SUPPORTING INFORMATION

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

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MATERIALS AND METHODS

Apparatus and electrodes

A model CHI812B potentiostat controlled by the CHI812B software was used for the electrochemical measurements and a Sunrise™ Tecan microplate reader with Magellan V 7.1 software was employed for the spectrophotometric measurements (ELISA methodology).

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 the corresponding specific cable connector (DRP-CAC), quadruple screen-printed carbon electrodes (SP_4CEs) with four carbon working electrodes (DRP-4W110, 2.95-mm Ø) and shared carbon auxiliary and Ag pseudo-reference electrodes and the specific cable connector (DRP-CONNECT4W) and octuple screen-printed carbon electrodes (SP_8CEs) with eight carbon working electrodes (DRP-8W110, 2.56-mm Ø) and the specific cable connector (CAC8X) were purchased from Metrohm-DropSens S.L. and used to perform all amperometric measurements.

Homemade polymethylmethacrylate (PMMA) casings with one or four Nd magnets (AIMAN GZ) embedded and a Teflon commercial one with eight Nd magnets embedded (MAGNET8X purchased from Metrohm-DropSens S.L.) were used to ensure stable and reproducible capture of the magnetic bioconjugates on the surface of the SPCEs, SP_4CEs and SP_8CEs working electrodes, respectively. Homemade glass electrochemical cells of 10 or 20 mL were also used.

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

Reagents and solutions

Dynabeads™ His-Tag Isolation and Pulldown-modified (His-tag-isolation MBs) (Cat. No.: 10103D, 1 µm Ø, 40 mg mL^{-1}) and Carboxylic Acid-modified (HOOC-MBs) (Cat. No.: M-270, 2.8 µm Ø, ~ $2 \cdot 10^9$ beads mL^{-1}) magnetic beads were purchased from Invitrogen™ Thermo Fisher Scientific. N-hydroxysulfosuccinimide (Sulfo-NHS), N-(3-dimethyl-aminopropyl)-N'-ethyl-carbodiimide (EDC), ethanolamine, hydroquinone (HQ) and hydrogen peroxide (H_2O_2, 30 % v/v) were acquired from Sigma-Aldrich.

Human La/SSB (La/SSB, 6His-tagged, Cat. No.: DAG264), Recombinant Human Small Nuclear Ribonucleoprotein 70kDa (U1) (U1-snRNP or SNRNP70, 6His-tagged, Cat. No.:

DAG604) and Recombinant Human TROVE Domain Family, Member 2 (TROVE2 or Ro/SS-A, 6His-tagged, Cat. No.: DAG624), RNP/Sm Antigen (smRNP, Cat. No.: DAG-T1223), SS-B(La) Antibody IgG ELISA kit (Cat. No.: DEIA1688), RNP 70 Ab ELISA Kit (Cat. No.: DEIA4147), SS-A (Ro) Antibody IgG ELISA kit (Cat. No.: DEIA1685) and RNP/Sm Antibody IgG ELISA Kit (Cat. No.: DEIA1690) were all purchased from Creative Diagnostics. These kits contain all calibrators, negative and positive controls (prepared in serum/buffer matrix), an enzyme conjugate solution containing rabbit anti-human polyclonal IgG labelled with horseradish peroxidase (HRP-antihIgG) and the washing buffer (WB, 50× or 10×) used.

Anti-Human IgA antibody HRP conjugated (Catalog # 109-035-011, HRP-anti-h-IgA), anti-Human IgM antibody HRP conjugated (Catalog # 109-035-043, HRP-anti-h-IgM) from Jackson Immunoresearch (Barcelona) and a mixture of polyclonal rabbit anti-human IgG, anti-human IgM and anti-human IgA antibodies labelled with horseradish peroxidase (HRP-anti-human IgG/ IgM/IgA mixture), provided in the dsDNA antibody ELISA Kit purchased from Creative Diagnostics (Cat. No. DEIA 1681), were also used.

IgG from human serum (hIgG, Cat. No.: I2511), albumin from human serum (HSA, Cat. No: A1653) and human hemoglobin (HB, Cat. No.: H7379) from Sigma-Aldrich, bovine serum albumin (BSA, Cat. No: 90604-29-8) from Gerbu Biotechnik, GmbH, AQP4-Abs (Cat. No: CSB-E13568h) from Cusabio, dsDNA-Abs (Cat. No: DEIA 1681) and CENPB-Abs (Cat. No: DEIA 1684) from Creative Diagnostics and BAFF (Cat. No: DY124-05) and APRIL (Cat. No: DY884B) from R&D Systems were used as standards in the selectivity studies. Sodium chloride, potassium chloride, sodium di-hydrogen phosphate, disodium hydrogen phosphate, 2-(N-morpholino)ethanesulfonic acid (MES) and Tris-hydroxymethyl-aminomethane-HCl (Tris-HCl) were provided from Scharlab. Commercial Blocker™ Casein in PBS (blocking buffer, BB) was acquired from Thermo Scientific (Cat. No.: 37528).

Purified water using the Milli-Q purification system (18.2 MΩ) was used to prepare all the buffer solutions: 10 mM phosphate buffer saline (PBS) pH 7.4, 25 mM MES buffer (MES) pH 5.0, 100 mM phosphate buffer solution (PB) pH 8.0, 100 mM Tris-HCl (Tris-HCl) pH 7.5 and 50 mM phosphate buffer solution (PB) pH 6.0.

Other solutions employed include: an EDC/Sulfo-NHS mixture solution (50 mg mL^{-1} each) prepared in MES buffer pH 5.0, and a 1.0 M ethanolamine solution prepared in 100 mM PB buffer pH 8.0 for HOOC-MB activation and blocking, respectively, and 100 mM HQ and 100 mM H_2O_2 solutions, both prepared in 50 mM PB pH 6.0, for amperometric detection.

Serum samples from healthy individuals and from patients diagnosed with SLE and SS were purchased from an online marketplace of human biospecimens (Central BioHub®) and stored (following supplier's recommendations) at −40 °C until use.

Procedures

Unless otherwise indicated, the protocols for the preparation of magnetic bioconjugates were performed in 1.5 mL microcentrifuge tubes, using 25 and 50 µL of the corresponding incubation and washing solutions, respectively. All incubation steps were performed at 25 °C under constant stirring (950 rpm). After each incubation step, the microcentrifuge tubes with the MBs suspension were placed in the magnetic concentrator for 2 min before removing the solution.

Implementation of indirect immunoassays on magnetic microsupports

The antigens were immobilized on the surface of commercial MBs using His-tag chemistry. In the case of not commercial availability of His-tagged antigen (smRNP), it was covalently immobilized through EDC/Sulfo-NHS chemistry, according to the following protocols:

For the immobilization of His-tagged antigens (La/SSB, Ro/SSA and U1snRNP70), the commercial His-tag isolation MBs suspension (2 µL) was washed twice with 10 mM PBS buffer pH 7.4, incubated for 15 min with the antigen solution (2.5, 5 or 10 µg mL^{-1} of Ro/SSA, U1snRNP70 or La/SSB antigen, respectively) prepared in 10 mM PBS buffer pH 7.4, and then washed twice with WB buffer.

