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

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Abstract

This work compares four protocols in the immobilization of the thrombin aptamer onto a graphite-epoxy composite electrode, in search of the most practical labeless impedimetric aptasensor. The immobilization protocols tested included: physical adsorption, avidin-biotin affinity interaction, amide covalent bond via electrochemical activation and via electrochemical grafting using 4- carboxybenzenediazonium coupling. The detection principle was based on the changes of the interfacial properties of the sensing surface which were probed in the presence of the reversible redox couple $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ using the obtained electrochemical impedance measurements. Different thrombin impedimetric aptasensors were therefore assayed, whereas the increase of the interfacial charge tranfer resistance (Rct) was noticed after the aptamer-thrombin interaction. Selectivity against common proteins in serum was also systematically characterized. Physical adsorption showed the lowest detection limit (4.5 pM), while avidin-biotin interaction allowed the highest selectivity and reproducibility (4.9%RSD in the pM range).

Keywords: Aptamer, Thrombin, Electrochemical Impedance Spectroscopy, immobilization, label-free.

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Introduction

Cardiovascular diseases are leading cause of death worldwide. Every year more people die from these diseases than from any 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 insoluble fibrin which forms the fibrin gel, both in physiological conditions and in a pathological thrombus [2]. Concentration levels of thrombin in blood are very low, and levels down to picomolar range are associated with disease; because of this, it is important to be able to assess this protein concentration at trace level, and with high selectivity [3].

In the recent years, there has been great interest in the development of aptasensors. Aptasensors are biosensors that use aptamers as the biorecognition element. 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 [4]. The first use of aptamers as biorecognition element in biosensors was in 1996, with an optical biosensor based on fluorescently labeled aptamers for IgG detection [5]. 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 [6]. Nowadays, among the different electrochemical techniques available, the use of Electrochemical Impedance Spectroscopy (EIS) has grown interest among biosensing studies [7-9]. EIS can be used as a characterization tool for characterization of sensor platforms, as it can also be the transduction technique to observe biorecognition events [10, 11]. It is an effective method for probing interfacial properties of modified electrodes such as capacitance, electron transfer resistance, blocking layers etc [12]. One of its main advantages is that it allows the design of biosensing protocols without the need of tagging of the intervening biomolecules; that is, permitting any labeless detection although without any the best detection limits [13].

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. The GEC

electrodes is a platform of general use in our laboratories and has been already extensively studied [14, 15]. The uneven surface of the graphite–epoxy electrode allows the immobilization of the aptamer onto its surface by physical adsorption, avidin-biotin affinity and amido-link covalent bonding via electrochemical activation [16] and/or electrochemical grafting [17]. 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 [18]. All different kinds of immobilization showed appropriate response behavior values to determine Thr in the picomolar range. The different procedures evaluated showed particular advantages; high sensitivity, simple instrumentation, low production cost, fast response and portability, depending the one considered.

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, hydrochloride acid, *N*-Hydroxysuccinimide (NHS), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), sodium nitrite, fibrinogen protein (Fbr), immunoglobulin G from human serum (IgG), avidin from egg white, albumin from bovine serum (BSA) and the target protein thrombin (Thr), were purchased from Sigma (St. Louis, MO, USA). Poly(ethylene glycol) 1.000 (PEG), sodium chloride and potassium chloride were purchased from Fluka (Buchs, Switzerland). 4-aminobenzoic acid (ABA) from Acros (Geel, Belgium). All reagents were analytical reagent grade. All-solid-state electrodes (GECs) were prepared using 50 μm particle size graphite powder (Merck, Darmstadt, Germany) and Epotek H77 resin and its corresponding hardener (both from Epoxy Technology, Billerica, MA, USA). All solutions were made up using MilliQ water from MilliQ System (Millipore, Billerica, MA, USA). The different modified aptamers (AptThr)[19] used in this study were prepared by TIB-MOLBIOL (Berlin, Germany). 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 used.

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 [7]. 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 [20, 21].

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 [8]. In this case, the composite material was cured at 40ºC for 1 week.

Protocol

<FIGURE 1>

The scheme of the experimental procedures is represented in Figure 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 [22].

