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Ahmad Alawad, Laure Latapie, David Evrard, Pierre Gros, Georges Istamboulie, et al.. SECM for Studying the Immobilization and Repartition of a Redox Anti-tetracycline Aptamer on Screen-printed Carbon Electrodes. *Electroanalysis*, 2021, 33 (2), pp.292-295. 10.1002/elan.202060182 . hal-03853257

HAL Id: hal-03853257

<https://hal.science/hal-03853257>

Submitted on 15 Dec 2023

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SECM for studying the immobilization and repartition of a redox anti-tetracycline aptamer on screen-printed carbon electrodes

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Abstract: Scanning electrochemical microscopy (SECM) is discussed as a versatile tool to provide a unique approach of localized electrochemical information in the context of biosensing research. The step-by-step immobilization of DNA aptamer with intrinsic redox activity on screen-printed carbon electrode (SPCE) was successfully monitored using SECM imaging tool. Control experiments were performed with a non-electroactive aptamer having a similar sequence length with the same proportion of A, T, G, and C bases. After immobilization of these aptamers on SPCE, SECM images showed the repartition of the electroactive anti-tetracycline aptamer when comparing with images produced for control and for all modification steps of SPCE. The possibility of tetracycline detection was also effectively proved by causing a decrease in recorded currents with SECM.

Keywords: Scanning electrochemical microscopy, electrochemical aptasensor, electroactive aptamer, tetracycline

Biosensors technology began when analytical scientists faced the challenge in developing novel methodologies for the detection and quantification of biomedical analytes and simple/small molecules [1, 2]. Over the past decade, many important technological advances have provided tools and materials required for the construction of various types of biosensor devices [3]. However, the selectivity and the sensitivity of a biosensor are primarily conditioned by its sensing layer [4]. Many different types of biological recognition elements (enzymes, antibodies, DNA/RNA...) have been employed for highly selective responses to particular analytes [4, 5]. Among affinity elements, aptamers are DNA or RNA oligonucleotides of particular interest since they fold into three-dimensional conformations capable of binding strongly and selectively to target molecules [6]. Biosensors in which aptamers substituted the common biological recognition elements are called aptasensors. Among various aptasensors reported in literature, electrochemical aptasensors have been demonstrated as rapid, sensitive, selective and low-cost devices [7, 8].

We have recently described the development of a label-free electrochemical aptasensor for the reagentless determination of tetracycline (TET) antibiotic in water. The sensing layer consisted in a specific 76-mer oligonucleotide [9] that was immobilized by carbodiimide coupling on a screen-printed carbon electrode (SPCE). Cyclic voltammetry experiments were carried out to characterize the sensor and revealed a very unusual and unexpected quasi-reversible signal, with oxidation and reduction peaks at +0.19 and -0.25 V vs silver / silver chloride (Ag/AgCl) reference electrode, respectively. Further experiments showed a specific decrease of this signal in presence of TET, demonstrating its proper binding by the aptamer. This remarkable behavior was exploited to detect TET at concentrations ranging from 0.11 to 45 nM, with a calculated limit of detection of 0.078 nM [10].

In the present work, we used scanning electrochemical microscopy (SECM) as an imaging technique to confirm the proper immobilization of intrinsically active anti-TET aptamers and to evaluate their repartition on the surface of a SPCE-based sensor. SECM is based on the current recorded at an ultramicroelectrode (UME) which is positioned close to a substrate to obtain its topography or local reactivity [11, 12]. SECM has already shown promising issues for the study of grafted organic layers [13], characterization of bio-receptors immobilization on modified electrode surfaces [14] and as useful technique for mapping of surface-confined DNA and visualization of DNA hybridization thanks to an

electroactive hybridization indicator [15]. Several studies confirmed that SECM is a suitable technique for imaging DNA-biosensors surface thanks to its good resolution and fast scanning rate [16-18].

SECM was used in this work in substrate generation/tip collection mode (SG/TC), where an electroactive species generated at the substrate surface (SPCE) is detected at the UME tip. As shown in Figure 1-a, the UME tip was fixed at a distance of 10 μm from the SPCE surface. The UME was moved along the x axis line at 10 $\mu\text{m/s}$ and the current was recorded point by point. The measurements were carried out in a buffer used as electrolyte without adding redox species in solution. SECM imaging was performed after each step required for aptamer immobilization in a scanning area of 2000 x 2000 μm^2 , with a step of 40 μm (Figure 1-b).