For the non-His-tagged smRNP antigen immobilization, 3 µL of the commercial HOOC-MBs suspension were washed twice with MES buffer (10 min each at 950 rpm), and the carboxyl groups activated by incubation for 35 min in the 50 mg mL^{-1} EDC/Sulfo-NHS mixed solution. Subsequently, the activated MBs were incubated for 15 min in a solution of 10 µg mL^{-1} smRNP prepared in MES and the remaining active groups on the smRNP-MBs were blocked by incubating for 60 min in 1.0 M ethanolamine solution and washed twice with 10 mM PBS buffer pH 7.4.

To perform the determination, the antigen-MBs were incubated in the solution of the Abs standard or the serum sample prepared in BB for 15 min to selectively capture the target Abs. After washing twice with BB, Abs-antigen-MBs were enzymatically labelled by incubation for 30 min in an HRP-antihIgG solution. The resulting magnetic bioconjugates were washed twice with BB and resuspended in 50, 5 or 22.5 µL of 50 mM PB buffer pH 6.0 to perform single, quadruple or octuple amperometric transduction, respectively.

For the determination of Abs against dsDNA, the bioconjugates were prepared as described previously (Arévalo et al., 2020).

Amperometric detection

The single and quadruple amperometric determinations were carried out in stirred solutions while octuple measurements were made using drops of quiescent solution. In both cases a potential of −0.20 V *vs.* Ag pseudo-reference electrode was applied.

The appropriate electrode (SPCE or SP_4CE) was placed in the corresponding PMMA housing with the embedded neodymium magnets to magnetically capture the suspension of the corresponding bioconjugates (HRP-antihIgG-Abs-antigen-MBs) on the WE surface of SPCE or SP_4CE (WE_1: La/SSB-Abs, WE_2: U1snRNP70-Abs, WE_3: Ro/SSA-Abs, WE_4: smRNP-Abs). The magnetic capture of each batch of bioconjugates on the corresponding working electrode was individually done with a micropipette (the spacing between the four SP_4CEs working electrodes allows it easily). Moreover, the appropriate distribution the four Nd magnets in the PMMA housing ensured that whatever was deposited on each working electrode remained on it. Then, the casing/electrode assembly with the captured magnetic bioconjugates was immersed into an electrochemical cell containing 10 or 20 mL of 50 mM PB buffer pH 6.0 supplemented with 100 or 200 µL of freshly prepared 100 mM HQ in the same buffer for single or simultaneous quadruple measurements, respectively.

When the background current was stabilized (approximately 100 s), 50 or 100 µL of the freshly prepared 100 mM H_2O_2 solution in 50 mM PB buffer pH 6.0 were added to the measuring cell for single or quadruple measurements, respectively, and the variation/s of the cathodic current/s produced by the HQ-mediated enzymatic reduction of the H_2O_2 was/were monitored until the steady state was reached. Amperometric responses were calculated as the difference between the current values before and after the addition of H_2O_2 and data given throughout the manuscript correspond to the mean values of three replicates with error bars estimated as three times the standard deviation of each set of replicates ($\alpha = 0.05$).

To perform the octuple detection, the SP_8CE was placed on the corresponding magnetic holder and 22.5 µL of the respective magnetic bioconjugate suspension (in 0.05 M phosphate buffer pH 6.0 containing 1.0 mM HQ) were deposited to cover the three electrodes of each of the 8 electrochemical cells. Once the background current was stabilized, 2.5 µL of freshly prepared 0.1 M H_2O_2 solution in the same buffer were added to the quiescent solution drop and the current variation was monitored until stabilization.

Analysis of serum samples

The developed bioplatform was applied to the determination of the target Abs in serum samples of healthy individuals as well as of patients diagnosed with SLE and SS purchased from Central BioHub®.

Once the absence of matrix effect was statistically confirmed for 1,000-fold diluted samples, the determination of the endogenous content of the four target Abs was performed by simple interpolation of the amperometric signals recorded for the diluted samples into each calibration plot constructed with the corresponding Abs standard solutions. To verify the accuracy of the results provided by the bioplatform, the same samples were analyzed by ELISA using the same immunoreagents.

ROC curves (Receiver Operating Characteristic Curve) of individual autoantibodies and in combination were obtained with R (version 3.6.2), using the "ModelGood" and the "Epi" packages to determine the diagnostic ability of the test (Torrente-Rodríguez et al., 2022). Cut-off values for separation of the indicated groups were calculated with GraphPad Prism 8 program (Torrente-Rodríguez et al, 2022).

RESULTS AND DISCUSION

Optimization of the experimental variables involved in the preparation of the biosensor

Larger ratios between the amperometric responses obtained in the presence (S) and in the absence (B) of target Abs standards were taken as the selection criterion. To obtain the S responses, the concentrations of standards tested were 12.5 U mL^{-1} La/SSB-Abs, 5 U mL^{-1} U1snRNP70-Abs and 10 U mL^{-1} of Ro/SSA-Abs and smRNP-Abs. The obtained results are shown in **Figs. S1-S4** and summarized in **Table S1**.

First, the volume of the HisTag-isolation MBs commercial suspension to prepare the bioconjugates involved in the La/SSB-Abs determination was optimized in the range 0.5-5.0 μL. According to the results displayed in **Fig. S1a**, a larger S/B ratio was reached by employing 2.0 μL. Since there was no significant difference between the B signals in the whole tested range, the decrease observed in the specific responses when working with volumes larger than 2.0 μL can be attributed to a worse efficiency of the immunorecognition processes when large amounts of MBs are used rather than to the non-conducting nature of the MBs. Considering also previous optimization for HOOC-MBs (Esteban-Fernández de Ávila et al., 2013), bioconjugates prepared from 2.0 and 3.0 μL of commercial suspensions of HisTag isolation-MBs and HOOC-MBs, respectively, were employed for each determination.

Regarding the optimal concentrations of immobilized antigen, although the optimal values were different for each of them, similar trends were observed (**Figs. S1b**, **S2a**, **S3a** and **S4a**). While the nonspecific responses did not change significantly over the whole tested range, the specific responses increased with the concentration of immobilized antigen up to a certain value and then decreased drastically, which can be attributed to a less efficient recognition of the target Abs due to steric hindrance (Arévalo et al., 2022). It is important to note that it was not possible to detect the presence of any of the target Abs (S/B~1) when no antigen was immobilized on the MBs (bars 0 in **Figs. S1b**, **S2a**, **S3a** and **S4a**), indicating the absence of nonspecific adsorption of both target Abs and HRP-antihIgGs under these conditions and that the capture of target Abs was produced only by selective recognition of the immobilized antigen on the MBs.

It is important to note that for the rest of the tested variables: incubation time for antigen immobilization and for the capture of the target Abs (15 min), volume of the secondary antibody solution (25 μL) and incubation time (30 min) for the enzymatic labelling of the Abs captured on the MBs, the same optimal values were found for the determination of the four target

antibodies, which facilitates the simultaneous preparation of the four types of bioconjugates. In addition, Figs S1-S4 show that, in general, the B responses increased and the S responses decreased drastically when long incubation times or large HRP-antihIgG concentrations were used. This behavior can be attributed to the increase of non-specific adsorptions and to possible aggregation phenomena under these conditions, which is detrimental to the efficiency of the immunorecognition reactions.