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 minutes with soft stirring. Finally, this was followed by two washing steps using PBS buffer solution for 10 minutes at room temperature, in order to remove any unadsorbed aptamer [23].

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

Electrochemical activation + covalent immobilization. To obtain an active surface with carboxyl groups, it was applied to the electrode a potencial of +0.8V versus reference electrode Ag/AgCl/KCl (sat.) in 1M $HClO_4$ solution during 5 hours [16]. After that, the electrode was immersed in 160 µl of aptamer in PBS solution with 1 mg of EDC and 0.5 mg of NHS during 24 hours [22], with the goal of covalent immobilization of the aptamer through the amide bond formation. This step was followed by two 10 minutes washing steps with PBS buffer solution.

Electrochemical grafting + covalent immobilization. GEC electrodes were modified with 4-aminobenzoic acid. Firstly, 30 mg of ABA was dissolved in 3ml of 1M HCl and cooled with ice. Then, the diazonium salt was prepared by adding 2mM $NaNO_2$ aqueous solution dropwise to this solution with constant stirring. Next, 570µl of $NaNO_2$ solution was added to the ABA solution. The electrode was immersed in the solution and 200 successive voltammetric cycles ranging between 0.0 and -1.0 V (v=200mV/s) were carried out [24]. 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 EDC and 0.5 mg of NHS during 12 hours, with the goal of covalent immobilization of the apatamer. This step was followed by two 10 minutes washing steps with PBS buffer solution [22].

Blocking step

After aptamer immobilization, the electrode was dipped in 160 µL of PEG 40 mM for 15 minutes 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 minutes.

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 minutes at room temperature. Then, the biosensor was washed twice with PBS buffer solution for 10 minutes at room temperature.

Equipment

Impedance measurements were performed with an IM6e Impedance Measurement Unit (BAS-Zahner, Kronach Germany) and an Autolab PGStat 20 (Metrohm Autolab B.V, Utrecht, The Netherlands). Thales (BAS-Zahner) and Fra (Metrohm Autolab) software, respectively, were used for data acquisition and control of the experiments. A three electrode configuration was used to perform the impedance measurements: a platinum-ring auxiliary electrode (Crison 52–67 1, Barcelona, Spain), an Ag/AgCl reference electrode and the constructed GEC as the working electrode. Temperature-controlled incubations were done using an Eppendorf Thermomixer 5436.

EIS detection

Impedimetric measurements were performed in 0.01M $[Fe(CN)_6]^{3-/4-}$ 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's plots ($-Z_i$ *vs.* Z_r) in the complex plane and fitted to a theoretical curve corresponding to the equivalent circuit with Zview software (Scribner Associates Inc., USA). A Randles' equivalent circuit was used to fit the obtained impedance spectra, figure 2. 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).

<FIGURE 2>

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 targeting. The charge transfer process is caused by the presence of the redox marker ($[Fe(CN)_6]^{3-/4-}$) in the bulk solution. Any modification of the electrode surface influences its electrochemistry [10, 25]. Thanks to this, it was possible to monitor each step of the biosensing just following the variation of R_{ct} without the need of labeling any of the participating biomolecules.

In order to compare the results obtained from the different electrodes used, and to obtain independent and reproducible results, a relative signal was used [8]. Thus, the Δ_{ratio} value was defined according to the following equations:

$$\Delta_{ratio} = \Delta_s / \Delta_p \quad (1)$$

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

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

where $R_{ct(AptThr\text{-}Thr)}$ 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 immobilization on the electrode, and $R_{ct\ (electrode\text{-}buffer)}$ was the electron transfer resistance of the blank electrode and buffer.

<FIGURE 3>

As can be seen in figure 3 , the R_{ct} value, visualized as the diameter of the semicircle, increased after each biosensing step. This was due to the electrochemical

reaction of 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: electrostatic repulsion and sterical hindrance [26] . When AptThr is immobilized onto the electrode surface, an initial layer is formed, where negatively charged phosphate groups of the AptThr skeleton are responsible of the electrical repulsion towards the negatively charged redox marker, thus producing the increase of the Rct value. The addition of target protein (Thr) resulted in the increment of the resistance value due to the hindrance caused by the formation of the complex AptThr-Thr.

