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Anti-double stranded DNA antibodies: Electrochemical isotyping in autoimmune and neurological diseases

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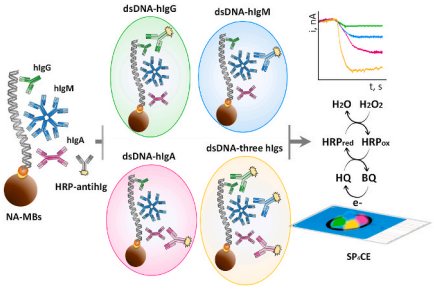
HIGHLIGHTS

- First bioplatform for isotyping of human anti-dsDNA antibodies (IgG, IgM, IgA and three Igs).
- Selective capture by magnetic microsupports modified with a laboratory-prepared biotinylated human dsDNA.
- Amperometric transduction at disposable screen-printed quadruple platforms.
- Quadruple determination within 1 h and 15 min.
- Analysis of serum samples from healthy individuals and from patients diagnosed with SLE and AD patients.

GRAPHICAL ABSTRACT



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ABSTRACT

This work reports the first amperometric biosensor for the simultaneous determination of the single or total content of the most relevant human immunoglobulin isotypes (hIgs) of anti-dsDNA antibodies, dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA and dsDNA-three hIgs, which are considered relevant biomarkers in prevalent autoimmune diseases such as systemic lupus erythematosus (SLE) as well as of interest in neurodegenerative diseases such as Alzheimer's disease (AD). The bioplatform involves the use of neutravidin-functionalized magnetic microparticles (NA-MBs) modified with a laboratory-prepared biotinylated human double-stranded DNA (b-dsDNA) for the efficient capture of specific autoantibodies that are enzymatically labeled with horseradish peroxidase (HRP) enzyme using specific secondary antibodies for each isotype or a mixture of secondary antibodies for the total content of the three isotypes. Transduction was performed by amperometry (−0.20 V *vs.* the Ag pseudo-reference electrode) using the H_2O_2/hydroquinone (HQ) system after trapping the resulting magnetic bioconjugates on each of the four working electrodes of a disposable quadruple transduction platform (SP_4CEs). The bioplatform demonstrated attractive operational characteristics for clinical application and was employed to determine the individual or total hIgs classes in serum from healthy individuals and from patients diagnosed with SLE and AD. The target concentrations in AD patients are provided for the first time in this work. In addition, the results for SLE patients and control individuals agree with those obtained by applying ELISA tests as well as with the clinical ranges reported by other authors, using individual detection methodologies restricted to centralized settings or clinical laboratories.

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1. Introduction

Natural autoantibodies or autoantibodies (Abs) are immunoglobulins (Igs) found in the plasma of healthy individuals and directed against self-proteins. Immunoglobulin-mediated humoral immunity is essential for host defence against pathogens and toxins, and dysregulation of this process leads to immunodeficiency and autoimmune diseases such as Sjögren's syndrome, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE). The field of Abs-associated diseases has been expanded in the recent years to encompass, in addition to cancerous and autoimmune diseases, neurodegenerative diseases such as Alzheimer's disease (AD) [1,2] and Parkinson's disease (PD) [3].

The effector functions of Abs are determined by their constant domain (Fc), which defines the antibody isotype and subclass. Autoantibodies can act as pathogenic molecules that mimic hormone stimulation of receptors, block neural transmission by binding to receptors, affect signalling pathways, lyse cells, and are associated with infections, neurodegeneration, autoimmunity and cancer, diseases characterized by the development of inflammatory processes [4,5]. These diseases can alter the isotype, structure, and function of Igs and consequently, their pathogenicity [6]. The B-cell response typically begins with the secretion of the pentameric IgM, after which a proportion of B cells undergoes the most prevalent and abundant isotype change and begin to produce IgG in serum, which is usually the most commonly used isotype in diagnostic tests showing personal specific signatures that tend to be stable over time [7], and in last step IgA, or IgE. It is also important to note that all these Ig isotypes appear years before the clinical manifestations of the diseases [8], so that their determination is of crucial importance for an early and reliable diagnosis or prognosis [9].

The serum level of Abs to nuclear antigens (ANAs-Abs) and, in particular of that bind to double-stranded DNA (dsDNA-Abs), is associated with a variety of rheumatologic diseases, mostly SLE, but also rheumatoid arthritis, Sjögren's syndrome or scleroderma and other conditions, including for example Graves' and Alzheimer's diseases [10,11]. In fact, these dsDNA-Abs represent one of the main diagnostic and classification criterion for SLE [12,13] and their hyperexpression is associated with multiple end-organ lesions in these patients, such as endothelial dysfunction in lupus nephritis, proatherogenic dyslipidaemia and accelerated atherosclerosis [13–15].

It is important to note that an accurate isotyping of these Abs is essential for a complete diagnosis, as it may have relevance in suggesting potentially common paths in response evolution, disease pathogenesis, and response to treatment [16]. For example, detection of dsDNA-hIgA seems to improve the ability to diagnose SLE and define the phenotype of lupus nephritis and active disease [12]. In contrast, dsDNA-hIgM might be protective for renal involvement [12,17,18], hIgG to hIgM (IgG/IgM) class ratio seems to be a significant parameter to distinguish patients with lupus nephritis from those without renal involvement [19], and dsDNA-hIgG induce cutaneous lupus erythematosus (CLE) [20].

These data support the hypothesis that clustering of dsDNA-Abs isotypes may help refine the diagnosis and prognosis of SLE [12]. Although these autoantibodies are more specific to autoimmune diseases, and particularly to SLE disorders, it is important to note that their association with inflammatory processes or the progression of neurodegenerative dementias has also been established [2,3,21].

A wide variety of methods have been reported for the determination of human dsDNA-Abs, mainly of the hIgG isotype, providing variable sensitivities depending on the bioassay setup, reagents and detection system used. Some of these methods involve fluorescence enzyme immunoassays (FEIA) [22,23] which, despite their good sensitivity, require complex instrumentation and cannot be miniaturized and transferred to point-of-care (POC) devices. Enzyme-linked immunosorbent assays (ELISAs) have also been used and many are commercially available, with wells precoated with dsDNA strands [https://eaglebio.com/product/anti-dsdna-elisa-assay-kit/] [https://www.creative-diagnostics.com/dsDNA-Antibody-EIA-Kit-103850-462.htm]. These methods exhibit nonlinear calibration plots as well as variation coefficient values larger than 4% and, although they are widely used in centralized settings and clinical laboratories, they require specialized instrumentation and personnel and are not affordable in all settings.

