). Belonging to ANAs, AAbs to native desoxyribonucleic acids are well-recognized diagnostic markers of Systemic Lupus Erythematosus (SLE), and their serum concentration is positively correlated to the severity of the disease. These antibodies were later found in patients with other inflammatory autoimmune diseases, as rheumatoid arthritis (RA), chronic active hepatitis or pancreatic cirrhosis (Cabiedes and Nuñez-Alvarez, 2010; Bai et al., 2017; Infantino et al., 2018; Wang and Xia, 2019). Additionally, different studies showed the increased level of anti-dsDNA AAbs in patients with RA treated with biological compounds (Eriksson et al., 2005; Yukawa et al., 2011).

A variety of methods for the detection of anti-dsDNA AAbs are described in the literature. The indirect immunofluorescence test is the reference method to screen for ANAs in patients with autoimmune diseases (Karamehic et al., 2007; Coople et al., 2012; Bizarro et al., 2018). Nevertheless, this technique requires complex instrumentation, is not compatible with decentralized analysis or point-of-care applications and is prone to false positive results. Methods using enzyme linked inmmunosorbent assays (ELISAs) for AAbs against dsDNA have been reported also (Janyapoon et al., 2005; Biesen et al., 2011), and, nowadays, different ELISA kits applicable for human serum or plasma are commercially available. For example, the anti-dsDNA AAbs ELISA kit of Creative Diagnostics is an indirect solid phase immunoassay involving well-coated human recombinant double-stranded DNA (dsDNA) and

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peroxidase-labeled IgG/M/A. This kit provides a non-linear calibration plot between 12.5 and 200 UI mL^{-1} anti-dsDNA with RSD values of 6.4% and 12.4% for intra-assays and inter-assays, respectively. The total assay time is 75 min. It is worth to remark that, although the ELISA methodology is long adopted in centralized laboratories, this technique is not compatible with multiplexed determinations and its implementation requires relatively expensive instrumentation (ELISA plate readers), hardly portable and miniaturizable.

In this context, the excellent inherent analytical characteristics of electrochemical biosensors, such as high sensitivity and selectivity, have positioned them as an attractive alternative for the detection of clinically relevant analytes. However, in the particular case of anti-dsDNA human antibodies, only one electrochemical biosensor has been reported to date (Rubin et al., 2014). It is a flow-through biosensor involving immobilization of native dsDNA on a porous membrane coupled to an eight-electrodes array. The biosensor provided a LOD value for anti-dsDNA human IgGs of 10 μg mL^{-1}. The assay time was 30 min and the biosensor was employed to perform the determination in 200 μL of 50-times diluted human serum samples of patients diagnosed with SLE. However, the preparation of the dsDNA coated membrane took ~16 h which is an important limitation of that strategy. Moreover, it is also important to mention that quantitative levels of anti-dsDNA antibodies in the samples were not provided but only the measured electrochemical currents. More recently, another electrochemical biosensor prepared by constant potential deposition of dsDNA onto SPCE has been reported for the determination of mouse (not human) anti-dsDNA antibodies (Fagúndez et al., 2018). This biosensor is able to perform the determination in a single 30-min step with a LOD of 8 μg mL^{-1} and was used for the determination in adult mouse 80-times diluted serum samples. It is important to note that both biosensors determined a single immunoglobulins (Igs) isotype (IgG) of anti-dsDNA antibodies and required a high amount of dsDNA to modify the porous membrane (90 μg) (Rubin et al., 2014) or the SPCE (20 μg) (Fagúndez et al., 2018).

This paper reports a fast and simple amperometric biosensing approach for the reliable determination of the three most common isotypes of Igs (IgG, IgM and IgA) AAbs against dsDNA in human serum samples. The biosensor involves for the first time the use of neutravidin-functionalized magnetic microparticles (NA-MBs) modified with a small amount of biotinylated-human dsDNA (b-dsDNA, 100 ng) prepared in the lab from human plasmid, as efficient magnetic microcarriers to selectively capture the target AAbs present in sera. Subsequently, the attached AAbs are detected with a commercial mixture of conventional HRP-labeled secondary antibodies for human IgG, IgA and IgM. The biorecognition event is monitored using amperometric transduction at −0.20 V (*vs* the Ag pseudoreference electrode) with the hydroquinone (HQ)/H_2O_2 system upon capturing the modified MBs on the surface of screen-printed carbon electrodes (SPCEs). The performance of the methodology was evaluated using anti-dsDNA AAbs standards provided in a commercial ELISA kit. In addition, the biosensor was successfully applied to quantify the serum levels of these AAbs in patients diagnosed with RA.

2. Materials and methods

2.1. Apparatus

Screen-printed carbon electrodes (SPCEs) (DRP-110, DropSens) consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode, and an Ag pseudoreference electrode, were used as electrochemical transducers. A specific cable connector (DRP-CAC, DropSens, S.L.) acted as interface between the SPCEs and the potentiostat. Amperometric measurements were carried out with a potentiostat model CHI812B (CH Instruments, Inc.) operated by the CHI812B software. A Bunsen AGT-9 Vortex, a Thermomixer MT100 constant temperature incubator shaker (Universal Labortechnik) and a magnetic separator DynaMag-2 Magnet (Thermo Fisher Scientific) were also employed. The modified MBs were captured onto the SPCEs surface by a neodymium magnet (AIMAN GZ) embedded in a homemade poly (methyl methacrylate) (PMMS) casing. All measurements were carried out at room temperature.