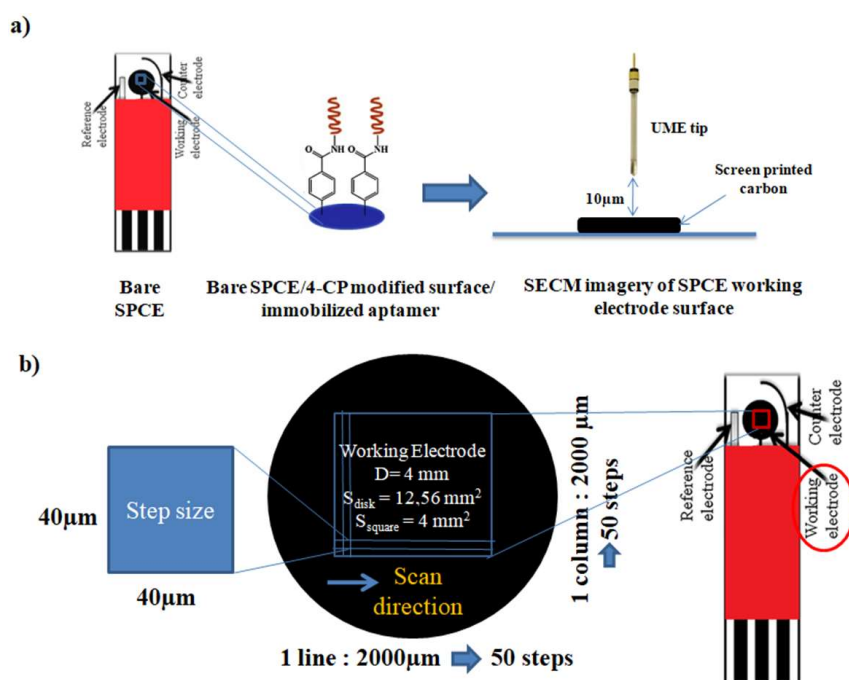


Fig. 1: a) Scheme of aptasensor preparation and probing mechanism, b) Scanning grid realized on SPCE working electrode

Figure 2 shows the different images resulting from SECM measurements over the aptamer immobilization steps. For each scan, the average current was calculated from the digital data excluding those of the first measurement line, strongly disturbed. Figure 2-a corresponds to the image obtained with a bare SPCE electrode immersed in binding buffer showing a homogenous repartition of current over the whole scanned area, with an average value of 15 pA. Figure 2-b shows the mapping of the electrode surface after its modification by 4-carboxyphenyl layer (4-CP) following the reduction of the diazonium salt. This activation step led to a decrease of the current down to values close to 0 pA over the scanning area, resulting from the passivation of the carbon surface as already observed by electrochemical impedance spectroscopy experiments [8, 10].

After immobilization of anti-TET aptamer by carbodiimide coupling, a new SECM experiment was performed. The resulting map shows a strong increase of the current up to a calculated average value of 50 pA (Figure 2-c) with an electrochemical reactivity less uniform than previously, where green and red spots corresponding to currents ranging from 100 to 280 pA reveal the presence of aptamer molecules on the surface. These results demonstrate that the studied aptamer can be detected on SPCE surface by SECM in SG/TC mode. Proper immobilization of aptamer was confirmed, allowing the global repartition of the aptamer on the electrode surface to be evaluated. In order to confirm this result, a similar sensor was elaborated with a random aptamer having the same size and nucleotide ratio than anti-TET aptamer. Figure 3 makes comparisons between the signals obtained with the anti-TET aptamer (Figure 3-a) and with the random one (Figure 3-b). The average current obtained in the presence of the anti-TET aptamer was 50 pA, while the average signal for random aptamer was 1 pA. Such a signal

was almost the same as that observed before immobilization (0 pA) (Figure 2-b). These results confirmed that green and red spots in figure 2-c and 3-a were due to the proper immobilization of the electroactive anti-TET aptamer on the SPCE surface.

A previous study using the TET aptasensor demonstrated that the aptamer electrochemical response decreased in presence of TET [10], probably due to a conformational change upon target binding. In order to confirm this observation, we evaluated the effect of TET binding using SECM by scanning the sensing area after one hour of incubation with 45 nM of TET. As shown in figure 2-d, the binding of TET induced a decrease in the average current from 50 to 2 pA, thus completely shutting off the aptamer electrochemical signal. This observation confirms the previous results obtained by cyclic voltammetry displaying that the sensor operates as a signal-off device [10].

