Specific label-free and real-time detection of oxidized low density lipoprotein (oxLDL) using an immunosensor with three monoclonal antibodies

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Abstract

Increased levels of plasma oxLDL, which is the oxidized fraction of Low Density Lipoprotein (LDL), are associated with atherosclerosis, an inflammatory disease, and the subsequent development of severe cardiovascular diseases that are today a major cause of death in modern countries. It is therefore important to find a reliable and fast assay to determine oxLDL in serum. A new immunosensor employing three monoclonal antibodies (mAbs) against oxLDL is proposed in this work as a quick and effective way to monitor oxLDL. The oxLDL was first employed to produce anti-oxLDL monoclonal antibodies by hybridoma cells that were previously obtained. The immunosensor was set-up by self- assembling cysteamine (Cyst) on a gold (Au) layer (4 mm diameter) of a disposable screen-printed electrode. Three mAbs were allowed to react with *N*-hydroxysuccinimide (NHS) and ethyl(dimethylaminopropyl)carbodiimide (EDAC), and subsequently incubated in the Au/Cys. Albumin from bovine serum (BSA) was immobilized further to ensure that other molecules apart from oxLDL could not bind to the electrode surface. All steps were followed by various characterization techniques such as electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV). The analytical operation of the immunosensor was obtained by incubating the sensing layer of the device in oxLDL for 15 minutes, prior to EIS and SWV. This was done by using standard oxLDL solutions prepared in foetal calf serum, in order to simulate patient's plasma with circulating oxLDL. A sensitive response was observed from 0.5 to 18.0 mg mL^{-1}. The device was successfully applied to determine the oxLDL fraction in real serum, without prior dilution or necessary chemical treatment. The use of multiple monoclonal antibodies on a biosensing platform seemed to be a successful approach to produce a specific response towards a complex multi-analyte target, correlating well with the level of oxLDL within atherosclerosis disease, in a simple, fast and cheap way.

1. Introduction

Coronary heart disease is the result of inadequate circulation of blood in the myocardium and occurs when the coronary arteries become partially blocked or obstructed.[1] Atherosclerosis is the main cause of cardiovascular disease and it is an in□ammatory condition characterized by thickening and hardening of the arterial wall, with the deposition of fatty substances, choles- terol, cellular waste products and other substances, leading to plaque formation, called atheroma, and causing hemodynamic alteration.[2,3] Furthermore, an atheroma, in later stages, can totally or partially block blood □ow through the arteries and cause plaque rupture with haemorrhage and development of thrombosis, which are the main causes of morbidity and mortality in westernized countries, leading to a considerable economic overload.[3-5]

LDL is the main carrier of cholesterol in the blood and exerts important physiological and cellular function and regulation of metabolic pathways. However, a large amount of clinical evidence, as well as epidemiological studies, correlate high concentrations of plasma cholesterol and increased risk of cardiovascular disease.[6] Furthermore, increased plasma levels of LDL, especially its oxidized form (oxLDL), are associated with atherosclerosis. oxLDL promotes vascular dysfunction by exerting direct cytotoxicity towards endothelial cells and increases chemotactic properties for monocytes and macrophages and consequent transformation into foam cells, which contribute to atherogenesis.[7-10]

Although the oxLDL level in circulating plasma is commonly recognized as an important predictive marker of risk of cardiovascular events, simple and cheap assays for oxLDL level

determination remain to be developed. There are many ways to determine oxLDL in the blood by ELISA (enzyme-linked immunosorbent assay) methods, such as employing different monoclonal and polyclonal antibodies that are capable of detecting oxLDL fractions involved in the atherogenesis.[11–13] Alternative methods employ anti-oxLDL Abs conjugated to gold nanoparticles and subsequent detection by liquid chromatography/tandem-mass spectrometry (LC-MS/MS)[13] or proteoheparan sulfate coated hydrophobic silica for subsequent ellipsometry readings.[14–16] These two approaches are however unsuitable for applications in point-of-care diagnosis, also being coupled to expensive equipment.

Despite the differences observed between different ELISA procedures used for oxLDL determination, which are directly correlated with the biological and clinical significance of oxLDL measures,[11] the gold standard methods to determine oxLDL are still carried out using commercial ELISA kits. These methods are also very expensive and time consuming, requiring highly qualified personnel and special handling procedures/storage conditions. Therefore, novel approaches with a simple procedure and low cost are highly necessary and would be greatly appreciated.

Biosensors could offer advantageous features compared to diagnostic tools like ELISA, due to their sensitivity, selectivity, simplicity, speed, low-cost, portability, and automation feasibility.[17–23] An easy approach towards biosensing design in this context is to include an antibody as a biological recognition element, usually recognized as an immunosensor.