2.2. Reagents and solutions

Neutravidin-modified magnetic microparticles (NA-MBs, Ø = 1 μm, 10 mg mL^{-1}) were from SpeedBeads™, GE Healthcare and Streptavidin-modified magnetic microparticles (Strept-MBs, Ø = 2.8 μm, 10 mg mL^{-1}) were from Invitrogen-ThermoFisher™. Standards, controls of anti-dsDNA AAbs (prepared in a serum/buffer matrix), a mixture of polyclonal rabbit anti-human IgG, anti-human IgM and anti-human IgA antibodies labeled with horseradish peroxidase (HRP-anti-human IgG/IgM/IgA mixture), sample buffer (Tris, NaN_3 <0.1% (w/w)) and washing buffer (PBS, NaN_3 < 0.1% (w/w)) were provided in the anti-dsDNA AAbs ELISA Kit purchased from Creative Diagnostics (Cat. No. DEIA 1681). Bovine serum albumin (BSA 100%, Type VH, Gerbu Biotechnik, GmbH), hydrogen peroxide (30% (v/v)), hydroquinone (HQ), human serum albumin (HSA), hemoglobin (HB), human IgG and human serum lyophilized powder (from clotted whole blood) were from Sigma-Aldrich. Other buffer solutions used were a 0.05 mol L^{-1} phosphate buffer solution, pH 6.0 (PB) and a 0.01 mol L^{-1} Tris−HCl (Scharlab) of pH 7.5 containing 1 mM EDTA and 2 mM NaCl (B&W buffer).

2.3. Procedures

2.3.1. Synthesis of biotinylated dsDNA from human plasmids (b-dsDNA)

Linearized 5′ biotinylated double-stranded DNA (b-dsDNA) was obtained by PCR using T7 biotinylated forward primer (5′-TAATACGACTCACTATAGGG-3′) and T7 terminator reverse primer (5′-GCTAGTTATTGCTCAGCGG-3′), with p53_GST, pANT7_cHalo or pDONR221 vectors as DNA template (Garranzo-Asensio et al., 2019, 2020). The final reaction volume (50 μL) contained 1 M betaine, 0.2 mM dNTP mix, 1x HF enzyme buffer, 0.5 μM forward primer, 0.5 μM reverse primer, 0.01 U μL^{-1} phusion high-fidelity enzyme (Thermo Fisher Scientific) and 125 ng of the corresponding vectors. The amplification program was: a denaturing hold of 5 min at 98 °C, followed by 35 cycles of 1 min at 98 °C and 3 min at 68 °C, and a final hold of 10 min at 72 °C. Then, two 50 μL PCRs were purified together by dsDNA precipitation with 2 vol of ethanol 100% and 11 μL of 3 M sodium acetate pH 5.0 during 30 min at -80 °C followed by centrifugation at 4 °C and 13,200 rpm during 15 min. Then, the supernatant was discarded and the precipitated dsDNA was cleaned twice with 500 μL of 70% ethanol during 20 min at -80 °C and, subsequently, centrifuged at 4 °C and 13, 200 rpm during 15 min. Then, the supernatant was discarded and the precipitated linearized 5′ b-dsDNA was resuspended in 30 μL of nuclease-free water and quantified with the NanoDrop 2000C (Thermo Scientific).

PCRs and purified dsDNA quality were verified by visualization onto a 0.7% agarose gel electrophoresis run at 120 V during 40 min and revealed with the UVP Transilluminator. The obtained results demonstrated that prepared b-dsDNA had no contamination with ssDNA, thus ensuring quantification only of anti-dsDNA AAbs (Makowski and Ramsby, 2003; Riboldi et al., 2005).

2.3.2. Isolation and enzyme labeling of the anti-dsDNA AAbs at b-dsDNA-NA-MBs

5 μL of NA-MBs were incubated with 25 μL of 100 ng b-dsDNA for 30 min. After washing 2 times with 50 μL of wash buffer, the resulting b-dsDNA-NA-MBs were incubated with 25 μL of the standard/control or serum sample (prepared/diluted in sample buffer) for 30 min. After two additional washings, the anti-dsDNA AAbs attached to the MBs were enzymatically labeled by incubation for 30 min with 75 μL of a HRP-anti-human IgG/IgM/IgA mixture. After two washing steps, the

modified MBs were resuspended in 45 μL of 0.05 mol L^{-1} PB pH 6.0 to perform the amperometric measurements.

All incubation steps were carried out under continuous stirring at 950 rpm and 25 °C, while washing operations were made using 50 μL of the B&W buffer for the incubation of b-dsDNA, while other washings were carried out with the wash buffer (provided in the commercial ELISA kit). Between each incubation step, the microtube containing the MBs suspension was placed on the magnetic separator for 2 min and then the supernatant was removed.

2.3.3. Amperometric measurements

A bare SPCE was positioned in the homemade PMMA casing with an encapsulated neodymium magnet and the 45 μL suspension of the modified MBs mentioned above were pipetted onto the working electrode (WE) surface. The SPCE/casing ensemble was connected to the potentiostat and immersed into an electrochemical cell containing 10 mL of 0.05 M PB solution (pH 6.0) supplemented with 1.0 mM fresh HQ. Amperometry in stirred solutions was carried out by applying a detection potential of −0.20 V (*vs* the Ag pseudoreference electrode) and adding 50 μL of a 0.1 M freshly prepared H_2O_2 solution. All the amperometric signals given through the manuscript corresponded to the difference between the steady state and the background currents and error bars were estimated as triple of the standard deviation of three replicates. Unless otherwise indicated, the presented data correspond to the average of at least three replicates and the confidence intervals were calculated for $\alpha = 0.05$.