Furthermore, in agreement with previous results (Arévalo et al., 2022), the S/B ratio was markedly better when the capture of the target Abs and their enzymatic labelling were performed in separate steps (**Figs. S1d**, **S2c**, **S3c** and **S4c**), which may be attributed to the lower capture efficiency of the target Abs when labelled with the secondary antibody.

The results obtained for the optimization of all these variables allow us to conclude that, starting from the MBs modified with the corresponding antigens, the quadruple determination of the four types of Abs involves only 2 incubation steps and can be completed in 45 min.

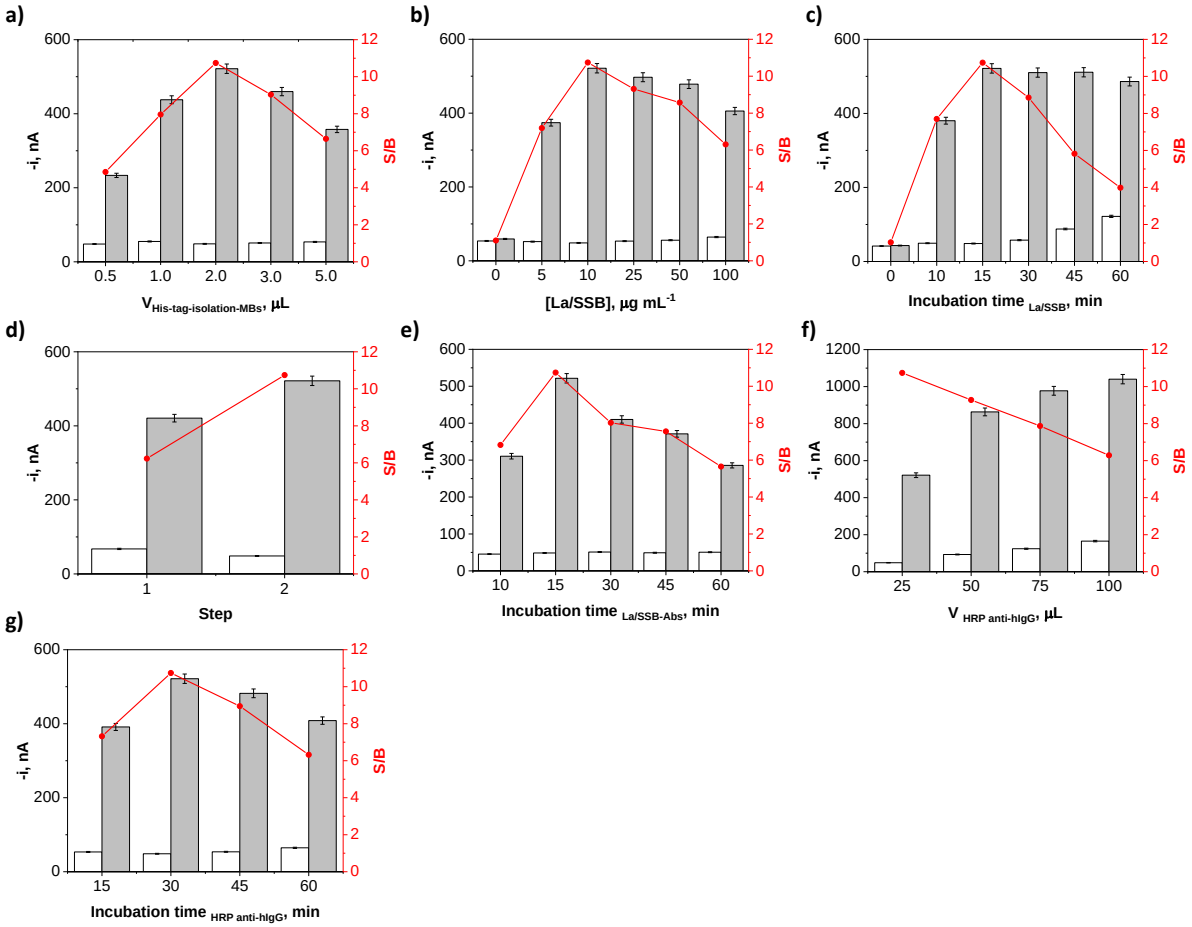

Fig. S1. Dependence of the amperometric responses obtained individually in the absence (blank, B; white bars) and in the presence of 12. 5 U mL^{-1} La/SSB-Abs standard solutions (signal, S; grey bars) with the bioplatforms constructed using bioconjugates prepared by varying the volume of the His-tag-isolation MBs suspension a), the La/SSB concentration b) and the incubation time c) for its immobilization through the histidine tag on the His-tag isolation MBs, the number of steps involved in the capture and enzymatic labelling of La/SSB-Abs on La/SSB-MBs d), the incubation time of La/SSB-MBs with La/SSB-Abs standard solution for their capture e) and the volume f) and incubation time g) with the HRP-antihIgG solution for the enzymatic labelling of La/SSB-Abs attached to the MBs. The values of S/B ratios are shown with circles and red lines.

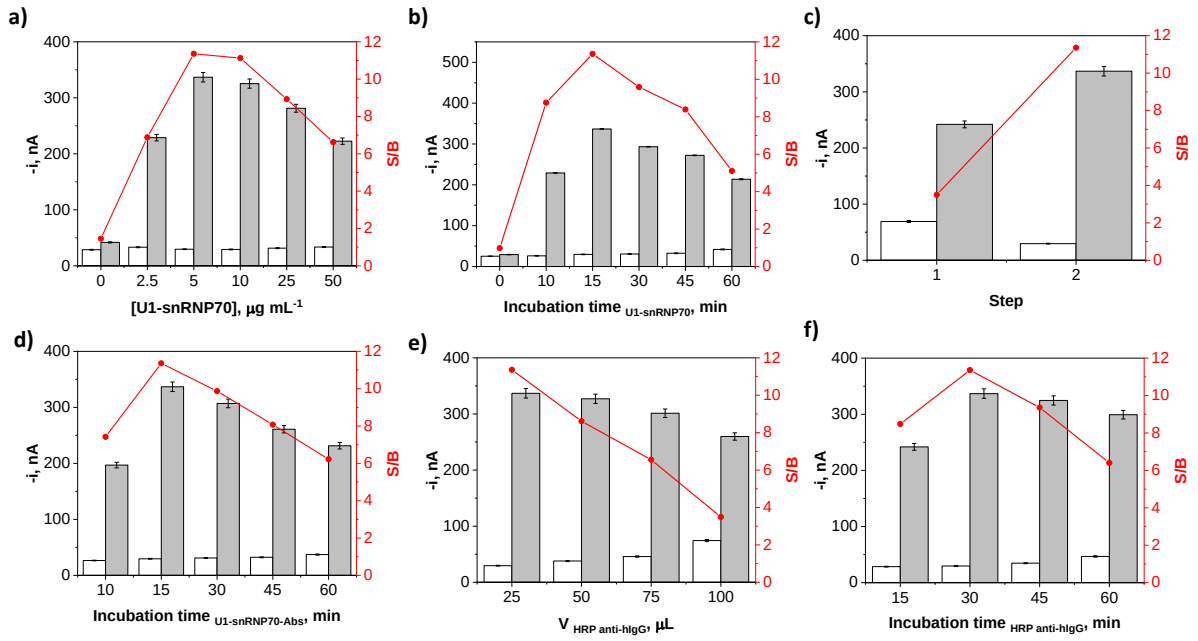

Fig. S2. Dependence of the amperometric responses obtained individually in the absence (blank, B; white bars) and in the presence of 5 U mL^{-1} U1snRNP70-Abs standard solutions (signal, S; grey bars) with the bioplatforms constructed using bioconjugates prepared by varying the U1snRNP70 concentration a) and the incubation time b) for its immobilization through the histidine tag on the His-tag isolation MBs, the number of steps involved in the capture and enzymatic labelling of U1snRNP70-Abs on U1snRNP70-MBs c), the incubation time of U1snRNP70-MBs with U1snRNP70-Abs standard solution for their capture e) and the volume f) and incubation time g) with the HRP-antihIgG solution for the enzymatic labelling of U1snRNP70-Abs attached to the MBs. The values of S/B ratios are shown with circles and red lines.

