ORIGINAL PAPER

A comparison of four protocols for the immobilization of an aptamer on graphite composite electrodes

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Abstract We have performed a comparative study on four protocols for the immobilization of the thrombin aptamer on a graphite-epoxy composite electrode with the aim to identify the most practical method for designing the corresponding impedimetric aptasensor. The protocols included (a) physical adsorption, (b) avidin-biotin affinity interaction, (c) electrochemical activation and covalent bonding via amide groups, and (d) electrochemical grafting using 4-carboxybenzenediazonium coupling. The properties of the sensing surface were probed by electrochemical impedance measurements in the presence of the (ferri/ferro)hexacyanide redox couple. An increase in the interfacial charge transfer resistance (R_{ct}) was noted in all cases after the aptamer-thrombin interaction had occurred. The selectivity of the aptasensor over common serum proteins was also systematically investigated. Physical adsorption resulted in the lowest detection limit of the probe (4.5 pM), while avidin-biotin interaction resulted in highest selectivity and reproducibility exhibiting a 4.9 % relative standard deviation at pM thrombin concentration levels.

Keywords Aptamer · Thrombin · Electrochemical impedance spectroscopy · Immobilization · Label-free

Introduction

Cardiovascular diseases are leading cause of death worldwide. Every year more people die from these diseases than from any

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other causes [1]. In these diseases thrombin (Thr) plays a central role. Thr is the last protease enzyme involved in the coagulation cascade and converts fibrinogen to fibrin in blood coagulation [2]. The precursor of Thr is the inactive zymogen prothrombin. This protein is synthesized in the liver and secreted into blood circulation, and it is activated by vascular injury by limited proteolysis following upstream activation of the coagulation cascade. Thrombin activity is regulated by serum inhibitors and by its own action. With procoagulant and anticoagulant functions, it plays a central role in thrombosis and haemostasis, and it is an agonist for a number of cellular responses during inflammation and wound repair. Many diseases including stroke and myocardial infarction involve thrombosis [3]. Concentration levels of thrombin in blood are very low, while values down to picomolar range are associated with disease; consequently, it is important to assess this protein concentration at trace level and with high selectivity [4].

In the recent years, there has been great interest in the development of aptasensors. Aptasensors are essentially biosensors based on aptamers as recognition elements. Aptamers are artificial DNA or RNA oligonucleotides selected in vitro which have the ability to bind to proteins, small molecules or even whole cells; these molecules are capable of recognizing their targets with affinities and specificities often matching or even exceeding those of antibodies [5]. The first use of aptamers as biorecognition element in biosensors was reported in 1996, with an optical biosensor based on fluorescently labeled aptamers for Immunoglobulin G detection [6]. In 2005 the first electrochemical aptasensor was described, based on a sandwich format, where aptamers were labeled with glucose oxidase for the detection of Thr by amperometry [7]. Recently, among the different electrochemical techniques available, electrochemical impedance spectroscopy (EIS) has been increasingly used in biosensing studies [8–10]. EIS can be used as a tool for characterization of sensor platforms, and it can

also be the transduction technique to observe biorecognition events [11, 12]. It is an effective method for probing interfacial properties of modified electrodes such as their capacitance, electron transfer resistance, blocking layers, etc. [13]. The important feature presented by EIS is that it does not require any labeled species (e.g. with fluorescent or enzyme tags) for the transduction; in this way, this detection technique can be used for designing label-free protocols thus avoiding more expensive and time-consuming procedures [14].

In this communication, we report a label-free electrochemical aptasensor for detection of Thr using graphite-epoxy composite electrodes (GEC), where different protocols for immobilization of the aptamer are evaluated and compared. GEC electrodes represent a platform for biosensing of general use in our laboratories and have been already extensively studied [15, 16]. The different immobilization protocols that have been studied in this work are: physical adsorption, avidin-biotin affinity, amido-link covalent bonding via electrochemical activation [17] and electrochemical grafting [18]. The transduction principle used is based on the change of electron-transfer resistance in the presence of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple, which can be measured by EIS [19]. All different kinds of immobilization showed appropriate response behavior values to determine Thr in the picomolar range. The different procedures evaluated showed particular advantages, such as, high sensitivity, simple instrumentation, low production cost, fast response and portability.