3. Results and discussion

Fig. 1 shows schematically the developed methodology. It is based on the efficient recognition of three immunoglobulin (Ig) isotypes (IgG, IgM and IgA) capable of recognizing human dsDNA using commercial NA-MBs modified with b-dsDNA prepared in the laboratory. After enzymatic tagging of the captured human Igs with HRP-conjugated secondary antibodies for each type of Ig, the modified MBs were captured on the SPCE working electrode and amperometric transduction was performed using the H_2O_2/HQ system. The cathodic current variation was proportional to the total concentration of the three Ig isotypes.

The reliability of the assay was tested by comparing the amperometric responses measured in the absence (B) and in the presence (S) of a standard provided in the commercial ELISA kit containing a total concentration of the three anti-dsDNA antibody isotypes of 50 IU mL^{-1}. The b-dsDNA-NA-MBs and NA-MBs (control without immobilized b-dsDNA) were used as well as the positive and negative controls provided in the ELISA kit using the b-dsDNA-NA-MBs. Discrimination of the anti-dsDNA AAbs presence was only possible when b-dsDNA-NA-MBs were used providing S/B ratios of 10.4 and 0.87 for the 50 IU mL^{-1} anti-dsDNA standard when using b-dsDNA-NA-MBs and NA-MBs, respectively. Moreover, the positive and negative controls provided in the commercial ELISA kit gave S/B values of 17.5 and 1.1 using the b-dsDNA-NA-MBs.

A comparison in terms of S/B ratio values was made using streptavidin-functionalized MBs (Strept-MBs) instead of NA-MBs as solid supports. The S/B ratio for the 50 IU mL^{-1} anti-dsDNA AAbs standard was 7.0 and 9.3, respectively, for Strept-MBs and NA-MBs. This result is in agreement with those reported by other authors (Zhao et al., 1993; Nguyen et al., 2012; Serafin et al., 2019), and can be attributed to the higher affinity of NA toward biotin, although a more comprehensive study would require comparing MBs of the same size and the same company. Accordingly, NA-MBs was selected as the appropriate solid substrate for developing the methodology.

3.1. Optimization of the experimental variables

The influence of other determining working variables was tested. Firstly, the influence of the size of b-dsDNA fragment immobilized on the NA-MBs on the assay performance was evaluated by comparing the amperometric responses provided in the absence and in the presence of 25 IU mL^{-1} anti-dsDNA AAbs standard. Different b-dsDNA fragments with lengths between 2.5 and 6 kilobases (see Fig. 2a)) were obtained using as PCR template, p53_cGST, pANT7_cHalo and pDONR221 human plasmids.

Fig. 2b) shows as the larger S/B ratios were achieved using the shortest b-dsDNA fragment prepared (p53_cGST human plasmid). This effect may be attributed to increased roll-up of longer immobilized b-dsDNA which makes it difficult the recognition for anti-dsDNA antibodies. Therefore, such shorter b-dsDNA was selected to develop the biosensing methodology.

Fig. 3 shows the results obtained for the test of other variables involved in the construction of HRP-anti-human IgG/IgM/IgA-anti-dsDNA AAbs-b-dsDNA-NA-MB/SPCEs biosensor. The variables checked were: loading (a) and incubation time (b) of b-dsDNA; number of steps involved (c); incubation time with anti-dsDNA AAbs standards (d) and volume (e) and incubation time (f) with HRP-anti-human IgG/IgM/IgA mixture. Larger S/B values measured in absence and in the presence of 25 IU mL^{-1} anti-dsDNA AAbs standard was taken as the criterion for selecting each variable.

As it is shown in Fig. 3a), the S/B values increased with the b-dsDNA loading immobilized onto NA-MBs up to 100 ng (amount selected for further work) and decreased drastically for larger loadings, probably due to hindered recognition by the antibodies when a high concentration of b-dsDNA was immobilized. A clear trend was not observed for the effect of the b-dsDNA incubation time with the NA-MBs (Fig. 3b)), but higher S/B value was observed for 30 min, setting this value for further studies. Since the approach involves the selective capture of anti-dsDNA human Igs isotypes and their further labeling with the mixture of HRP-labeled secondary antibodies, the performance of two different protocols involving one or two steps from the preparation of b-dsDNA-NA-MBs was evaluated. In the two-steps assay, the b-dsDNA-NA-MBs were firstly incubated with 25 μL of the anti-dsDNA AAbs standard (25 IU mL^{-1}, 30 min) followed by incubation with 100 μL of HRP-anti-human IgG/IgM/IgA mixture (15 min). In the one-step assay, the b-dsDNA-

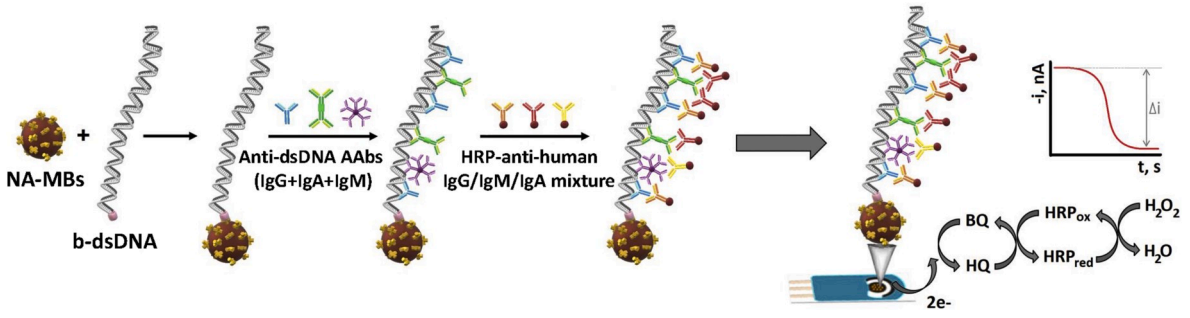

Fig. 1. Schematic display of the different steps involved in the preparation of the biosensor developed for the determination of anti-dsDNA AAbs as well as the used amperometric transduction.

