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Development of impedimetric and optical calcium biosensor by using modified gold electrode with porcine S100A12 protein

Maria D.L. Oliveira[a], Celso P. de Melo[b], Glaucius Oliva[c], Cesar A.S. Andrade[d,*]

[a] Departamento de Bioquímica, Universidade Federal de Pernambuco, 50670-901 Recife, PE, Brazil
[b] Departamento de Física, Universidade Federal de Pernambuco, 50670-901 Recife, PE, Brazil
[c] Instituto de Física de São Carlos, Universidade de São Paulo, 13560-970 São Carlos, SP, Brazil
[d] Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, 55608-680 Vitória de Santo Antão, PE, Brazil

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ABSTRACT

We describe the development of a label free method to analyze the interactions between Ca^{2+} and the porcine S100A12 protein immobilized on polyvinyl butyral (PVB). The modified gold electrodes were characterized using cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), scanning electron microscopy (SEM) and surface plasmon resonance (SPR) techniques. SEM analyses of PVB and PVB-S100A12 showed a heterogeneous distribution of PVB spherules on gold surface. EIS and CV measurements have shown that redox probe reactions on the modified gold electrodes were partially blocked due the adsorption of PVB-S100A12, and confirm the existence of a positive response of the immobilized S100A12 to the presence of calcium ions. The biosensor exhibited a wide linear response to Ca^{2+} concentrations ranging from 12.5 to 200 mM. The PVB-S100A12 seems to be bound to the gold electrode surface by physical adsorption; we observed an increase of 1184.32 m° in the SPR angle after the adsorption of the protein on the PVB surface (in an indication that 9.84 ng of S100A12 are adsorbed per mm^2 of the Au-PVB electrode), followed by a further increase of 581.66 m° after attachment of the Ca^{2+} ions. In addition, no SPR response is obtained for non-specific ions. These studies might be useful as a platform for the design of new reusable and sensitive biosensing devices that could find use in the clinical applications.

1. Introduction

Calcium ions regulate many biological processes through their interactions with specific receptor binding proteins [1], several of which have been identified in various cellular environments in eukaryotic cells [2]. For instance, a recent report has examined how the calcium concentration present in blood and saliva could be used in the evaluation of dental development [3].

In addition, there are many methods for detection of calcium such as biosensors [4–6], continuous flow analysis (CFA) [7], HPLC [8], electrochemical methods [9–11], X-ray fluorescence [12], ion-selective electrodes [13] and ion chromatography [14]. Today, calcium levels in human fluids are clinically determined using either atomic adsorption spectroscopy or colorimetry. In fact, titration methods are becoming gradually obsolete, and the ion-selective electrode (ISE) method is nowadays commonly used for the quantitative determination of calcium cations not bound to proteins or other complexing agents, even though interferences from accompanying ions, hydrogen ions and ionic strength differences continue to be problematic. As illustrative examples, we note that Buckley and Russell [15] reviewed those techniques for assay of serum calcium cation, including non-potential analyses (such as spectrophotometry), potential techniques (such as ISE other electrochemical studies), and some reference methods, for example, colorimetric assay [16], while Freaney et al. [17] have described an automatic method for determining calcium in blood, serum and plasma.

Calcium-binding proteins (CaBPs) are involved in the regulation of several biological processes [18,19]. S100 proteins constitute the largest subfamily of EF-hand proteins (i.e., those CaBPs that present a helix-loop-helix structural domain) and interact either in a Ca^{2+}-dependent or in an independent way with proteins involved in cell proliferation and differentiation, cellular architecture, signal transduction, and intracellular metabolism [20,21]. Most S100 proteins occur as non-covalent homodimers [22] and are characterized by the presence of two EF-hand motifs per monomer, which are ordered in N-terminal EF-hand (helix I–loop I–helix II) with a flexible linker region that connects helix II to helix III of the C-terminal EF-hand (helix III–loop II–helix IV). The linker region and C-terminal extension show the least amount of sequence conservation among S100 proteins [23,24]. Different conformational changes are triggered by calcium binding to S100 proteins in the two EF-hands, and they exhibit distinctive affinities for calcium [25].

* Corresponding author. Tel.: +55 81 3523 3351; fax: +55 81 3523 3351.
E-mail address: csrandrade@gmail.com (C.A.S. Andrade).

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The C-terminal EF-hand contains the canonical Ca^{2+}-binding loop common to all CaBPs. Porcine S100A12 (also known as calgranulin c) is a member of the S100 protein subfamily that consists of 91 amino acids (approximately 10.7 kDa and an acid pI of 5.8) and is characterized by the presence of two EF-hands motifs and an additional binding site (Lis-x-x-x-His), with high affinity for Zn^{2+} on the c-terminal region [26].