Experimental

Reagents and solutions

Potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6]$, potassium dihydrogen phosphate, sodium monophosphate, methanol, perchloric acid, hydrochloric acid, N-Hydroxysuccinimide, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, sodium nitrite, fibrinogen protein, immunoglobulin G from human serum, avidin from egg white, albumin from bovine serum (BSA) and the target protein thrombin (Thr), were purchased from Sigma (St. Louis, MO, USA, www.sigmaaldrich.com). Poly(ethylene glycol) 1.000 (PEG), sodium chloride and potassium chloride were purchased from Fluka (Buchs, Switzerland, www.sigmaaldrich.com). 4-aminobenzoic acid from Acros (Geel, Belgium, www.acros.com). All reagents were analytical reagent grade. All-solid-state electrodes (GECs) were prepared using 50 μm particle size graphite powder (Merck, Darmstadt, Germany, www.merck.com) and Epotek H77 resin and its corresponding hardener (both from Epoxy Technology, Billerica, MA, USA, www.epotek.com). All solutions were made up using MilliQ water from MilliQ System (Millipore, Billerica, MA, USA, www.millipore.

com). The different modified aptamers (AptThr) [20] used in this study were prepared by TIB-MOLBIOL (Berlin, Germany, www.tib-molbiol.com). Their sequences and modifications are shown below:

AptThr: 5′-GGTTGGTGTGGTTGG-3′
NH$_2$-AptThr: 5′-NH$_2$- GGTTGGTGTGGTTGG-3′
Bio-AptThr: 5′-Bio- GGTTGGTGTGGTTGG-3′

Stock solutions of aptamer, thrombin and other proteins were diluted with sterilized and deionised water (to prevent any DNA cleavage), separated in fractions and stored at −20 °C until required. The buffer employed was PBS (187 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.76 mM KH_2PO_4, pH 7.0). Stock solutions of aptamer and thrombin were diluted with sterilized and deionised water, separated in fractions and stored at −20 °C until use.

Electrode preparation procedures

Graphite-epoxy composite (GECs) electrodes used were prepared using a PVC tube body (6 mm i.d.) and a small copper disk soldered at the end of an electrical connector. The working surface is an epoxy-graphite conductive composite, formed by a mixture of graphite (20 %) and epoxy resin (80 %), deposited on the cavity of the plastic body [8]. The composite material was cured at 80 °C for 3 days. Before each use, the electrode surface was moistened with MilliQ water and then thoroughly smoothed with abrasive sandpaper and finally with alumina paper (polishing strips 301044–001, Orion) in order to obtain a reproducible electrochemical surface [21, 22].

Avidin modified graphite-epoxy composite electrodes (AvGECs) were prepared with the same protocol as that used to prepare the graphite epoxy composite. The composite is formed by 2 % lyophilized avidin, 18 % graphite and 80 % epoxy resin [9]. In this case, the composite material was cured at 40 °C for 1 week.

Protocol

The scheme of the experimental procedures is represented in Fig. 1, with specific steps described below. Before any immobilization, the aptamer solution was heated at 80–90 °C for 3 min to promote the loose conformation of the aptamer. Then, the solution was dipped in a bath of cold water to obtain the proper detecting conformation [23].

Aptamer immobilization

Physical adsorption After getting the proper aptamer conformation, the electrode was immersed in 160 μL of AptThr solution, where the adsorption took place at room temperature for 15 min with soft stirring. Finally, this step was followed by

Fig. 1 Experimental scheme of the different immobilization techniques and biosensing steps. [1], Physical adsorption; [2], Electrochemical activation; [3], Electrochemical grafting; [4], Avidin-Biotin affinity
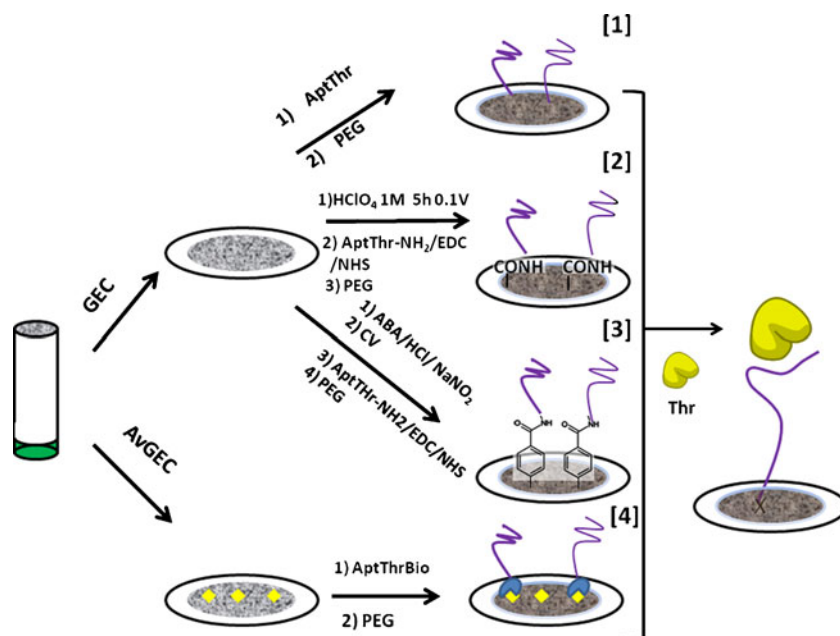
two washing steps using PBS buffer solution for 10 min at room temperature, in order to remove any unadsorbed aptamer [24].