Although immunosensors display several attractive features, there are two major difficulties hindering the performance of immunosensing devices. One difficulty is to ensure that the Fragment antigen-binding (Fab) is suitably exposed for antigen binding. Many studies report random binding of Abs, preventing many of the Fab fragments in the receptor platform from binding to the antigen. To solve this problem, several authors reported complex chemical systems driving the Abs connected to the solid support in a specific orientation,[24–28] but in this case the many reactions involved turn this a very complex process. Also, the effectiveness of each stage of chemical modification conditioning such an orientation is unknown, for which this process may not be as effective as expected in the theoretical approach.

The other major difficulty is to obtain a specific response towards the antigen. The use of monoclonal antibodies (mAb) may turn out to be a successful approach to obtain such a specific response towards a selected target antigen.[28–31] This is particularly relevant when the analyte is not a single structure but a mixture of several/complex target compounds. This is the case for oxLDL, obtained by random oxidation of native LDL.

Thus, this work proposes the development of an immunosensor for oxLDL by employing simple chemistry strategies that ensure suitable orientation of the mAb on top of a gold sensing layer of screen-printed electrodes (SPEs). Furthermore, three different mAbs were merged in the same platform to ensure specific antigen binding towards a target of multiple chemical structures. The optimization of the sensor design, the subsequent analytical performance and its real application are presented further.

2. Materials and methods

All procedures involving animals or humans were approved and conducted according to the Institutional Ethics Committee on research involving human (CEPSH of ICB/USP) (Protocol no. 1033/CEP) and the Committee for Animal Care and Utilization (CEUA of ICB/USP) (Protocol no. 116; book 02; page 119).

2.1 Equipment

The electrochemical measurements were conducted in a potentiostat/galvanostat from Metrohm Autolab/PGSTAT302N, impedimetic module and controlled by NOVA software. AuSPEs were purchased from DROPSENS (DRP-C220AT), consisting of working and counter electrodes made of gold and the reference electrode and electrical contacts made of silver. The diameter of the working electrode was 4 mm. The SWV measurement was conducted in 5.0 mmol L^{-1} of $[Fe(CN)_6]^{3-}$ and 5.0 mmol L^{-1} of $[Fe(CN)_6]^{4-}$, prepared in PBS pH 7. In SWV potentials were changed from −0.7 to +0.7 V, corresponding to a frequency of 50 Hz and a step height of 150 mV. EIS assays were conducted with the same redox couple $[Fe(CN)_6]^{3-/4-}$ at a standard potential of 0.12 V, using a sinusoidal potential perturbation with an amplitude of 0.01 V (RMS) and the number of frequencies equal to 50, logarithmically distributed over a frequency range of 0.1–100 kHz. The impedance data were fitted to a Randles equivalent circuit using the implemented NOVA software.

2.2 LDL isolation

Blood samples were collected from healthy volunteers of both sexes, after fasting for 12 hours, with cholesterol and triglyceride concentrations in plasma within the normal range (cholesterol <240 mg dL^{-1} and triglycerides <160 mg dL^{-1}, as confirmed by laboratorial data). Blood was collected in blood collection vacuum tubes (BD Vacutainer), containing 0.054 mL of anticoagulant, ethylenediaminetetraacetic acid (EDTA, disodium salt), 4.5 mL volume for suction. The blood of each volunteer was separately centrifuged (1000 × *g*, 4 °C, 10 minutes) and was used to create a pool of plasma. Then, a mixture of preservatives with 2 mM benzamidine, 0.5% gentamycin, 0.25% chloramphenicol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 units per mL aprotinin was added to this pool.

LDL (d ¼ 1006 ± 1063 g mL^{-1}) was isolated by sequential ultracentrifugation at 100 000*g* for 20 h at 4 °C,[32] using a 50Ti rotor, L-8 Ultracentrifuge, Beckman (VLDL: 12 h, density ¼ 1.006 g mL^{-1}; LDL: 20 h, density < 1.063 g mL^{-1}; HDL: 40 h, density < 1.21 g mL^{-1}). LDL was dialyzed for 48 h in PBS–EDTA 0.01% pH 7.4 (0.9% NaCl, 0.3% Na_2HPO_4, 0.2% H_2PO_4; NaOH 0.38% and 0.01% EDTA) and filtered (0.22 mm in diameter, Millipore). LDL preparations were stored at 4 °C. The protein content was measured by Lowry's method.[33]

2.3 LDL oxidation

For the preparation of oxLDL, native LDL from 4 to 6 mg mL^{-1} was incubated with a solution of copper sulphate ($CuSO_4$), at a

nal concentration of 20 mM for 18 h at 37 °C. The reaction was blocked with the addition of 1 mM EDTA (Fernvik *et al.*, 2004; Zaratin *et al.*, 2002). To assess the extent of the LDL oxidation, the malondialdehyde (MDA) content was analyzed by measuring the thiobarbituric acid reactive substances (TBARSs).[34,35] MDA was formed as a consequence of the peroxidation of LDL particles.