Considering this background and the mentioned limitations, the opportunities offered by electroanalytical bioplatforms for the determination of clinical biomarkers in terms of simplicity, affordable cost, and POC applicability, have been applied to the single [24–26] or multiplexed [27,28] determination of hIgG Abs of relevance in autoimmune [24–26,28] and/or degenerative [27] diseases. This work expands the application of one of these previous works [24] to the development of a bioplatform for the quadruple determination of both the individual most prevalent isotypes (hIgG, hIgM and hIgA) and their total content (three hIgs) of human dsDNA-Abs. The bioplatform involves the simultaneous preparation of four neutravidin-modified commercial magnetic microcarriers batches on which a biotinylated dsDNA prepared in the laboratory from a human plasmid is immobilized and used to selectively capture the dsDNA-Abs. The bioconjugates were enzymatically labeled with HRP-conjugated secondary antibodies for hIgG, hIgA or hIgM, for the determination of each isotype, or with a mixture of the three secondary antibodies for the determination of the total content of the three hIgs classes. Each bioconjugates batch was trapped on one of the four working electrodes available at the disposable multi-transduction platform and the quadruple determination was performed by amperometry in the presence of the H_2O_2/hydroquinone (HQ) system and applying a potential of −0.20 V (vs. the Ag pseudo-reference electrode). After characterizing its analytical performance, the bioplatform was applied to the quadruple amperometric determination of the individual and total content of the three human dsDNA-Abs isotypes in serum samples from healthy subjects and from patients with SLE or AD.

2. Experimental section

2.1. Reagents and solutions

The dsDNA-Abs standards (prepared in a serum/buffer matrix), a mixture of horseradish peroxidase (HRP)-labeled rabbit polyclonal anti-human IgG, anti-human IgM and anti-human IgA antibodies (HRP-anti-human IgG/IgM/IgA mixture), sample buffer (5 × , Tris, NaN_3 < 0.1% (w/w)) and washing buffer (50 × , PBS, NaN_3 < 0.01% (w/w)) were supplied in the dsDNA Antibody ELISA Kit from Creative Diagnostics (Cat. No. DEIA1681). HRP-anti-human IgG (HRP-antihIgG, Cat. No. ab97225) was purchased from Abcam, HRP-anti-human IgM (HRP-antihIgM, Cat. No. 109-035-129) and HRP-anti-human IgA (HRP-antihIgA, Cat. No. 109-035-011) were from Jackson ImmunoResearch. Lyophilized human serum from clotted whole blood (Cat. No. S2257) purchased from Sigma Aldrich was used in the matrix effect studies.

Neutravidin-modified magnetic microparticles (NA-MBs, Ø = 1 μm, 10 mg mL^{-1}, Cat. No. GE78152104010350) were purchased from SpeedBeads™.

Human serum IgG (hIgG, Cat. No. I2511), human serum albumin (HSA, Cat. No. A1653) and human hemoglobin (HB, Cat. No. H7379) from Sigma-Aldrich were used for selectivity studies. Hydroquinone (HQ) and hydrogen peroxide (H_2O_2, 30% v/v), purchased from Sigma-Aldrich, were used for amperometric transduction.

Biotinylated dsDNA from human plasmid was prepared as previously reported [24].

Sodium chloride, potassium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate and Tris-hydroxymethyl-aminomethane-HCl (Tris-HCl) were from Scharlab. Purified water using the Milli-Q purification system (18.2 MΩ) was used to prepare all buffer solutions: 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA and 2 mM NaCl (B&W buffer) and 50 mM phosphate buffer (PB) solution pH 6.0 (measurement buffer). Commercial Blocker™ Casein in PBS (blocking buffer,

BB) was acquired from Thermo Fisher Scientific (Cat. No. 37528).

The solutions used to perform amperometric detection include 100 mM HQ and 100 mM H_2O_2 and were prepared fresh in 50 mM PB pH 6.0.

2.2. Apparatus and electrodes

All the amperometric measurements were performed at room temperature using a CHI812B potentiostat (CH Instruments, Inc.) controlled by CHI812B software, and employing screen-printed carbon electrodes (SPCEs) with one carbon working electrode (SPCE, DRP110, 4 mm Ø), a carbon auxiliary electrode and a Ag pseudo-reference electrode with its corresponding specific cable connector (DRP-CAC) and quadruple screen-printed carbon electrodes (SP_4CEs) with four carbon working electrodes (DRP-4W110, 2.95 mm Ø) with shared carbon auxiliary and Ag pseudo-reference electrodes and the specific cable connector (DRP-CONNECT4W), for single or quadruple determination respectively, supplied by Metrohm-DropSens S.L. Glass 10- or 20-mL electrochemical cells, and homemade polymethylmethacrylate (PMMA) housings with one or four neodymium magnets (AIMAN GZ) embedded where SPCEs or SP_4CEs were placed, were used to ensure stable and reproducible capture of magnetic bioconjugates on the working electrodes.

A DynaMag-2 magnetic concentrator (Cat. No: 12321D, Dynabeads®, Invitrogen™ Thermo Fisher Scientific), a Bunsen AGT-9 vortexer, a MT100 Thermo-shaker (Universal Labortechnik), a Crison pH-meter model Basic 20+, a Heidolph Reax Top homogenizer for small samples, and an MPW-65R centrifuge (Med. Instruments) were also employed.

Spectrophotometric ELISA measurements were performed with a Sunrise™ Tecan microplate reader using the Magellan V 7.1 software.

2.3. Procedures

2.3.1. Preparation of the magnetic bioconjugates

The protocols for the modification of the magnetic microsupports with the b-dsDNA and their use for the selective capture of the dsDNA-Abs are similar to those reported previously [24] with changes regarding the enzymatic labeling of the captured dsDNA-Abs. Incubation and washing steps were carried out with 25 and 50 μL, respectively, assisted by a thermostatic incubator with agitation (950 rpm, 25 °C) or a magnetic concentrator. Briefly, for each determination, 5 μL of the commercial suspension of NA-MBs were modified by incubation for 30 min with 100 ng b-dsDNA (prepared in B&W buffer). After two washings with wash buffer, the as prepared conjugates were incubated for 30 min with the standard solution or the sample to be analyzed (prepared in sample buffer) for the selective capture of the dsDNA-Abs. The resulting Abs-dsDNA-b-NA-MBs were incubated with 75 μL of the commercial mixture HRP-antihIgG/IgM/IgA or with 25 μL of 1.0 μg mL^{-1} HRP-antihIgG or 1000-fold diluted HRP-antihIgM or HRP-antihIgA (all prepared in BB) for 30, 15 and 45 min to interrogate dsDNA-three hIgs, hIgG, and hIgM/hIgA, respectively.