In the present study we describe the development of biosensor that uses a porcine S100A12 modified polyvinyl butyral (PVB) electrode that relies in the sensitiveness of electrochemical impedance spectroscopy (EIS) and surface plasmon resonance (SPR) signals for calcium detection. To determine the ability of the porcine S100A12 in recognizing calcium ions, our assay was based on registering the differences of the biosensor responses in the absence and presence of these ions in the testing media. The modified electrodes were characterized by use of cyclic voltammetry (CV), EIS, scanning electron microscopy (SEM) and SPR techniques.

Due to the introduction of sensitive bench top instruments and the expansion in the range of strategies for studying biological or biochemical systems [27] in more recent times, SPR based techniques have found expanded use and recently they have been introduced in clinical studies [28]. The competitive advantages of such techniques include the fact that SPR allows both real-time qualitative and quantitative assessments of the prevailing biomolecular interactions while eliminating the need of labeling reagents; in addition, the SPR signal is highly sensitive to even small changes in the refractive index at the interface with a thin noble metal film [29].

On its turn, EIS has long been established as a sensitive technique to monitor the electrical response of a solid/liquid system subjected to the application of a periodic small amplitude AC signal. Analysis of the system response provides information concerning the solid/liquid interface and on the eventual occurrence of reactions at this local region [29,30].

2. Experimental

2.1. Materials

S100A12 protein was obtained by synthetic gene assembly, cloning and, subsequent recombinant S100A12 expression [31]. $CaCl_2$, ethylenediamine tetraacetic acid (EDTA) and PVB were purchased from Sigma Chemical (St. Louis, MO, USA). Potassium ferri- and ferrocyanide were obtained from VETEC (Brazil). All chemicals and solvents were of analytical grade and they have been used as received, without further purification. Water used was obtained from a Milli-Q plus (Billerica, USA) purification system.

2.2. Apparatus

Electrochemical measurements were carried out on a PGSTAT 302N potentiostat (Autolab, Eco Chemie, The Netherlands) interfaced with an analyzer controlled by a computer. A three-electrode setup with an Ag/AgCl (saturated KCl) reference electrode was employed throughout the investigation. All potentials are referred to this electrode. A platinum wire and a modified gold disc (d = 2 mm) were used as auxiliary and working electrodes, respectively.

2.3. Porcine S100A12 immobilization

First, the gold disc electrode was mechanically polished with 0.05 μm α-Al_2O_3 powder, and washed ultrasonically in distilled water. A sol–gel method was employed to modify the electrode [32]. Porcine S100A12 solution (200 μg/mL) was mixed with 300 μL of polyvinyl butyral (PVB)–ethanol solution (2%, v/v), quickly added into a beaker (4 °C) and kept under stirring for 10 min. After the electrode was incubated into the above solution for 10 min and air-dried, it was ready for use in the calcium detection investigation.

2.4. Calcium–S100A12 interaction

The porcine S100A12 modified electrode (Au-PVB-S100A12) was rinsed with water to remove unbound protein and then exposed for 10 min to different $CaCl_2$ solutions, which were previously diluted in 10 mM pH 7.4 PBS solution, so as to obtain final calcium concentrations in the 12.5–200 mM range. All the above procedures were performed at room temperature (ca. 26 °C).

2.5. Electrochemical measurements

The impedance spectra were recorded in the frequency range of 100 mHz to 100 kHz. The amplitude of the applied sine wave potential was 10 mV, while the direct current (dc) potential was limited at the open circuit potential measured just before the application of the sine wave potential. Cyclic voltammetry (CV) was performed with a potential sweeping between +0.7 and −0.2 V at a scan rate of 50 mV s^{-1}. CV and EIS measurements, performed in the presence of a 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1) solution used as a redox probe in PBS (pH 7.4), were carried out at different stages of the preparation of the modified electrode. All electrochemical measurements were performed at room temperature and inside a Faraday cage.

2.6. SPR instrumentation and measurements

All SPR measurements have been made by use of an Esprit double channel SPR instrument (Autolab, Eco Chemie, The Netherlands) setup in the Kretschmann configuration [33]. Gold plated sensor surfaces (Autolab, Eco Chemie, The Netherlands) were used for the SPR measurements, and the corresponding signal was recorded as a function of calcium concentration for the Au-PVB-S100A12 system. The SPR signal was first recorded using a running solution to stabilize the electrode and allow the setting up of the baseline. During each run, the first 200 s represent the base line, after which the sample was added and allowed to interact with the electrode. The response of the SPR sensor was automatically monitored in a PC by a digital converter. For the quantitative assessment, the increase in the value of SPR angle after the total process (i.e., the net increase in the final baseline from the initial baseline) corresponds to the amount of bound analyte as the other processing conditions are kept constant.