Avidin-Biotin affinity The AvGEC electrode was dipped in 160 μL of biotinylated AptThr solution for 15 min at room temperature with soft stirring. This was followed by two washing steps using PBS buffer solution for 10 min.

Electrochemical activation + covalent immobilization First, carboxyl groups were electrochemically generated on the electrode surface, and next the aptamer was immobilized through amide bonding. To obtain an active surface with carboxyl groups, it was applied to the electrode a potential of +0.8 V versus reference electrode Ag/AgCl/KCl (sat.) in 1 M $HClO_4$ solution for 5 h [17]. After that, the electrode was immersed in 160 μL of aptamer in PBS solution with 1 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and 0.5 mg of N-hydroxysuccinimide for 24 h [23], with the goal of covalent immobilization of the amino-ended aptamer through the amide bond formation. This step was followed by two 10 min washing steps with PBS buffer solution.

Electrochemical grafting + covalent immobilization In this case, electrochemical grafting employing 4-aminobenzoic acid followed by aptamer immobilization through amide bonding was performed. Firstly, 30 mg of 4-aminobenzoic acid was dissolved in 3 mL of 1 M HCl and cooled with ice. Then, the diazonium salt was prepared by adding 570 μL of 2 mM $NaNO_2$ aqueous solution dropwise to the 4-aminobenzoic acid solution, with constant stirring. The electrode was immersed in this solution, and 200 successive voltammetric cycles ranging between 0.0 and −1.0 V ($v = 200$ mV s^{-1}) were

performed [25], generating a carbon-carbon bond. The modified electrodes (benzoic acid modified carbon) were washed thoroughly with water and methanol and dried at room temperature. Finally, the electrodes were immersed in 160 μL of aptamer solution with 1 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and 0.5 mg of N-hydroxysuccinimide during 12 h, with the goal of covalent immobilization of the aptamer through the amide formation. This step was followed by two 10 min washing steps with PBS buffer solution [23].

Blocking step

After aptamer immobilization, the electrode was incubated in 160 μL of PEG 40 mM for 15 min at room temperature with soft stirring to minimize any possible nonspecific adsorption. This was followed by two washing steps using PBS buffer solution for 10 min.

Thrombin assay

The last step was the recognition of Thr by the immobilized AptThr. For this, the electrode was dipped in a solution with the desired concentration of Thr. The incubation took place for 15 min at room temperature. Then, the biosensor was washed twice with PBS buffer solution for 10 min at room temperature, and the EIS measurement performed.

Equipment

Impedance measurements were performed with an IM6e Impedance Measurement Unit (BAS-Zahner, Kronach Germany,

www.zahner.de) or an Autolab PGStat 20 (Metrohm Autolab B.V, Utrecht, The Netherlands, www.ecochemie.nl). Thales (BAS-Zahner) and FRA (Metrohm Autolab) software were used for data acquisition and control of the experiments, respectively. A three electrode configuration was used to perform the impedance measurements: a platinum-ring auxiliary electrode (Crison 52–67, Barcelona, Spain, www.crisoninstruments.com), a Ag/AgCl pseudo-reference electrode prepared in the laboratory and the constructed GEC as the working electrode. Temperature-controlled incubations were done using an Eppendorf Thermomixer 5436 (Hamburg, Germany, www.eppendorf.com).

EIS detection

Impedimetric measurements were performed in 0.01 M $[Fe(CN)_6]^{3-/4-}$ solution prepared in PBS at pH 7. The electrodes were dipped in this solution and a potential of +0.17 V (vs. Ag/AgCl) was applied. Frequency was scanned from 10 kHz to 50 mHz with a fixed AC amplitude of 10 mV. The obtained spectra were represented as Nyquist plots ($-Z_i$ vs. Z_r) in the complex plane and fitted to a theoretical curve corresponding to the equivalent circuit with Z_{view} software (Scribner Associates Inc., USA, www.scribner.com). The equivalent circuit was formed by one resistor/ capacitor element in series with a resistance. In the equivalent circuit, the resistance in series with the capacitor element, R_1, corresponds to the resistance of the solution, the resistance in parallel with the capacitor element, R_{ct}, is the charge transfer resistance between the solution and the electrode surface, while the capacitor element is the constant phase element (CPE) associated with the double-layer capacitance. The use of a CPE instead of a capacitor is required to optimize the fit to the experimental data, and this is due to the nonideal nature of the electrode surface. Any diffusional term was neglected as it was not the object of this study. The chi-square goodness-of-fit was calculated for each fitting and in all cases the calculated values for each circuit were <0.2, much lower than the tabulated value for 50 degrees of freedom (67.505 at 95 % confidence level).

