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Electrochemical biosensor for the simultaneous determination of rheumatoid factor and anti-cyclic citrullinated peptide antibodies in human serum

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This paper reports a dual electrochemical biosensor involving carboxylated- or neutravidin-functionalized magnetic microbeads and dual screen-printed carbon electrodes for the simultaneous determination of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCPA) autoantibodies used as biomarkers for the detection of rheumatoid arthritis autoimmune disease. Sandwich-type biosensors involving Fc fragments of IgG Fc(IgG) and biotinylated cyclic cytrullinated peptide (CCP-biotin) to form CCP-biotin-Neutr-MBs for the specific immobilization of RF and CCPA, respectively, as well as conjugation with HRP-IgM and HRP-IgG for RF and CCPA, respectively, were prepared. Amperometric detection was performed at −0.20 V *vs.* Ag pseudo-reference electrode using the H_2O_2/hydroquinone (HQ) system upon capturing the bioconjugates onto the corresponding working electrode (WE1 or WE2) of SPCdEs. The dual biosensor exhibits high sensitivity for RF and CCPA with LOD values of 0.8 and 2.5 IU mL^{-1}, respectively. The simultaneous determination can be completed in about two hours using a simple protocol and a sample volume (25 μL) four times smaller than that required by the ELISA method. The dual electrochemical biosensor was used for the determination of both target biomarkers in human serum.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease causing pain, swelling and stiffness, characterized by inflammation of the joints.[1,2] Early diagnosis of RA is extremely important for applying suitable treatment to prevent irreversible joint damage. Serum of RA patients shows frequent presence of autoantibodies, with IgM-rheumatoid factor (RF) as the most prevalent, and anti-citrullinated protein antibodies (CPAs).[3] RF is an antibody targeting the Fc fragment of the patient's own IgG molecules while CPAs comprise a group of highly specific RA antibodies, detected during the early stages of the disease, which recognize various peptides and proteins such as filaggrin or fibrinogen, whose arginyl residues are post-translationally transformed into citrullyl residues. These autoantibodies play an important role in inducing inflammation and joint damage, releasing pro-inflammatory cytokines from monocytes/macrophages. It is reported that 70–80% of RA patients are CPA positive. Therefore, high levels of CPA are associated to bad prognosis of the disease.[4,5] In addition, most of patients with RA are also positive to RF, although this factor is not specific for RA as it is produced in a wide range of autoimmune rheumatic diseases and infections.[6,7] Citrullinated fibrinogen, citrullinated a-enolase and mutated citrullinated vimentin can be used as the selective antigens for CPAs monitoring in RA diagnosis, but the most common antigen is cyclic citrullinated peptide (CCP), which is based on cyclic citrullinated sequences derived from filaggrin[8,9] and is specific for anti-cyclic citrullinated peptide antibodies (CCPA).

The development of sensitive and accurate methods for the detection of disease biomarkers is an essential tool for achieving an effective early and reliable clinical diagnosis. In this context, multianalyte strategies provide more valuable information. Therefore, faster and cost effective multiplexed assays are of great significance. Regarding RA biomarkers, the development of reliable methodologies for the determination of RF and CPA autoantibodies is of great interest, as their monitoring is challenging due to the low concentration ranges found in biological samples. The established cut-off values are 14 IU mL^{-1} for RF and 20 IU mL^{-1} for CCPA.[10] These biomolecules are usually determined individually by flocculation or agglutination of IgG-coated particles or cells (particularly in the case of RF), or by ELISA methods. Indeed, various colorimetric

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ELISA kits for the single determination of RF or CPPA are commercially available. As an example, the protocol involved in the RF IgM ELISA kit (RFM31-K01) from Eagle Biosciences Inc. for the determination of RF in serum [https://eaglebio.com/wp-content/uploads/data-pdf/rfm31-k01-rf-igm-elisa-assay-kit-insert.pdf] implies wells coated with IgG to bind with RF and detection with an HRP conjugated anti-human IgA. The assay time is around 1 h 45 min, and the logarithmic calibration plot (non-linear) ranges between 1 and 300 IU mL^{-1}. ELISA kits for the determination of CCPA use CCP-coated microtiter plates to bind the antibody followed by detection with HRP-labelled IgG. As an example, the protocol involved in the Medizym® anti-CCP 3860 CCP31-K01-A kit from Eagle Biosciences Inc. [https://eaglebio.com/wp-contentuploads/data-pdf/ccp31-k01-anti-ccp-elisa-assay-kit-package-insert.pdf], exhibits a logarithmic calibration plot with a non-linear dynamic range between 1 and 2000 IU mL^{-1}, and an assay time of about 2 h 15 min.

Regarding biosensors, a label free impedimetric immunosensor for the determination of RF was reported using the rare earths oxide YbY_xO_y sensing membranes and covalent immobilization of the autoantibody, providing a calibration range from 10^{-7} to 10^{-3} M (1.54–1.54 × 10^4 IU mL^{-1}).[11] More recently, Chinnadayyala *et al.*[12] proposed an impedimetric-interdigitated wave type microelectrode array (IDWμE) functionalized with a self-assembled monolayer (SAM) of thioctic acid for antigen immobilization and determination of RF in serum. The covalent immobilization of IgG Fc fragment onto the SAM modified electrode array provided a linear calibration plot between 1 and 200 IU mL^{-1} and a LOD value of 0.6 IU mL^{-1}. Regarding the detection of CCPA, a multiwalled carbon nanotube-polystyrene based electrochemical transducer using as the recognition element a chimeric fibrin-filaggrin synthetic peptide covalently immobilized to the surface of the electrode was reported.[13] Although these two latter biosensors were used for direct measurements in human serum, no data on the calibration plots and the analytical characteristics of the methods were reported.