2.4 Monoclonal antibodies (mAbs)

The mAb-producing hybridomas anti-oxLDL, previously obtained inside the group, were tested against the product of oxLDL oxidation separated by molecular exclusion chromatography (superdex 200), by an ELISA indirect method and by competitive ELISA with synthetic peptides of apoB.[36] The hybridomas were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) containing 10% foetal calf serum (FCS), 100 mg mL^{-1} L-glutamine and 50 mg mL^{-1} gentamicin. The isotypes of the monoclonal antibodies (73, 77 and 87), present in supernatant fluid of hybridoma cultures, were stored at −20 °C until purification.

The isotypes of the mAbs were determined by an ELISA indirect method, using the kit of Mouse Monoclonal Antibody Isotyping Reagents (SIGMA, Saint Louis, Missouri, USA). Briefly, the ELISA procedure was conducted with the anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM or IgA produced in goat, used at diluted 1 : 1000 to coat the wells of microtiter plates and left overnight at 4 °C. Non-specific reactions were blocked with skim milk (5% w/v in PBS; Molico Milk, Nestle) for 2 hours at 37 °C. After that, the mAb73, 77 or 87 were added at a concentration of 250 mg mL^{-1} and left for 1 hour at 37 °C. Then, anti-mouse Ig horseradish peroxidase linked whole Ab (Amersham LIFE SCIENCE) diluted 1 : 1000 were added into every well and kept for 1 hour at 37 °C. This was followed by the addition of *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) with hydrogen peroxide as a substrate. Between all steps, the plate was washed four times with PBS–Tween containing 0.05% Tween 20 (PBS-SM-T20) and three times with PBS. The reaction was stopped with sulphuric acid 2 N and read using a spectrophotometer at 492 nm.

2.5 Antibody purification

The mAbs in the supernatant fluid were purified by affinity chromatography using Protein G (Protein G Sepharose 4 Fast Flow-Sigma-Aldrich). For this, 2 mL of Protein G were added to each 500 mL of hybridoma supernatant fluid, and the resulting mixture was subjected to mild agitation for rv6 hours, to allow the antibodies to bind to Protein G. After stirring, the flask was allowed to stand for the precipitation of Protein G. The supernatant was removed and Protein G was washed with PBS until the solution stayed clear. The non-adsorbed proteins were removed by washing with PBS. The mAbs were eluted from the column with 0.5 M acetic acid, and collected in 10 drops of eluent flow, every 5 seconds, in tubes containing 250 mL of Tris 1 M of pH 8.8, per well (to neutralize pH of the samples). The presence of proteins (antibodies) in each tube was detected by the Bradford method. After this, the aliquots were gathered to create a pool with a higher amount of protein, and subsequently dialyzed in PBS for 18 h. The samples were stored at 4 °C, protected from light. The as-purified mAbs were assayed for protein content by Lowry's method,[33] separated in smaller vials, and stored at −20 °C until use.

2.6 Building of the immunosensor

The immunosensor was setup by self-assembling Cyst (Cysteamine ($C_9H_{13}N_3O_5$) 50 mM) for 1 hour on the working 4 mm f gold (Au) layer of a SPE. Antibodies were left to react with NHS (*N*-hydroxysuccinimide ($C_4H_5NO_3$) 25 mM) and EDAC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) for 15 minutes, and subsequently incubated in the Au/Cys. A drop of BSA (0.5 mg mL^{-1}) was then added to the sensing layer, for 1 hour, to avoid non-specific binding. The modified electrodes were finally incubated with oxLDL in buffer solution for 15 minutes. All these steps of chemical modification were followed by EIS and SWV.

2.7. Optimization of the immunosensor

The kind and amount of mAbs were tested first. Biosensors were prepared with single mAb (73, 77 or 87) or multiple mAbs (73, 77 and 87 in the same device). The concentration of mAbs was always 50 mg mL^{-1} (in single mAb and in the pool of 3 different species of mAbs). Overall, the biosensor with a single mAb and three mAbs were produced with the same concentration of Ab.

The obtained biosensors were calibrated by adding successive oxLDL standard solutions of increasing concentrations, and using the same conditions of electrical operation aforementioned for EIS and SWV studies.

The selectivity of the biosensor was assessed for native LDL, myoglobin equine skeletal muscle (Sigma-Aldrich) and haemoglobin human (Sigma-Aldrich), solutions prepared in PBS pH 7, with high concentrations of 100 mg mL^{-1}, for all negative controls. The time of contact given to these solutions was 1 hour.

To simulate the use of the immunosensor with patient's plasma with circulating oxLDL, the lowest concentration of oxLDL tested was diluted in FCS and the response was checked by EIS and SWV.

All electrochemical tests were carried out in triplicate.

3. Results and discussion

3.1. Oxidation of native LDL

To measure the extent of LDL oxidation, thiobarbituric acid measurements were employed. This compound is an important indicator of lipid peroxidation. Its concentration increased with the incubation time of LDL in copper sulphate, up to a maximum value where the oxidation no longer proceeded. The best time for oxidation was found at an 18 h incubation period.