It is important to note that for the determination of both individual and total content of the 3 isotypes, the dsDNA-Abs calibrators supplied in the dsDNA Antibody ELISA Kit from Creative Diagnostics (Cat. No. DEIA1681) were used as standards.

The resulting bioconjugates were washed twice with 50 μL of wash buffer and resuspended in 50 or 5 μL of 50 mM PB buffer pH 6.0 to perform single or quadruple amperometric transduction, respectively.

2.3.2. Amperometric detection

The suspension of the magnetic microparticles modified with the bioconjugates was deposited on the corresponding working surface of each electrode (SPCE or SP_4CEs), which was previously placed in a homemade PMMA casing with the embedded neodymium magnets, allowing the magnetic trapping of the microparticles.

Amperometric transduction was carried out under constant stirring (400 rpm) by immersing the casing/electrode assembly with the captured magnetic bioconjugates in a cell containing 10 or 20 mL of 50 mM PB buffer pH 6.0 and 100 or 200 μL of a freshly prepared 100 mM HQ solution in the same measuring buffer, for single or quadruple detection, respectively, so that the electrodes were oriented towards the place where the H_2O_2 addition was made. A constant potential of −0.20 V (*vs.* the Ag pseudo-reference electrode) was applied, and when the background current was stabilized (approximately 50 s), 50 or 100 μL of a 100 mM H_2O_2 solution (freshly prepared in PB 50 mM pH 6.0) were added, producing a cathodic current variation attributed to enzymatic reduction of the H_2O_2 mediated by HQ, which again reached the steady state.

The amperometric responses given in the manuscript corresponded to the difference between the steady state current reached after the addition of H_2O_2 and the background current and are the mean values of three replicates. The error bars shown in the figures were estimated as three times the standard deviation of the replicates ($\alpha = 0.05$).

It is important to note that we performed amperometric measurements in stirred solutions instead of using drops of quiescent solutions to avoid the "false increase" of the cathodic current that occurs in quiescent solutions after the addition of H_2O_2 because of the increase of its local concentration near the electrode surface which decreases as the diffusion progresses. Nevertheless, previous works with SPCEs [29] and SP_4CEs [30] showed that there was no significant difference between the results obtained with the two ways of measurement.

2.3.3. Analysis of serum samples

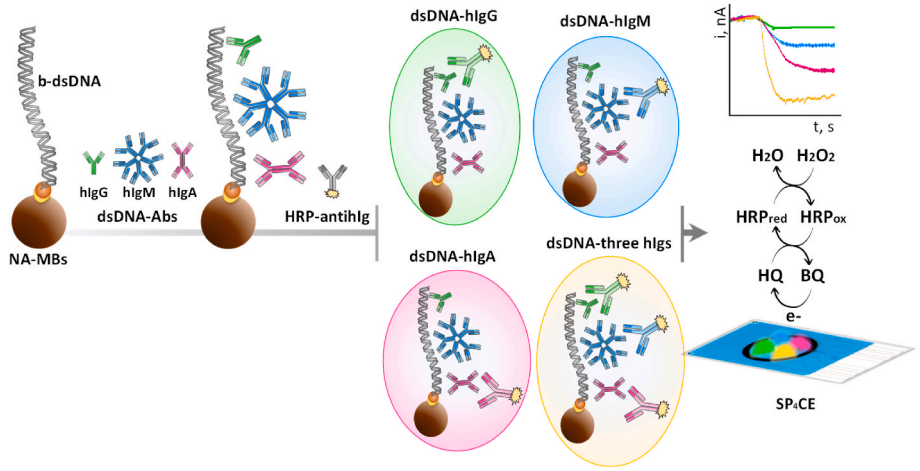
The developed biosensing multiplatform was applied to the determination of the individual and total content of the mentioned dsDNA-Abs isotypes in real human serum samples from healthy individuals and from patients diagnosed with SLE and AD. Serum samples from healthy individuals and SLE patients were purchased from an online marketplace for human biospecimens (Central BioHub®), while serum samples from AD patients were obtained from the BT-CIEN. The samples were stored at −40 °C or −80 °C, respectively, until use. Written informed consents were obtained from all subjects and patients participating in the study. All experiments were performed in compliance with all ethical aspects and relevant guidelines and regulations of the involved institutions.

After confirming the absence of matrix effect in serum samples diluted 100-fold or more, the determinations were performed by interpolation of the amperometric responses provided by the bioplatforms for 100-fold diluted serum samples into the calibration plots constructed with the dsDNA-Abs calibrator standards.

The serum samples were also analyzed using the same reagents by single ELISA kits for each target. ROC curves (Receiver Operating Characteristic Curve) of individual and in combination autoantibodies were obtained with R (version 3.6.2), using the "ModelGood" and the "Epi" packages to determine the diagnostic ability of the test. Cut-off values for separation of the indicated groups were calculated with GraphPad Prism 8 program [31].

3. Results and discussion

This work reports the first bioplatform that allows both individual and total determination of the three most relevant isotypes of human dsDNA-Abs (hIgG, hIgM and hIgA). For this purpose, and as it is illustrated in Scheme 1, dsDNA-Abs were efficiently captured on commercially available NA-functionalized magnetic microcarriers modified with a b-dsDNA produced in the laboratory from a human plasmid (dsDNA-b-NA-MBs). The Abs captured on the dsDNA-b-NA-MBs were enzymatically labeled with HRP-conjugated secondary antibodies selective to each isotype or with a mixed solution of all of them. Thus, for quadruple transduction, four batches of magnetic bioconjugates were prepared simultaneously and differentially enzymatically labeled to allow the determination of dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA or dsDNA-three hIgs. These batches were captured on each of the working



Scheme 1. Fundamentals of the bioplatform functioning developed for the single or total amperometric multidetermination of the human dsDNA-Abs (hIgG, hIgM, and hIgA isotypes) on disposable platforms. The reactions involved in the amperometric transduction are also displayed.

electrodes of a SP_4CEs, and amperometry at $E_{app} = -0.20$ V *vs.* Ag pseudo-reference electrode, in stirred solutions and in the presence of the H_2O_2/HQ system was employed to measure cathodic current variations which were proportional to the concentration of each target.