3. Results and discussion

3.1. Characterization by SEM

In Fig. 1a we show a SEM image of a gold electrode coated with PVB, where a heterogeneous distribution of PVB spherules can be seen on top of a PVB film. An image of a gold electrode coated with PVB and S100A2 is shown in Fig. 1b: the film covers the entire surface, a fact that was confirmed by both EIS and CV analyses, which indicated the presence of a resistive layer at the gold/solution interface.

3.2. Electrochemical detection of calcium at PVB-S100A12 modified electrode

In Fig. 2 we show CV curves of the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probe, dissolved in phosphate buffer (pH 7.4), obtained by use of a bare gold electrode and of a modified gold electrode. When the electrode surface is modified, the electron transfer

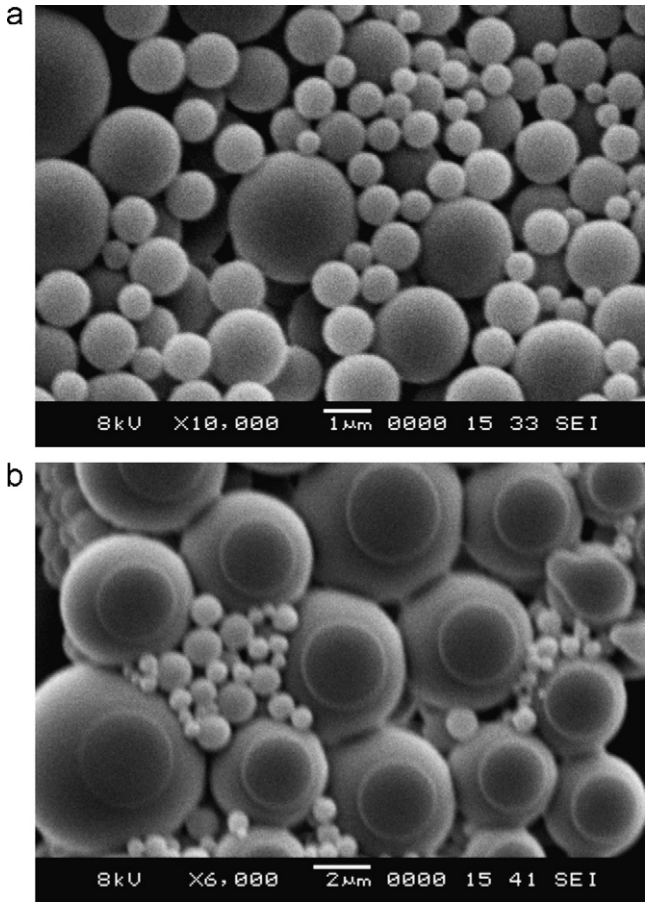

Fig. 1. SEM images of the gold electrode coated with PVB (a) and PVB-S100A12 (b).

kinetics of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probe is perturbed. In Fig. 2 we can find cyclic voltammograms corresponding to the cases of different electrodes (bare gold, PVB-modified, PVB-S100A12-modified, PVB-S100A12-calcium modified electrode and PVB-S100A12-calcium modified electrode incubated with EDTA in a 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ PBS solution), all of them obtained by use of a scanning rate of 50 mV s^{-1}.

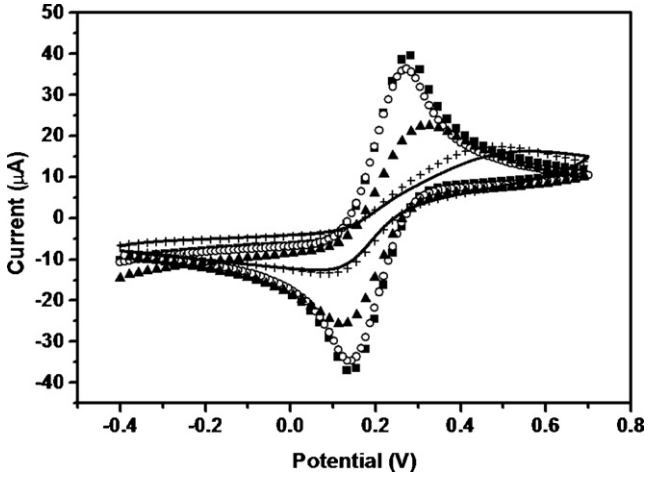

Fig. 2. Cyclic voltammograms (CVs) of the working electrode at different stages: bare gold electrode (■), Au-PVB (○), Au-PVB-S100A12 (▲), Au-PVB-S100A12-Ca^{2+} (—) and Au-PVB-S100A12-Ca^{2+}-EDTA (+). Supporting electrolyte: 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 1:1 + 0.15 M NaCl in 10 mM pH 7.4 solution; scan rate of 50 mV s^{-1}.

