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Early and differential autoimmune diseases diagnosis by interrogating specific autoantibody signatures with multiplexed electrochemical bioplatforms

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ABSTRACT

This work reports the first bioplatform to assist in the early and reliable diagnosis of autoimmune diseases by quadruple determination of autoantibodies (Abs) produced against extractable nuclear antigens (ENAs): La/SSB-Abs, Ro/SSA-Abs, U1snRNP70-Abs and smRNP-Abs. The bioplatform involves indirect immunoassays on the surface of magnetic microcarriers (independent batches for each of the target autoantibodies) and amperometric transduction using the H_2O_2/hydroquinone (HQ) system on a disposable multiple electrode platform. The magnetic microcarriers were modified with the corresponding antigens using His-tag and carbodiimide/succinimide chemistries and employed for the selective capture of the corresponding autoantibodies. Thereafter, they were enzymatically labelled with a secondary antibody conjugated with horseradish peroxidase (HRP) and magnetically captured on each of the working surfaces of the quadruple platform. The evaluation of the analytical and operational characteristics of the bioplatform for the amperometric determination of standards, performed under optimized experimental conditions, confirmed the bioplatform competitiveness in terms of sensitivity and point-of-care application compared to commercially available ELISA methodologies for the single determination of target Abs. The developed bioplatform was applied to the analysis of serum samples from healthy individuals and from patients with two prevalent autoimmune diseases (systemic lupus erythematosus, SLE, and Sjögren's syndrome, SS). The obtained results proved the potential of the bioplatform for the differential diagnosis of these two autoimmune diseases through the accurate, simple, and rapid multidetermination of the four target Abs.

1. Introduction

Almost 8% of the world's population is affected by one or more than 80 different autoimmune diseases, representing a huge social and health economic impact, greater than that of cancer (Orlov et al., 2020). In the last decade the incidence of these diseases has even increased which is attributed to genetic causes or environmental factors, such as lifestyle choices or pollution (Zharkova et al., 2017) (Barbero Mazzucca et al., 2021). It is important to note that the strong overlap of signs and symptoms among autoimmune diseases can be responsible for delays in diagnosis and appropriate treatment. According to a survey made by the Autoimmune Diseases Association, it takes up to 4.6 years and almost 5 visits to the doctor to receive a proper diagnosis of an autoimmune disease (Doghramji).

The onset and progression of autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), rheumatoid arthritis (RA), multiple sclerosis (MS) or Alzheimer's disease (AD) appear to be associated with B- and T-cell abnormalities, protein misfolding and aggregation, and dysregulation of specific autoantigens and their corresponding autoantibodies (Abs). Among them, Abs against nuclear antigens (ANAs) and extractable nuclear antigens (ENAs) (ANAs-Abs and ENAs-Abs, respectively) should be highlighted (Alsubki

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et al., 2020). However, it is important to note that these autoantibodies are also dysregulated in other clinical conditions such as cancer or acute tissue damage (Marshall et al., 2018). Hence, it is critical to know the role of autoantibodies, the actual significance of their presence in each disease, and to establish characteristic autoantibody signatures that allow early (patients can carry Abs many years before manifesting clinical symptoms (Bizzaro, 2008)) and unequivocal identification of each disease.

Currently, the diagnosis of highly heterogeneous autoimmune diseases is based on invasive biopsies that are not easily accessible to many patients (Zhang et al., 2021) and on the determination by laboratory testing of anti-nuclear antibody (ANA) patterns that react with antigens in the nucleus, nucleolus, cytoplasm, and mitotic cell apparatus (Kiriakidou and Ching, 2020). ANA testing has a high sensitivity, ranging from 90% to 95% in patients with SLE (Emlen and O'Neill, 1997), which the prototypical systemic autoimmune disease and one of the most heterogeneous diseases (Dörner and Furie, 2019). However, ANA tests show relatively low specificity, being positive in 30% of healthy controls (Damoiseaux and Tervaert, 2006) (Nagele et al., 2013), especially in elderly (Oke and Wahren-Herlenius, 2013). Therefore, these tests should be complemented by identification of autoantibodies against specific ENAs-Abs. Among them, La/SSB-Abs, Ro/SSA-Abs, U1snRNP70-Abs and smRNP-Abs stand out due to their potential to be used as biomarkers for diagnosis, prognosis and monitoring of autoimmune diseases (Wu et al., 2017).