3.1. Optimization of the experimental variables

The working conditions for the amperometric transduction on SPCEs using the HRP/H_2O_2/HQ system were previously optimized [32]. Similarly, the conditions used for the preparation of dsDNA-b-NA-MBs,

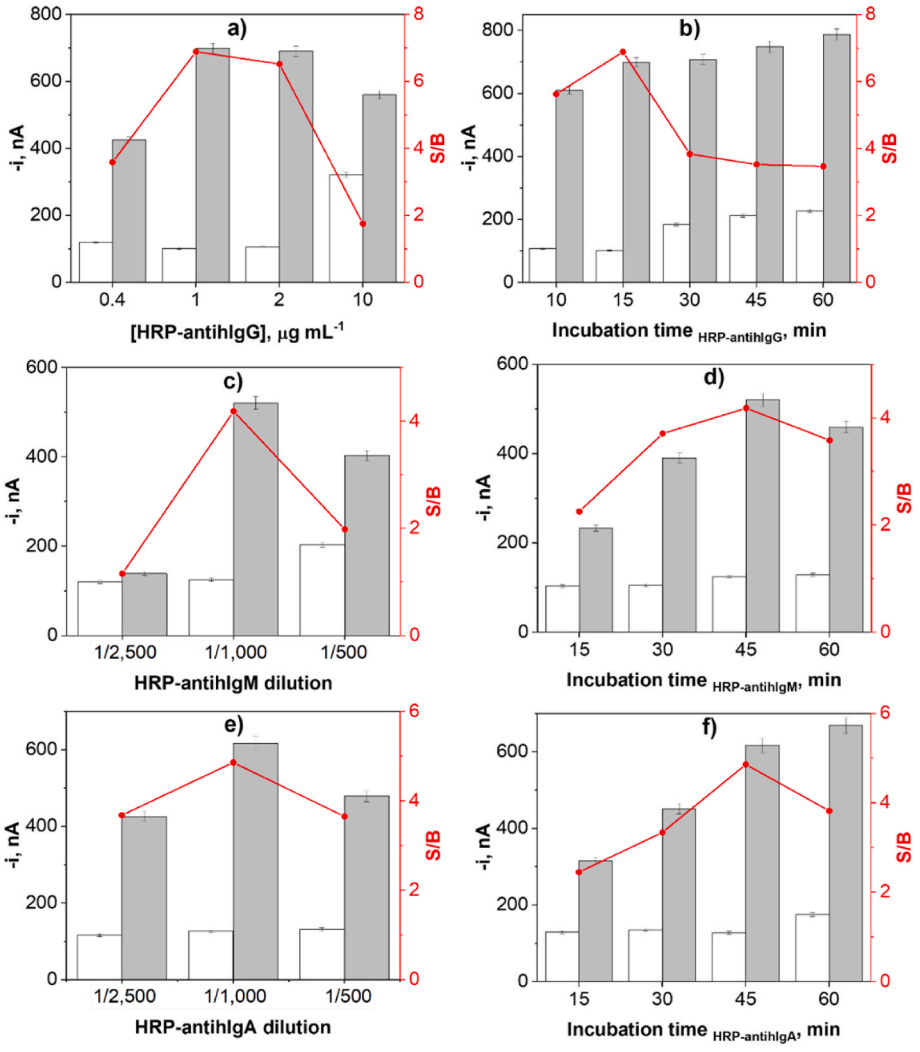


Fig. 1. Amperometric responses for 0 and 25 U mL^{-1} of the dsDNA-Abs calibrator measured with the bioplatforms for the individual determination of human isotypes of dsDNA-Abs (hIgG, hIgM and hIgA) upon variation of the concentration/dilution (a, c, e) and incubation time (b, d, f) of the HRP-labeled secondary antibodies (a and b: HRP-antihIgG; c and d: HRP-antihIgM; e and f: HRP-antihIgA). The values of the corresponding S/B ratios are shown in circles and red lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Experimental variables evaluated for the amperometric determination of human isotypes of dsDNA-Abs.

Variable	dsDNA-Abs isotype	Tested range	Selected value
HRP-antihIg concentration/ dilution	hIgG	0.5–10 μg mL^{-1}	1.0
	hIgM	1/2500–1/ 500	1/1000
	hIgA		
HRP-antihIg incubation time, min	hIgG	10–60	15
	hIgM	15–60	45
	hIgA		

the capture of dsDNAs-Abs and the joint enzymatic labeling of the three isotypes were optimized before [24]. Therefore, only the conditions involved in the enzymatic labeling of each individual isotype, i.e., concentration/dilution and incubation time with the corresponding secondary antibody were checked for the development of the quadruple bioplatform. For this purpose, the amperometric responses provided by the SPCEs bioplatforms for each isotype for 0 (B signal) and 25 (S signal) U mL^{-1} of the dsDNA-Abs calibrator supplied in the corresponding ELISA kit, were measured and the S/B ratio calculated. The obtained results are shown in Fig. 1 and summarized in Table 1.

As can be seen in Fig. 1, similar trends were observed for both variables concerning the three HRP-secondary antibodies. The S/B ratios increased with the HRP-labeled secondary antibody concentration and the incubation time up to a value, and showed a decrease for larger concentrations or longer incubation times due to the noticeable increase of the non-specific responses compared to the specific ones. It is important to note that better ratios for both variables were attained at the same value for the secondary antibodies used to recognize hIgM and hIgA isotypes (1/1000 dilution and 45 min incubation time). In the case of the hIgG isotype, better S/B ratios were obtained by incubating the Abs-dsDNA-b-NA-MBs for 15 min in a 1.0 μg mL^{-1} of HRP-antihIgG solution.

3.2. *Analytical and operational characteristics for the individual and total quadruple amperometric determination of the three isotypes of dsDNA-Abs*

The feasibility of the multiplexing approach allowing the simultaneous determination of the individual and total content of the three target human isotypes of dsDNA-Abs, was confirmed by the results shown in Fig. 2. The quadruple amperometric determination involved the preparation of independent magnetic conjugates for the determination of dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA and dsDNA-three IgG, according to the selected conditions summarized in Table 1. Once the four independent batches were prepared, the quadruple amperometric transduction at the SP_4CEs was performed. It is worthy to mention that the amperometric responses recorded at the SP_4CEs are 70–80% smaller than those obtained by capturing the same bioconjugates on SPCEs due to the differences in the geometric area of the working electrodes (6.8 mm^2 for SP_4CEs vs. 12.6 mm^2 for SPCEs). However, there were no significant differences between the S/B ratios calculated using the SPCE or the SP_4CEs (4.4 *vs.* 4.3 for dsDNA-hIgG; 2.4 *vs.* 2.4 for dsDNA-hIgM; 2.4 *vs.* 2.3 for dsDNA-hIgA; 3.6 *vs.* 3.5 for dsDNA-three hIgs, respectively). In addition to the feasibility of multiplexing, the obtained results show that no significant cross-reactivity occurred between the four adjacent working electrodes of the SP_4CEs.