The isolation and purification of the oxLDL in solution was made by ionic chromatography. As in any oxidation, carboxylic functions were introduced by oxidation and these carry an anionic charge under physiological pH. So, the oxLDL fraction after an 18 h oxidation period was retained longer than the native LDL in an ion exchange column (Resource Q).

3.2. Antibody–antigen interactions

All tests made regarding the mAbs showed that these were isotyped as IgG1. The mAb was tested against the product of oxLDL separated by molecular exclusion chromatography (superdex 200) by ELISA method.[36] By competitive ELISA with synthetic peptides of apoB, only the mAb77 showed slightly decreased binding to oxLDL. Further information regarding these antibodies has been presented by Freitas, 2004.[36]

The size distribution profile of oxLDL and the size of formation of the antigen (oxLDL)–antibody (mAb anti-oxLDL) complex was determined by dynamic light scattering. This was done simply by placing a drop of the solutions under study in Avid Nano DLS equipment.

The oxLDL solutions displayed nanostructures with an average size of 67.7 nm f, with a standard deviation of 65.6 nm. This unusually high standard deviation was expected, because the oxidized nanostructures were produced by random oxidation of the native LDL.[37] The expected average molecular weight of oxLDL was 85 013.13 kDa, corresponding to a polydispersion % of 0.94.

The antigen–antibody (Ag–Ab) complex had an average size of 208.9 nm f, with a standard deviation of 130.1 nm. Considering that each mAb may bind to two Ag species, and that the variation in the size of Ag was 65.6 nm, a variation in 130.1 nm of the Ag–Ab complex demonstrated the great ability of the Ab to bind to the target structures, independent of their size or exact structure. This was also expected because the mAbs had been targeted for this oxLDL mixture. The expected average molecular weight of oxLDL was 440 162.20 kDa, corresponding to a polydispersion% of 0.39.

3.3. Immunosensor design

The immunosensor design consisted of creating an amine monolayer on a gold support to bind to the Ab through its Fc terminal. This would allow both paratope regions of the Ab to be free for subsequent antigen binding. The overall process can be seen in Fig. 1.

The amine monolayer was created by incubating the gold support on cysteamine: the thiol group bound to the gold and led to the formation of a compact amine monolayer (Fig. 1A). The presence of this layer on gold could be confirmed by the increase in the charge-transfer resistance (R_{ct}) observed after the gold clean support was incubated in cysteamine (Fig. 2). The R_{ct} corresponded to the diameter of the semicircle, and its increase corresponded to an increased resistance in the electron

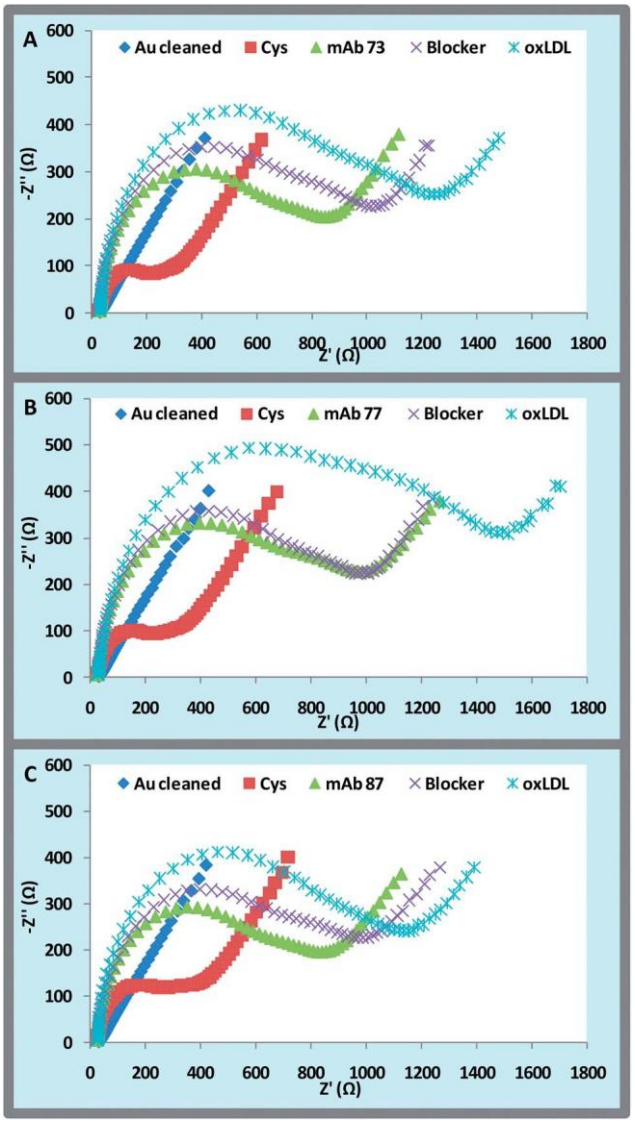

Fig. 2 The EIS response of the immunosensor assembly with single mAb (A) mAb73, (B) mAb77 and (C) mAb87.