In this paper, we report an electrochemical biosensor for the simple, fast and reliable simultaneous determination of RF and CCPA. Dual screen-printed carbon electrodes (SPCdEs) and carboxyl- or neutravidin-functionalized magnetic beads (cMBs or Neutr-MBs) were used as the substrates for the immobilization of the specific recognition elements for RF and CCPA, respectively. A Fc fragment of native human IgG protein (Fc(IgG)) was covalently immobilized onto cMBs and the target RF was sandwiched with a HRP-labelled detection antibody (HRP-IgM). In the case of CCPA, a biotinylated CCP peptide was immobilized onto Neutr-MBs conjugated to CCPA antibody and sandwiched with HRP-IgG. The MBs bearing the resulting bioconjugates (HRP-IgM-RF-Fc(IgG)-cMBs and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs) were magnetically captured on the SPCdE electrode surfaces (W1 and W2, respectively). Quantification of RF and CCPA was accomplished by amperometry at −0.2 V *vs.* Ag pseudo-reference electrode after addition of hydrogen peroxide in the presence of hydroquinone (HQ).

2. Experimental

2.1. Apparatus and electrodes

Amperometric measurements were made with a CH1 1030B potentiostat (Chemical Instruments) equipped with the CH1 1030B software. SPCEs (DRP-110) with a 4 mm-Ø carbon working electrode and dual SPCdEs (DRP-X1110) consisting of two elliptic carbon working electrodes with surface area of 5.6 mm^2 (Metrohm-DropSens), were used. These electrodes include a carbon counter electrode and a silver pseudo-reference electrode. The specific DRP-CAC and DRP-BICAC connection cables were also from Metrohm-Dropsens. Magnetic separation during the incubation/washing steps was performed using a DynaMag™-2 (Dynal Biotech ASA, Thermo Fisher) magnetic particle concentrator. A neodymium magnet (AIMAN GZ) was used to control the attraction of the modified MBs to the SPCE surface. A Velp Scientific Vortex was used for the homogenization of the solutions. An Optic Ivymen System (Comecta), a Crison model Basic 20+ pH-meter, an Elmasonic S-60 (Elma) ultrasonic bath, and an MPW-65R centrifuge from MPW (Med. Instruments) were also employed.

2.2. Reagents and solutions

Human IgG Fc fragment (Fc(IgG), ab90285), rheumatoid factor IgM (RF, ab178653), and peroxidase labelled anti-human IgM (HRP-IgM, ab178653) were from the anti-rheumatoid factor IgM ELISA kit (ab178653) from Abcam. Biotinylated cyclic cytrullinated peptide (CCP-biotin) was also used. Human cyclic cytrullinated peptide antibody (CCPA) and HRP labelled anti-human IgG antibody (HRP-IgG) prepared in buffered serum matrix were from the commercial ELISA kit DE7760 (Demeditec). Carboxyl-functionalized magnetic microbeads (cMBs, 2.8 μm, 10 mg mL^{-1}, Dynabeads® M-270 carboxylic acid) were purchased from Dynal Biotech ASA (Thermo Fisher Scientific). Neutravidin-modified-magnetic microparticles (Neutr-MBs, 1 μm-Ø, 10 mg mL^{-1}, SpeedBeads™) were from GE Healthcare. Deionized water was from a Millipore Milli-Q purification system (18.2 MΩ cm). Other reagents used were HQ and hydrogen peroxide (30%, w/v), both from Sigma.

Buffer solutions were: 25 mM MES of pH 5.0 prepared from 2-(*N*-morpholine) ethano-sulfonic acid (Gerbu); a 0.05 M potassium phosphate buffer of pH 6.0 (PB); PBS, consisting of a 0.05 M sodium phosphate buffer of pH 7.2 supplemented with 0.0920 g NaCl (Labkem) and 0.0020 g KCl (Scharlau) in 100 mL deionized water; PBST, prepared by addition of 0.1000 g Tween 20 (Aldrich) to 100 mL PBS.

2.3. Procedures

2.3.1. Preparation of the biosensor for the determination of RF. A 3 μL-aliquot of the cMBs suspension was transferred to a 1.5 mL eppendorf tube and two washings were made with 50 μL of 25 mM MES buffer solution of pH 5.0 by incubating for 10 minutes at 25 °C with constant stirring at 950 rpm. After each washing, the tube was placed in the magnetic concentrator for 2 min then removing carefully the supernatant. Thereafter, 25 μL of a solution containing 50 mg mL^{-1} EDC

and NHSS were added to activate the carboxylic groups, allowing incubation for 30 minutes at 25 °C with constant stirring. Subsequently, two washings with 50 μL of 25 mM MES buffer solution of pH 5.0 were made, and 25 μL of a 25 μg mL^{-1} Fc (IgG) solution in 25 mM MES buffer solution of pH 5.0 were added, allowing incubation for 30 min. Then, the bio-functionalized MBs were separated, the supernatant discarded, and two washings with 50 μL of 25 mM MES buffer solution of pH 5.0 were made. Next, 25 μL of the analyte (RF) solution (or the sample) were added and incubated for 60 minutes at 37 °C with constant stirring at 950 rpm. Then, the particles were separated using the magnetic separator, the supernatant discarded, and two further washings were performed with 50 μL of the MES buffer solution. 25 μL of the commercial HRP-IgM conjugate were added and incubated for 60 minutes at 25 °C with constant stirring at 950 rpm. Once the particles were magnetically separated, two washings were performed with 50 μL of the washing solution included in the anti-rheumatoid factor IgM ELISA kit. The HRP-IgM-RF-Fc(IgG)-cMBs conjugates were resuspended in 25 μL of a 0.05 M PB 6.0 buffer solution and kept in the eppendorf tube until the measurements were carried out.