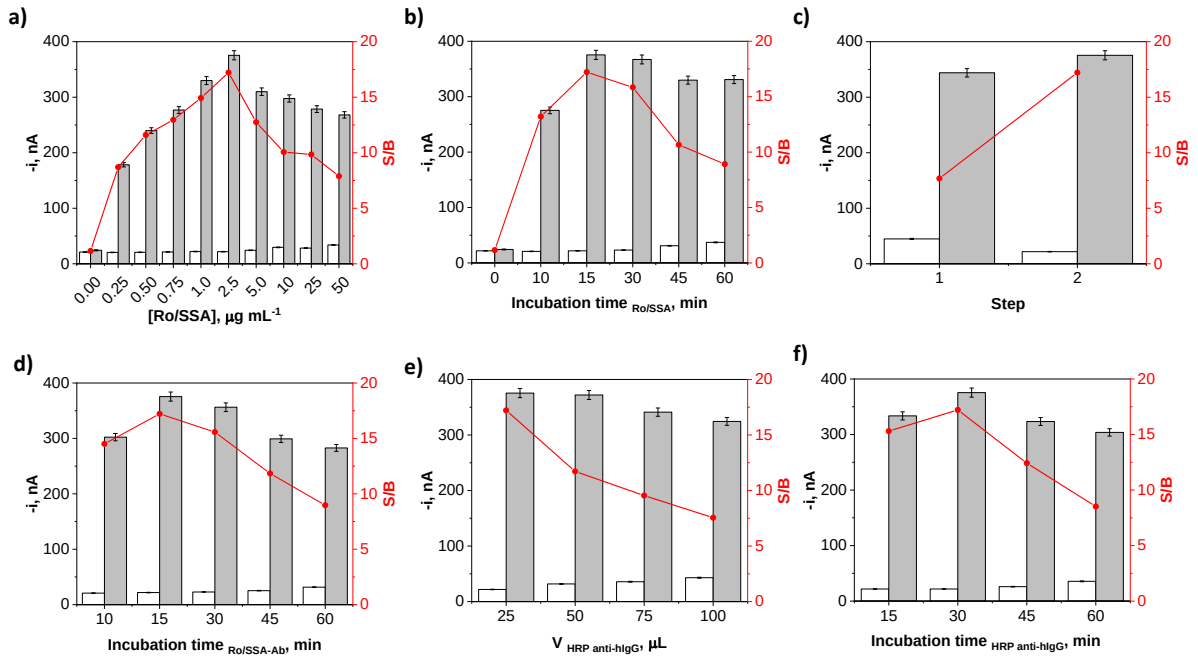

Fig. S3. Dependence of the amperometric responses obtained individually in the absence (blank, B; white bars) and in the presence of 10 U mL^{-1} Ro/SSA-Abs standard solutions (signal, S; grey bars) with the bioplatforms constructed using bioconjugates prepared by varying the Ro/SSA concentration a) and the incubation time b) for its immobilization through the histidine tag on the His-tag isolation MBs, the number of steps involved in the capture and enzymatic labelling of Ro/SSA-Abs on Ro/SSA-MBs c), the incubation time of Ro/SSA-MBs with Ro/SSA-Abs standard solution for their capture e) and the volume f) and incubation time g) with the HRP-antihIgG solution for the enzymatic labelling of Ro/SSA-Abs attached to the MBs. The values of S/B ratios are shown with circles and red lines.

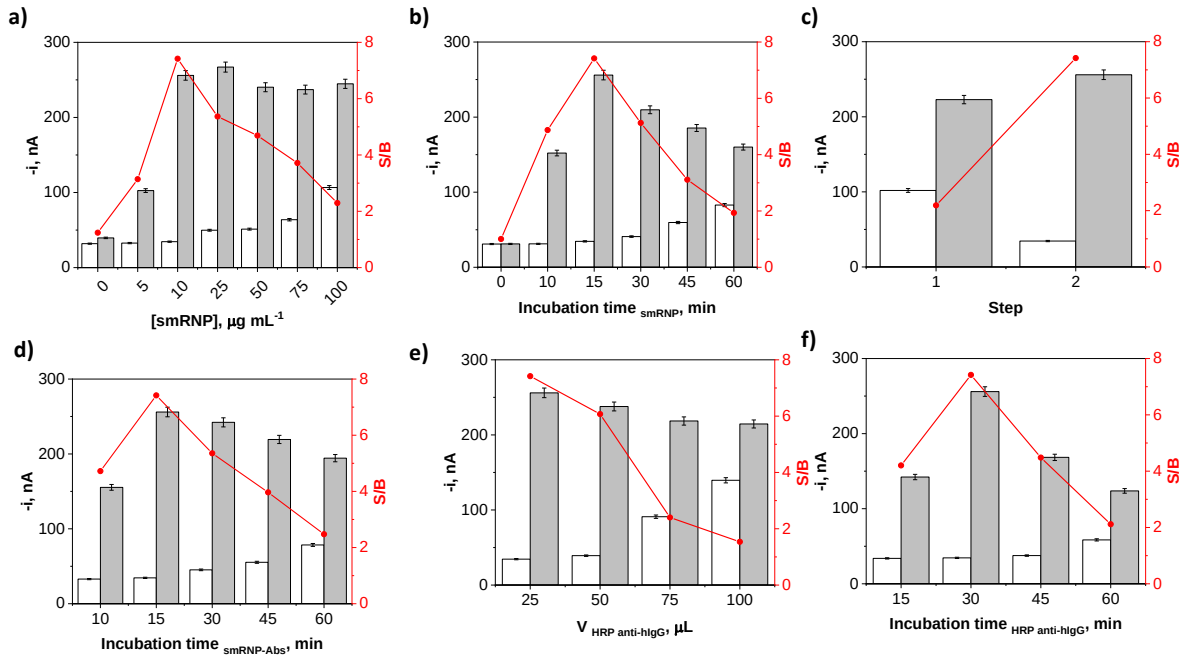

Fig. S4. Dependence of the amperometric responses obtained individually in the absence (blank, B; white bars) and in the presence of 10 U mL^{-1} smRNP-Abs standard solutions (signal, S; grey bars) with bioplatforms constructed using bioconjugates prepared by varying the smRNP concentration a) and the incubation time b) for its covalent immobilization using EDC/Sulfo-NHS on HOOC-MBs, the number of steps involved in the capture and enzymatic labelling of smRNP-Abs on smRNP-MBs c), the incubation time of smRNP-MBs with smRNP-Abs standard solution for their capture e) and the volume f) and incubation time g) with the HRP-antihIgG solution for the enzymatic labelling of smRNP-Abs attached to the MBs. The values of S/B ratios are shown with circles and red lines.