For example, smRNP-Abs, known as antibodies to Smith antigen, have been included in the serologic criteria for diagnosing patients with SLE because of their high sensitivity (Migliorini et al., 2005). These Abs are almost always associated with U1snRNP70-Abs used as follow-up biomarkers, because they develop later in the course of the disease and are also considered the serological hallmark of "mixed connective tissue disease" (MCTD) and Raynaud's phenomenon (Tani et al., 2014) (Migliorini et al., 2005). The presence of La/SSB-Abs and/or Ro/SSA-Abs is a hallmark of SS and SLE (Agmon-Levin et al., 2017). Ro/SSA-Abs identify pregnant women who are at increased risk of having a child with Neonatal Lupus Syndrome (NLS) (Gryka-Marton et al., 2021), and may also be the only autoantibodies present in a subset of asymptomatic patients with negative ANA tests (Hayashi et al., 2008).

Currently, enzyme-linked immunosorbent assays (ELISA, FEIA), indirect immunofluorescence (IIF) and Western blot (WB) are the most common methods employed in ANA testing for autoimmune disorders (Zhang et al., 2017) (Campuzano et al., 2019). However, these techniques are time-consuming, and require sophisticated instruments. Moreover, their sensitivity is limited, and they are sometimes only able to detect an autoimmune disease when an irreversible tissue damage has already occurred (Zhang et al., 2017). Considering these limitations, the development of novel and reliable techniques for Abs detection is very important for the early and reliable diagnosis of autoimmune diseases.

In this sense, electrochemical biosensors are currently considered as cutting-edge tools, competitive with well-established and/or state-of-the-art technologies, for the determination of clinical biomarkers due to their simplicity of handling, affordable cost, adequate sensitivity and compatibility with multiplexed and point-of-care determinations (Nguyen et al., 2015) (Campuzano et al., 2019). In addition, they have recently successfully incurred in the determination of relevant autoantibodies in cancer, neurodegenerative and autoimmune diseases (Garranzo-Asensio et al., 2020) (Montero-Calle et al., 2021) (Valverde et al., 2021) (Arévalo et al., 2020) (Arévalo et al., 2022a).

Being aware of the important social and economic repercussions of having tools that contribute to the early and reliable detection of autoimmune diseases, this work reports an electrochemical bioplatform for the simultaneous determination of four specific ENAs-Abs: La/SSB-Abs, Ro/SSA-Abs, U1snRNP70-Abs and smRNP-Abs. Although bioplatforms have been developed for the determination of multiple antibodies related to cancer (Garranzo-Asensio et al., 2020) (Montero-Calle et al., 2021) and Alzheimer's disease (Valverde et al., 2021), to our knowledge this is the first report for the multi-determination of autoantibodies related to autoimmune diseases. The developed bioplatform involves the use of antigen-specific modified magnetic microparticles (MBs) taking advantage of His-tag or carbodiimide/hydroxysuccinimide (EDC/NHS) chemistries, for the capture of the corresponding autoantibodies (IgGs) that are enzymatically labelled with HRP-conjugated secondary antibodies followed by amperometric transduction using the H_2O_2/HQ system on disposable platforms for quadruple detection. The bioplatform performance is compatible with clinical application and exhibits potential to discriminate between healthy individuals and patients diagnosed with autoimmune diseases through the simple and reliable determination of the four autoantibodies serum level in only 45 min.

2. Materials and methods

Apparatus and electrodes, Reagents and solutions and all the Procedures used (Implementation of indirect immunoassays on magnetic microsupports, Amperometric detection, Analysis of serum samples) are described in detail in the Supporting Information (SI).