Results and discussion

In the comparison of immobilization protocols, we observed the change of the charge transfer resistance (R_{ct}) between the solution and the electrode surface after each electrode modification, and after the Thr sensing. The charge transfer process monitored was that of the redox marker ($[Fe(CN)_6]^{3-/4-}$), as usual in electrochemical impedance studies. In it, any modification of the electrode surface, i.e. the aptamer immobilization, the PEG blocking or the biosensing event altered the electrochemical process of the redox marker [11, 26]. Thanks

to these variations, it was possible to monitor each step of the biosensing, in this case by following the variation of R_{ct}.

In order to compare the results obtained from the different electrodes used, and to obtain independent and reproducible results, the relative signal was used, Δ_{ratio} [9]. Thus, the Δ_{ratio} value was defined according to the following equations:

$$\Delta_{ratio} = \Delta_s / \Delta_p \tag{1}$$

$$\Delta_s = R_{ct(AptThr-Thr)} - R_{ct(electrode-buffer)} \tag{2}$$

$$\Delta_p = R_{ct(AptThr)} - R_{ct(electrode-buffer)} \tag{3}$$

where $R_{ct (AptThr-Thr)}$ was the electron transfer resistance value measured after incubation with the thrombin protein; $R_{ct (AptThr)}$ was the electron transfer resistance value measured after aptamer inmobilization on the electrode, and $R_{ct (electrode-buffer)}$ was the electron transfer resistance of the blank electrode and buffer.

As can be seen in Fig. 2, the R_{ct} value, visualized as the diameter of the semicircle, increased after each biosensing step. This change was due to the inhibition of the electrochemical reaction of the redox marker at the electrode surface, caused by the presence of blocking layers. Two different factors should be taken into account to properly explain this effect: electrostatic repulsion and sterical hindrance [27]. When the AptThr is immobilized onto the electrode surface, an initial blocking layer is formed, where negatively charged phosphate groups of the AptThr skeleton are responsible for electrical repulsion towards the negatively charged redox marker, thus producing the increase of the R_{ct} value. The addition of target protein (Thr) resulted in the increment of the resistance value due to the sterical hindrance caused by the formation of the complex AptThr-Thr.

Detection of thrombin: comparison between different immobilization protocols

The goal of these experiments was to study and compare the best immobilization technique using a graphite-epoxy composite. All steps of these experiments have been optimized separately (data not shown). The obtained Thr calibration curves are represented in Fig. 3.