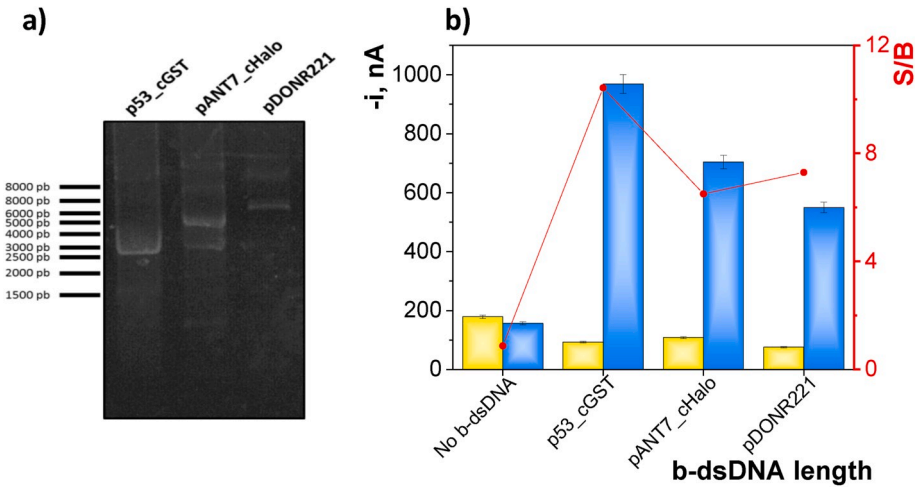

Fig. 2. Gel electrophoresis of b-dsDNAs obtained by PCR using as template the indicated human plasmids (a). Effect of the b-dsDNA fragment length immobilized on the NA-MBs on the amperometric responses measured in the absence (yellow bars) and in the presence (blue bars) of 25 IU mL^{-1} anti-dsDNA AAbs standards, and the corresponding S/B values (red circles) (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

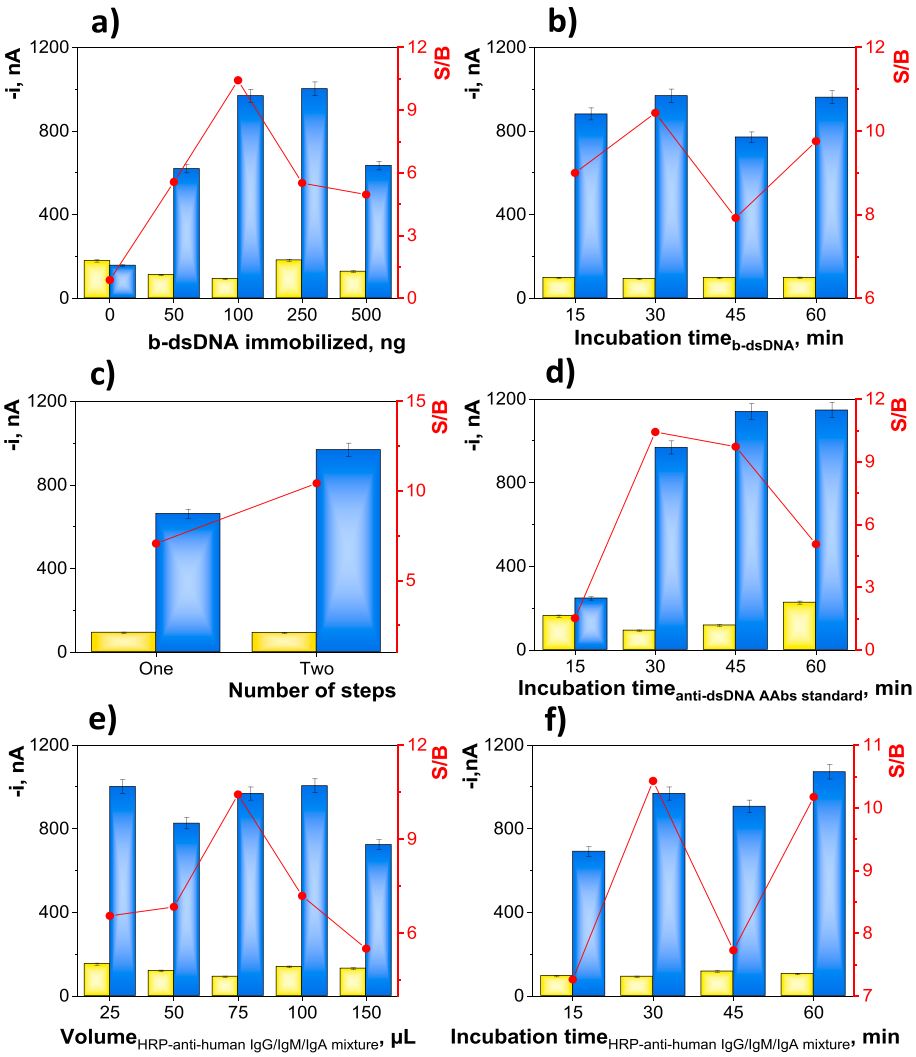

Fig. 3. Effect on the amperometric responses measured with the biosensors in the absence (yellow bars) and in the presence (blue bars) of 25 IU mL^{-1} anti-dsDNA AAbs standards of b-dsDNA loading (a) and incubation time (b), number of steps involved in the preparation of the biosensor (c), incubation time with anti-dsDNA AAbs (d) and volume (e) and incubation time (f) of HRP-anti-human IgG/IgM/IgA mixture, The resulting S/B values are shown as red circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Neutravidin-MBs were 45-min incubated with a mixture solution containing 25 µL of the anti-dsDNA antibodies standard and 100 µL of HRP-anti-human IgG/IgM/IgA mixture. Fig. 3c) shows as S/B values of 7.1 and 10.4 were obtained for the assays with one or two steps, respectively, which can be attributed to a poorer recognition of the immobilized b-dsDNA when target Igs are marked with their corresponding

HRP-labeled secondary antibodies. Therefore, the protocol was implemented in two steps to get higher sensitivity. Nevertheless, it is important to note that a one-step simpler protocol is also possible, although slightly sacrificing sensitivity, thus rendering the developed approach more compatible with automatization.