3. Results and discussion

This work reports the first electrochemical bioplatform for the simultaneous determination of four ENAs-Abs: La/SSB-Abs, SSA-Abs, U1snRNP70-Abs and smRNP-Abs. Fig. 1 shows as the bioplatform is based on the use of MBs modified with the corresponding antigens by exploiting two different chemistries conditioned by the commercial availability of the antigens (His-Tag chemistry for La/SSB, Ro/SSA and U1snRNP70 or EDC/NHS chemistry for smRNP) for the capture of the corresponding Abs and the enzymatic labelling of the captured IgGs using an HRP-conjugated secondary antibody (HRP-anti-hIgG). The prepared magnetic immunoconjugates for each interrogated Abs were then captured on each working electrode of an SP_4CE, and the four amperometric responses were simultaneously monitored in the presence of the H_2O_2/HQ system. According to the used assay format, the cathodic current variations obtained from the enzymatic reduction of H_2O_2 mediated by HQ were directly proportional to the concentration of each type of target Abs.

To develop the multiplexed bioplatform providing the best analytical performance for the target Abs, we optimized independently the key variables involved in the preparation of the bioconjugates for the determination of each target Abs by performing their individual amperometric transduction on single SPCEs. The results of all these optimizations are presented and discussed in the SI (Figs. S1–S4 and Table S1).

3.1. Analytical and operational characteristics for quadruple determination of anti-ENAs Abs

Once the conditions for the preparation of the bioconjugates for each target Abs were selected, the possibility of simultaneous determination was evaluated by comparing the amperometric responses recorded at SPCEs and SP_4CEs. As expected, Fig. S5 (in the SI) shows that the amperometric responses were 70–85% smaller at the SP_4CEs compared to SPCEs, due to the SP_4CEs working electrode smaller surface area (6.8 *vs.* 12.6 mm^2). Nevertheless, no significant differences were observed in the S/B ratios (ratios of amperometric responses obtained in the presence, S, and absence, B, of target Abs standards) when performing single or quadruple measurements. The results obtained with the quadruple platform further confirmed the absence of cross-reactivity between adjacent working electrodes. All these results confirmed the feasibility of performing the simultaneous determination of the four target Abs on the same bioplatform with the advantages that this entails.

The analytical and operational characteristics achieved with the quadruple bioplatform were evaluated. Semi-logarithmic calibration graphs constructed under the previously selected working conditions for

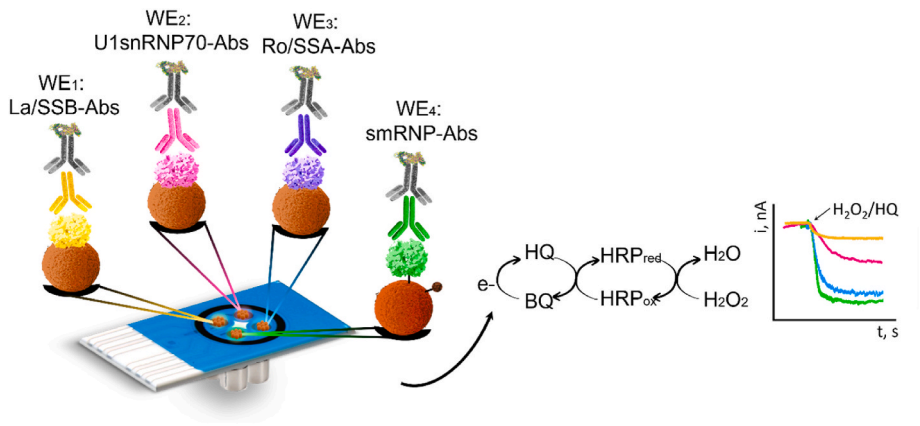

Fig. 1. Schematic diagram of the quadruple biosensing platform assisted by the use of MBs for the amperometric determination of La/SSB-Abs, U1snRNP70-Abs, Ro/SSA-Abs and smRNP-Abs.

the amperometric determination of standards of the four target Abs are displayed in Fig. 2, and the corresponding parameters summarized in Table 1.