The first technique used was physical adsorption. This immobilization protocol is based on a direct adsorption of AptThr through weak, labile bonds with active substrate sites, in this case on a graphite-epoxy composite electrode. Adsorption is the simplest method to immobilize aptamers on electrodes. It does not require additional reagents or special nucleic acid modifications, thus resulting in a rapid, simple and low cost protocol for the aptamer immobilization [24].
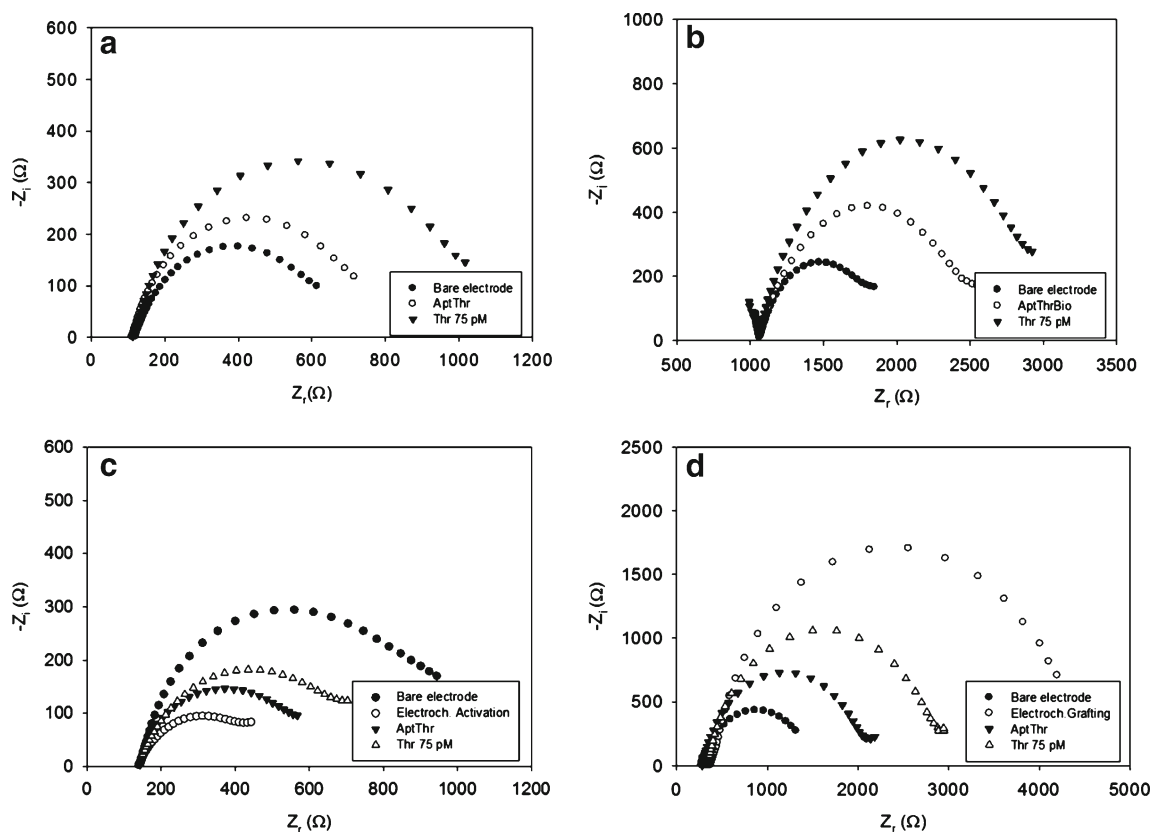
Fig. 2 Nyquist Diagram obtained for the Thrombin aptasensor prepared by: **a** physical adsorption, **b** avidin-biotin affinity, **c** covalent bonding via electrochemical activation and **d** covalent bonding via electrochemical grafting. All experiments were performed in PBS solution and all EIS measurements were performed in PBS solution containing 0.01 M $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$

This method presented high sensitivity and low detection limits, with a value for S/N=3 of 4.5 pM, see Fig. 3a.

The subsequent technique that was used to immobilize AptThr was through avidin-biotin interaction of the properly terminal-modified aptamer. The basis of this technique is the strong affinity, in this case, between avidin and biotin to form a complex ($K_a = 1 \cdot 10^{15}$ M^{-1}) [28]. The stability of this interaction is nearly equal to that of a covalent bond. In fact, it can only be broken under very extreme conditions. As we can see in Fig. 3b, this method presented high sensitivity and a low detection limit, 4.7 pM for S/N=3.

Next, two different covalent bond immobilizations were used. These covalent bonds on the electrode surface can provide the benefits by structural flexibility and chemical stability, thus improving hybridization efficiency. The first technique used was through an electrochemical activation of the electrode surface and carboxyl moieties formation. For its operation, it needed application of a potential of +0.8 V vs Ag/AgCl to the carbon electrode surface for a duration of 5 h in perchloric acid 1 M. Due to the extreme conditions of the approach, the electrodes were surface-modified with carboxyl groups [17]. After that, this surface-confined carboxyl group were activated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride / N-Hydroxysuccinimide to

link amino groups of the properly terminated aptamers through the carbodiimide reaction [29]. The other covalent bond method used was electrochemical grafting [18]. This method consists of anchoring 4-aminobenzoic acid molecules to the electrode surface through diazonium salt reaction and C-C bond formation. The modification steps could be followed through EIS, see Fig. 2d. The electron-transfer resistance showed first a high increase due to the formation of anchor points on the electrode surface, in the form of benzoic acid functionalities; then a decrease in its value was observed due to the immobilization of the amino ended aptamer on the surface through the carbodiimide reaction, this decrease was a consequence of electrostatic repulsion. These two covalent immobilization methods presented narrower linear working ranges although the sensitivities were higher; these can be observed on the Fig. 3c and d. The highest net signal is obtained with the electrochemical activation approach.