Table S1. Optimization of key experimental variables involved in the preparation of magnetic bioconjugates for the amperometric determination of each target Abs.

Variable		Target Abs	Results displayed in Figure	Tested range	Selected value
MBs	HisTag-isolation MBs	La/SSB	S1a	0.5 – 5.0	2.0
		U1snRNP70	--	--	
		Ro/SSA	--	--	
	HOOC-MBs	smRNP	--	--	3.0
[Antigen], µg mL^{-1}		La/SSB	S1b	0 – 100	10

	U1snRNP70	S2a	0 – 50	5
	Ro/SSA	S3a	0 – 50	2.5
	smRNP	S4a	0 – 100	10
Antigen incubation time, min	La/SSB	S1c	0 – 60	15
	U1snRNP70	S2b		
	Ro/SSA	S3b		
	smRNP	S4b		
Number of steps involved for the capture and enzymatic labelling of target Abs	La/SSB	S1d	1 or 2	2
	U1snRNP70	S2c		
	Ro/SSA	S3c		
	smRNP	S4c		
Abs standard incubation time, min	La/SSB	S1e	10 – 60	15
	U1snRNP70	S2d		
	Ro/SSA	S3d		
	smRNP	S4d		
Volume HRP-antihIgG, µL	La/SSB	S1f	25 – 100	25
	U1snRNP70	S2e		
	Ro/SSA	S3e		
	smRNP	S4e		
Incubation time with HRP-antihIgG solution, min	La/SSB	S1g	15 – 60	30
	U1snRNP70	S2f		
	Ro/SSA	S3ef		
	smRNP	S4ef		

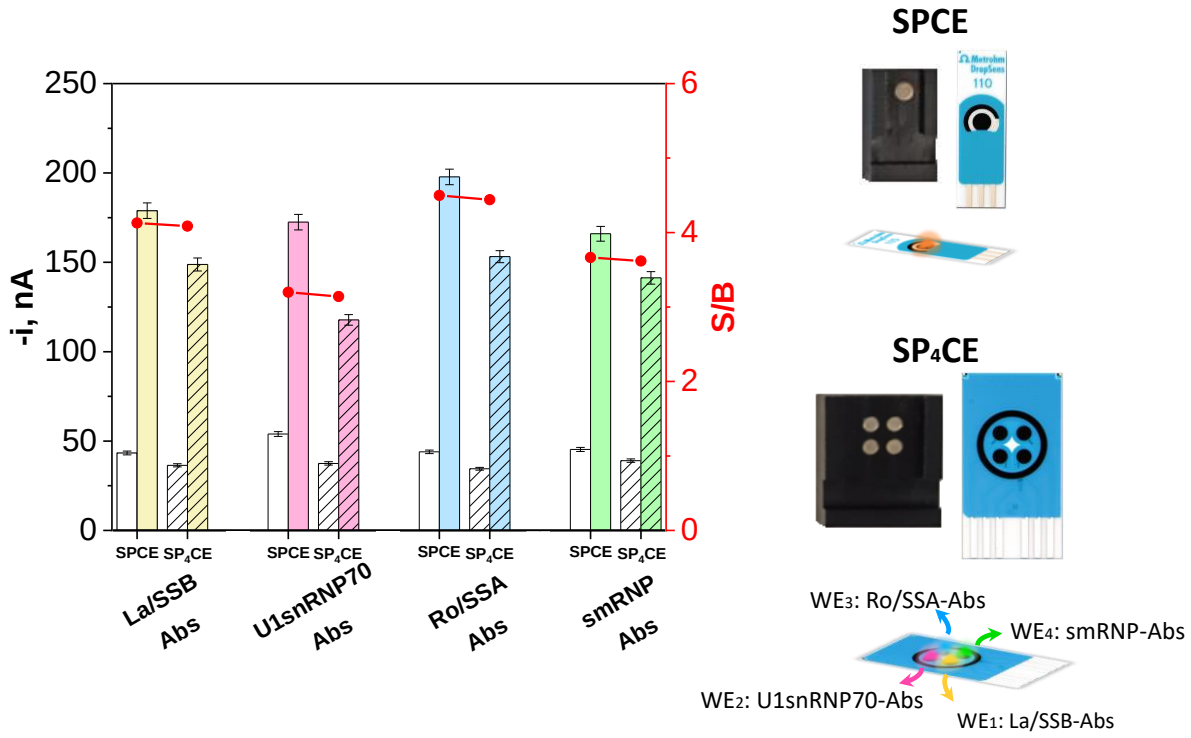

Fig. S5. Comparison of the amperometric responses obtained for 0 (white bars) and 25 U mL^{-1} La/SSB-Abs (yellow bars), U1snRNP70-Abs (pink bars), Ro/SSA-Abs (blue bars) or smRNP-Abs (green bars) standards solutions with bioplataforms constructed for the single (SPCEs, unstripped bars) or quadruple (SP_4CEs, stripped bars) measurements.