The lack of bioelectroanalytical platforms reported in the literature for the determination of the target ENAs-Abs limits the comparison of the quadruple bioplatform analytical performance to that of claimed for ELISA kits commercialized for the single determination of these Abs. According to their specifications [https://www.4adi.com/product/pdf/3220-SSB-Human-anti-SS-B-IgG-ELISA-kit-manual.pdf] [https://www.4adi.com/product/pdf/3300-120-RNG-Human-Anti-RNP-70-IgG-ELISA-kit-manual.pdf] [https://www.4adi.com/product/pdf/3210-Anti-Sjogren-syndrome-typeA-antigen-ELISA-kit-manual.pdf][https://www.4adi.com/product/pdf/3300-110-SRG-Human-RNP-Sm-IgG-ELISA-kit-manual.pdf][http://img.creative-diagnostics.com/pdf/DEIA1688,SS-B.pdf] [https://www.creative-diagnostics.com/pdf/DEIA4147.pdf] [https://www.creative-diagnostics.com/pdf/DEIA1685.pdf], the ELISA kits achieve LODs of 1 U mL^{-1} for the four target Abs, which are between 100 and 20 times larger than those obtained with the quadruple bioplatform. It is important to remark that the linear ranges provided by the bioplatform for the determination of the four target Abs, comprising in most cases three orders of magnitude, are slightly wider but similar than those obtained with the commercial ELISA kits for their individual determination (1−200 U mL^{-1} according to the specifications of the commercial ELISA kits). Moreover, it should be noted that, although there is no considerable difference between the time involved in each determination, unlike ELISA kits, the bioplatform allows the simultaneous determination of the four Abs using affordable and applicable point-of-care instrumentation.

Table 1
Analytical features obtained when using the developed quadruple bioplatform for the simultaneous amperometric determination of the four target Abs.

	Target Abs			
Analytical characteristic	La/SSB	U1snRNP70	Ro/SSA	smRNP
Linear range, U mL^{-1}	0.05–50	0.1–100	0.1–100	0.2–100
Slope, nA per decade of concentration (U mL^{-1})	192 ± 3	108 ± 2	90 ± 2	66.5 ± 0.4
Intercept, nA	-1 ± 4	-23 ± 3	45 ± 2	42.0 ± 0.6
LOD[a], U mL^{-1}	0.01	0.03	0.04	0.05
LOQ**, U mL^{-1}	0.05	0.09	0.1	0.2
RSD, % (n = 5)	2.4 (10 U mL^{-1})	2.5 (25 U mL^{-1})	2.1 (25 U mL^{-1})	2.4 (25 U mL^{-1})

[a] 3 × s_b/m and **10 × s_b/m where s_b is the standard deviation of 10 blank measurements and m the calibration plot slope value.

It is also important to highlight that the achieved LOD values are significantly lower than the cut-off value established in the commercial