Detection of thrombin: comparison between different immobilization protocols

<FIGURE 4>

The goal of these experiments was to obtain the best immobilization technique using a graphite-epoxy composite. All steps of these experiments have been optimized separately (data not show). The obtained Thr calibration curves are represented in Figure 4.

The first technique used was physical adsorption. This protocol consists of direct adsorption of AptThr through weak, labile bonds with active substrate sites, in this case on a graphite-epoxy composite. Adsorption is the simplest method to immobilize aptamers on electrode surfaces. It does not require additional reagents or special nucleic acid modifications, thus resulting in a rapid, simple and low cost protocol for the AptThr immobilization [23]. This method presented high sensitivity and low detection limits, with a value for S/N=3 of 4.5 pM, see figure 4(a).

Subsequently, the technique used to immobilize AptThr was through avidin-biotin interaction. 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}) [27]. 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 figure 4 b), this method presented high sensitivity and a low detection limit, 4.7 pM for S/N=3.

<TABLE 1>

Next, two different covalent bond immobilizations were used. These covalent bonds on the electrode surface can benefit by structural flexibility and chemical stability, thus improving hybridization efficiency. The first technique used was through the electrochemical activation of the electrode surface. This method consists of the application of a potential of +0.8V to the carbon electrode surface for a duration of 5 hours in perchloric acid 1M. Due to the extreme conditions of the approach, the electrodes were functionalized with carboxyl groups [16]. After that, the surface-confined carboxyl group were activated with EDC/NHS to link amino groups of the functionalized aptamers through the carbodiimide reaction [28]. The other covalent bond method used was electrochemical grafting [17]. This consists of anchoring ABA molecules to the electrode surface through diazonium salt reaction and C-C bond formation. The modification steps could be followed through EIS. The transfer-electron resistance showed first a high increase due to the formation of anchor points on the electrode surface; then its decrease was observed due to the immobilization of AptThr on the surface through the carbodiimide reaction, as a consequence of electrostatic repulsion. These two covalent immobilization methods presented reduced linear working ranges although with high sensitivity; these can be observed on the figures 4 c) and d). The highest net signal is obtained with electrochemical activation approach.

All numerical results are represented in Table 1. As we can see, the highest sensitivity was attained using affinity immobilization due to the strong affinity of avidin with biotinylated AptThr, therefore this method presents more efficiency on the biorecognition, rather than the covalent immobilization methods. Apart, its performance might be fine-tuned, if the amount of avidin on the AvGECs is optimized for each specific application. Again, the best reproducibility was achieved using the avidin modified biocomposite platform, this could be due to the surface presenting less heterogeneity among different electrodes compared to 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 technique. Finally, the best detection limit corresponds to physical adsorption followed by affinity method. Probably the high sensitivity can be attributable to the correct orientation of the receptor, while the reduced linear working range is to be defined by the amount of recognition sites present on the electrode surface.

An important feature of 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.

Cross- Reactivity

Majoritary proteins (BSA, Fbr and IgG) which may be present in serum together with Thr [29], were tested as potencial interfering substances for the aptasensor response towards Thr.

The first protein studied was BSA. BSA is found in serum at a level from 3500 to 5000 mg/dL, representing more than 60% of the total protein present. To perform the test, the highest concentration in serum was used, 5000 mg /dL [30]. When the aptamer was incubated with this protein, electron interfacial resistance did not increase, in this case a slight decrease was observed. Therefore, it was proved that albumin was not recognized by the AptThr, and it did not interfere with AptThr-Thr system, independently of the immobilization considered.

Next, the protein studied was Fbr. Fbr is a fibrillar 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 [31]. It was observed that the electron interfacial resistance increased as 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 IgG. IgG is a globular protein that is synthesized 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 to 1550 mg/dL in serum, with a reference value of 1250 mg/dL. IgG also acted as an interferent, which is proved from the increase of the resistance R_{ct}. This increase, as it also happened in the case of Fbr, may be due to some biological interaction between the aptamer and these proteins, not yet described.