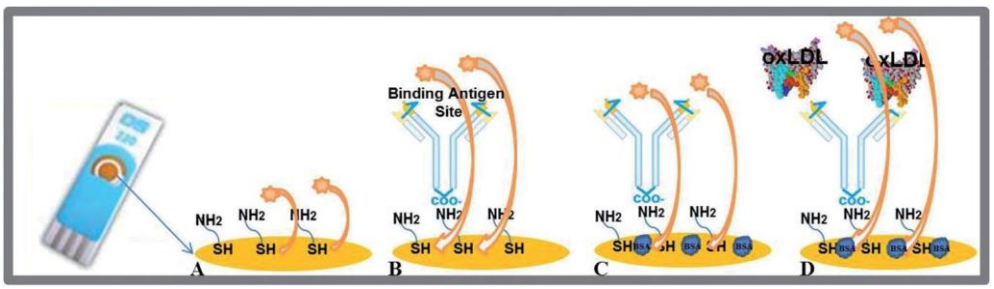

Fig. 1 Immunosensor assembly. A gold electrode with self-assembled cysteamine (A); antibody binding (B); BSA binding (C) and the modified electrodes were finally incubated with oxLDL (D).

transfer kinetics of the iron redox-probe, accounting for the formation of a resistive layer.

In order to ensure an extensive Ab binding to the amine surface under mild conditions, the –COOH groups of the Ab had to be activated first by conventional EDAC–NHS chemistry.[38] Once activated, the antibodies were connected to the support by covalent amide functional groups (Fig. 1B), promoting a significant increase in the R_{ct} of the Au/NH_2 support (Fig. 2). This behaviour was consistent for all mAbs used.

All regions of the Au/NH_2/mAb support different from paratope sites could allow binding of other chemical species besides oxLDL. To avoid this, the biomaterial was incubated in BSA (Fig. 1C). Again, a significant increase in R_{ct} was observed (Fig. 2), thus confirming that BSA was bound to the Au/amine/mAb surface and indicating that a great number of non-specific response sites had been blocked.

The Ag present in the immunosensor was expected to bind to the paratope sites of the Ab (Fig. 1D). As oxLDL particles were mainly negatively charged, the subsequent R_{ct} should decrease, considering that the iron redox probe used to check the charge transfer capability had the same charge. This was observed for all immunosensors (Fig. 2).

3.4. Single and poly-monoclonal antibody response

Different mAbs obtained from the same oxLDL mixture were called mAb73, mAb77 and mAb87. Each is expected to have different epitopes for the complex targeted mixture, meaning that immunosensors assembled with individual or combined mAb mixtures could have different electrical operation.

The immunosensor was first assembled in a single Ab arrangement, immobilizing only mAb73 or mAb77 or mAb87 on the Cys layer of the Au-SPE. Overall, each of these displayed similar EIS spectra, responding well to the oxLDL antigen diluted in buffer solution (Fig. 2). The EIS spectra of the assembly of the biosensor with multi-mAbs (the three kinds together in a single SPE) are shown in Fig. 3A. Its analytical performance (Fig. 3B) was similar to that displayed by the single mAb SPEs, which is in clear agreement with the simple fact that the area of the working layer to which the Ab may bind is the same, independent of the number or kind of Ab binding to it. The as-obtained device showed a linear response from 5 to 20 mg mL^{-1}, with slope and intercept values of 41.33 (U × mL mg^{-1}) and 1267.4 U, respectively. Each device was produced in triplicate and the results obtained showed good reproducibility, always below 5%.

Overall, the single or multi-immunosensing devices displayed similar features. Still, only multi-mAb was used in further studies, because it was reasonable to assume that different epitopes for the same target mixture comprising multiple oxidised nanostructures of LDL would favour the specificity of the analytical response.

3.5. Selectivity study

To check the ability of the mAb to discriminate oxLDL nanostructures from the parent or coexisting compounds in serum, the response of the multi-mAb biosensor was checked against