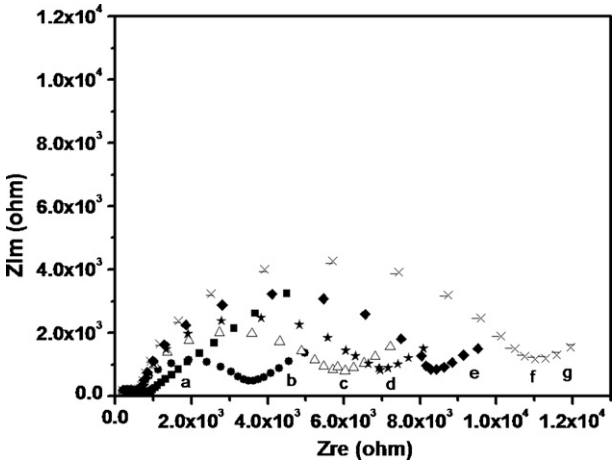

Fig. 3. Nyquist plots of the steps immobilization of S100A12: bare gold electrode (■), Au-PVB-S100A12 (●), the Au-PVB-S100A12-$Ca^{2+}_{(5\,min)}$ (△), Au-PVB-S100A12-$Ca^{2+}_{(10\,min)}$ (*) and Au-PVB-S100A12-$Ca^{2+}_{(15\,min)}$ (♦), Au-PVB-S100A12-$Ca^{2+}_{(20\,min)}$ (−) and Au-PVB-S100A12-$Ca^{2+}_{(25\,min)}$ (×) modified electrode. The impedance spectra were taken in 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 1:1 + 0.15 M NaCl in 10 mM pH 7.4 in the frequency range from 100 mHz to 100 kHz.

As shown in Fig. 2, while the curve for the bare electrode has the typical shape of a diffusion-controlled redox process, the stepwise assembly of different materials on the gold electrode seems to be accompanied by both a progressive decrease in the amperometric response of the electrode and an increase in the peak to peak separation between the cathodic and anodic waves of the redox probe. However, after the S100A12 molecules are coupled to the calcium ions, an obvious disappearance of the anodic and cathodic peaks occurs. This can be attributed to the fact that the calgranulin–calcium complex can act as an inert electron and mass transfer blocking layer that hinders the diffusion of ferricyanide towards the electrode surface. To verify if this calcium binding process would be reversible, the system was immersed in the EDTA aqueous solution during 20 min. As shown in the corresponding curve in Fig. 2, where an increase in the amperometric response can be observed, the calcium is quelled in the presence of EDTA.

EIS is an effective method for examining the interfacial properties of the sensor surface during the process of successive modifications. In EIS, the semicircle diameter of the spectrum equals the charge transfer resistance, R_{CT}. This resistance controls the electron transfer kinetics of the redox-probe at the electrode surface (Fig. 3). The EIS curve corresponding to the bare gold electrode shows a very small semicircle domain, implying the existence of a very low charge transfer resistance (R_{CT}) to the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probe in the electrolyte solution. However, after the gold electrode is immersed in the PVB solution, the EIS curve shows a high interfacial R_{CT}, indicating that the deposited PVB layers obstruct the charge transfer through the electrochemical probe. Finally, the PVB-S100A12-modified gold electrode was obtained by dipping the gold electrode into the homogeneous mixing solution containing protein and PVB. After immobilization of the S100A12 protein, the interfacial resistance of the modified electrode increased again, reaching levels corresponding to approximately three times those of the PVB-modified gold electrode. The impedance change of the modification process also showed that the protein had been immobilized to the electrode surface. In Fig. 3 we also show additional curves representing the S100A12 interaction with calcium the different incubation times (5, 10, 15, 20 and 25 min).