<TABLE 2>

<TABLE 3>

To evaluate the sensitivity of the aptasensor we compared the calibration plots for the different proteins by the different kinds of immobilization. Table 2, 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 IgG and also for Fbr. In addition, EC_{50} values for each type of immobilizations, table 3, and % Cross Response (% CR) for all interfering proteins were calculated [24]. The lowest EC_{50} value obtained corresponded to electrochemical grafting immobilization for thrombin protein, $2.30 \cdot 10^{-11}$M, and the larger, $8.480 \cdot 10^{-5}$ M, to electrochemical activation immobilization for IgG. Overall % CR values ranged from $1.910 \cdot 10^{-3}$ to $6.275 \cdot 10^{-5}$ %, where the largest % CR corresponded to Fbr in adsorption immobilization. Therefore, it was demostrated that the aptasensor showed a much higher sensitivity to Thr, regarding potential interfering proteins, which displayed this effect due to the high level of concentration in which they are present in serum. Given all recognition is due to the same aptamer, secondary response shown here has to be attributable to incomplete blocking by PEG or by better orientation for biosensing. This is probably detected in the covalent immobilization (with largest EC_{50}) or in some displacement effect in the case of physical adsorption.

Conclusions

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

All immobilization techniques showed low detection limits, good ranges of concentration for thrombin detection and high sensitivity; among them, avidin-biotin was the best overall method displaying high affinity with a sensitivity value of $1.530 \cdot 10^{10}$ M^{-1}, a linear range of 0.75-100 pM and a reproducibility of 4.9% RSD. The best detection limit obtained was 4.5 pM by physical adsorption method, although this was also the one with poorer selectivity.

In addition, the interference produced by serum proteins, fibrinogen and immunoglobulin G was characterized, displaying some limitations in the operation of the aptasensor, although usable given the concentration excess at which they manifest.

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

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

Table 2. Comparison among sensitivity values for interfering proteins in serum, obtained with different protocols for AptThr immobilization (Each sensitivity value obtain for calibration n=4 in the linear range).

Immobilization	**Thr Sensitivity (M^{-1})**	**Fbr Sensitivity (M^{-1})**	**IgG Sensitivity (M^{-1})**	**BSA Sensitiviy (M^{-1})**
Physical Adsorption	$1.106 \cdot 10^{10}$	$3.698 \cdot 10^{5}$	$2.385 \cdot 10^{4}$	--
Av-Bio Affinity	$1.530 \cdot 10^{10}$	$1.499 \cdot 10^{4}$	$1.082 \cdot 10^{5}$	--
Electrochemical Activation	$1.206 \cdot 10^{10}$	$3.022 \cdot 10^{5}$	$1.132 \cdot 10^{4}$	--
Electrochemical grafting	$8.756 \cdot 10^{9}$	$8.103 \cdot 10^{5}$	$1.605 \cdot 10^{4}$	--

Table 3. Comparison among EC_{50} values and % Cross-Response for interfering proteins in serum, obtained with different protocols for AptThr immobilization. (% CR = 100 x EC_{50} Thr/ EC_{50} interfering protein).

Immobilization	**EC_{50} (M)**		
	Thr	**Fbr**	**IgG**
Physical Adsorption	$4.400 \cdot 10^{-11}$	$2.300 \cdot 10^{-6}$	$3.400 \cdot 10^{-5}$
Av-Bio Affinity	$4.600 \cdot 10^{-11}$	$8.190 \cdot 10^{-6}$	$7.330 \cdot 10^{-5}$
Electrochemical Activation	$4.390 \cdot 10^{-11}$	$8.650 \cdot 10^{-6}$	$8.480 \cdot 10^{-5}$
Electrochemical grafting	$2.280 \cdot 10^{-11}$	$8.930 \cdot 10^{-6}$	$5.000 \cdot 10^{-5}$
Immobilization	**%CR**		
	Thr	**Fbr**	**IgG**
Physical Adsorption	-	$1.910 \cdot 10^{-3}$	$1.290 \cdot 10^{-4}$
Av-Bio Affinity	-	$5.620 \cdot 10^{-4}$	$6.275 \cdot 10^{-5}$
Electrochemical Activation	-	$5.080 \cdot 10^{-4}$	$5.170 \cdot 10^{-5}$
Electrochemical grafting	-	$2.550 \cdot 10^{-4}$	$4.560 \cdot 10^{-5}$