The analytical and operational characteristics of the bioplatform for the quadruple amperometric determination of dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA and dsDNA-three hIgs were established. The calibration plots constructed using the dsDNA-Abs calibrators of the commercial kit are shown in Fig. 3 and the corresponding parameters summarized in Table 2.

The data summarized in Table 2 show that the quadruple bioplatform exhibit concentration ranges and LOD values suitable for the determination of these biomarkers in serum from patients diagnosed with autoimmune diseases. It is important to note that the most widely accepted cut-off value in serum for the discrimination of patients with SLE is 100 U mL^{-1} of dsDNA-Abs [33], which is about two orders of magnitude larger than the LOD values achieved with the developed bioplatform. It is also important to remark the great reproducibility of the whole process involving both the bioconjugates manufacturing and the quadruple amperometric transduction at SP_4CEs. Interestingly, an excellent storage stability of the dsDNA-b-NA-MBs was obtained showing that no significant loss of sensitivity of the as prepared bioplatforms was observed after 40 days of the dsDNA-b-NA-MBs preparation. Importantly, and counting since this preparation, the quadruple determination (dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA and dsDNA-three hIgs) can be completed in only 1 h and 15 min.

To our knowledge, only two others electrochemical bioplatforms have been reported so far for the determination of dsDNA-Abs. One of these works reports the single determination of dsDNA-hIgGs [34], while other deals with the determination of dsDNA-three hIgs [24]. The work of Rubin et al. describes a continuous flow biosensor involving immobilization of native dsDNA on a porous membrane coupled to an eight-electrode array and claimed a 5-times lower LOD than that achieved with the developed bioplatform (10 *vs.* 50 μg mL^{-1}). However, such biosensor required 16 h and 90 μg of dsDNA for preparing the dsDNA-coated membrane compared to the 30 min and 100 ng of dsDNA required for the preparation of the dsDNA-b-NA-MBs. In addition, the obtained results evidenced that the transduction on the quadruple platforms had a very scarce influence on the achieved LOD for dsDNA-three hIgs, when compared with the single transduction (0.56 vs 0.30 U mL^{-1} [24]), which, as commented above, must be attributed to the smaller area of the SP_4CEs working electrodes compared to the SPCEs.

When the comparison is made versus the commercially available ELISA methodologies, it is important to note that most of them determine the total IgG, IgM and IgA class Abs [Abbexa, Cat. No. abx364947; Creative Diagnostics, Cat. No. DEIA1681; MyBiource, Cat No.

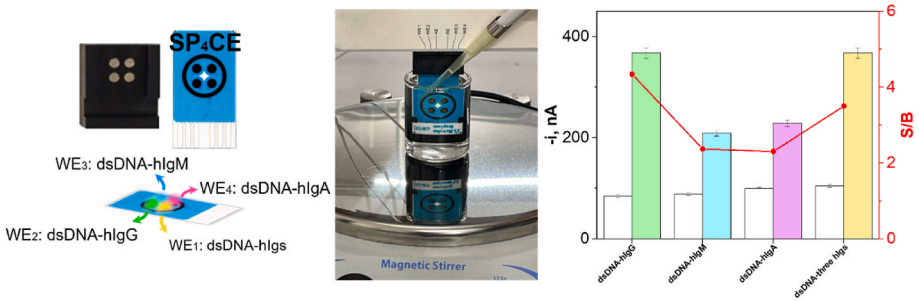


Fig. 2. Drawing with the type of magnetic bioconjugates captured on each working electrode of the SP_4CEs to perform the quadruple amperometric determination of the target antibodies and real image of the electrode setup used for quadruple amperometric determinations with the developed bioplatform. Amperometric responses measured for 0 (white bars, B signal) and 25 (grey bars, S signal) U mL^{-1} of the enzymatically marked dsDNA-Abs calibrator for determination of dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA and dsDNA-three hIgs at SP_4CEs. The values of the corresponding S/B ratios are shown in circles and red lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

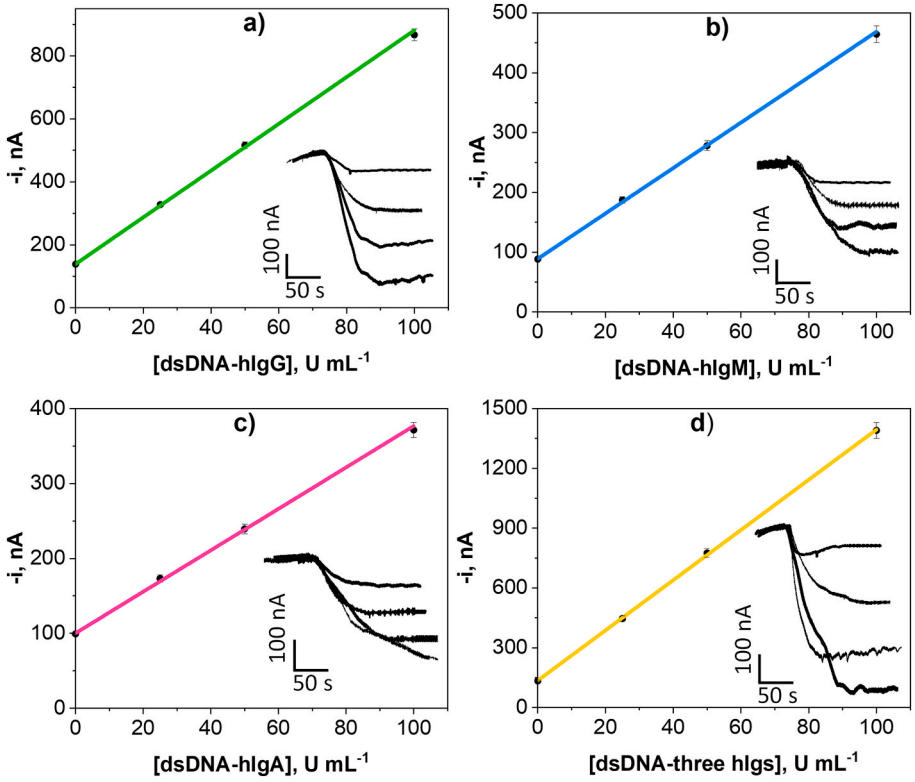


Fig. 3. Calibration graphs and real amperometric traces recorded with the quadruple bioplatform for the amperometric determination of dsDNA-hIgG a), dsDNA-hIgM b) dsDNA-hIgA c) and dsDNA-three hIgs.