The general equivalent electric circuit (Randles) [32] includes the ohmic resistance of the electrolyte solution (R_Ω), the Warburg impedance (W) resulting from ion diffusion from the bulk elec-

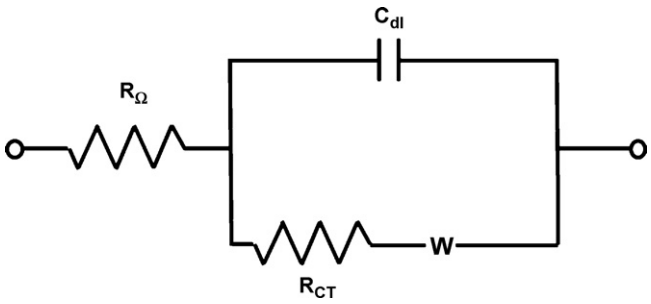

Fig. 4. Equivalent circuit adopted to fit the impedance data in the presence of redox pair of $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$: R_Ω is the ohmic resistance of the electrolyte solution, C_{dl} the double layer capacitance, Z_W the Warburg impedance, and R_{CT} the electron-transfer resistance.

trolyte to the electrode interface, the double layer (C_{dl}), and the charge-transfer resistance (R_{CT}), which are admitted to exist when the electrolyte solution contains a redox probe. In the PVB-S100A12 system, R_Ω and W denote, respectively, the bulk properties of the electrolyte solution and the diffusion features of the redox probe in the solution. When a Randles model (Fig. 4) [32] is used as an equivalent circuit to describe the data of electrochemical impedance measurements, the fitting of the measured spectra is shown in Fig. 5, where a good agreement can be observed over the entire measurement frequency range.

The best EIS parameters for this system were calculated from the data shown in Fig. 3 and are presented in Table 1. As discussed before, the stepwise increase of the electron-transfer resistance (R_{CT}) for PVB, PVB-S100A12 and PVB-S100A12-Ca^{2+} indicate the formation of insulating films of PVB and protein on the gold electrode. Our results show the occurrence of a high R_{CT} value for the PVB-S100A12-Ca^{2+} system. Since it is well known that high R_{CT} values are usually associated to the blockage of the electrode surface [32], we can assume that we have identified that a specific binding of Ca^{2+} exists in our system. Conversely, the low R_{CT} value obtained for the PVB-S100A12-Mg^{2+} seems to be due to unspecific binding (Fig. 5). Finally, when the PVB-S100A12-Ca^{2+} system is taken in contact with the EDTA, it was observed a decrease in the R_{CT}, which is probably due to the quelling of the ions, in agreement to what we had previously noticed in the cyclic voltammetry measurements.

In summary, the sequence of measurements for the pure PVB, PVB-S100A12, and PVB-S100A12-ion electrodes are consistent with the occurrence of progressive blockage of the interface, confirming that the amount of material immobilized and/or adsorbed on the electrode surface directly correlates with the impedance.

The results presented in Fig. 5 clearly show that S100A12 remain capable to recognize the calcium ion, as revealed by the increase of charge transfer resistance, R_{CT}. Hence, the performance of the modified electrode for detection of ions may be evaluated through the relative variation of this parameter (ΔR_{CT}), according to the

Table 1
Fitted impedance results to modified electrodes.

Modified electrode	R_{CT}/kΩ[a]	C_{dl}/μF[a]
With PVB	1.22	1.18
With PVB-S100A12	3.21	3.98
With PVB-S100A12-$Ca^{2+}_{(5\,min)}$	5.59	3.28
With PVB-S100A12-$Ca^{2+}_{(10\,min)}$	6.80	3.39
With PVB-S100A12-$Ca^{2+}_{(15\,min)}$	7.98	2.25
With PVB-S100A12-$Ca^{2+}_{(20\,min)}$	10.82	2.66
With PVB-S100A12-$Ca^{2+}_{(25\,min)}$	10.90	2.56
With PVB-S100A12-Ca^{2+}-EDTA	5.33	3.42
With PVB-S100A12-Mg^{2+}	3.72	3.54

[a] Values of the equivalent circuit elements from fitted impedance results.