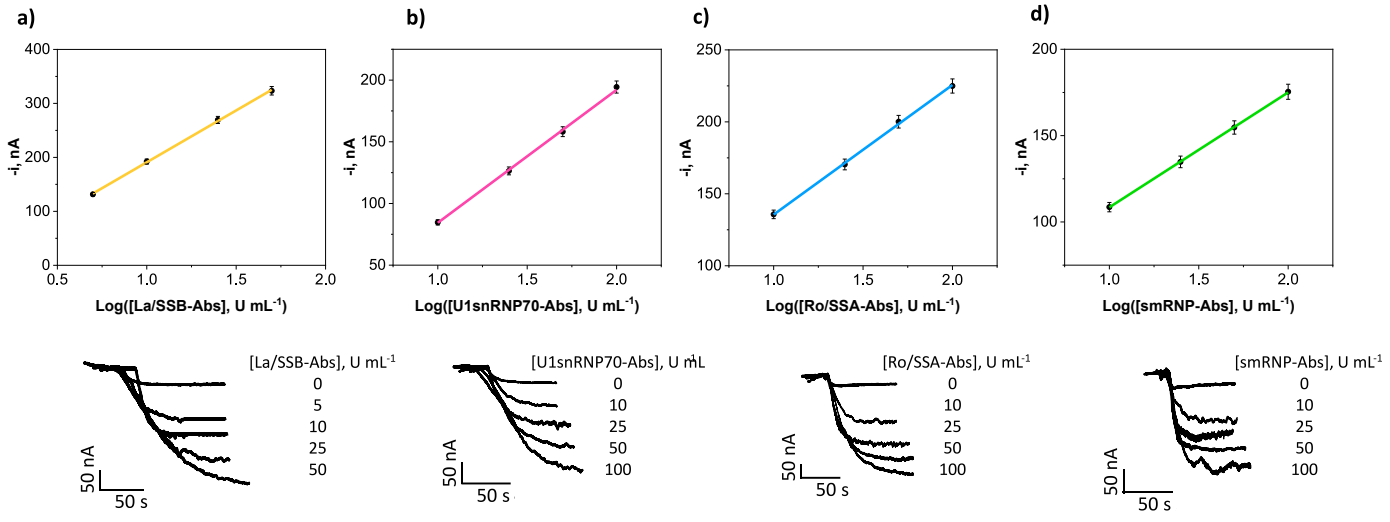

Fig. 2. Calibration graphs (top) and amperometric responses (bottom) obtained when using the quadruple bioplatform for measurements of standard solutions of La/SSB-Abs a), U1snRNP70-Abs b), Ro/SSA-Abs c) and smRNP-Abs d).

kits to consider a positive result probably related to a pathological state (25 U mL^{-1} for the four target Abs), thus allowing the developed bioplatform to be used for discrimination between negative and positive individuals to this type of Abs.

In addition, Table 1 shows as all the calculated RSD values are smaller than 3%. The values shown were obtained from the amperometric responses measured with 5 different quadruple bioplatforms for 10 U mL^{-1} La/SSB-Abs and 25 U mL^{-1} U1snRNP70-Abs, Ro/SSA-Abs and smRNP-Abs. Such RSD values confirmed the good reproducibility of the protocols used in the implementation of the bioassays on MBs and in the amperometric transduction at the SP_4CEs.

The storage stability of antigen-modified MBs kept at 4 °C in microcentrifuge tubes re-suspended in 50 μL 10 mM filtered PBS (pH 7.4) was evaluated. Fig. S6 (in the SI) shows the amperometric responses provided by the quadruple bioplatforms prepared each control day from the stored bioconjugates, in the absence and in the presence of the indicated concentration of target Abs. The obtained results confirmed the possibility of using the stored bioconjugates 35 days after their preparation without significant loss of sensitivity.

3.2. Selectivity

The behaviour of bioplatform was evaluated in the presence of other proteins and Abs which can coexist in the samples with the target Abs. The amperometric responses provided by the bioplatforms for 0 and 12.5 La/SSB-Abs, 50 U1snRNP70-Abs, 10 Ro/SSA-Abs and 25 smRNP-Abs U mL^{-1} standards were compared with those in the presence of the potential interferents at the expected concentrations in serum samples of healthy individuals.

Fig. S7 (in the SI) shows as only the presence of human IgG (hIgG) affected the determination of the four target Abs and human serum albumin (HSA) also affected that of smRNP-Abs. This behaviour was reported previously (Arévalo et al., 2020) (Arévalo et al., 2022a) (Arévalo et al., 2022b) (Muñoz-San Martín et al., 2022) and attributed to nonspecific adsorptions of hIgGs on the surface of MBs, which may also be persistent in the not sufficiently too purified HSA. Nevertheless, the interference practically disappeared for 100-fold smaller hIgG or HSA concentrations. Therefore, the analytical characteristics of the bioplatform for the determination of the four target Abs together with the established cut-off value in serum (25 U mL^{-1}), ensured the suitability of the bioplatform to analyze diluted samples where the concentrations of hIgGs and HSA do not affect the determination of the target Abs.

3.3. Diagnostic potential for the detection and discrimination of autoimmune diseases and versatility of the developed bioplatform

The bioplatform was applied to the determination of target Abs in serum samples, acquired from an online market (Central BioHub®), corresponding to four healthy individuals and four and six patients diagnosed with SLE and SS, respectively.

Previously, and to evaluate the accuracy of the results obtained with the bioplatform, the positive and negative controls (prepared in a serum/buffer matrix) provided in the commercial ELISA kits that supply the used immunoreagents were analyzed. Table S2 (in the SI) compares the obtained concentration values confirming the high accuracy of the results obtained with the bioplatform for the determination of the four autoantibodies.