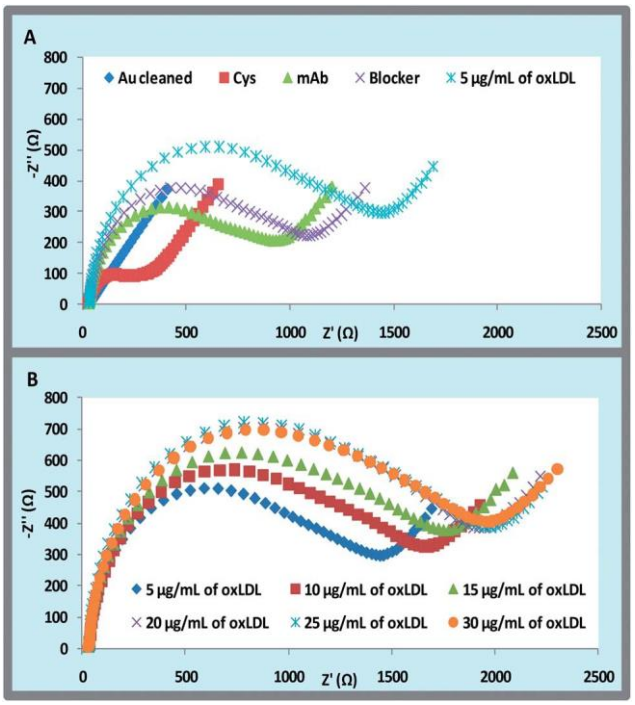

Fig. 3 EIS spectra obtained on the assembly of the multi-mAb immunosensor ((A) including a cleaned Au surface, Cys, mAb73, 77 and 87 together (mAb), block with BSA and the response to the first standard of oxLDL, 5 mg mL^{-1}) and its subsequent calibration with oxLDL ranging from 5 to 30 mg mL^{-1} (B).

native LDL, myoglobin and haemoglobin. This was done by placing a drop of the corresponding solutions on the platform that had been blocked by BSA. This study was made with foreign solutions having a high concentration of the target species, 100 mg mL^{-1}, and the time given for affinity reaction with the platform was 1 hour. This procedure was the same for the negative control made with buffer. Repeated applications of solutions with foreign species were made, ensuring that the effects of their abnormally high levels in serum would also be checked.

In general, the immunosensor incubated in foreign species showed the same response as that shown by the immunosensor after BSA incubation (Fig. 4), confirming that they were unable to react with the mAbs placed in the outer surface of the biosensor. None of the previous foreign species at any of the concentrations tested were able to change the baseline response of the immunosensor. Even native LDL was unable to change the response of the device, thus confirming the specificity of the dimensional platform towards the targeted oxLDL material. The biosensor did not respond to other antigens at 15 minutes and even for contact times up to 1 h.

Overall, the biosensor demonstrated excellent results in terms of sensitivity and specificity. Tests with nonspecific antigens as native LDL, myoglobin and hemoglobin, made with FCS without human oxLDL, also grant avoiding or masking the response of the device with monoclonal antibodies. The results showed the feasibility of the immunosensor to be applied to patients with an over concentration of side-antigens.

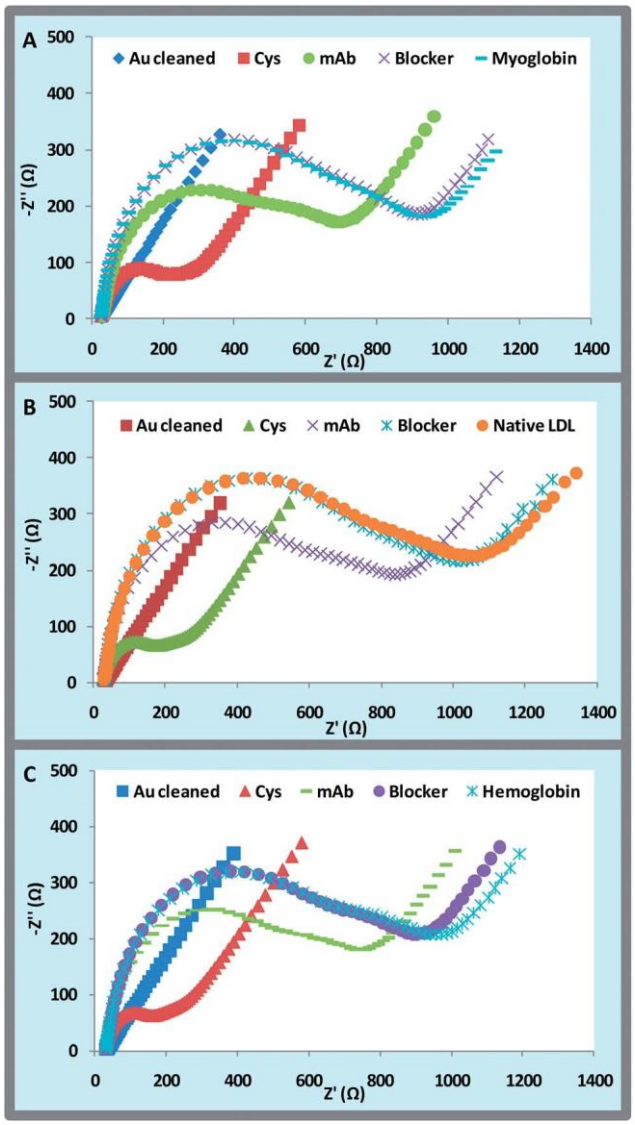

Fig. 4 Selectivity test against proteins in serum that coexist with oxLDL. (A) myoglobin, (B) native LDL and (C) haemoglobin.