FIGURES

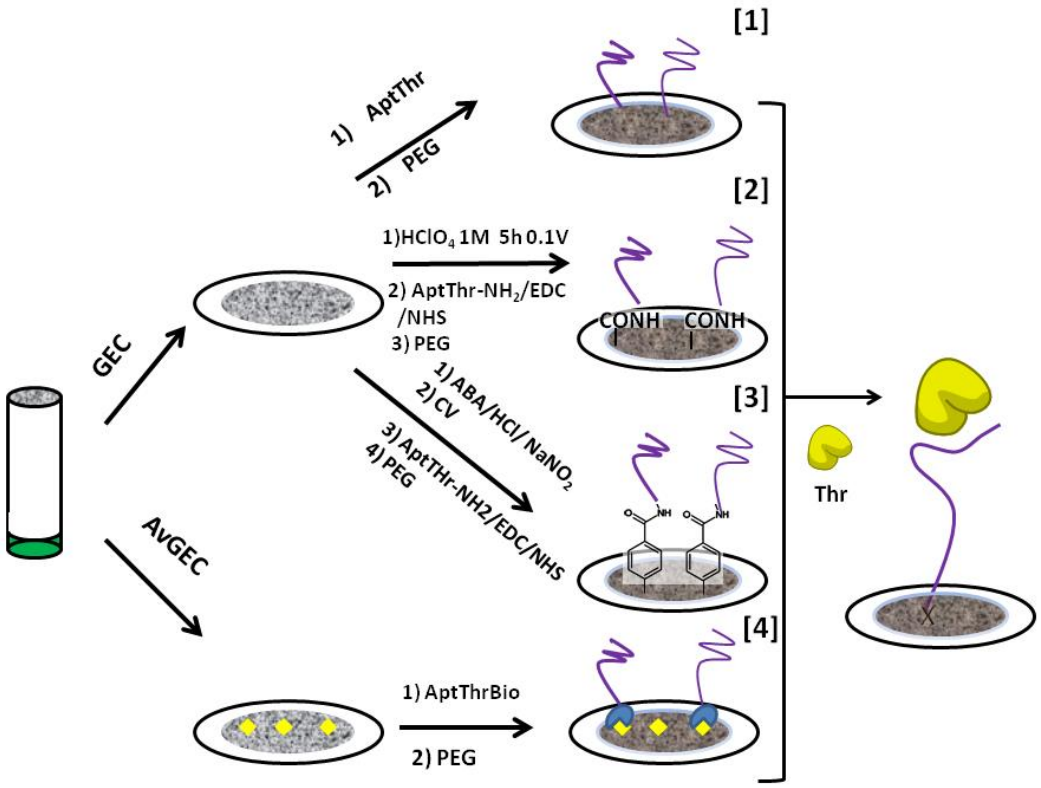


Fig.1 Experimental scheme of the different immobilization techniques and biosensing steps. [1], Physical adsorption; [2], Electrochemical activation; [3], Electrochemical grafting; [4], Avidin-Biotin interaction.

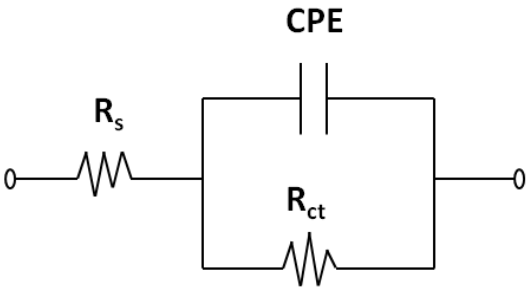


Fig. 2 Equivalent circuit used for the data fitting. R_s is the resistance of the solution, R_{ct} is the electron-transfer resistance and CPE, the capacitive contribution, in this case as a constant phase element.