All numerical results are represented in Table 1. The highest sensitivity was attained using affinity immobilization due to the strong affinity of avidin with biotinylated AptThr. This method presents more efficiency on the biorecognition than the covalent immobilization methods. In addition, its performance can be fine-tuned, by optimizing the amount of avidin on the AvGECs for each specific application. Also, the
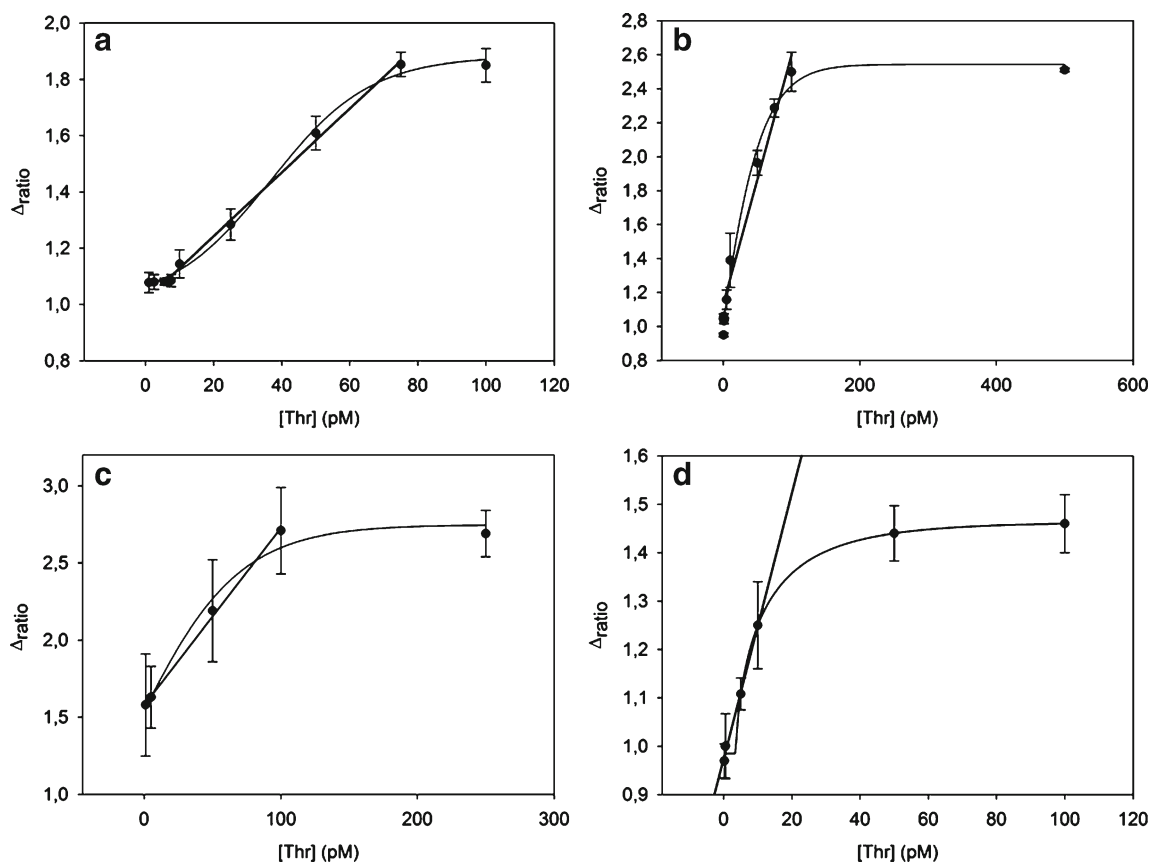
Fig. 3 Calibration curves and regression lines for the four immobilization procedures tested: **a** physical adsorption, **b** avidin-biotin affinity, **c** electrochemical activation and **d** electrochemical grafting. Uncertainly values correspond to five replicate experiments

best reproducibility was achieved using the avidin-modified biocomposite platform, probably due to the surface presenting less heterogeneity among different electrodes compared to electrodes prepared using covalent bonding techniques. The response with highest linearity was obtained using the electrochemical grafting method, what suggests a proper interaction of the bonded aptamer but the broadest linear range corresponds to the affinity process. Largest impedimetric signal (Δ_{ratio}) is observed for the electrochemically activated-amide bond immobilization, probably this is the configuration with best space orientation, optimal proximity of ligand to electrode and favorable steric effects. Finally, the lowest detection limit was yielded by the physical adsorption followed by the affinity method. Probably the high sensitivity can be

attributable to the correct orientation of the receptor, while the reduced linear working range could be defined by the amount of recognition sites present on the electrode surface.

Apart of these response features, an important asset for the electrochemical grafting is that immobilization can be electrically addressed; this feature can be of high significance if preparing assays of aptamer biosensors for multiplex formats.