2.3.2. Preparation of the biosensor for the determination of CCPA. A 3 μL aliquot of the Neutr-MBs suspension was deposited in a 1.5 mL microcentrifuge tube. The functionalized microparticles were washed twice with 50 μL PBST of pH 7.2. Each washing involved placing the tube in the magnetic concentrator for 2 min and further removing of the supernatant. Thereafter, the Neutr-MBs were incubated with 25 μL of CCP-biotin prepared in PBS of pH 7.2 for 30 min. After two washings with 50 μL of the same buffer, the supernatant was removed and 25 μL of CCPA solution (or the sample) were incubated with the CCP-biotin-Neutr-MBs during 60 min. Thereafter, 25 μL of HRP-IgG solution were incubated for 60 min onto CCPA-CCP-biotin-Neutr-MBs. The resulting HRP-IgG-CCPA-CCP-biotin-Neutr-MBs bioconjugates were washed twice as indicated above and resuspended in 50 μL of 0.05 M PB (pH 6.0) just before the amperometric measurement.

2.3.3. Individual determination of RF or CCPA. The single amperometric determination of RF or CCPA was performed by depositing the HRP-IgM-RF-Fc(IgG)-cMBs or HRP-IgG-CCPA-CCP-biotin-Neutr-MBs bioconjugates on the SPCE working electrode surface previously placed on a housing containing an encapsulated neodymium magnet to ensure magnetic capture in a stable and reproducible manner. Then, the SPCE bearing the functionalized MBs was immersed in a measuring cell containing 10 mL of 0.05 M PB (pH 6.0) and 1 mM HQ solution prepared immediately before the electrochemical measurement. The current was measured in stirred solution after applying a constant potential of −0.2 V *vs.* Ag pseudo-reference electrode. Once the background current was stabilized (100 s approximately), 50 μL of the 0.1 M H_2O_2 solution were added to the electrochemical cell and the change in the recorded current as a consequence of the enzymatic reduction of H_2O_2 mediated by HQ was monitored once the steady state was reached (aprox. 200 s). The biosensor responses were measured as the difference between the steady state current and the background current.

2.3.4. Simultaneous determination of RF and CCPA. The procedures described above for the individual determination of RF and CCPA were also used for their simultaneous

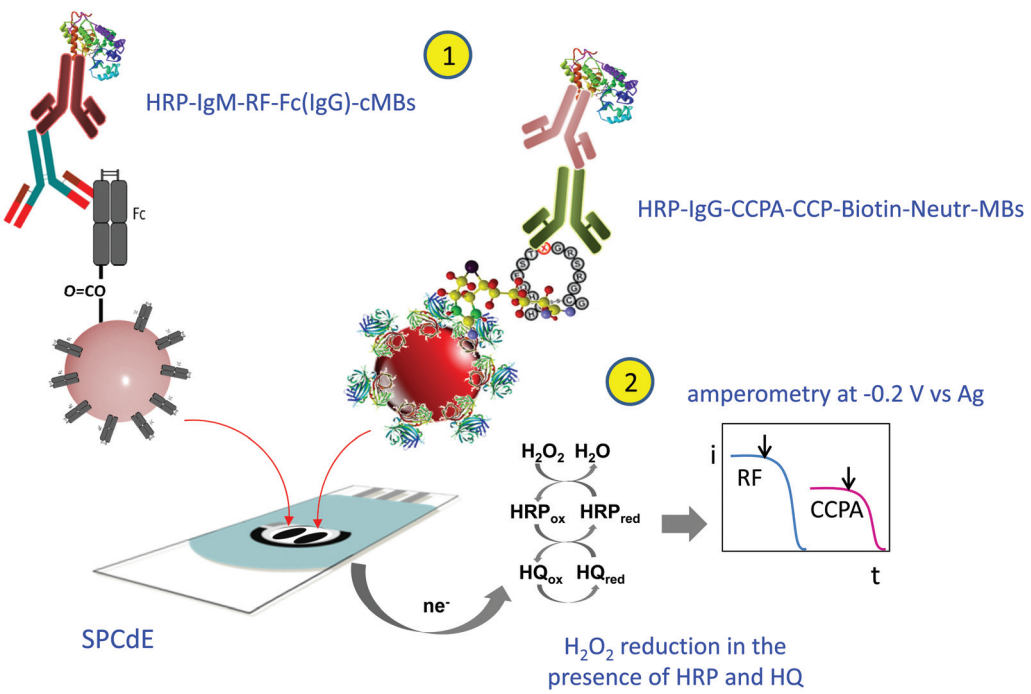

Fig. 1 Graphics showing (1) the addition of HRP-IgM-RF-Fc(IgG)-cMBs and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs conjugates to the SPCdE and (2) the reactions involved in the amperometric detection.

amperometric determination through deposition of the HRP-IgM-RF-Fc(IgG)-cMBs or HRP-IgG-CCPA-CCP-biotin-Neutr-MBs on the W1 or W2 working electrode surface of the SPCdE, respectively (Fig. 1).