The effect of the b-dsDNA-NA-MBs incubation time with the anti-dsDNA AAbs standards to ensure the efficient capture of the target Igs is shown in Fig. 3d). As it can be seen, larger S/B value was obtained for 30 min incubation. Longer times provoked a significant decrease in the S/B ratio due to the significant increase in the non-specific current. The effect of the volume and incubation time of the HRP-anti-human IgG/IgM/IgA mixture used to label the capture Igs is shown in Fig. 3e) and f), respectively. Larger S/B values were obtained using a volume of 75 μL and an incubation time of 30 min, which were selected for further work. While similar S/B values were obtained for incubation times longer than 30 min, the lower S/B values for volumes larger than 75 μL can be attributed to a less efficient recognition process when the volume ratio between incubation solution and MBs suspension is very high. Table S1 (in the Supporting Information) summarizes the ranges studied for the tested experimental variables and the values selected for further work. Other experimental variables such as the detection potential, the HQ/H_2O_2 concentration, the pH/composition of the supporting electrolyte solution and the NA-MBs amount were optimized previously (Eguilaz et al., 2010; Conzuelo et al., 2012; Gamella et al., 2012; Serafín et al., 2019).

3.2. Analytical characteristics for the determination of anti-dsDNA AAbs

The calibration plot constructed with the biosensors prepared under the selected experimental conditions for anti-dsDNA AAbs standards is displayed in Fig. 4a) together with some of the recorded amperograms (Fig. 4b). A good correlation (R^2 = 0.993) was found between the variation in the cathodic current and the concentration of anti-dsDNA antibodies from 1 to 200 IU mL^{-1}. The regression equation was -i (nA) = (17.6 ± 0.3) [anti-dsDNA AAbs] (nM) + (273 ± 44). The LOD value was estimated according to the 3 × s_b criterion, where s_b was calculated as the standard deviation (n = 10) for measurements carried out in the absence of anti-dsDNA AAbs. The calculated LOD was 0.3 IU mL^{-1} (equivalent to 24.1 μg mL^{-1} anti-dsDNA human IgGs considering the weight of these immunoglobulins corresponding to one IU (Humphrey and Batty, 1974)).

When comparing these analytical characteristics with those claimed for the only electrochemical biosensor reported for the determination of anti-dsDNA human AAbs (Rubin et al., 2014), one can note that the LOD is slightly higher (24.1 vs 10 μg mL^{-1}) and the test time is slightly longer (45 vs 30 min). However, the biosensor reported in this work does not need time-consuming protocols to immobilize the dsDNA in a porous membrane and it uses 900 times less dsDNA (100 ng vs 90 μg). In addition, the developed biosensor was used to determine the three anti-dsDNA AAbs isotypes (IgG + IgA + IgM). Moreover, the biosensor reported here provides a similar sensitivity and assay time (starting from the preparation of the ds-DNA modified support) than those of commercial ELISAs. However, the biosensor involves a linear signal-to-concentration dependence instead of 4-Parameter-Fit with lin-log coordinates. Importantly, the biosensor has important advantages derived from the electrochemical detection and the use of SPCEs including affordable cost and compatibility with multiplexed detection and point-of-care use.

Table S2 (in the Supporting information) provides a rough snap shot of the available methodologies for the determination of anti-dsDNA human AAbs compared to the one developed in this work. As it can be seen, most are ELISAs, which provide non-linear calibration plots, require 100 μL sample/reagents volume and relatively expensive instrumentation, and are of limited applicability in centralized settings.

The reproducibility of the amperometric responses was evaluated through the comparison of the current provided by five different biosensors prepared in the same manner on same or different days for 50 IU mL^{-1} anti-dsDNA AAbs. The relative standard deviation (RSD) values obtained were 3.2 and 4.3%, respectively, thus indicating the good reproducibility of the protocols used to prepare the biosensors and perform the amperometric transduction.

3.3. Selectivity

The effect of potentially interfering compounds present in serum (HSA, BSA, HB and human IgGs) on the biosensor response was tested. Fig. 5 shows the measured amperometric responses at the concentrations levels indicated in the Figure caption as well as in the absence (Blank) and the presence of 50 IU mL^{-1} anti-dsDNA AAbs. As can be seen, BSA gave a similar signal to that of the blank. However, HSA and HB showed a slight interference, the latter one probably because of the HB endogenous peroxidase activity (Grigorieva et al., 2013). As expected, a high signal was obtained for human IgGs considering the fundamentals of the developed assay. As it is discussed in the next section, the interference of HB is negligible when working with 100-times diluted samples and that of human IgGs which do not recognize dsDNA can be easily corrected.