3.6 Analytical performance

The immunosensor with multi-mAbs was assembled next to check its response against oxLDL, for concentrations up to 50 mg mL^{-1}. The overall response showed that the biosensor responded well after 5 mg mL^{-1} oxLDL, this being the concentration level included in the linear response range of the device. The linear response was observed up to 20 mg mL^{-1}, being expressed as R_{ct} (U) ¼ 41.33 × oxLDL (mg mL^{-1}) + 1267.4, with a squared correlation coefficient of 0.994. The standard deviation of replicate measurements was below 5%.

3.7. Serum reading and practical application of the device

Considering that the targeted biosensor was meant to operate well in serum, it was important to check the performance of the biosensor under close-to-real conditions. This was done by replacing the buffer solution by FCS, prepared in diluted or non-diluted (equal to biological composition) solutions. FCS was used to simulate serum because it displays great similarity to human's serum.[39] SWV and EIS studies were conducted for this intended application.

The EIS spectra of the immunosensor tested with oxLDL diluted in FCS showed clear evidence of the presence of the affinity between oxLDL and the mAbs (Fig. 5A). To ensure that FCS was not responsible for this R_{ct} increase, a blank signal was measured for FCS without oxLDL (Fig. 5B). This negative control showed that all biological species present in FCS were unable to interact with the immunosensor surface. This attributed the device a specific response to human oxLDL, which is mostly related to the specific affinity between the mAbs and oxLDL, combined with the immunosensor design.

The analytical performance of the immunosensor in FCS solutions was checked by calibrating the device with oxLDL standards prepared in FCS. Since all samples would need at least one dilution step prior to analysis, diluted FCS was used in this case. The oxLDL concentration range was set to 0.50–50 mg mL^{-1}. Under these conditions, the immunosensor was able to detect human oxLDL from 0.50 to 18.0 mg mL^{-1}. The slope and intercept values were, respectively, 62.43 (U × mL mg^{-1}) and 540.2 U, with a squared correlation coefficient of 0.9951 (typical spectra in Fig. 6A and B). The limit of detection was 0.22 mg mL^{-1}, calculated as the interception between the linear and non-liner parts of the calibration curve.

The performance of the immunosensor in diluted FCS was also checked by SWV. The voltammograms so obtained can be seen in Fig. 6. In general, the assembly of the device decreased

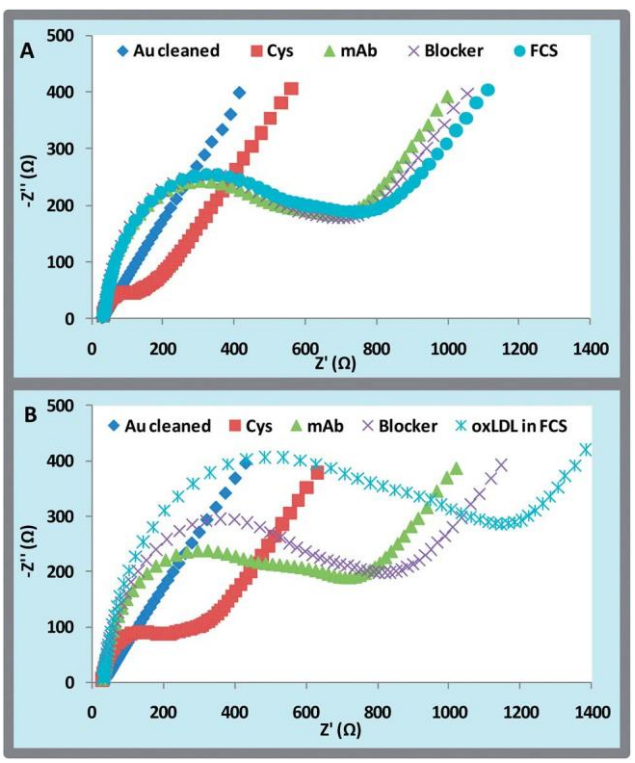

Fig. 5 EIS spectra of the immunosensor with FCS (A) or oxLDL diluted in FCS (B).