In order to judge the interest of the comparison done, the four immobilization protocols were compared with other label-free biosensors using aptamers for Thr detection. The label-free techniques considered were differential pulse voltammetry, potentiometry, and EIS. The details are shown in Table 2, where sensitivity info is presented as the LOD of each

Table 1 Calibration curves results obtained with different AptThr immobilizations. % RSD values correspond to five replicate experiments at 75 pM thrombin concentration

Immobilization	Sensitivity (M^{-1})	Reproducibility (RSD %)	Linear range (pM)	LOD (pM)	Correlation coeficient (r)
Physical adsorption	$1.106 \ 10^{10}$	7.2	7.5–75	4.5	0.998
Av-Bio affinity	$1.530 \ 10^{10}$	4.9	0.75–100	4.7	0.999
Electrochemical activation	$1.206 \ 10^{10}$	8.3	2.5–100	10.5	0.996
Electrochemical grafting	$8.756 \ 10^{9}$	7.2	0.2–10	7.3	0.994

Table 2 Comparison of the proposed Thr aptasensor with other recent reported label-free aptasensors in the literature

Transduction technique	Platform	LOD	Interferents tested	RSD%	Ref
Differential pulse votammetry	Gold	10 nM	BSA, α-fetoprotein, carcinoembryonic antigen	–	[33]
Differential pulse voltammetry	gold	3 nM	Human serum	3	[34]
Potentiometry	FET	6.7 nM			[35]
EIS	Gold	80 pM	BSA	10	[36]
EIS	Gold recordable compact discs	5 nM	Human serum	1	[37]
EIS	MWCNT	105 pM	Human serum	7.5	[38]
EIS	Platinum/ poly(pyrrole-NTA)/Cu^{2+}	4.4 pM	BSA, lysozime, Immunoglobulin G	7.3	[39]
EIS	GEC/Physical Adsorption	4.5 pM	Fibrinogen, Immunoglobulin G, BSA	7.2	This work
EIS	AvGEC	4.7 pM	Fibrinogen, Immunoglobulin G, BSA	4.9	This work
EIS	GEC/Electrochem. Activation	10.5 pM	Fibrinogen, Immunoglobulin G, BSA	8.3	This work
EIS	GEC/Electrochem. Grafting	7.3 pM	Fibrinogen, Immunoglobulin G, BSA	7.2	This work
EIS	Chemically modified graphene	10 nM	Immunoglobulin G, BSA, Avidin	–	[40]

biosensor compared. Our proposed aptasensor is on the first range of lowest LOD and % RSD value, specially the avidin-biotin affinity method. These data demonstrate that our graphite-epoxy composite electrodes can be sensing platforms of choice for the development of aptasensors, in this case, for detecting thrombin.

Cross-reactivity

Majoritary proteins in serum (BSA, Fibrinogen and Immunoglobulin G) which may accompany Thr [30], were tested as potential interfering substances for the different aptasensors towards Thr compared in the study, see the different EIS spectra on the Supplementary Information.

The first protein studied was BSA. BSA is found in serum at a level from 3,500 to 5,000 mg·dL^{-1}, representing more than 60 % of the total protein present. To perform the test, the

highest concentration in serum was used, 5,000 mg dL^{-1} [31]. When the aptamer was incubated with this protein, the electron interfacial resistance did not increase, and instead, in this case a slight decrease was observed. Therefore, it was proven that albumin was not recognized by the AptThr, and it did not interfere with AptThr-Thr system, regardless of the immobilization method used.

Next, the protein studied was Fibrinogen. Fibrinogen is a fibrous protein involved in the blood clotting process. By the action of thrombin, fibrin is degraded and results in the formation of a clot. This protein is present in human serum in a concentration range of 200 to 400 mg dL^{-1} [32]. It was observed that the electron interfacial resistance increased as

Table 3 Comparison among sensitivity values for interfering proteins in serum, obtained with different protocols for AptThr immobilization (Each sensitivity value obtain for calibration, of the single protein, $n = 4$ in the linear range)

Immobilization	Thr sensitivity (M^{-1})	Fibrinogen sensitivity (M^{-1})	Immunoglobulin G sensitivity (M^{-1})	BSA sensitivity (M^{-1})
Physical adsorption	$1.106\ 10^{10}$	$3.698\ 10^5$	$2.385\ 10^4$	–
Av-Bio affinity	$1.530\ 10^{10}$	$1.499\ 10^4$	$1.082\ 10^5$	–
Electrochemical activation	$1.206\ 10^{10}$	$3.022\ 10^5$	$1.132\ 10^4$	–
Electrochemical grafting	$8.756\ 10^9$	$8.103\ 10^5$	$1.605\ 10^4$	–

Table 4 Comparison among EC$_{50}$ values and % Cross-Response for interfering proteins in serum, obtained with different protocols for AptThr immobilization. (% CR=100 (EC$_{50}$ Thr/ EC$_{50}$ interfering protein)