2.3.5. Determination of RF and CCPA in serum. The simultaneous determination of RF and CCPA in human serum was performed by applying the procedure described above to aliquots of undiluted serum samples and interpolation of the measured amperometric responses into the calibration plots of RF and CCPA standard solutions.

3. Results and discussion

As it is described in the Experimental section, hIgG Fc fragments (Fc(IgG)) immobilized onto carboxylated MBs were used for the specific binding of RF. Fc(IgG) possesses high affinity for RF and as demonstrated by Kanmert *et al.*,[14] the use of Fc fragments implies that no other parts of IgG interfere with RF binding, thereby reducing the risk of false-positives, and also allowing for precise control of the Fc-antigens at the molecular level, which provides greater reproducibility. Regarding CCPA, Neutr-MBs were used as supports to immobilize biotinylated CCP for further binding with CCPA. The use of neutravidin- instead streptavidin- or avidin functionalized MBs has some advantages.[15] Neutravidin possesses higher affinity toward biotin, almost reaching the theoretical maximum activity (14 μg biotin per mg protein).[16] In addition, the nearly neutral charge of neutravidin minimizes non-specific interactions, thus providing calibration plots with smaller intercepts. Furthermore, since neutravidin is a deglycosilated form of avidin, the unions to lectins are practically undetectable.

3.1. Optimization of the experimental variables

The influence of the variables involved in the preparation of the RF and CCPA biosensors on the respective amperometric responses was evaluated. The working value of each variable was selected according to the criterium of a better specific-to-unspecific current (S/N) ratio. Regarding the biosensor for RF, the loading of Fc(IgG) and the time for incubation on cMBs were tested (Fig. 2). As it can be seen, a larger S/N ratio was found for 25 μg mL^{-1} Fc(IgG) (Fig. 2A), and for an incubation time of 30 min (Fig. 2B). Therefore, these values were selected for further work.

The effect of the HRP-IgM dilution on the amperometric responses of the biosensor is shown in Fig. 2C. The biosensor specific response decreases as the conjugate dilution factor increases while the nonspecific current remains almost constant. Thus, a larger S/N ratio was achieved using undiluted conjugate solution. Moreover, Fig. 2D shows that a larger S/N ratio was obtained for 60 min as the incubation time of HRP-IgM onto RF-Fc(IgG)-cMBs/SPCE.