3.4. Determination of anti-dsDNA AAbs in serum from RA patients

The practical applicability of the developed biosensing platform was evaluated by determining the anti-dsDNA IgG, IgM and IgA isotypes

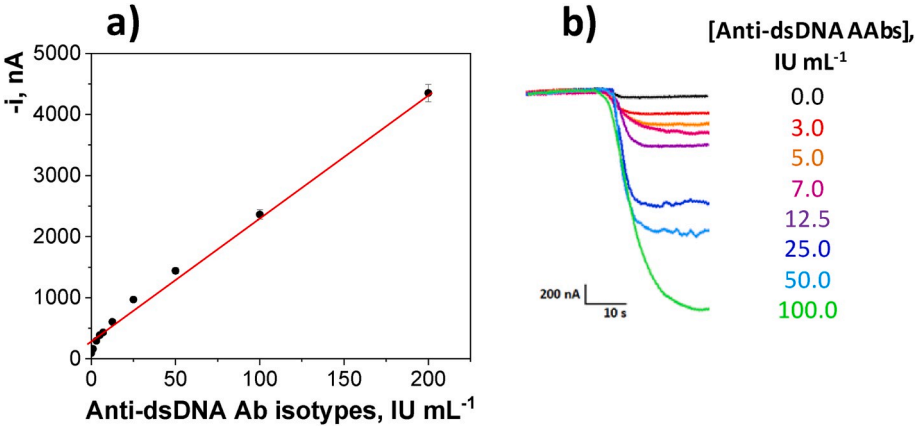

Fig. 4. Calibration plot for anti-dsDNA AAbs standards constructed with the developed biosensor (a). Amperometric responses provided by the biosensors for different anti-dsDNA AAbs concentrations (b).

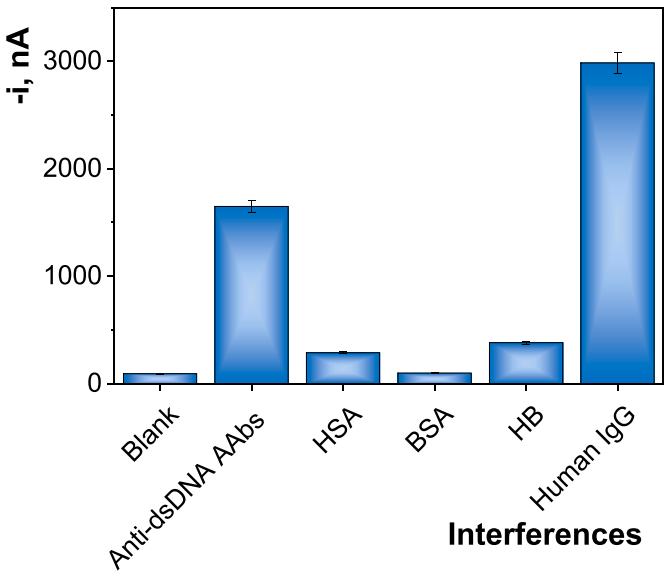

Fig. 5. Amperometric responses provided by the developed biosensors in the absence (blank signal) and in the presence of 50 IU mL^{-1} anti-dsDNA AAbs, 50 mg mL^{-1} HSA, 5 mg mL^{-1} BSA, 5 mg mL^{-1} HB and 1 mg mL^{-1} of human IgG standards.

levels in serum of healthy individuals and patients diagnosed with RA. The potential existence of matrix effect was checked by comparing the slope values of the calibration plots constructed with buffered standard solutions and in diluted human serum samples from healthy individuals. The slope values for both calibration plots were not statistically different (17.6 ± 0.3 nA mL IU^{-1} and 17 ± 2 nA mL IU^{-1}; $t_{exp} = 0.07 < t_{tab} = 2.776$) when working with 100-time diluted samples. However, the intercept value of the calibration plot constructed in a diluted serum sample from a healthy individual was about 4-times higher than that expected considering the anti-dsDNA AAbs endogenous concentration. To elucidate whether this is due to the presence of human Igs (as discussed in section 3.3) or to some endogenous peroxidase in the serum, the amperometric responses obtained for 100-fold diluted serum samples using b-dsDNA-NA-MBs and NA-MBs (with no immobilized b-dsDNA) in the absence and in the presence of HRP-anti-human IgG/IgM/IgA mixture were compared. Results shown in Fig. S1 (in the Supporting Information) and Fig. 5 led us to assign this interference to the high concentration of human Igs present in serum of adult individuals: 0.7−4.0 (IgA), 7−16 (IgG) and 0.4−0.23 (IgM) mg mL^{-1} (Gonzalez-Quintela et al., 2008). Therefore, the determination of 100 times-diluted serum samples was made simply by interpolating into the calibration plot prepared with anti-dsDNA AAbs standards (Fig. 4) the amperometric measurements obtained after subtracting the one recorded for a commercial synthetic human serum sample. Table 1 summarizes the obtained results according to this protocol as well as the comparison with those provided by the ELISA test. As expected, a significantly higher level of anti-dsDNA AAbs was found in patients with RA with respect to the levels for healthy individuals. The results are in agreement with the cut-off value of 25 IU mL^{-1} established to discriminate between positive and negative samples for anti-dsDNA IgG, IgA and IgM isotypes, thus allowing a clear discrimination between healthy individuals and RA patients. Table 1 shows also the good agreement between the biosensing and the ELISA methodologies. The paired samples t-test demonstrated that no significant differences existed for the results provided by the two methods ($\alpha = 0.05$, p-values of 0.36 and 0.28 for healthy individuals and RA patients, respectively), with a correlation plot for the results obtained by both methods with slope and intercept values of (1.03 ± 0.03) and (-21 ± 38) IU mL^{-1}, respectively.

Table 1
Concentration of anti-dsDNA AAbs (IgG + IgM + IgA isotypes) (in IU mL^{-1}) found in the serum samples analyzed with the developed biosensor and the ELISA test.