MBS269122; ELISAKITS.co.uk, Anti-dsDNA Screen ELISA Kit] or the individual IgG class content [ImmunoDiagnostics Limited, Cat. No. 31A040; Signosis, Cat. No. EA-500; Eagle Biosciences, SKU: DSD31-K01; Cusabio, Cat. No. CSB-E04911h; MyBiource, Cat No. MBS2601760; ELISAKITS.co.uk, Anti-dsDNA IgG ELISA Kit]. Nevertheless, some companies, such as ELISAKITS.co.uk and MyBiource, offer different kits that allow the individual or total determination of hIgG, hIgM, and hIgA isotypes. These kits have adequate sensitivity with LOD values of around 1 U mL^{-1}. However, to our knowledge, there are no kits available that allow the simultaneous determination of individual isotypes and their total content. Moreover, the ELISA kits must be used with instrumentation that is difficult to miniaturize and by trained personnel, aspects in which the proposed bioplatform could lead the way.

3.3. Selectivity

As it was previously reported [24], the significant interference observed in the presence of non-dsDNA-related hIgs for the determination of dsDNA-related hIgs, which was attributed to the not target hIgs non-specific adsorption on the magnetic microcarriers, was efficiently corrected by subtracting the current measured for the appropriately diluted commercial synthetic serum sample from the response of a real sample. Therefore, in this work we only evaluated the influence of the presence of HSA and HB on the determination of hIgGs, hIgMs and hIgAs. The amperometric responses obtained for 0 and 25 U mL^{-1} of the dsDNA-Abs calibrators, prepared in the absence and in the presence of both interferents at their normal concentrations in serum are shown in Fig. 4. As it can be observed, the presence of HB did not interfere with the determination of the three isotypes. However, the presence of HSA did affect the determination of hIgGs and hIgAs. This effect can be attributed to the presence of hIgG in the not completely purified HSA [35]. The fact that HSA interfered for hIgG and hIgA and not for hIgM may be justified by the different secondary antibodies used for their enzymatic labeling and/or to the structures of these isotypes which are more similar, monomeric and/or dimeric, in the case of hIgG and hIgA and more different, pentameric, in hIgM. However, it is

Table 2
Analytical, operational and storage characteristics of the quadruple bioplatform constructed for the individual and total amperometric determination of dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA and dsDNA-three hIgs.

Characteristic	dsDNA-Abs			
	hIgG	hIgM	hIgA	Three hIgs
Linear range, U mL^{-1}	3.4–100	5.0–100	5.9–100	1.9–100
Slope, mL (nA U)$^{-1}$	7.4 ± 0.1	3.80 ± 0.06	2.76 ± 0.06	12.6 ± 0.1
Intercept, nA	139 ± 2	89 ± 1	100 ± 2	136 ± 7
LOD*, U mL^{-1}	1.01	1.49	1.77	0.56
LOQ**, U mL^{-1}	3.4	5.0	5.9	1.9
RSD, % (n = 5, 25 U mL^{-1} dsDNA-Abs calibrators)	2.2	3.0	2.8	2.9
Storage stability of dsDNA-b-NA-MBs (S/B for 25 U mL^{-1} dsDNA-Abs calibrators)**	40 days			

*3 × s_b/m and ** 10 × s_b/m, s_b is the standard deviation of 10 blank measurements and m is the slope of the calibration plots. **Stored in 10 mM filtered PBS (pH 7.4) at 4 °C.

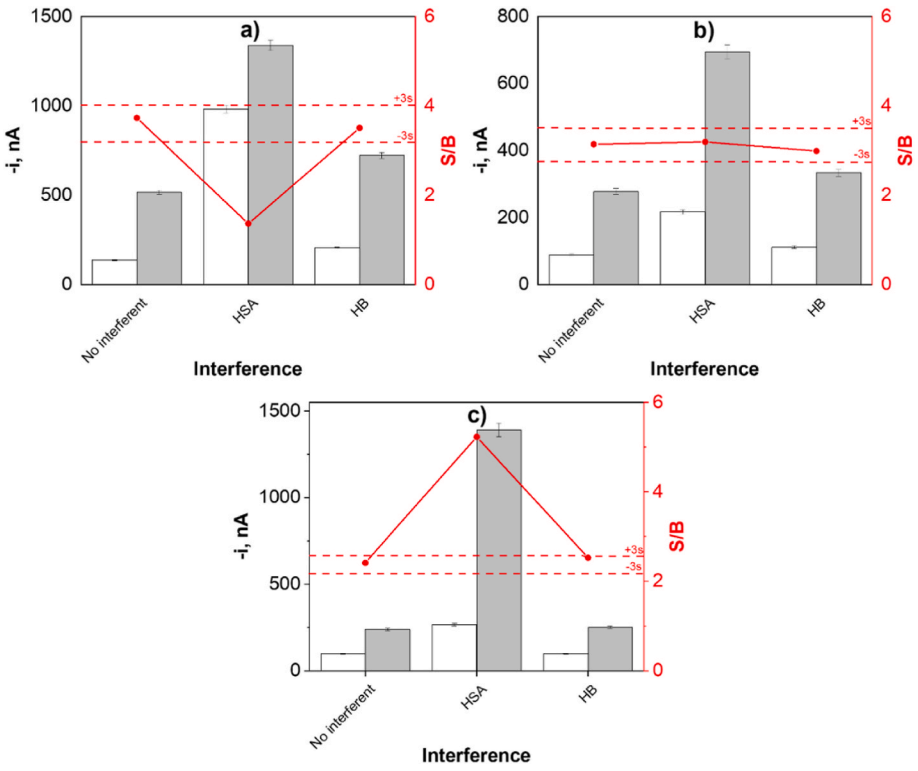


Fig. 4. Comparison of the amperometric responses (and the corresponding S/B ratios in red) for dsDNA-hIgG a), dsDNA-hIgM b) and dsDNA-hIgA c) provided by the developed quadruple bioplatform for 0 and 25 U mL^{-1} of the dsDNA-Abs calibrators prepared in the absence and in the presence of 50 mg mL^{-1} HSA and 5 mg mL^{-1} HB. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

important to note that, as it was previously reported for the interference of hIgs non-specific to dsDNA, the interference of HSA became negligible upon a 100-fold sample dilution.

3.4. Application to the analysis of serum samples

The developed quadruple bioplatform was applied to the determination of the individual and total content of the target hIgs in eight different serum samples (3 from healthy individuals, 3 from SLE patients and 2 from AD patients).