Immobilization	Thr	EC$_{50}$ (M) fibrinogen	Immunoglobulin G
Physical adsorption	$4.400\ 10^{-11}$	$2.300\ 10^{-6}$	$3.400\ 10^{-5}$
Av-Bio affinity	$4.600\ 10^{-11}$	$8.190\ 10^{-6}$	$7.330\ 10^{-5}$
Electrochemical activation	$4.390\ 10^{-11}$	$8.650\ 10^{-6}$	$8.480\ 10^{-5}$
Electrochemical grafting	$2.280\ 10^{-11}$	$8.930\ 10^{-6}$	$5.000\ 10^{-5}$

Immobilization	Thr	%CR fibrinogen	Immunoglobulin G
Physical adsorption	100	$1.910\ 10^{-3}$	$1.290\ 10^{-4}$
Av-Bio affinity	100	$5.620\ 10^{-4}$	$6.275\ 10^{-5}$
Electrochemical activation	100	$5.080\ 10^{-4}$	$5.170\ 10^{-5}$
Electrochemical grafting	100	$2.550\ 10^{-4}$	$4.560\ 10^{-5}$

a result of some type of recognition by the AptThr. Therefore, this protein could act as interference for the system.

Lastly, the protein studied was Immunoglobulin G. Immunoglobulin G is a globular protein synthesized by the immune system in response to the invasion of any bacteria, virus or fungi. It is present in human serum over a range of concentrations from 950 mg dL^{-1} to 1,550 mg dL^{-1} in serum, with a reference value of 1,250 mg dL^{-1}. Immunoglobulin G also acted as an interferent, which is proven by the increase in the resistance R_{ct} value. This behavior, which was also exhibited by the aptasensor towards fibrinogen, may be due to some biological interaction between the aptamer and these proteins. These interactions are not yet fully described in the literature.

To evaluate the sensitivity of the aptasensor we compared the calibration plots for the different proteins, pure solution of single protein, by the different kinds of immobilization. Table 3, summarizes the parameters for each protein. The first thing one can see is that all types of immobilization showed the highest sensitivity for its target molecule, Thr, with its slope of response being five to six orders of magnitude greater than the slope for immunoglobulin G and also for fibrinogen. In addition, EC$_{50}$ values for each type of immobilizations and % Cross Response (% CR) for all interfering proteins were calculated, Table 4 [25]. The EC$_{50}$ value corresponds to the inflection point of the calibration curve towards the interfering species and represents the concentration of protein that provides 50 % of the Δ_{ratio} saturation value. Thus, the lower is the EC$_{50}$ value, the greater is the affinity (or the interference) of the considered protein. The lowest EC$_{50}$ value obtained, 2.30 10^{-11} M, corresponded to electrochemical grafting immobilization for thrombin protein, and the larger, 8.480 10^{-5} M, to electrochemical activation immobilization for immunoglobulin G. In overall, the % CR values, which states the degree of response in comparison to primary analyte thrombin, ranged from 1.910 10^{-3} to 6.275 10^{-5} %; these are openly low relative values, where the largest % CR corresponded to fibrinogen in adsorption immobilization. Therefore, it was demonstrated that the aptasensor showed a much higher sensitivity to Thr, regarding potential interfering proteins, that when they display some effect is due to the high level of concentration at which they are present in serum. Given all recognition events are due to the same aptamer used, the differences in interfering effects are attributable to incomplete blocking by PEG in some of the four protocols or to better immobilization orientation of the aptamer for biosensing. The latter is probably the case in the covalent immobilization (showing the lowest % CR) or some displacement effect in the case of physical adsorption.

Conclusions

In this paper, we have presented the use of aptasensors for the detection of thrombin based on graphite-epoxy composite electrodes. Results obtained with four kinds of AptThr immobilization (physical adsorption, avidin-biotin affinity, covalent bonding via electrochemical activation and covalent bonding via electrochemical grafting) were compared.

With the different methods reported, low detection limits (in the pM level), ample ranges of response for thrombin concentration (more than one decade) and low % RSD values were achieved. Among the four methods proposed, avidin-biotin affinity was the best overall method displaying high affinity with a sensitivity value of 1.530 10^{10} M^{-1}, a linear range of 0.75–100 pM and a reproducibility of 4.9 % RSD. However, it is an expensive method, as it needs the incorporation of the expensive avidin on the biocomposite. The lower detection limit attained was 4.5 pM by physical adsorption method, although the latter was also the one with poorer selectivity (highest % CR values). Considering interfering effects produced by serum proteins, fibrinogen and immunoglobulin G demonstrated some effect, which may restrict the utility of the aptasensor; still, given the high concentration values at which they interfere and the low % CR determined (lower than 0.001 %), these interfering effects may be tolerated. In the case a maximum accuracy is sought, a double recognition sandwich scheme, or an amplification scheme might be the solution.

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