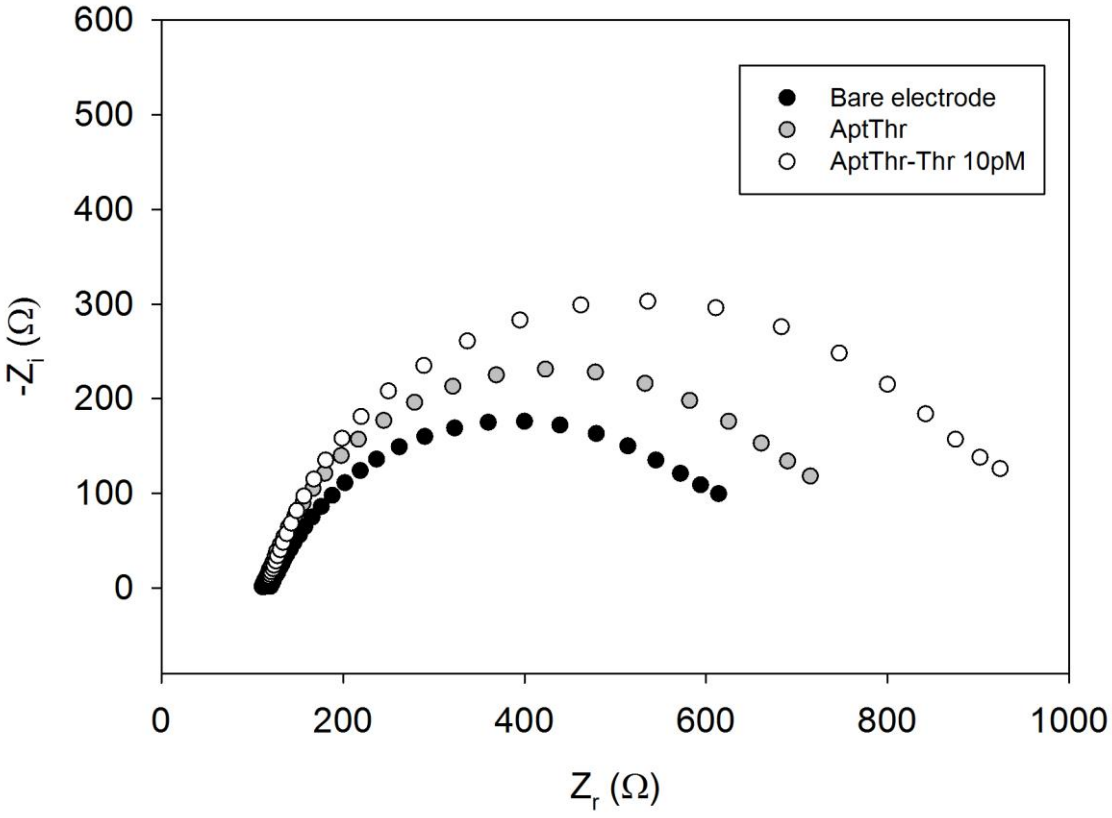
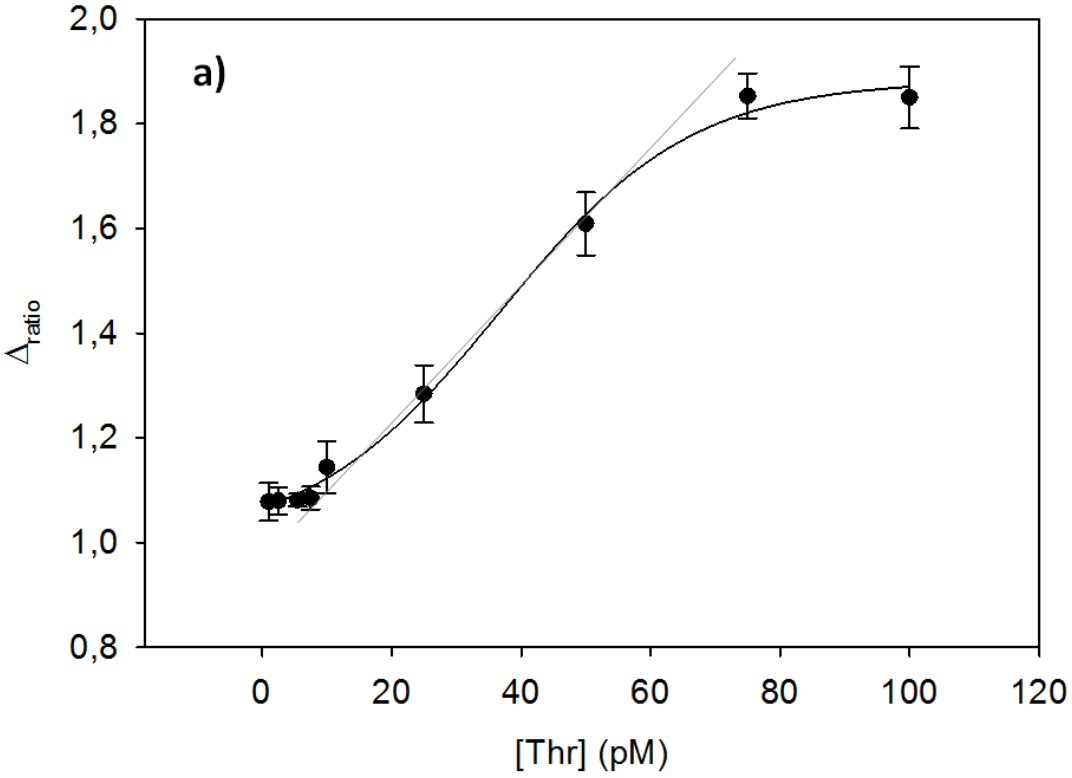