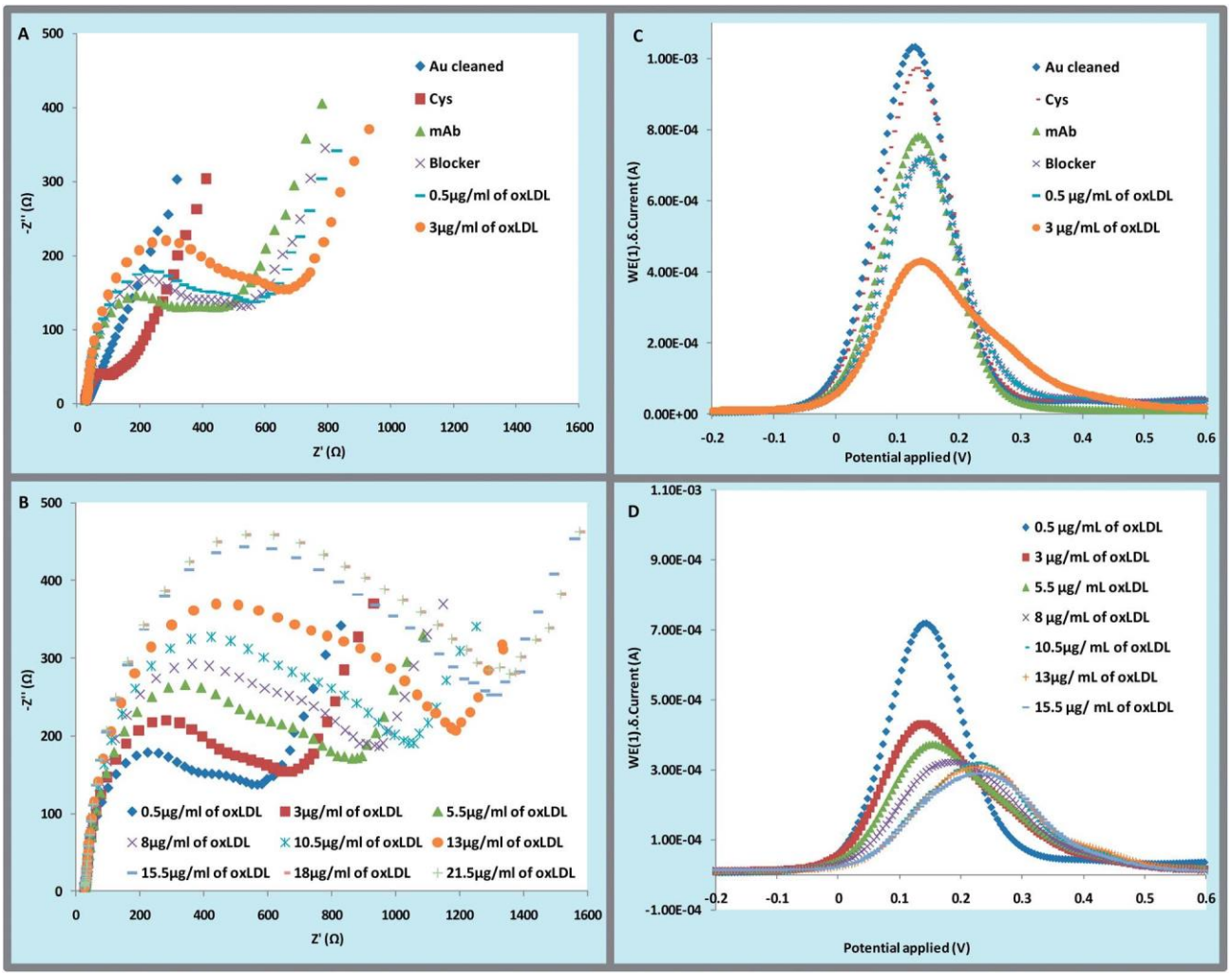

Fig. 6 EIS spectra of the immunosensor assembly and oxLDL response (A) and its operation when diluted in FCS, calibrated with oxLDL ranging from 0.5 to 21.5 mg mL^{-1}, and the corresponding SWV data (C and D, respectively).

the peak current of the redox probe (Fig. 6C), which was consistent with the previous EIS data. The calibration in SWV showed linear responses ranging from 3.0 to 10.5 mg mL^{-1} (Fig. 6D), pointing to that EIS seemed to be a more suitable technique for analytical application.

4. Conclusions

The use of monoclonal antibodies on a biosensing platform was an effective approach to monitor the level of oxLDL fraction in the blood, a parameter of major relevance in atherosclerosis disease. EIS was a suitable technique to follow the affinity reaction between the mAbs immobilized on the screen-printed electrode and oxLDL. The immunosensor displayed an unusual speci□city for the targeted analyte, being able to detect it when present in real serum matrices, at concentrations within physiological levels.

Reading in real time is achieved within 20 minutes, including binding of the antibody to the antigen and EIS reading, and avoiding unnecessary use of conjugated antibodies for detection purposes. The screen-printed electrode device proposed here is expected to cost less than **V**10, excluding here the price associated with the □rst production of the mAbs, because the necessary hybridoma cells have already been established and these cells are able to produce antibodies "inde□nitely".

The immobilization of the antibody on the screen-printed electrode is also a quick process, taking only a few hours, unlike the typical overnight incubation in the diagnostics standards. In addition, the Ab immobilization in ELISA does not guarantee the Fab region free to bind the antigen. So, to have a good grip of the antibody in an ELISA plate, it is necessary that the board is of the type "high binding plates", but these boards are quite expensive and even so the correct antibody orientation is not guaranteed.

In brief, compared to the gold standard protocol of ELISA, the proposed device provides a much faster response at a much lower cost, also aiming at a suitably oriented antibody binding. Overall, the produced device is simple, fast, cheap, and portable, showing great potential to improve the current diagnosis of atherosclerosis in point-of-care testing.

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