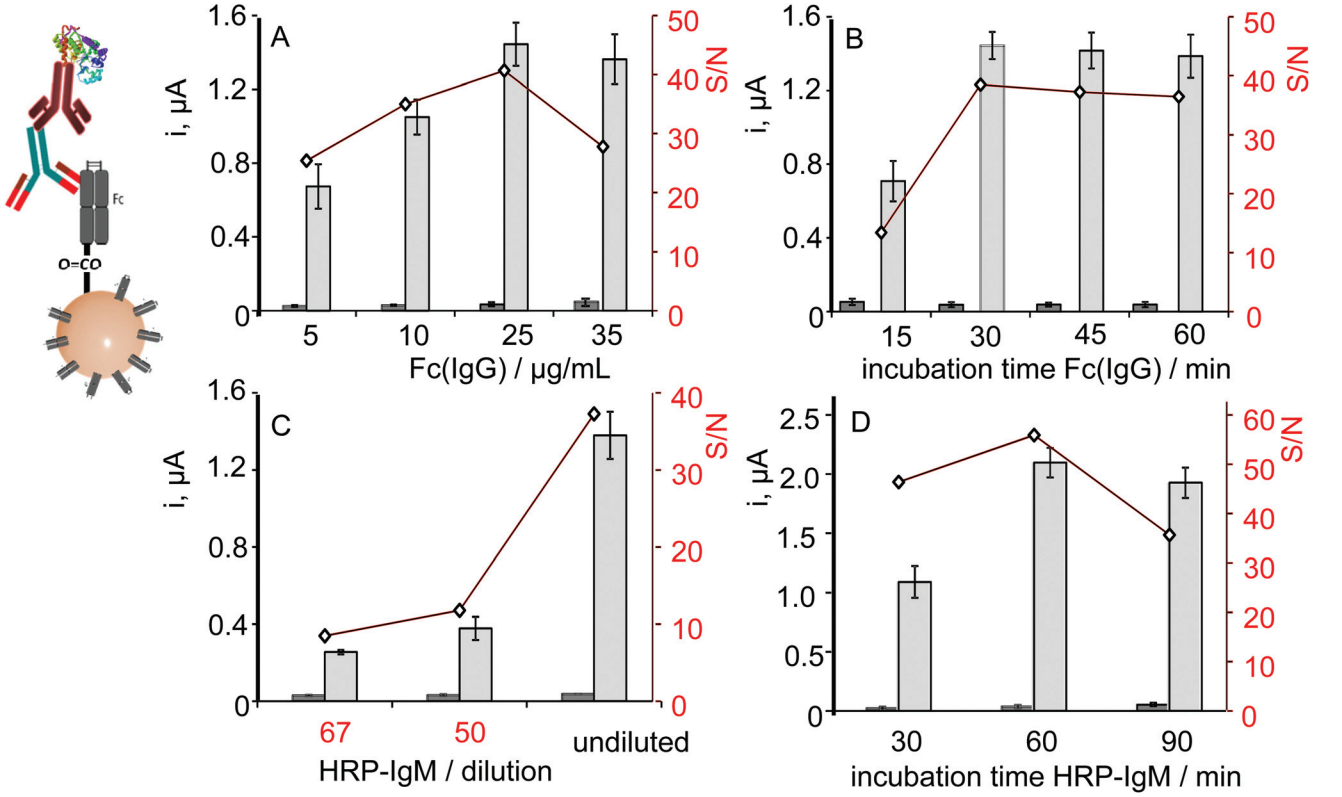

Fig. 2 Effect of loading (A) and incubation time (B) of Fc(IgG) onto cMBs /SPCE; dilution (C) and incubation time (D) of HRP-IgM immobilized onto RF-Fc(IgG)-cMBs/SPCE on the amperometric responses of the HRP-IgM-RF-Fc(IgG)-cMBs/SPCE biosensor. 3 μL cMBs; 0 U mL^{-1} (dark grey) or 300 U mL^{-1} (light grey) RF, 60 min.

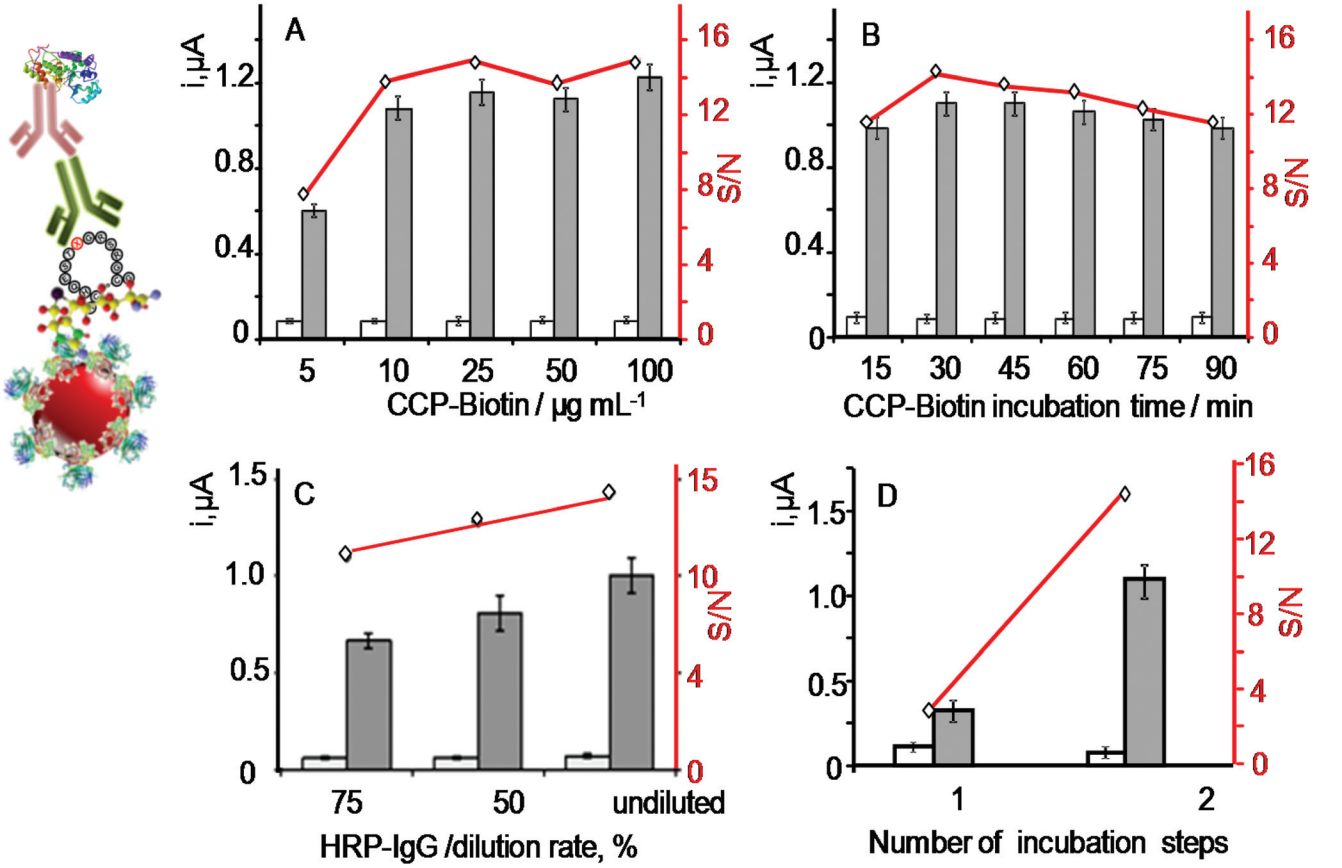

Fig. 3 Effect of: loading (A) and incubation time (B) of CCP-biotin onto Neutr-MBs/SPCE; dilution rate of HRP-IgG (C) immobilized onto CCPA-CCP-biotin-Neutr-MBs/SPCE; number of incubation steps involved in the biosensor preparation (D) on the amperometric responses of the HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCE biosensor. 3 μL Neutr-MBs; 0 U mL^{-1} (white) or 20 U mL^{-1} (grey) CCPA, 60 minutes.