Sample		Biosensor	$RSD_{n=3}$, %	ELISA	$RSD_{n=3}$, %
Healthy volunteers	1	8 ± 2	1.1	8 ± 1	4.4
	2	13 ± 3	3.8	15 ± 3	3.2
	3	19 ± 4	3.4	16 ± 6	5.4
AR patients	4	1002 ± 45	4.4	1105 ± 59	5.3
	5	1772 ± 93	5.3	1739 ± 75	4.3
	6	2426 ± 54	2.2	2311 ± 135	5.8

Mean value $\pm$ ts/$\sqrt{n}$ (n = 3; $\alpha = 0.05$).

When the biosensors were applied to the controls supplied in the commercial ELISA kit, and the amperometric measurements for the undiluted controls were interpolated into the calibration plot constructed with standards (Fig. 4), the calculated contents were 5.3 and 73 IU mL^{-1} anti-dsDNA AAbs. These results are consistent with the ranges indicated in the Quality Control Certificate enclosed to the purchased ELISA kit (Positive Control < 10 IU mL^{-1} and Negative Control: 60−90 IU mL^{-1}), which confirmed the suitability of the measurements carried out with the bioplatform.

4. Conclusions

This paper describes the first biosensing strategy implemented on the surface of MBs for the determination of anti-dsDNA human AAbs. The method proposes the use of NA-MBs modified with b-dsDNA, prepared in the laboratory by PCR from human plasmid DNA, for the efficient capture of human anti-dsDNA IgG, IgM and IgA class AAbs. The three Igs isotypes captured on the b-dsDNA-NA-MBs are enzymatically labeled with a mixed solution of HRP-conjugated secondary antibodies. Amperometric detection is carried out upon the capture of the magnetic bioconjugates on the surface of the SPCEs. The method provides a linear range between 1 and 200 IU mL^{-1} with a LOD value of 0.3 IU mL^{-1} for anti-dsDNA AAbs standards and is used to determine anti-dsDNA AAbs in 100-times diluted serum samples from RA patients in only 75 min. The low amount of b-dsDNA (100 ng) and the short time required for its immobilization on the NA-MBs (30 min) together with the possibility of providing quantitative levels in serum of anti-dsDNA human Igs are competitive advantages of the developed biosensors. Moreover, the biosensor shows also advantages with respect to the ELISA method in terms of cost and possibility of performing multiplexed determinations at the point-of-care by non-specialized personnel. In addition, the developed methodology can easily be adapted to the individual determination of each Ig isotype simply by labelling with the corresponding secondary antibody or to the determination of other disease-relevant autoantibodies by immobilizing the appropriate antigens on the MBs surface. Therefore, the developed biosensors offer the prospect of greatly expand autoantibody analysis, a hallmark of autoimmune and cancer diseases, into the practical routine at both primary care and subspecialty decentralized settings within clinically actionable times.

Declaration of interest statement

This paper reports a fast and simple amperometric biosensing approach for the reliable determination of the three most common isotypes of Igs (IgG, IgM and IgA) AAbs against dsDNA in human serum samples. The method proposes the use of NA-MBs modified with b-dsDNA, prepared in the laboratory by PCR from human plasmid DNA, for the efficient capture of anti-dsDNA human Igs isotypes which are enzymatically labeled with a mixed solution of HRP-conjugated secondary antibodies. The cathodic current variation occurring upon the capture of the resultant magnetic bioconjugates on the surface of the SPCEs provides a linear range between 1 and 200 IU mL^{-1} with a LOD value of 0.3 IU mL^{-1} for anti-dsDNA AAbs standards. The low amount of b-dsDNA (100 ng) and the short time required for the immobilization on the NA-MBs (30 min) together with the possibility of providing

quantitative levels in serum of anti-dsDNA human Igs are competitive advantages of the developed biosensors. Moreover, the biosensor shows also advantages with respect to the ELISA method in terms of cost and possibility of performing multiplexed determinations at the point-of-care by non-specialized personnel. In addition, the developed methodology can be applied to the individual determination of the three types of human Igs against ds-DNA simply by labelling with the secondary antibody for the isotype of interest instead of using the mixture solution of the three secondary antibodies. The methodology can be easily transferred to the determination of other disease-relevant autoantibodies by convenient immobilization of the appropriate antigens on the MBs surface. Therefore, the developed biosensors offer the prospect of greatly expand autoantibody analysis, a hallmark of autoimmune and cancer diseases, into the practical routine at both primary care and subspecialty decentralized settings within clinically actionable times.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Beatriz Arévalo: Conceptualization, Investigation, Writing - review & editing, Writing - original draft, Funding acquisition. **Verónica Serafín:** Conceptualization, Investigation, Writing - review & editing, Writing - original draft. **Marta Sánchez-Paniagua:** Conceptualization, Investigation, Writing - review & editing, Writing - original draft. **Ana Montero-Calle:** Investigation, Resources. **Rodrigo Barderas:** Supervision, Resources, Writing - review & editing, Writing - original draft, Funding acquisition. **Beatriz López-Ruíz:** Conceptualization, Writing - review & editing, Writing - original draft. **Susana Campuzano:** Conceptualization, Supervision, Writing - review & editing, Writing - original draft, Funding acquisition. **Paloma Yáñez-Sedeño:** Conceptualization, Supervision, Writing - review & editing, Writing - original draft, Funding acquisition. **José M. Pingarrón:** Writing - review & editing, Writing - original draft, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2020.112233.