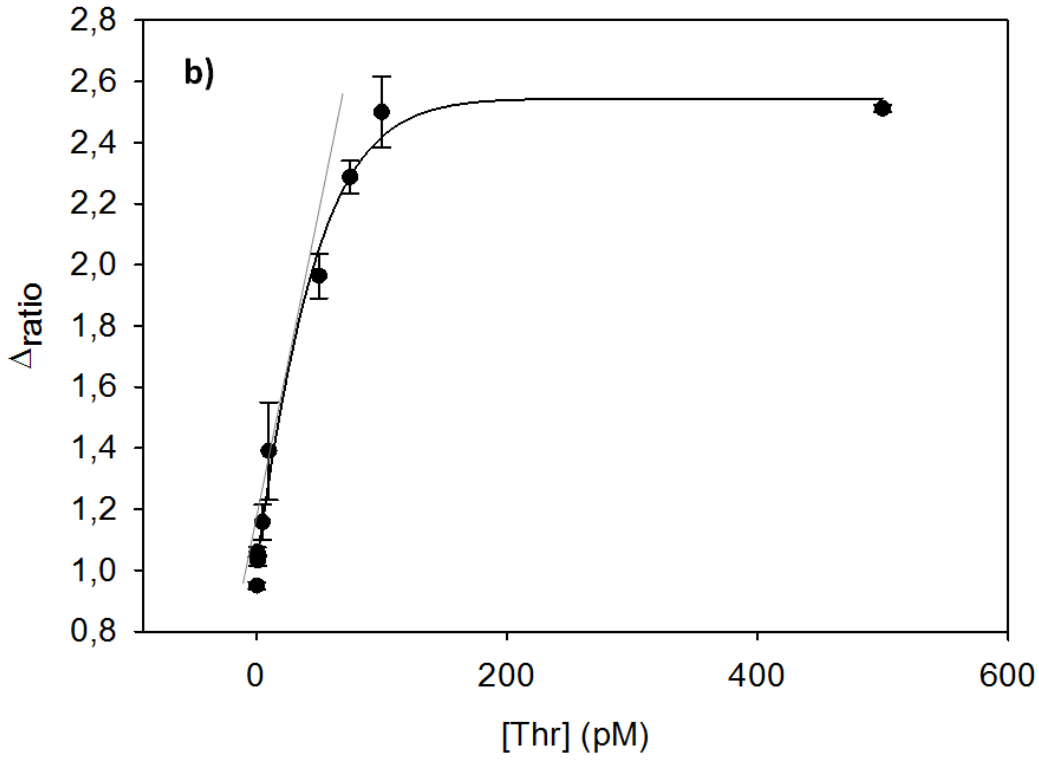