Table 3 compares the slope values of the calibration plots constructed with buffered standards and in a 100-fold diluted human commercial serum sample. The statistical comparison confirmed the absence of matrix effect under these conditions. Accordingly, the individual and total endogenous contents of the target hIgs were determined by interpolation of the responses resulting of subtracting the current measured for the 100-fold diluted commercial synthetic serum sample from the response of analyzed sample (also 100-fold diluted) [24] into the calibration plots for the dsDNA-Abs standards in buffered solutions.

Table 3
Statistical comparison between the slope values (mL nA U^{-1}) of the calibration plots constructed with the multiplexed bioplatform for the dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA, and dsDNA-three hIgs prepared in buffered solutions and in a 100-fold diluted commercial human serum sample.

	Buffered solutions	100-fold diluted commercial human serum	t_{exp}*
dsDNA-hIgG	7.4 ± 0.1	7.2 ± 0.3	0.255
dsDNA-hIgM	3.80 ± 0.06	3.5 ± 0.2	1.126
dsDNA-hIgA	2.76 ± 0.06	2.67 ± 0.02	0.649
dsDNA-three hIgs	12.6 ± 0.1	12.3 ± 0.2	1.608

*t_{exp} estimated by comparing the slope value obtained for standards prepared in the corresponding matrix and in buffered solutions, $t_{tab} = 2.776$, $n = 4$, $\alpha = 0.05$.

The results obtained in the analysis of the serum samples are shown in Fig. 5 and summarized in Table 4, which also shows the concentrations found when the same samples were analyzed using individual ELISA kits involving the same immunoreagents.

As expected, the contents of the three isotypes are significantly larger in patients diagnosed with SLE and AD compared to healthy individuals.

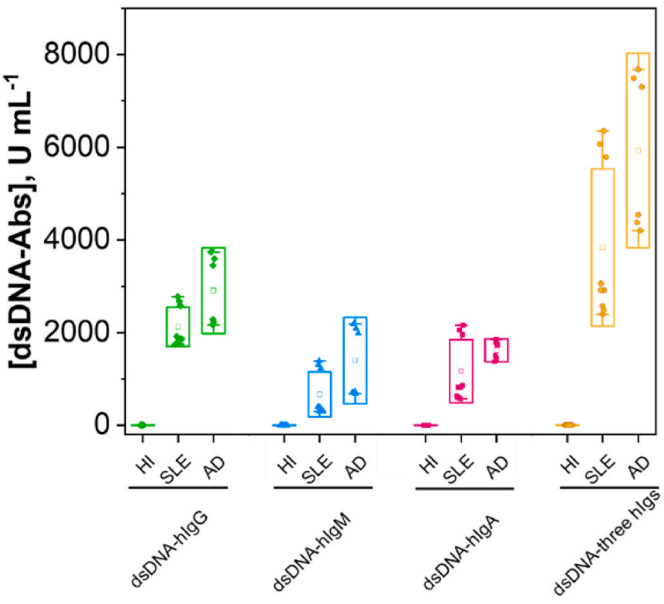


Fig. 5. Endogenous content (including values of all replicas) of dsDNA-hIgG, dsDNA-hIgM, dsDNA-hIgA and dsDNA-three hIgs provided by the multiplexed bioplatform for the analysis of serum samples grouped into pools of healthy individuals (HI), SLE and AD patients.

Table 4
Endogenous content (in U mL^{-1}, mean value ± ts/$\sqrt{n}$; n = 3; α = 0.05) of the dsDNA-Abs (hIgG, hIgM, hIgA and three hIgs) provided by the quadruple bioplatform developed in this work as well as by using the individual ELISA methodologies for serum samples from healthy individuals and patients diagnosed with SLE and AD.

		Multiplexed bioplatform				ELISA			
		hIgG	hIgM	hIgA	Three hIgs	hIgG	hIgM	hIgA	Three hIgs
Control	1	5.3 ± 0.4	1.5 ± 0.2	2.2 ± 0.2	9.2 ± 0.5	5.1 ± 0.4	1.4 ± 0.3	2.1 ± 0.2	8.7 ± 0.8
	2	2.6 ± 0.2	2.4 ± 0.2	1.0 ± 0.1	6.2 ± 0.5	2.6 ± 0.3	2.5 ± 0.3	1.0 ± 0.2	6.0 ± 0.8
	3	4.6 ± 0.8	2.9 ± 0.4	1.5 ± 0.2	9.0 ± 0.6	4.6 ± 0.8	3.0 ± 0.5	1.4 ± 0.2	9.5 ± 0.8
SLE	4	2677 ± 242	1308 ± 198	2056 ± 247	6070 ± 701	2614 ± 493	1324 ± 244	2060 ± 261	6035 ± 802
	5	1921 ± 137	312 ± 28	862 ± 116	3056 ± 356	1851 ± 240	301 ± 33	842 ± 141	3096 ± 520
	6	1808 ± 143	387 ± 40	602 ± 72	2843 ± 217	1735 ± 264	384 ± 48	614 ± 121	2636 ± 309
AD	7	3594 ± 362	2088 ± 245	1796 ± 148	7490 ± 464	3316 ± 583	2128 ± 367	1707 ± 242	6763 ± 1344
	8	2224 ± 149	706 ± 57	1439 ± 158	4373 ± 425	2119 ± 348	664 ± 92	1485 ± 248	4180 ± 862

The results are also consistent with the fact that hIgG is the predominant Ig class in human serum. Furthermore, the concentration determined for the three subclasses agreed with the arithmetic sum of the individual contents.

As occurred with other biomarkers, the cut-offs and reference values established for dsDNA-Abs are dependent on the method, testing laboratory and bioreagents involved, which reveals a lack of standardization [36]. Nevertheless, the results provided by the bioplatform are consistent with the serum cut-off value established in most commercial ELISA kits for diagnosing SLE by interrogating dsDNA-Abs or dsDNA-hIgG (20−25 U mL^{-1}) [Creative Diagnostics, Cat. No. DEIA1681; Immuno-Diagnostics Limited, Cat. No. 31A040; ELISAKITS.co.uk, Anti-dsDNA Screen ELISA Kit; ELISAKITS.co.uk, Anti-dsDNA IgG ELISA Kit]. The results are also consistent with those reported by Gargiulo et al. [33] and Abdel-Rahman Elsayed et al. [37] (>100 and 49 U mL^{-1} for dsDNA-Abs, respectively), by González-Rodríguez for the determination of dsDNA-hIgG (10–45 U mL^{-1}) [36]), and by Villalta et al., 73.5, 69.5 and 28 U mL^{-1} for hIgG, hIgM and hIgA, respectively, to discriminate between positive and negative SLE samples [12]. It is also important to note that some commercial ELISA kits (e.g. Quanta Lite™ dsDNA Kit, INOVA Diagnostic Inc, CA, USA and Warde Medical Cat. No 3000200 Laboratory), classify the sample results as: negative: 0−200 U mL^{-1}; equivocal: 201−300 U mL^{-1}; moderately positive: 301−800 U mL^{-1} and strongly positive >801 U mL^{-1} [10]. According to this criterion, the contents obtained with the bioplatform for the analyzed patients would be strongly positive.