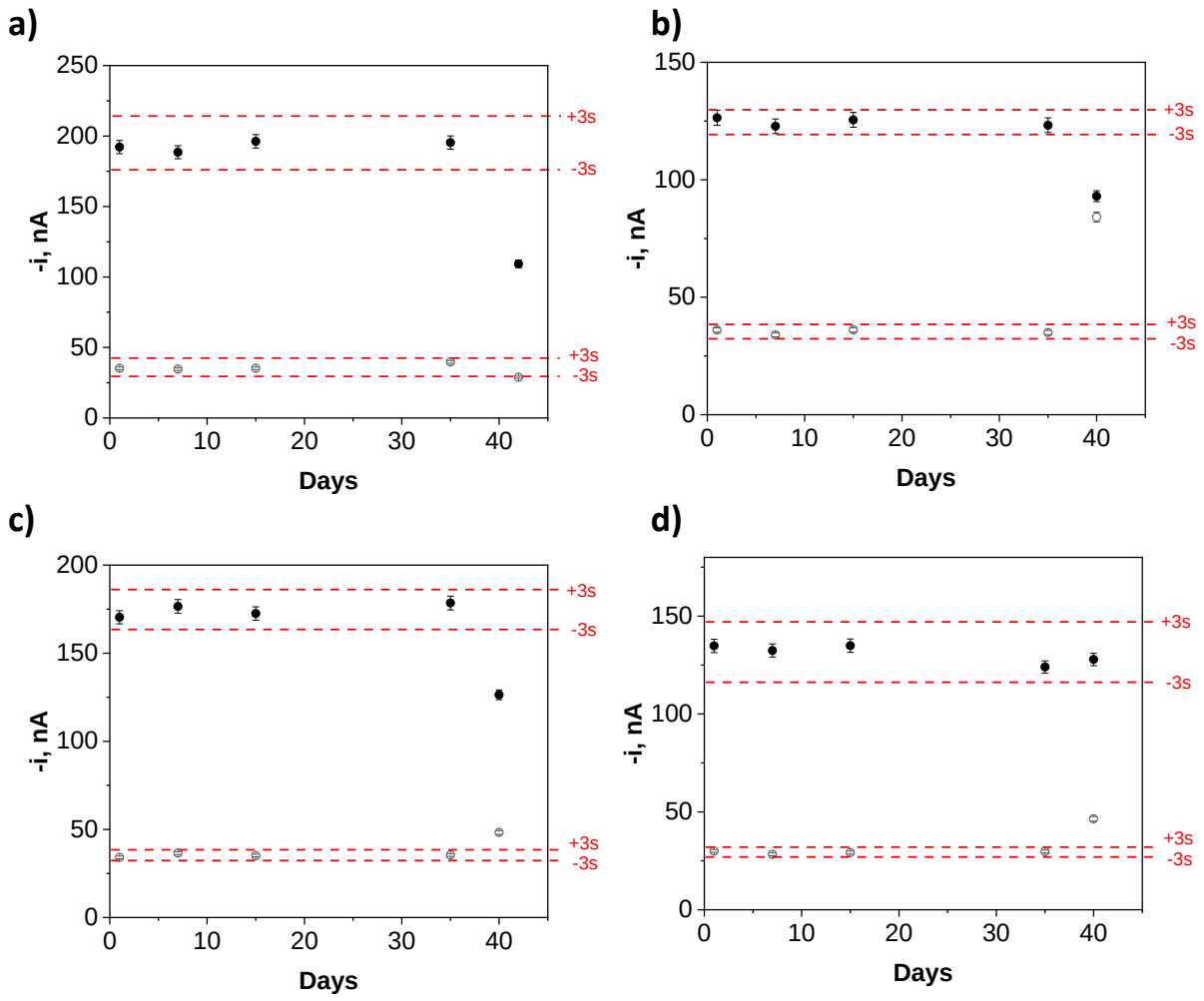

Fig. S6. Storage stability of the La/SSB-MBs a), U1snRNP70-MBs b), Ro/SSA-MBs c) and smRNP-MBs d) stored at 4 ºC in filtered PBS. Amperometric responses obtained in the absence (empty circles) and in the presence (full circles) of 12.5 U mL^{-1} La/SSB-Abs and 25 U mL^{-1} of U1snRNP70-Abs, Ro/SSA-Abs or smRNP-Abs standard solutions. Control limits (dashed lines) were set as ±3s of the mean value of three measurements obtained on the day of bioconjugates preparation.

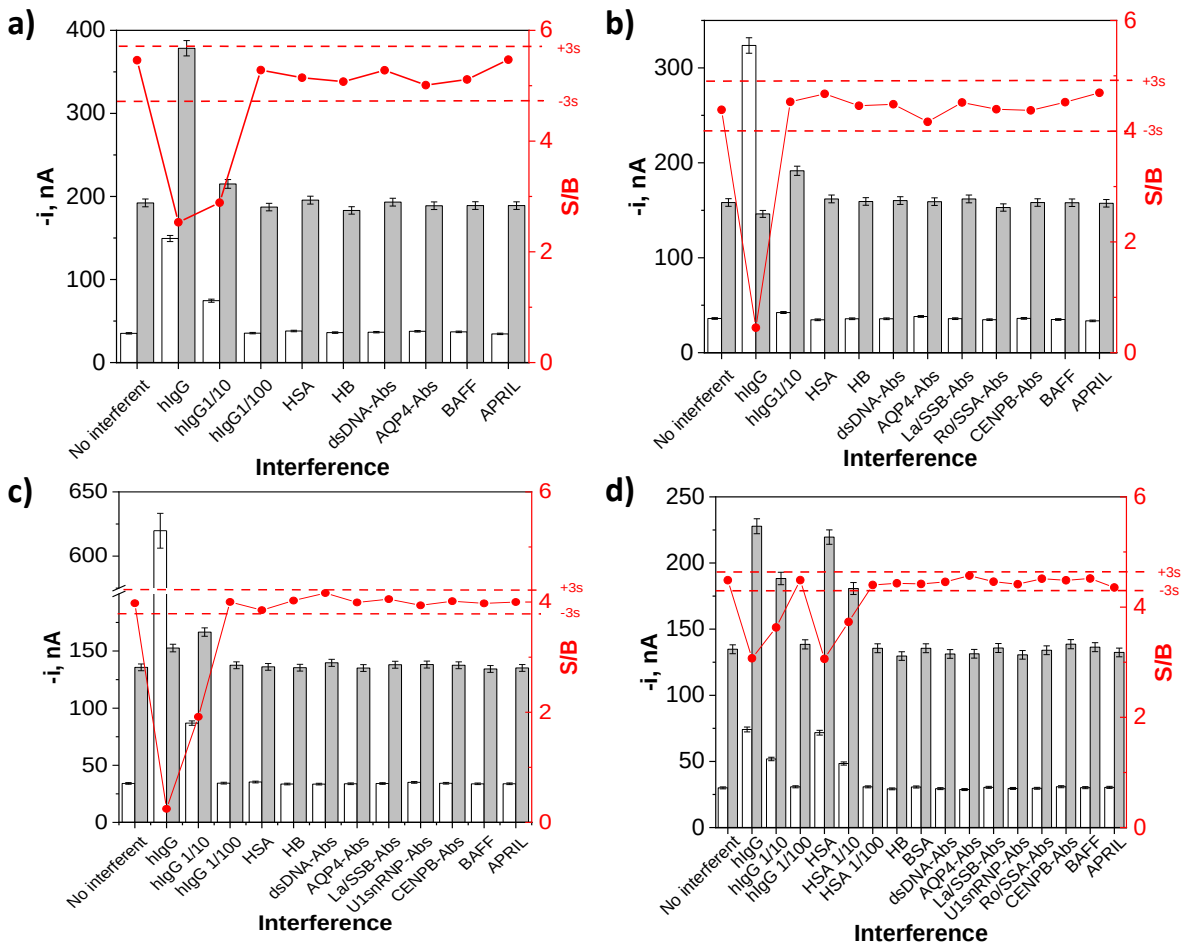

Fig. S7. Comparison of the amperometric responses provided by the quadruple bioplatform for 0 (white bars) and 12.5, 50, 10 or 25 (grey bars) U mL^{-1} La/SSB-Abs a), U1snRNP70-Abs b), Ro/SSA-Abs c) or smRNP-Abs d), prepared in the absence and in the presence of 1, 0.1 (1/10) and 0.01 (1/100) mg mL^{-1} hIgG; 50 mg mL^{-1} HSA; 5 mg ml^{-1} HB; 5 mg mL^{-1} BSA; 25 U mL^{-1} dsDNA-Abs; 1 ng mL^{-1} AQP4-Abs; 12.5 U mL^{-1} La/SSB-Abs; 10 U mL^{-1} U1snRNP70-Abs; 10 U mL^{-1} Ro/SSA-Abs; 10 U mL^{-1} CENPB-Abs; 1 ng mL^{-1} BAFF and 1 ng mL^{-1} APRIL.

Table S2. Concentration of target autoantibodies (mean value ± ts/√n; n = 3; α = 0.05) obtained with the developed quadruple bioplatform for the analysis of the positive and negative controls supplied in the commercial ELISA kits as well as the values indicated in the specifications.