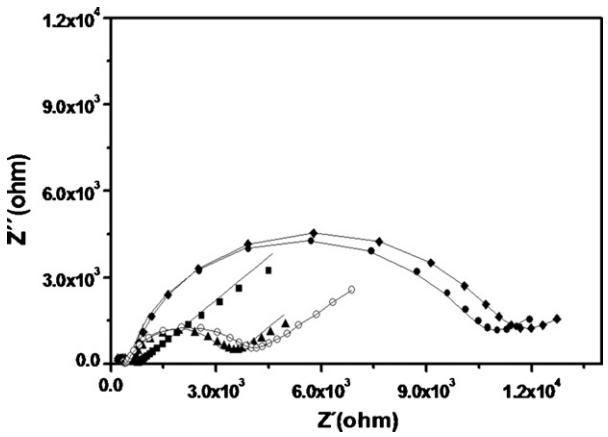

Fig. 5. Nyquist plots of the steps immobilization of S100A12: bare gold electrode (■), Au-PVB-S100A12 (▲), Au-PVB-S100A12-Mg^{2+} (○), Au-PVB-S100A12-Ca^{2+}-EDTA (●) and Au-PVB-S100A12-Ca^{2+} (♦). Solid lines correspond to the fitting using the obtained Randles parameters.

equation:

$$\Delta R_{CT}(\%) = \frac{R_{CT}(\text{S100A12-ion}) - R_{CT}(\text{S100A12})}{R_{CT}(\text{S100A12})}$$

here, while R_{CT}(S100A12) is the value of the charge-transfer resistance of the PVB-S100A12 modified electrode, R_{CT}(S100A12-ion) is the value of the charge-transfer resistance of the PVB-S100A12 after exposing it to solution containing calcium.

A more detailed study was carried out for different calcium concentrations (12.5 mM, 25 mM, 50 mM, 100 mM and 200 mM), which suggest that the interaction between S100A12 and ion can be quantitatively assessed by the modified electrode. As expected, the ΔR_{CT} increases linearly with calcium concentration, in an indication that the interactions between S100A12 protein and calcium can be detected by the modified electrode (Fig. 6). The performance of PVB-S100A12 system is shown in Table 2, for fixed Ca^{2+} and Mg^{2+} concentrations, where once more is confirmed the capability of S100A12 in recognizing calcium ions after adsorption. We call attention to the fact that only a small increase in the R_{CT} value (to 3.8 kΩ) is observed when the S100 protein is exposed to magnesium ions, a value to be compared to the charge transfer resistance (~11.0 kΩ) estimated when Ca^{++} ions are used instead. These results are a strong indication that this system could be applicable to the construction of a biosensor for calcium levels at human blood serum.

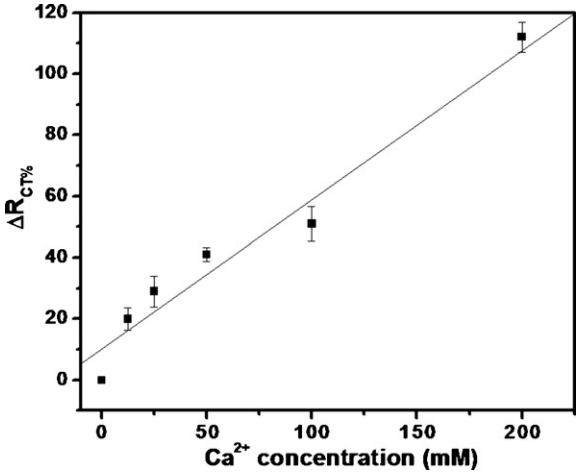

Fig. 6. Diagrams show ΔR_{CT}% for the Au-PVB-S100A12 case after exposing the electrode to different concentrations of calcium ions dissolved in H_2O.

Table 2
Fitted impedance results to S100A12-Ca^{2+} interaction from Fig. 5.

Modified electrode	S100A12-ion interaction			$\Delta R_{CT}(\%)_{(calcium)}$	$\Delta R_{CT}(\%)_{(magnesium)}$
	Before[a] (R_{CT}/Ω)	After[b]	After[c]		
Au-PVB-S100A12	3.21	7.98	3.72	149.0	16.0

[a] Before contact to ions.
[b] After contact to calcium.
[c] After contact to magnesium.

3.3. SPR characterization of S100A12–PVB modified electrode

Fig. 7 shows an illustrative sensorgram for PVB, PVB-S100A12 and PVB-S100A12-Ca^{2+}, PVB-S100A12-Mg^{2+} modified gold electrodes. When the solution of 0.1% PVB was injected in a flow cell during approximately 2.5 min, the resonance angle shifted about 287.06 m° with a low coefficient of variation (CV = 10.87%). The use of ethanol instead of water or buffers to dilute PVB promoted a more homogeneous layer. The PVB layer can act like an immobilization matrix. In this assay, the incubation time was controlled at 6 min and the reproducibility achieved in three assays showed a good value of CV (7.23%). Subsequently, the adsorption of S100A12 protein on Au-PVB array was accomplished by changing the resonance angle under continuous monitoring through the SPR system, in three replicates analyses. As a result of the fact that the PVB-S100A12 was attached to the gold electrode surface, we observed an increase of 1184.32 m° in the SPR angle after modification of PVB with S100A12, and a further increase of 581.66 m° after Ca^{2+} attachment. The S100A12 protein binds the calcium ions through linking by the aminoacid groups. A variation of 120 m° represents a change in the quantity of surface protein of approximately 1 ng/mm^2 or in the bulk refractive index of approximately 10^{-3} [33], indicating that 9.84 ng of S100A12 had adsorbed per mm^2 of the Au-PVB electrode. Finally, when EDTA was added to the system to sequestrate the calcium ions, a shift of 590.66 m° in the angle was observed. Also, in agreement to the CV and EIS measurements, the association with Mg^{2+} did not produce any modification in the optical response of PVB-S100A12 system.