The loading of CCP-biotin conjugate and the time for incubation on Neutr-MBs were optimized by measuring the amperometric responses of the HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCE biosensor. Fig. 3A shows as a concentration of 25 μg mL^{-1} CCP-biotin provided a larger ratio between the current measured in the presence (S) of 20 U mL^{-1} CCPA and its absence (N). In addition, an incubation time of 30 min yielded a larger S/N ratio (Fig. 3B). The effect of the HRP-IgG dilution on the electrochemical response of the biosensor is depicted in Fig. 3C. The specific response decreased as the dilution factor increased while the nonspecific current kept practically constant. Therefore, a better S/N ratio was obtained when using undiluted conjugate solution. Moreover, 60 min showed to be an adequate incubation time of HRP-IgG onto CCPA-CCP-biotin-Neutr-MBs/SPC (results not shown).

In addition, the number of steps involved in the assay was checked in order to short the time needed for the preparation of the biosensor. Two different procedures were evaluated: (1) a single 60 min incubation step involving both the target CCPA and HRP-IgG solution on the CCP-biotin-Neutr-MBs, or (2) two steps involving 60 min incubation of CCP-biotin-Neutr-MBs in the CCPA solution and a further 60 min incubation of CCPA-CCP-biotin-Neutr-MBs in HRP-IgG solution. As it can be seen in Fig. 3D, both a larger specific response and a better S/N ratio were obtained using two sequential incubations. This is more likely due to a smaller biorecognition efficiency of the target antibody and the peptide immobilized onto the Neutr-MBs due to the steric hindrance occurring when CCPA was mixed with HRP-IgG in homogeneous solution. A similar behavior was observed for sandwich-type immunosensors involving the use of biotinylated detection antibodies conjugated with strep-HRP.[17]

Table 1 summarizes the results obtained in the optimization studies. Other experimental conditions such as the composition of the H_2O_2/HQ system or the detection potential were optimized previously.[15] It is worth mentioning the high S/N values, near 50 and 15, achieved with the HRP-IgM-RF-Fc (IgG)-cMBs/SPCE and CCPA-CCP-biotin-Neutr-MBs/SPCE biosensors, respectively. This behavior is probably due to the use of Fc(IgG)-cMBs and Neutr-MBs conjugates as immobilizing substrates because of the special characteristics of Fc(IgG) and neutravidin commented above, as well as to the use of MBs implies that the reagents not specifically linked or the non-targeted components of the sample can be easily and efficiently removed by magnetically controlled washings thus leading to lower matrix effects.

Table 1 Experimental variables selected for the simultaneous determination of RF and CCPA with the dual biosensor

Target	Variable	Studied range	Selected value
RF	cMBs, μL	—	3
	Fc(IgG) loading, μg mL^{-1}	5–35	25
	Incub. time Fc(IgG), min	15–60	30
	HRP-IgM, dilution %	67, 50, undiluted	Undiluted
	Incub. time HRP-IgM, min	30–90	60
CCPA	Neutr-MBs, μL	—	3
	CCP-biotin loading, μg mL^{-1}	5–100	25
	Incub. time CCP-biotin, min	15–90	30
	HRP-IgG, dilution %	75, 50, undiluted	Undiluted
	Incub. time HRP-IgG, min	60; 1 or 2 steps	60; 2 steps

3.2. Analytical characteristics of the dual biosensor

Fig. 4 displays the calibration plots and some amperometric responses for RF and CCPA standards obtained with the SPCdE biosensor under the optimized working conditions summarized in Table 1. The current *vs.* RF concentration followed the adjusted equation: i, nA = (848 ± 36) log([RF], IU mL^{-1}) − (315 ± 59), with R^2 = 0.995, and a range of linearity extended between 3 and 300 IU mL^{-1}. The LOD value was calculated according to the $3S_b$ criterion, where S_b was estimated as the standard deviation expressed in concentration units from ten amperometric responses of the biosensor in the absence of antibody. The LOD value was 0.8 IU mL^{-1}. Regarding CCPA, the equation for the calibration plot is: i, nA = (1409 ± 54) log[CCPA, U mL^{-1}] − (1179 ± 113) (R^2 = 0.996). The range of linearity ranged between 10 and 1000 U mL^{-1} CCPA, with a LOD value of 2.5 U mL^{-1}.

The repeatability between the amperometric responses for both biomarkers was calculated using ten different dual immunosensors prepared at the same day. The RSD values for measurements without RF and CCPA were 6.1% and 5.4%, respectively, while RSD values calculated from solutions containing 300 IU mL^{-1} RF and 20 IU mL^{-1} CCPA were 6.8% and 5.7%. Similar RSD values were obtained from measurements made on different days in the absence or in the presence of both targets, which confirmed the reproducibility of the working procedure including the preparation of the sandwich immunocomplexes on the MBs, their magnetic capture on the SPCdEs and the dual amperometric detection.

The storage stability of Fc(IgG)-cMBs and CCP-biotin-Neutr-MBs was tested by preparing the same day various conjugates and storing them at 8 °C in 50 μL PBS. Different biosensors prepared using the stored conjugates were used for a daily control by measuring 300 IU mL^{-1} RF or 1000 IU mL^{-1} CCPA. The obtained results (not shown) revealed that the responses of the dual biosensor kept within the control limits set at ±3 times the standard deviation of the measurements (n = 3) carried out the first day of the study, for the next 20 days.