Target Abs	Controls provided in the commercial ELISA Kits			
	Negative, U mL^{-1}		Positive, U mL^{-1}	
	Reference value	Bioplatform	Reference value	Bioplatform
La/SSB-Abs	0.5	0.49 ± 0.05	15	14.8 ± 0.8
U1snRNP70-Abs	2	2.0 ± 0.2	55	53 ± 5
Ro/SSA-Abs	2	2.2 ± 0.1	75	76 ± 6
smRNP-Abs	4	4.0 ± 0.3	75	75 ± 10

Table S3. Comparison between the slope values (nA per decade of concentration (U mL^{-1})) of the calibration plots constructed with the quadruple immunoplatform for the target Abs standards prepared in buffer solution and in a representative 1000-fold diluted serum sample.

Target Abs	Buffered solution	1000-times diluted serum	t_{exp}^{*}
La/SSB-Abs	192 ± 3	186 ± 2	1.717
U1snRNP70-Abs	108 ± 2	109 ± 2	0.362
Ro/SSA-Abs	90 ± 2	88 ± 1	0.818
smRNP-Abs	66.5 ± 0.4	59 ± 3	2.102

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

Table S4. Parameters of the correlation plots displayed in **Fig. 3b**.

	La/SSB-Abs	U1snRNP70-Abs	Ro/SSA-Abs	smRNP-Abs
R^2	0.997	0.993	0.990	0.991
Slope	0.99 ± 0.01	1.01 ± 0.02	0.98 ± 0.02	0.98 ± 0.02
Intercept	-0 ± 2	1 ± 3	1 ± 3	1 ± 3

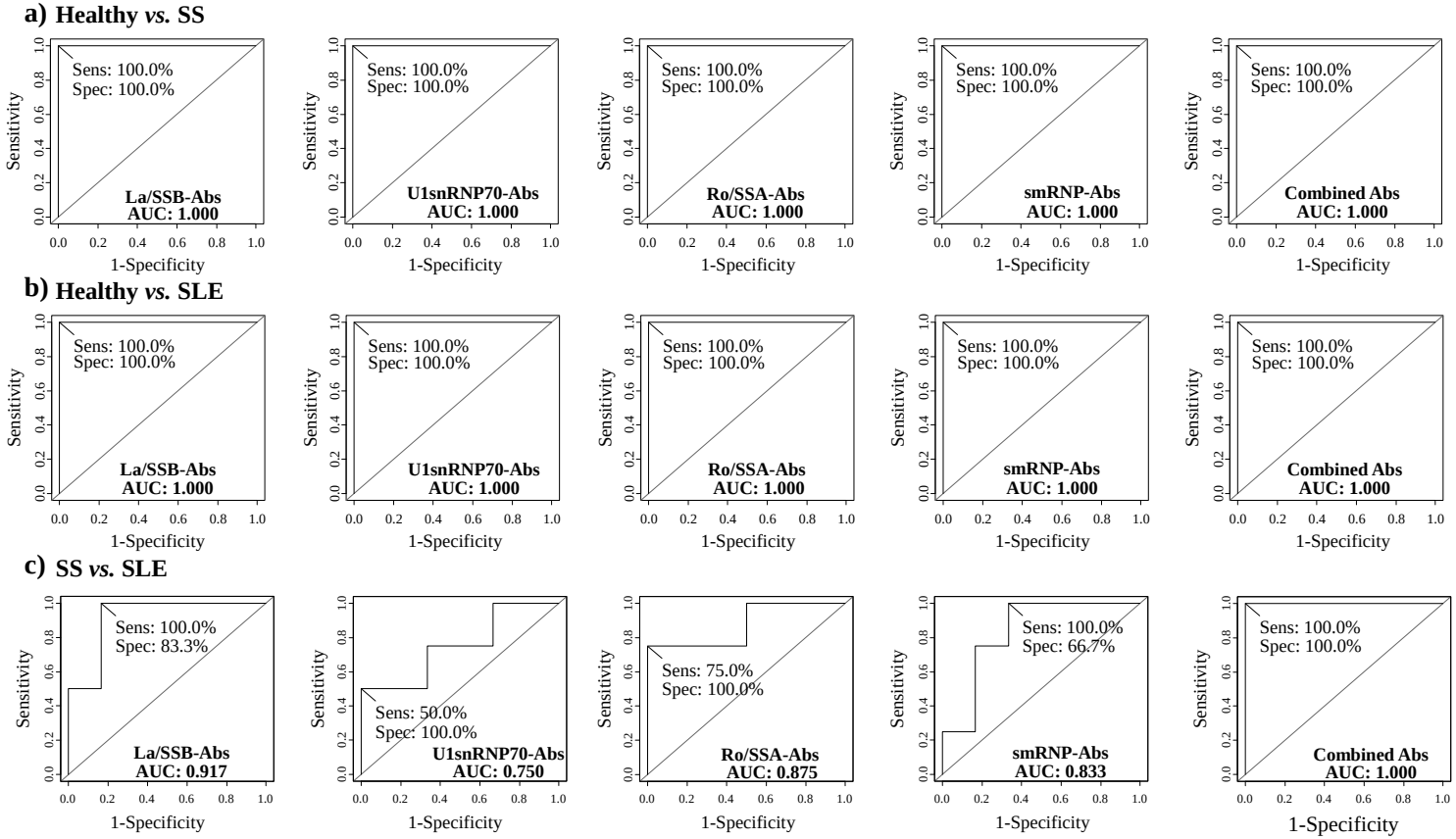

Fig. S8. ROC curves of the diagnostic value of the quadruple bioplatform for the diagnosis of SLE a) and SS b) and discrimination between SLE and SS patients c) by interrogating serum levels of La/SSB-Abs, U1snRNP70-Abs, Ro/SSA-Abs and smRNP-Abs. For the construction of these curves, the results of all serum samples summarized in Table 2 corresponding to 4 healthy individuals, 4 patients with SLE and 6 patients with SS have been considered.

Versatility of the developed bioplatform

Regarding its versatility, the proposed quadruple bioplatform can be employed to determine the content of total Igs and of the different isotypes (IgG, IgM and IgA) against a specific antigen. To check this, we tested the quadruple determination of total Igs, IgG, IgM and IgA isotypes against the La/SSB antigen. Four different batches of La/SSB-MBs were prepared and, after incubation with the diluted serum sample, they were enzymatically labelled with HRP-labelled secondary antibodies able to recognize each Ig isotype or with a mixture containing three secondary antibodies capable of recognizing the three isotypes (in the case of the determination of total Igs). **Fig. S9** shows the results obtained in the analysis of a sample from a SS patient (sample 13 in **Table 2**) providing mean contents (3 replicates) of 519 (total Igs), 398 (IgGs), 94 (IgAs) and 17 (IgMs) U mL^{-1}. These results agree with that reported in the literature confirming the presence of the three isotypes in this type of patients [Pournar et al., 1999].