In Fig. 8 we carried out a SPR study for different concentrations of calcium. Our results suggest that the interaction between S100A12 and Ca^{2+} ions can be quantitatively assessed by the SPR technique. Similar to the ΔR_{CT} results, SPR increases linearly with calcium concentration.

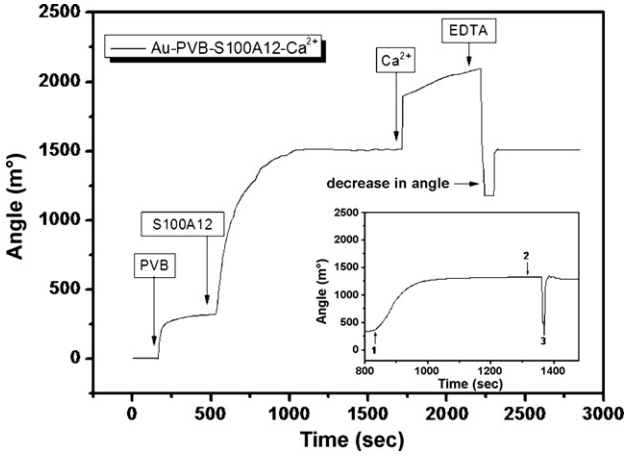

Fig. 7. Typical sensorgram to Au-PVB-S100A12-Ca^{2+}. Inset: SPR response of PVB-S100A12 system (position 1, base line immobilization), injection of Mg^{2+} ions (position 2, negative control) and PBS buffer (position 3).

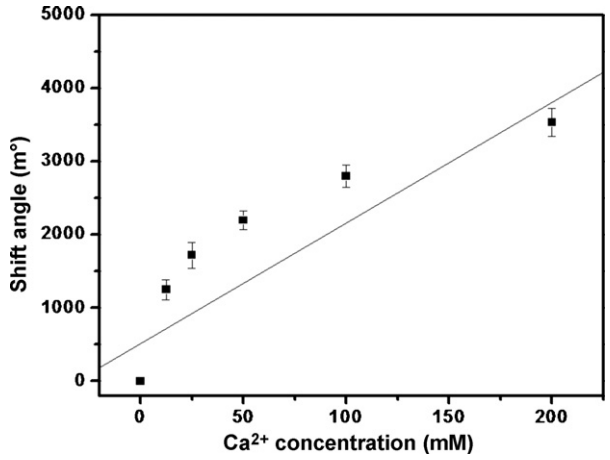

Fig. 8. SPR response for PVB-S100A12 system at different concentrations of calcium ions.

4. Conclusions

The PVB-S100A12 system was capable to detect Ca^{2+} exhibiting a relatively rapid response. EIS and CV were applied to evaluate the protein-ion reaction in the presence of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probe. EIS and CV measurements showed that redox probe reactions on the modified gold electrodes were partially blocked due the adsorption of PVB-S100A12. This system exhibited a wide linear response to Ca^{2+} concentrations in the 12.5 mM–200 mM range. The results confirm the positive responses of the S100A12 to the presence of calcium ions and therefore the capacity of calgranulin to retain its biological activity even upon adsorption to a solid substrate. In the PVB-S100A12 electrode this protein is bound to the gold electrode surface, and an increase of 1184.32 m° in SPR angle after modification of PVB with S100A12 is observed, indicating that 9.84 ng/mm^2 of S100A12 adsorbs on Au-PVB electrode, with a further increase of 581.66 m° found after Ca^{2+} attachment. We believe that the fabrication of this Au-PVB-S100A12 calcium biosensor is of considerable interest due to the simplicity of its operation and the possibility of further optimization under practical applications. Efforts are in progress to increase the detection range and to estimate the amount of Ca^{2+} ions in blood serum.