The determination in serum implies a 1000-fold sample dilution. This dilution factor allowed the inclusion of the amperometric responses within the linear ranges of the calibrations obtained with standards (Fig. 2a) and avoided any matrix effect (Table S3 in the SI). Therefore, the endogenous content of the four target Abs in serum was determined by simple interpolation of the amperometric responses recorded for the diluted samples into such calibrations. The obtained results are depicted in Fig. 3a and summarized in Table 2. In addition, the results obtained by applying the individual ELISA methodologies for the determination of the target Abs are included in Table 2. Fig. 3b and Table S4 (in the SI) show the correlation between the results provided by the quadruple bioplatform and the ELISA method. As it can be deduced, no significant differences between both methods were apparent.

As expected, Fig. 3a shows as significantly larger concentrations of the four target Abs were observed for patients with autoimmune diseases compared to healthy individuals. Moreover, all the SLE and SS patients exhibited larger contents than the cut-off value established in serum (25

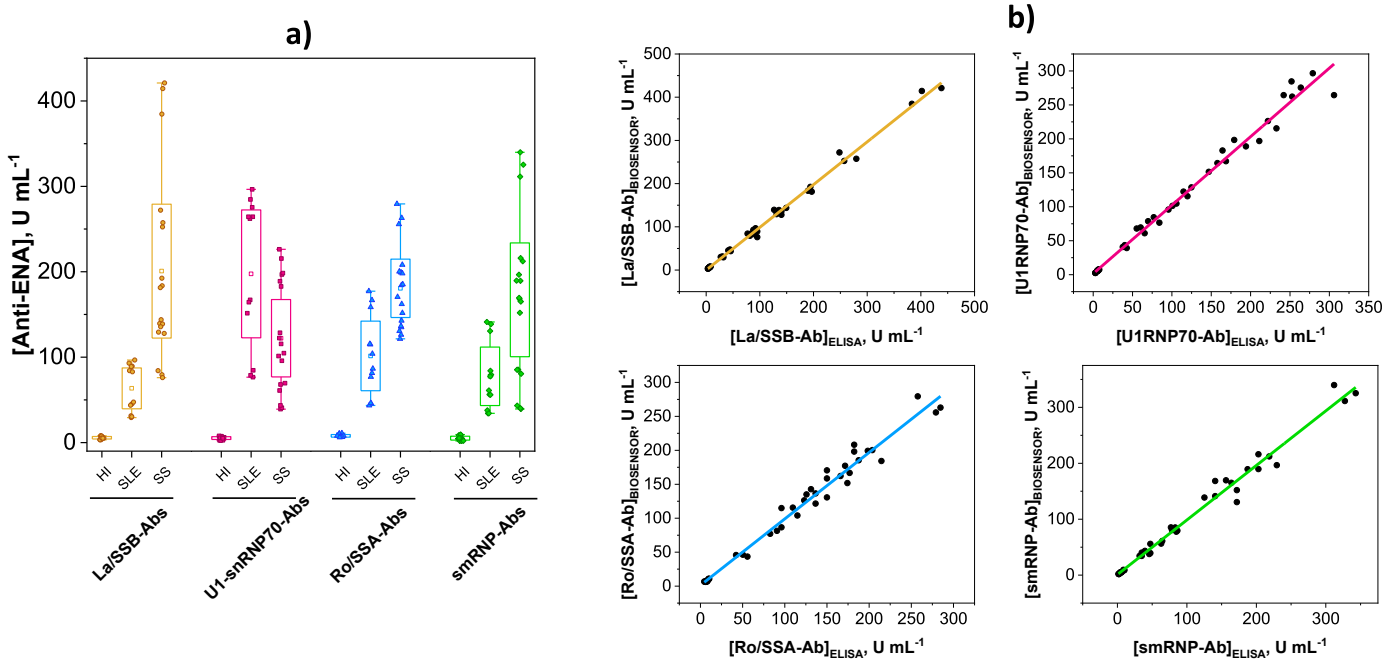

Fig. 3. Target Abs concentrations obtained with the quadruple bioplatform for serum samples grouped into pools of healthy individuals (4) and SLE (4) and SS patients (6) (a). Correlation plots between the concentrations obtained with the bioplatform and the ELISA methods available for their individual determination (b). All graphs include the values of the three replicates performed for each determination.