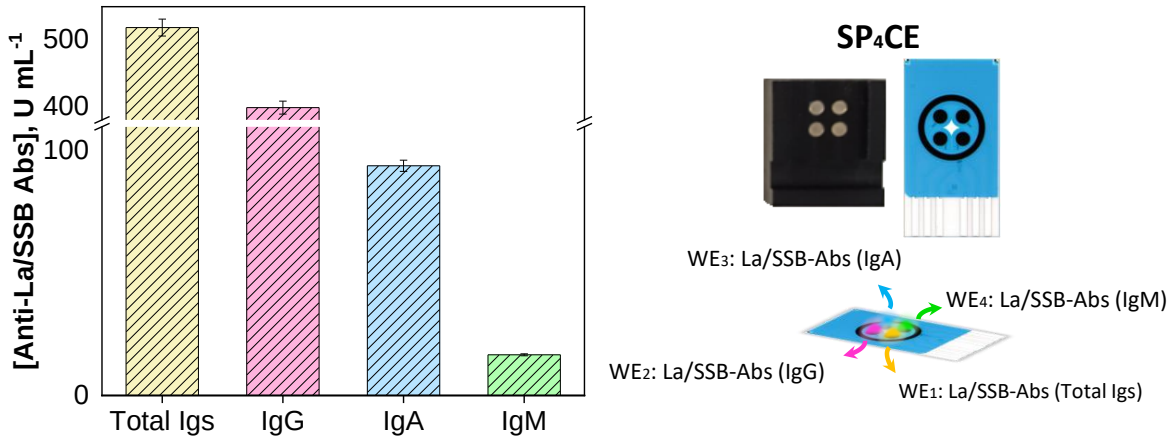

Fig. S9. Concentrations of total Igs and IgG, IgM and IgA isotypes against La/SSB found with the quadruple bioplatform in the serum of a SS patient.

Another potential multiplexed application would be the determination in the same assay of the IgG content against 4 different antigens (La/SSB, U1snRNP70, Ro/SSA and smRNP) and both the total content and the individual isotypes against a fifth antigen (dsDNA) using in this case an octuple detection platform. **Fig. S10** compares the amperometric responses obtained in the analysis of representative samples from a healthy individual and a patient with SLE (samples 1 and 5 in Table 3, respectively). As expected, significantly larger contents of the eight Abs were found for the SLE patient compared to the healthy individual.

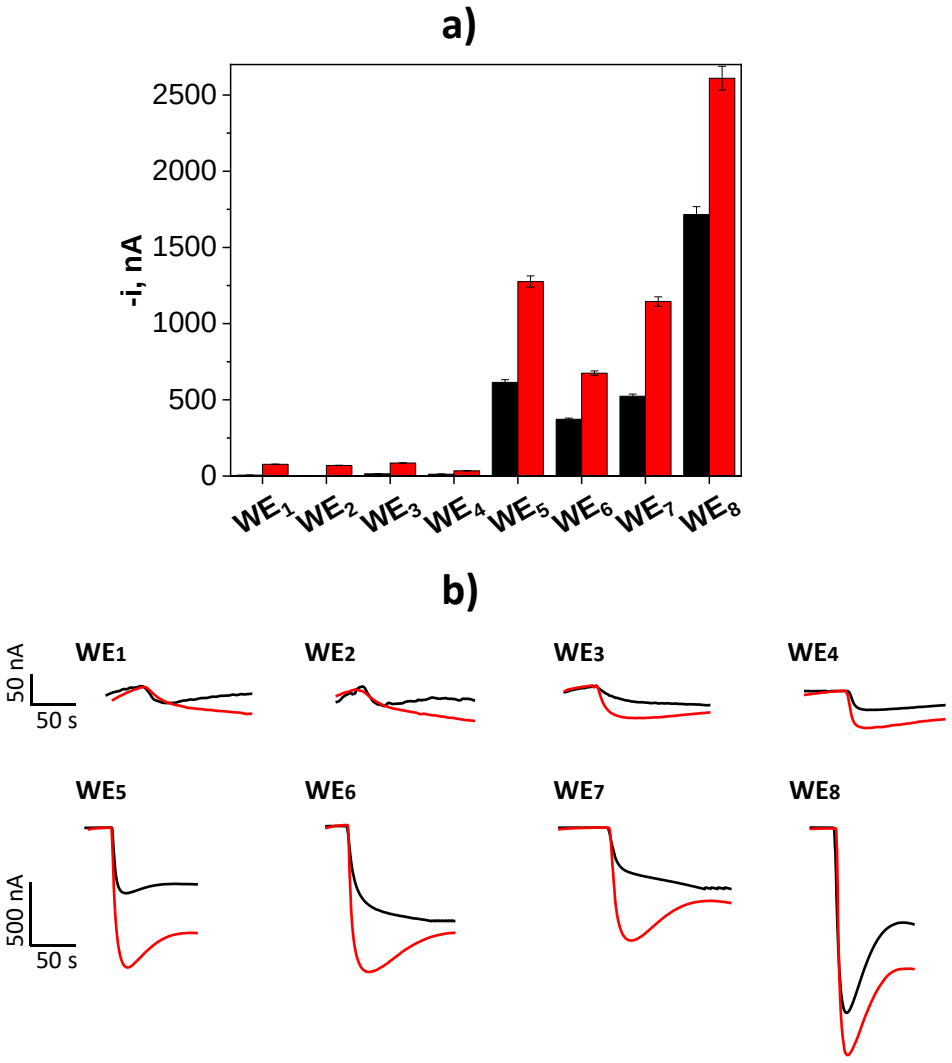

Fig. S10. Amperometric responses obtained in the octuple determination at SP_8CEs of Abs (IgGs) against La/SSB, U1snRNP70, Ro/SSA and smRNP (WE_1-WE_4) and of total IgGs (WE_5) and individual isotypes (WE_6: IgGs, WE_7: IgAs and WE_8: IgMs) against dsDNA in serum samples from a healthy individual (in black) and from a patient with SLE (in red).

References

Arévalo, B., Serafín, V., Sánchez-Paniagua, M., Montero-Calle, A., Barderas, R., López-Ruíz, B., Campuzano, S., Yañez-Sedeño, P., Pingarrón, J.M., 2020. Fast and sensitive diagnosis of autoimmune disorders through amperometric biosensing of serum anti-dsDNA autoantibodies. Biosens. Bioelectron. 160, 112233. https://doi.org/10.1016/j.bios.2020.112233.

Arévalo, B., Blázquez, M., Serafín, V., Montero-Calle, A., Calero, M., Valverde, A., Barderas, R., Campuzano, S., Yáñez-Sedeño, P., Pingarrón, J.M., 2022. Unraveling autoimmune and neurodegenerative diseases by amperometric serological detection of antibodies against aquaporin-4. Bioelectrochemistry 144, 108041. https://doi.org/10.1016/j.bioelechem.2021.108041.

Esteban-Fernández de Ávila, B., Escamilla-Gómez, V., Campuzano, S., Pedrero, M., Salvador, J.P., Marco, M.P., Pingarrón, J.M., 2013. Ultrasensitive amperometric magnetoimmunosensor for human C-reactive protein quantification in serum. Sens. Actuators B Chem. 188, 212–220. http://dx.doi.org/10.1016/j.snb.2013.07.026.

Torrente-Rodríguez, R.M., Montero-Calle, A., San Bartolomé, C., Cano, O., Vázquez, M., Iglesias-Caballero, M., Corral-Lugo, A., McConnell, M.J., Pascal, M., Mas, V., Pingarrón, J.M., Barderas, R., Campuzano, S., 2022. Towards Control and Oversight of SARS-CoV-2 Diagnosis and Monitoring through Multiplexed Quantitative Electroanalytical Immune Response Biosensors. Angew Chem Int Ed 61, e202203662. http://dx.doi.org/10.1002/anie.202203662.