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References

[1] M.J. Berridge, M.D. Bootman, P. Lipp, Nature 395 (1998) 645.
[2] J.P. Glusker, Adv. Protein Chem. 42 (1991) 1.
[3] W.Z. Hu, H. Haraguchi, Anal. Chim. Acta 289 (1994) 231.

[4] E. Akyilmaz, O. Kozgus, Food Chem. 115 (2009) 347.
[5] M. Mank, D.F. Reiff, N. Heim, M.W. Friedrich, A. Borst, O. Griesbeck, Biophys. J. 90 (2006) 1790.
[6] X.Y. Bi, W.L. Wong, W.J. Ji, A. Agarwal, N. Balasubramanian, K.L. Yang, Biosens. Bioelectron. 23 (2008) 1442.
[7] R. Traversi, S. Becagli, E. Castellano, V. Maggi, A. Morganti, M. Severi, R. Udisti, Anal. Chim. Acta 594 (2007) 219.
[8] B. Paull, M. Macka, P.R. Haddad, J. Chromatogr. A 789 (1997) 329.
[9] A. Bratov, N. Abramova, C. Dominguez, A. Baldi, Anal. Chim. Acta 408 (2000) 57.
[10] Z.L. Chen, M.A. Adams, Talanta 47 (1998) 779.
[11] K.Y. Chumbimuni-Torres, L.T. Kubota, J. Food Compos. Anal. 19 (2006) 225.
[12] R.A. Bugallo, S.R. Segade, E.F. Gomez, Talanta 72 (2007) 60.
[13] J.F. van Staden, R.I. Stefan, Talanta 49 (1999) 1017.
[14] B.S. Yu, Q.G. Yuan, L.H. Nie, S.Z. Yao, J. Pharm. Biomed. Anal. 25 (2001) 1027.
[15] B.M. Buckley, L.J. Russell, Ann. Clin. Biochem. 25 (1988) 447.
[16] E. Chapoteau, B.P. Czech, W. Zazulak, A. Kumar, Clin. Chem. 1820–1 (1993) 824.
[17] R. Freaney, O.M. Casey, T. Quinn, F.P. Muldowney, J. Autom. Chem. 8 (1986) 197.
[18] R.M. Tufty, R.H. Kretsinger, Science 187 (1975) 167.
[19] Kretsing, C.E. Rh, Nockolds, J. Biol. Chem. 248 (1973) 3313.
[20] L. Santamaria-Kisiel, A.C. Rintala-Dempsey, G.S. Shaw, Biochem. J. 396 (2006) 201.
[21] T. Hatakeyama, M. Okada, S. Shimamoto, Y. Kubota, R. Kobayashi, Eur. J. Biochem. 271 (2004) 3765.
[22] O.V. Moroz, G.G. Dodson, K.S. Wilson, E. Lukanidin, I.B. Bronstein, Microsc. Res. Tech. 60 (2003) 581.
[23] B.C.M. Potts, J. Smith, M. Akke, T.J. Macke, K. Okazaki, H. Hidaka, D.A. Case, W.J. Chazin, Nat. Struct. Biol. 2 (1995) 790.
[24] R. Donato, Biochim. Biophys. Acta: Mol. Cell Res. 1450 (1999) 191.
[25] C.W. Heizmann, J.A. Cox, Biometals 11 (1998) 383.
[26] E.C. Dellangelica, C.H. Schleicher, J.A. Santome, J. Biol. Chem. 269 (1994) 28929.
[27] R.J. Green, R.A. Frazier, K.M. Shakesheff, M.C. Davies, C.J. Roberts, S.J.B. Tendler, Biomaterials 21 (2000) 1823.
[28] Z. Matharu, G. Sumana, M.K. Pandey, V. Gupta, B.D. Malhotra, Thin Solid Films 518 (2009) 719.
[29] S. Helali, H. Ben Fredj, K. Cherif, A. Abdelghani, C. Martelet, N. Jaffrezic-Renault, Mater. Sci. Eng. C 28 (2008) 588.
[30] J.R. Macdonald, Impedance Spectroscopy: Emphasizing Solid Materials and Systems, Wiley, New York, 1987.
[31] A.F. Garcia, W. Garcia, M.C. Nonato, A.P. Araujo, Biophys. Chem. 134 (2008) 246.
[32] M.D.L. Oliveira, M.T.S. Correia, F.B. Diniz, Biosens. Bioelectron. 25 (2009) 728.
[33] R.F. Dutra, L.T. Kubota, Clin. Chim. Acta 376 (2007) 114–120.