Fig. 3 Nyquist Diagram obtained for the thrombin aptasensor prepared by physical adsorption: (**a**) Bare electrode ●, (**b**) Aptamer of thrombin (AptThr) ◍, and (**c**) AptThr-Thr ○ 10 pM [Thr]**.**


a)
2,0
1,8
1,6
1,4
1,2
1,0
0,8
Δratio
0
20
40
60
80
100
120
[Thr] (pM)


b)
2,8
2,6
2,4
2,2
2,0
1,8
1,6
1,4
1,2
1,0
0,8
Δratio
0
200
400
600
[Thr] (pM)

(cont)

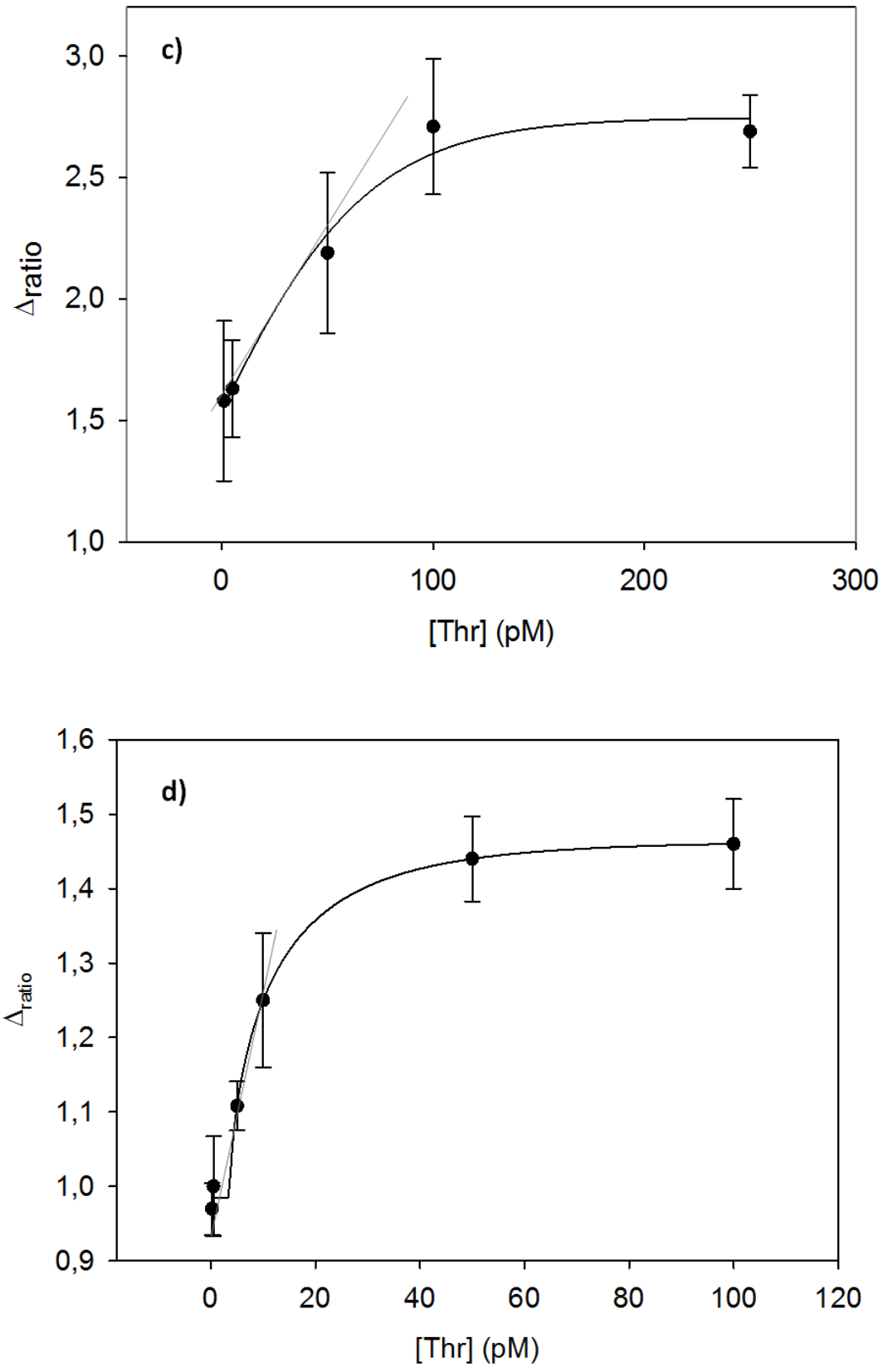


Fig. 4 Calibration curves and regression lines for the four immobilization procedures tested: a) adsorption physical, b) avidin-biotin affinity, c) electrochemical activation and d) electrochemical grafting. Uncertaintly values correspond to n=5 replicate experiments.