Table 2
Endogenous concentrations (in U mL^{-1}, mean value ± ts/$\sqrt{n}$; n = 3; α = 0.05) of the target autoantibodies obtained using the developed quadruple bioplatform as well as using the individual ELISA methodologies for serum samples of healthy individuals and patients diagnosed with SS and SLE.

		Bioplatform				ELISA			
		La/SSB-Abs	U1snRNP70-Abs	Ro/SSA-Abs	smRNP-Abs	La/SSB-Abs	U1snRNP70-Abs	Ro/SSA-Abs	smRNP-Abs
Healthy	1	6.4 ± 0.4	5.8 ± 0.6	10.6 ± 0.6	4.7 ± 0.4	6 ± 2	5.7 ± 0.8	10 ± 1	4.4 ± 0.9
	2	7.8 ± 0.5	7.5 ± 0.8	6.8 ± 0.5	9 ± 1	8 ± 2	7 ± 2	6.6 ± 0.9	9 ± 1
	3	3.3 ± 0.2	2.6 ± 0.7	7.9 ± 0.4	2 ± 0.3	3.5 ± 0.7	2.8 ± 0.4	8.1 ± 0.7	2.0 ± 0.5
	4	5.2 ± 0.3	5.3 ± 0.7	6.4 ± 0.4	5.2 ± 0.7	5 ± 1	5 ± 1	6.1 ± 0.9	6 ± 1
SLE	5	45 ± 5	80 ± 10	45 ± 4	58 ± 7	44 ± 6	77 ± 18	49 ± 9	58 ± 8
	6	93 ± 9	267 ± 18	82 ± 12	137 ± 14	92 ± 9	253 ± 27	90 ± 17	146 ± 36
	7	85 ± 8	282 ± 40	168 ± 12	35 ± 5	88 ± 10	279 ± 67	166 ± 35	38 ± 9
	8	30 ± 2	161 ± 21	111 ± 16	80 ± 8	30 ± 6	158 ± 27	107 ± 17	83 ± 10
SS	9[a]	80 ± 10	41 ± 5	266 ± 30	41 ± 5	85 ± 23	40 ± 6	274 ± 35	41 ± 8
	10	132 ± 16	122 ± 16	128 ± 17	84 ± 7	133 ± 17	120 ± 12	128 ± 18	80 ± 8
	11	261 ± 25	213 ± 37	161 ± 23	162 ± 22	262 ± 40	222 ± 27	163 ± 31	159 ± 28
	12	139 ± 10	66 ± 11	137 ± 15	183 ± 29	142 ± 17	60 ± 12	139 ± 24	182 ± 27
	13	407 ± 48	190 ± 19	197 ± 30	326 ± 35	409 ± 57	179 ± 37	198 ± 40	327 ± 39
	14	186 ± 14	101 ± 11	194 ± 20	208 ± 26	200 ± 36	101 ± 12	191 ± 28	217 ± 33

[a] According to Central BioHub specifications the concentrations of La/SSB-Abs and Ro/SSA-Abs in these samples are 76.0 and > 200 U mL^{-1}, respectively.

U mL^{-1} for all target Abs) by commercial ELISA kits.

The potential of the bioplatform and the selected Abs signature for the diagnosis and discrimination of the autoimmune diseases was checked by constructing ROC curves from the bioplatform results summarized in Table 2. Fig. S8 (in the SI) displays the resulting ROC curves with the corresponding parameters summarized in Table 3. These parameters show that the bioplatform allowed a full discrimination between healthy individuals and SS or SLE patients (AUC, specificity, and sensitivity 100% for each of the autoantibodies). In contrast, none of the single autoantibodies was able to discriminate SS from SLE patients, but with specificities above 66.7% and AUCs above 75%. However, in combination, the four autoantibodies against La/SSB, U1snRNP70, Ro/SSA and smRNP showed full ability to discriminate either healthy *vs.* SS or SLE individuals, or SS *vs.* SLE individuals. It is important to note that, to our knowledge, there is not any reported technology able to discriminate patients with SLE and SS. Indeed, the ELISA methodology routinely employed for the determination of single target Abs, is only claimed to discriminate healthy individuals from patients with autoimmune diseases, not for the differential diagnosis of the latter, which is considered particularly relevant for the personalized and efficient diagnosis of each patient.