References

Bai, Y., Tong, Y., Liu, Y., Hu, H., 2017. Clin. Exp. Immunol. 191, 1–10.
Biesen, R., Dahnrich, C., Rosemann, A., Barkhudarova, F., Rose, T., Jakob, O., Bruns, A., Backhaus, M., Stocker, W., Burmester, G.-R., Schlumberger, W., Egerer, K., Hiepe, F., 2011. Arthritis Res. Ther. 13, R26. https://doi.org/10.1186/ar3250.
Bizzaro, N., Brusca, I., Previtali, G., Grazia Alessio, M., Daves, M., Platzgummer, S., Cinquanta, L., Paura, G., Infantino, M., Manfredi, M., Faricelli, R., Bassetti, D., Musso, M., Deleonardi, G., Trevisan, M.T., Radice, A., Liguori, M., Imbastaro, T., Pesente, F., Fabris, M., Tonutti, E., 2018. Autoimmun. Rev. 17, 541–547.
Cabiedes, J., Nuñez-Alvarez, C.A., 2010. Reumatol. Clínica 6, 224–230.
Conigliaro, P., Chimenti, M.S., Triggianese, P., Sunzini, F., Novelli, L., Perricone, C., Perricone, R., 2016. Autoimmun. Rev. 15, 673–683.
Conzuelo, F., Gamella, M., Campuzano, S., Pinacho, D.G., Reviejo, A.J., Marco, M.P., Pingarrón, J.M., 2012. Biosens. Bioelectron. 36, 81–88.
Copple, S.S., Giles, S.R., Jaskowski, T.D., Gardiner, A.E., Wilson, A.M., Hill, H.R., 2012. Am. J. Clin. Pathol. 137, 825–830.
Eguílaz, M., Moreno-Guzmán, M., Campuzano, S., González-Cortés, A., Yáñez-Sedeño, P., Pingarrón, J.M., 2010. Biosens. Bioelectron. 26, 517–522.
Eriksson, C., Engstrand, S., Sundqvist, K.-G., Rantapaa-Dahlqvist, S., 2005. Ann. Rheum. Dis. 64, 403–407.
Fagúndez, P., Brañas, G., Cairoli, E., Laiz, J., Tosar, J.P., 2018. Analyst 143, 3874–3882.
Gamella, M., Campuzano, S., Conzuelo, F., Reviejo, A.J., Pingarrón, J.M., 2012. Electroanalysis 24, 2235–2243.
Garranzo-Asensio, M., Guzmán-Aránguez, A., Povés, C., Fernández-Aceñero, M.J., Montero-Calle, A., Ceron, M.A., Fernandez-Diez, S., Rodríguez, N., Gómez de Cedrón, M., Ramírez de Molina, A., Domínguez, G., Barderas, R., 2019. Sci. Rep. 9, 13547. https://doi.org/10.1038/s41598-019-49960-x.
Garranzo-Asensio, M., San Segundo-Acosta, P., Povés, C., Fernández-Aceñero, M.J., Martínez-Useros, J., Montero-Calle, A., Solís-Fernández, G., Sanchez-Martinez, M.C., Rodríguez, N., Cerón, M.A., Fernandez-Diez, S., Domínguez, G., de los Ríos, V., Peláez-García, A., Guzmán-Aránguez, A., Barderas, R., 2020. J. Proteonomics. 214, 103635. https://doi.org/10.1016/j.jprot.2020.103635.
Gonzalez-Quintela, A., Alende, R., Gude, F., Campos, J., Rey, J., Meijide, L.M., Fernández-Merino, C., Vidal, C., 2008. Clin. Exp. Immunol. 151, 42–50.
Grigorieva, D.V., Gorudko, I.V., Sokolov, A.V., Kosmachevskaya, O.V., Topunov, A.F., Buko, I.V., Konstantinova, E.E., Cherenkevich, S.N., Panasenko, O.M., 2013. Bull. Exp. Biol. Med. 155, 118–121.
Humphrey, J.H., Batty, I., 1974. Immunochemistry 11, 759.
Infantino, M., Manfredi, M., Merone, M., Grossi, V., Benucci, M., Gobbi, L., Bandinelli, F., Damiani, A., Soda, P., 2018. Immunol. Res. 66, 340–347.
Janyapoon, K., Jivakanont, P., Surbrsing, R., Siriprapapan, W., Tachawuttiwat, T., Korbsrisate, S., 2005. Pathology 37, 63–68.
Karamehic, J., Subasic, D., Gavrankapetanovic, F., Zecevic, L., Eminovic, I., Memic, S., Seric, N., Drace, Z., 2007. Med. Arh. 61, 16–19.
Makowski, G.S., Ramsby, M.L., 2003. Ann. Clin. Lab. Sci. 33, 142–148.
Nguyen, T.T., Sly, K.L., Conboy, J.C., 2012. Anal. Chem. 84, 201–208.
Riboldi, P., Gerosa, M., Moroni, G., Radice, A., Allegri, F., Sinico, A., Tincani, A., Meroni, P.L., 2005. Autoimmunity 38, 39–45.
Rubin, R.L., Wall, D., Konstantinov, K.N., 2014. Biosens. Bioelectron. 51, 177–183.
Serafín, V., Arevalo, B., Martinez-Garcia, G., Aznar-Poveda, J., Lopez-Pastor, J.A., Beltran-Sanchez, J.F., Garcia-Sanchez, A.J., Garcia-Haro, J., Campuzano, S., Yañez-Sedeño, P., Pingarrón, J.M., 2019. Sensor. Actuator. B Chem. 299, 126934. https://doi.org/10.1016/j.snb.2019.126934.
Wang, X., Xia, Y., 2019. Front. Immunol. 10, 1–14.
Yukawa, N., Fujii, T., Kondo-Ishikawa, S., Yoshifuji, H., Kawabata, D., Nojima, T., Ohmura, K., Usui, T., Mimori, T., 2011. Arthritis Res. Ther. 13, R213. https://doi.org/10.1186/ar3546.
Zhao, S., Walker, D.S., Reichert, W.M., 1993. Langmuir 9, 3166–3173.