Importantly, the obtained results allow the use of the dual biosensor for the determination of RF and CCPA at clinically relevant levels according to the cut-off values of 14 IU mL^{-1} for RF and 20 IU mL^{-1} for CCPA.[10] The comparison of the analytical characteristics achieved with the dual biosensor with those claimed for the individual assays using ELISA kits leads to the following discussion. The method using the RF IgM ELISA kit (RFM31-K01) from Eagle Biosciences [https://eaglebio.com/wp-content/uploads /data-pdf/rfm31-k01-rf-igm-elisa-assay-kit-insert.pdf] claims 1 IU mL^{-1} as the lowest RF concentration detected, while the anti-CCP ELISA assay kit 31-K01 [https://eaglebio.com/wp-content/uploads/data-pdf/ccp31-k01a-anti-ccp-elisa-assay-kit-package-insert.pdf] gives a CCPA concentration of 2 IU mL^{-1} as the functional sensitivity. These values are very similar to the LOD values achieved with the dual biosensor. Moreover, similar analytical characteristics were also found when the comparison is made with the few biosensors reported in the literature.[11,12] Obviously, the main advantage of the biosensor developed in this work is the possibility of performing the simultaneous determination of two relevant rheumatoid arthritis biomarkers, which is not possible using the ELISA methodology and has not been described so far for

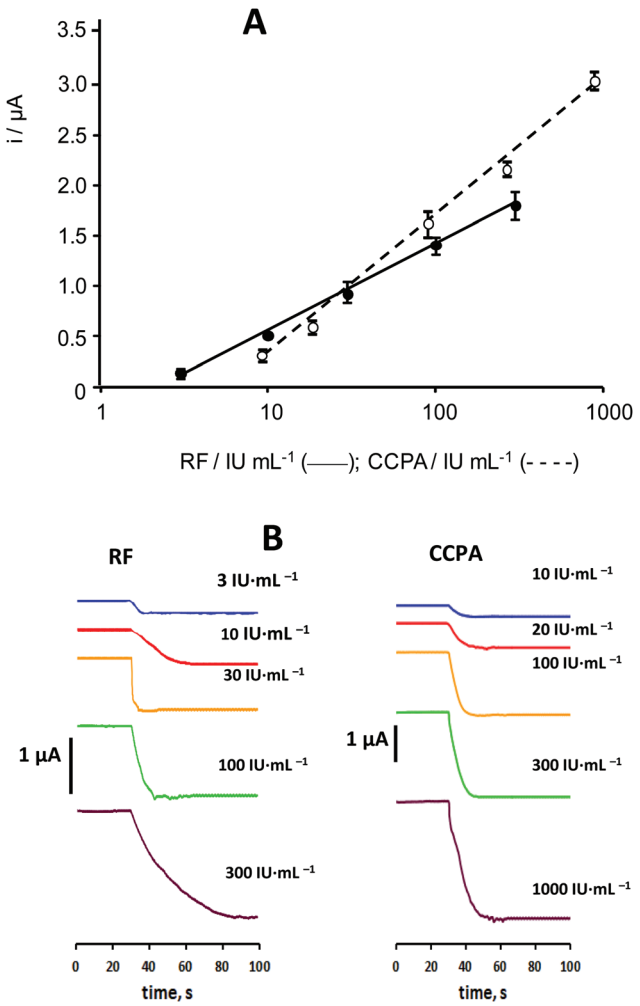

Fig. 4 (A) Calibration plots constructed with the dual biosensor for RF (black) and CCPA (white); (B) amperometric responses at the SPCdE biosensor. Working conditions are summarized in Table 1. Error bars estimated as triple of the standard deviation of three replicates.

any other biosensor design for the target antibodies. In fact, the dual biosensor allows the determination of RF and CCPA in an assay time of two hours approximately, counting from the immobilization of the autoantibodies on the respective Fc (IgG)-cMBs and CCP-biotin-Neutr-MBs conjugates. An important issue is the simultaneous determination can be accomplished at similar sensitivity levels and LOD values than those claimed for the single determination of the target autoantibodies using other methodologies. In addition, only 25 μL of sample solutions are required in the dual assay in comparison with the 100 μL required by the ELISA method.

3.3. Selectivity

The selectivity of the dual biosensor was tested by comparing the amperometric responses measured for 0 and 30 IU mL^{-1} RF or 0 and 20 IU mL^{-1} CCPA standard solutions in the absence and in the presence of various proteins coexisting in serum samples. Fig. 5 shows that no significant interference was apparent in the determination of RF and CCPA in the presence of serum proteins at the expected concentration levels for healthy patients. These results evidenced the high specificity of the Fc(IgG) and CCP peptide employed for the recognition of the target compounds as well as the excellent selectivity of the amperometric transduction at the optimized working conditions.

A potential major risk in the preparation of multiplexed biosensors is the possible cross-talking between adjacent working electrodes. To check this, the amperometric responses