The results in Table 3 also agreed with the presence of La/SSB-Abs and/or Ro/SSA-Abs being considered a hallmark of SS and SLE (Agmon-Levin et al., 2017), these 2 target Abs being the ones for which the best individual ROC curve parameters are obtained.

In addition, the analysis of the quantitative 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 3 for each target Abs to discriminate patients with SLE or SE from healthy subjects or what is even more challenging between SS and SLE patients.

Moreover, the results of the experiments shown and discussed at the end of the SI (Figs. S9 and S10) are a proof of the bioplatform's versatility for the on-demand determination of 4–8 Abs (total Igs or individual isotypes) by simply changing the antigen immobilized on the MBs, the secondary antibody for the enzymatic labelling and the type of disposable electrochemical platform.

4. Conclusions

To assist in the early diagnosis and discrimination of autoimmune diseases, we report in this work the first electrochemical bioplatform for the multideterminación of Abs against four specific ENAs: La/SSB, U1snRNP70, Ro/SSA and smRNP. The bioplatform exploits the use of magnetic microcarriers modified with the corresponding antigens by means of His-tag or covalent EDC/NHS immobilization chemistry and amperometric transduction on quadruple disposable platforms.

The developed method exhibits an analytical performance in terms of sensitivity, selectivity, simplicity and quick response compatible with its clinical applicability for the determination of the four target antibodies in both centralized and point-of-care settings, and has been tested in a selected cohort of individuals including healthy subjects and patients diagnosed with SLE and SS. The obtained results demonstrate the potential of the bioplatform as well as of the biomarker signature selected for the differential diagnosis of these two autoimmune diseases of high and increasing prevalence, establishing cut-off values in serum for each of the targets Abs. Moreover, the versatility of the developed bioplatform for the determination of autoantibodies against other antigens (both total content of Igs or individual isotypes) was proved. These capabilities hold tremendous promise for demonstrating the diagnostic value of IgGs against certain antigens to assess the contribution of less studied isotypes, such as IgAs, and to identify characteristics molecular signatures that contribute to the early, reliable and minimally invasive diagnosis of autoimmune diseases.

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Table 3
Potential of the four target autoantibodies (1: La/SSB-Abs, 2: U1snRNP70-Abs, 3: Ro/SSA-Abs and 4: smRNP-Abs) considered individually or in combination to discriminate autoimmune diseases and serum cut-off values.

	Healthy *vs.* SS					Healthy *vs.* SLE					SS *vs.* SLE				
Parameter	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	91.7	75	87.5	83.3	100
Sensitivity	100	100	100	100	100	100	100	100	100	100	100	50	75	100	100
Specificity	100	100	100	100	100	100	100	100	100	100	83.3	100	100	66.7	100
Cut-off (U mL^{-1})	43.9	24.25	69.3	25.0	–	18.9	43.75	27.8	22.0	–	112.5	240.0	119.5	149.5	–

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

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CRediT authorship contribution statement

Beatriz Arévalo: Methodology, Investigation, Writing – review & editing, Writing – original draft. **Verónica Serafín:** Methodology, Investigation, Writing – review & editing, Writing – original draft. **María Garranzo-Asensio:** Investigation, Writing – review & editing, Writing – original draft. **Rodrigo Barderas:** Writing – review & editing, Writing – original draft, Funding acquisition. **Paloma Yáñez-Sedeño:** Conceptualization, Supervision, Resources, Writing – review & editing, Writing – original draft, Funding acquisition. **Susana Campuzano:** Conceptualization, Supervision, Resources, Writing – review & editing, Writing – original draft, Funding acquisition. **José M. Pingarrón:** Supervision, Resources, Writing – review & editing, Writing – original draft.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biosx.2023.100325.

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