Regarding the results on patients with AD, although the literature echoes the relationship of dsDNA-Abs with this disease [10,11], no cut-off values have been reported to confront the results obtained with the developed bioplatform. In fact, the provided results are the first contents described in the literature for this type of patients. Therefore, an exhaustive validation of these biomarkers is still necessary for AD patients.

Importantly, the correlations plots displayed Fig. 6 between the results obtained with the developed bioplatform and the individual ELISA kits, with slope and intercept values including 1 and 0, respectively, confirmed the excellent agreement of the results provided by both methodologies.

Despite the limited size of the patient's cohort, an analysis of the results by means of ROC curves was performed (Table 5). The resulting parameters showed that the bioplatform allowed a full discrimination between healthy individuals and SS or AD patients with AUCs, sensitivity and specificity of 100% for individual hIgG, hIgM, or hIgA isotypes and all three hIgs. However, the bioplatform was unable to fully

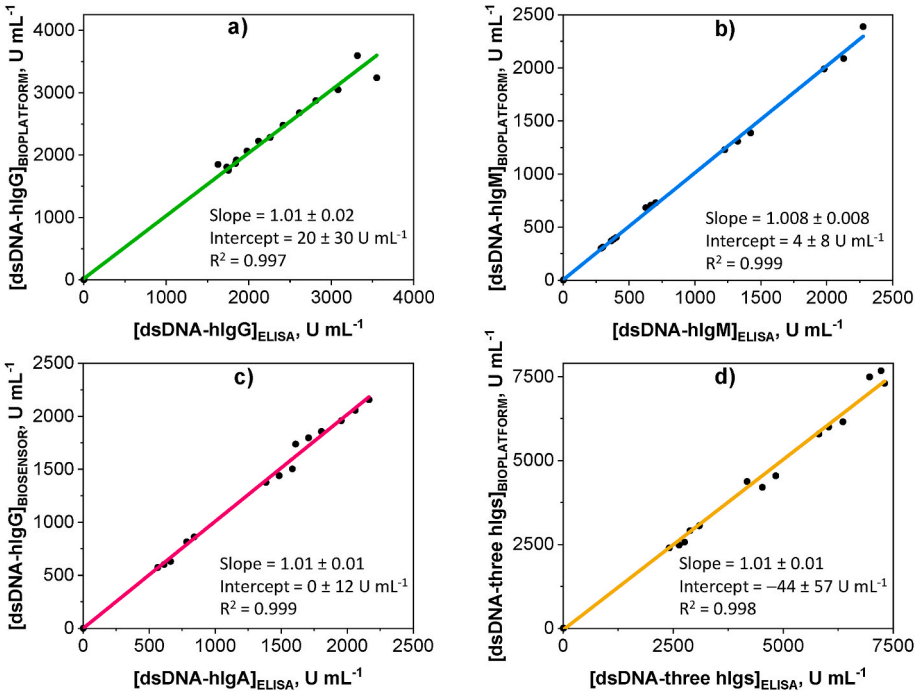


Fig. 6. Correlation plots between the concentrations obtained by the developed multiplexed bioplatform and the individual ELISA methodologies for the determination of the target dsDNA-Abs.

Table 5
Potential of the four-target dsDNA-Abs (1: hIgG, 2: hIgM, 3: hIgA and 4: three hIgs) considered individually or in combination to discriminate autoimmune and neurological diseases and serum cut-off values.

Parameter	Healthy *vs.* SS					Healthy *vs.* AD					SLE *vs.* AD				
	1	2	3	4	All[a]	1	2	3	4	All[a]	1	2	3	4	All[a]
AUC	100	100	100	100	100	100	100	100	100	100	83.3	83.3	66.7	83.3	100
Sensitivity	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Specificity	100	100	100	100	100	100	100	100	100	100	66.7	66.7	66.7	66.7	100
Cut-off (U mL^{-1})	907	157	302	1426	–	1115	354	721	2191	–	2073	546	1151	3715	–

[a] Considering the results of the four target Abs together.

discriminate SLE from AD patients, due to the partial overlapping in the concentration of patients. However, sensitivities of 100%, specificities larger than 66.7% and AUCs of 83.3% for hIgG, hIgM and all three hIgs, with a lower value for hIgA, which is the less concentrated immunoglobulin in blood, were achieved. Therefore, in combination, the four-target dsDNA-Abs showed full ability to discriminate either healthy individuals *vs.* SLE or AD patients, or SLE *vs.* AD individuals. The analysis of the results obtained with the developed quadruple bioplatform by means of ROC curves allowed us to establish the cut-off values shown in the last row of Table 5 for each target dsDNA-Abs isotype to discriminate patients with SLE or AD from healthy individuals, and between SLE and AD patients.

4. Conclusions

The first bioplatform allowing the simultaneous determination of the individual and total content of the three most common isotypes of dsDNA-Abs is reported. The strategy is based on the efficient capture of dsDNA-Abs, assisted by magnetic microsupports modified with a human dsDNA prepared in the laboratory, their differential enzymatic labeling using individual secondary antibodies or a mixture of them, and their trapping on the working electrodes of a disposable multiplexing platform to perform amperometric quadruple transduction.

The developed quadruple bioplatform exhibits suitable analytical and operational operability characteristics for the diagnosis of SLE patients according to the most widely accepted cut-off values in serum. The bioplatform was applied to the individual and total analysis of the target hIgs subclasses in serum samples from healthy individuals and from patients diagnosed with SLE and AD. The obtained results show that the bioplatforms are bioanalytical tools competitive in terms of simplicity, speed, cost, information provided and applicability by low-specialized personnel and at the POC, with other available methodologies to perform the isotyping of this type of serum antibodies in patients with SLE and AD, thus contributing to the advance towards a more personalized diagnosis and to investigate the clinical potential that these antibodies and the different isotypes can play in the diagnosis and prognosis of many diseases other than SLE.

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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.

Data availability

Data will be made available on request.

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