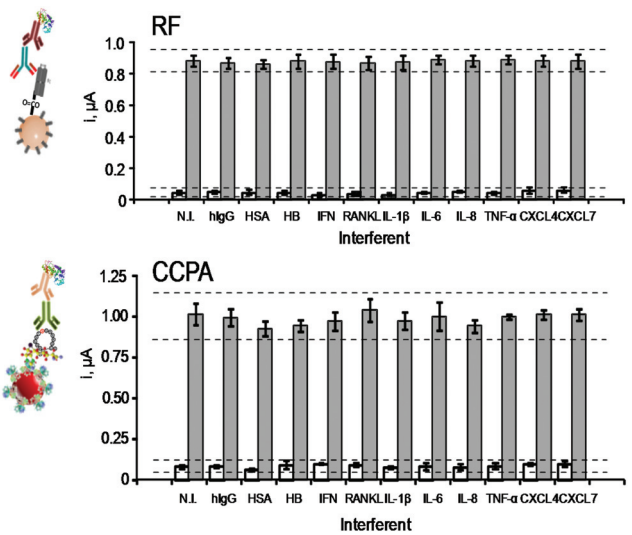

Fig. 5 Amperometric measurements obtained with the dual biosensor for 0 (white), and 30 IU mL^{-1} RF (up) or 20 IU mL^{-1} CCPA (down) (grey) prepared in the absence and in the presence of 1 mg mL^{-1} human IgG; 50 mg mL^{-1} human serum albumin (HSA); 5 mg mL^{-1} hemoglobin (HB); 100 pg mL^{-1} interferon gamma (IFN); 50 pg mL^{-1} receptor activator of nuclear factor-κB ligand (RANKL); 50 pg mL^{-1} interleukin 1 β (IL-β); 300 pg mL^{-1} interleukin 6 (IL-6); 300 pg mL^{-1} interleukin 8 (IL-8); 200 pg mL^{-1} tumor necrosis factor α (TNF-α); 200 pg mL^{-1} chemokine (C–X–C motif) ligand 4 (CXCL4) and 50 ng mL^{-1} chemokine (C–X–C motif) ligand 7 (CXCL7). Error bars are estimated as three times the standard deviation value of three replicates.

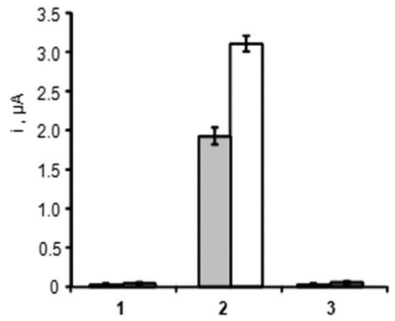

Fig. 6 Simultaneous amperometric responses measured with the HRP-IgM-RF-Fc(IgG)-cMBs/SPCdE (W1, grey) and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCdE (W2, white) biosensors for standard mixtures containing: (1) 0 IU mL^{-1} of both biomarkers; (2) 300 IU mL^{-1} RF + 1000 IU mL^{-1} CCPA; (3) 1000 IU mL^{-1} CCPA + 300 IU mL^{-1} RF. Error bars estimated as triple of the standard deviation of three replicates.

measured with different biosensors for different mixtures of RF and CCPA were compared (Fig. 6). As it is clearly shown, no significant cross-talking occurred between the two working electrode surfaces thus endorsing the viability of the dual bioplatform for the simultaneous determination of both biomarkers.

3.4. Simultaneous determination of RF and CCPA in human serum

The dual biosensor was applied to the quantification of RF and CCPA in human serum samples from Abcam (ab178653) and Demeditec (DE7760) provided by the respective ELISA kits as negative and positive controls for both targets. The possible existence of matrix effects was tested by comparing the slope values of the calibration plots constructed with RF or CCPA buffered standards solutions (Fig. 4) with those calculated from calibration plots prepared in serum by successive addition of standard aliquots to the samples. The application of the Student t test at a significance level of 0.05 and five degrees of freedom, provided $t_{exp} = 2.219 < t_{tab} = 2.571$ for RF, and $t_{exp} = 0.689 < t_{tab} = 2.571$ for CCPA, thus revealing the absence of matrix effect. Therefore, quantification was carried out by simple interpolation into the standards calibration plots.

Table 2 shows as the simultaneous determination of RF and CCPA with the dual biosensor provided concen-

Table 2 Determination of RF and CCPA in serum by means of the RF and CCPA dual biosensor and comparison with the results obtained with ELISA kits for the single determination of each autoantibody[a]

	RF, IU mL^{-1}		CCPA, IU mL^{-1}	
Control	Biosensor ($n = 6$)	ELISA ($n = 3$)	Biosensor ($n = 6$)	ELISA ($n = 3$)
Negative	4.3 ± 0.5	4 ± 1	9 ± 1	9 ± 2
Positive	62 ± 6	64 ± 9	263 ± 5	265 ± 10

[a] Mean value ± ts ($\alpha = 0.05$).

tration values in good agreement with those obtained by ELISA kits for the individual determination of each autoantibody involving colorimetric detection with the TMB/H_2O_2 system.

4. Conclusions

In this work, the first electrochemical dual biosensor for the simultaneous determination of RF and CCPA, two relevant biomarkers related with rheumatoid arthritis, is reported. The developed methodology relies on the preparation of sandwich type bioassays for the target compounds between Fc(IgG) or biotin-CCP captured onto carboxylated MBs (cMBs) or neutravidin-functionalized MBs (Neutr-MBs), respectively, and HRP-labeled detector antibodies. The dual biosensor implies the use of dual SPCEs as the electrochemical platform. High sensitivities were achieved with both IgG HRP-IgM-RF-Fc(IgG)-cMBs/SPCdE and HRP-IgG-CCPA-CCP-biotin-Neutr-MBs/SPCdE biosensors with LOD values of 0.8 IU mL^{-1} RFA and 2.5 IU mL^{-1} CCPA, respectively. In addition, the biosensor exhibits a great selectivity. The quantification of both targets can be completed in about two hours (counting from the immobilization of the targets on the respective conjugates) involving a simple working protocol and a sample volume (25 μL for each biomarker) four times smaller than that required with ELISA kits. All these results clearly improve the characteristics of the ELISA methods, also taking into account that this technique only allows individual but not simultaneous determinations.

Conflicts of interest

There are no conflicts to declare.

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