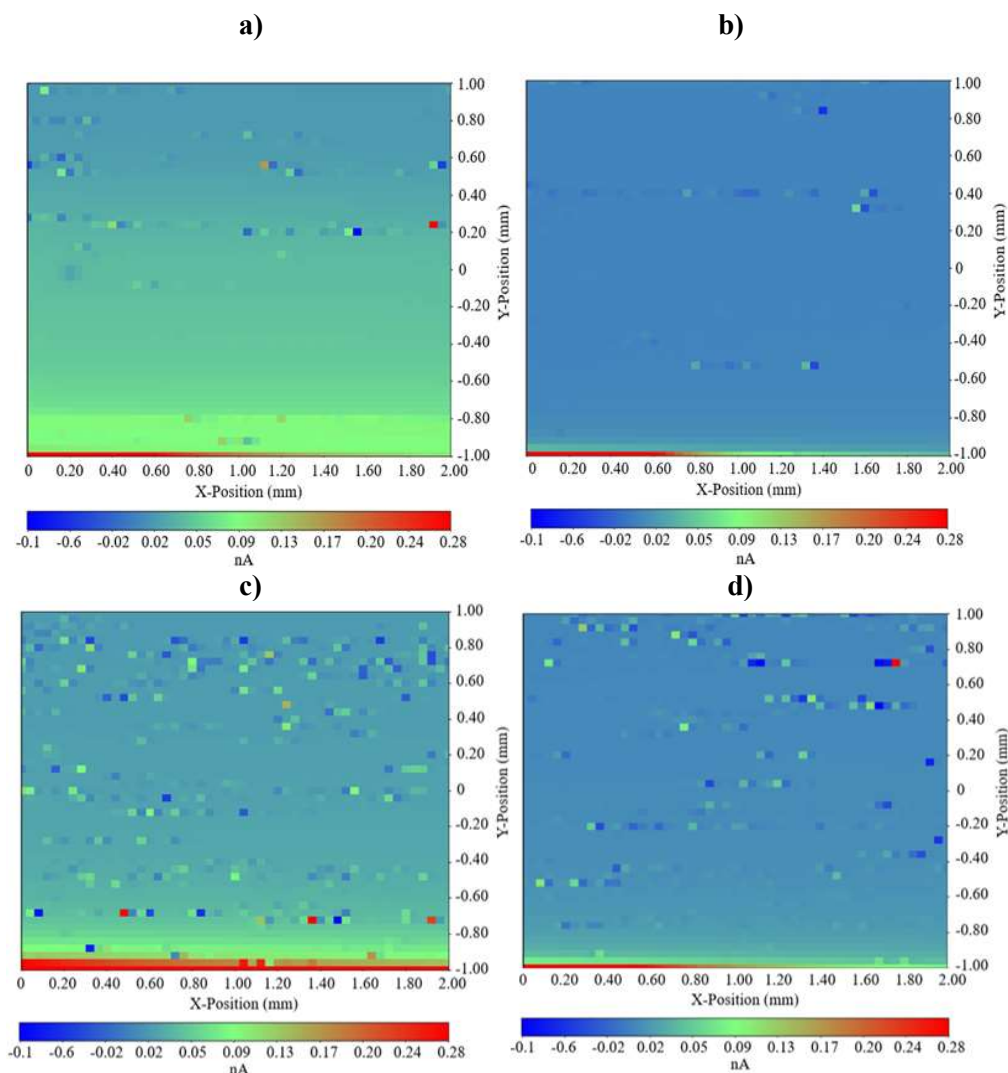


Fig.2. SECM images realized in binding buffer of: a) bare SPCE, b) 4-CP-modified SPCE, c) aptasensor for tetracycline detection, d) aptasensor response after incubation with 45nM of tetracycline. The scanned surface was $2000 \times 2000 \mu\text{m}^2$ with step size $x=y=40 \mu\text{m}/\text{point}$, $z = 10 \mu\text{m}$; $E_{UME} = 0.5 \text{ V vs Ag/AgCl}$; $E_{SPCE} = -0.3 \text{ V vs Ag/AgCl}$

a)

b)

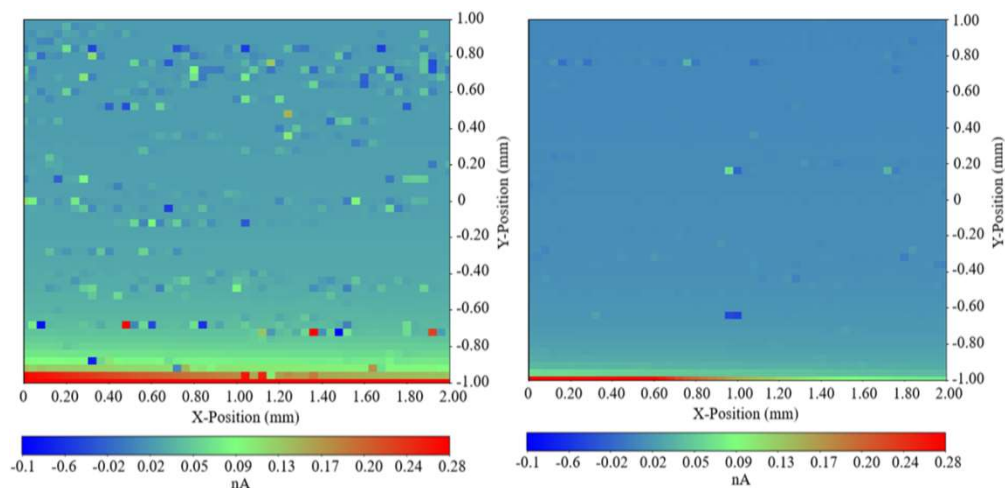


Fig. 3. SECM images realized in binding buffer of: a) anti-TET aptamer immobilized on 4-CP-modified SPCE, b) random aptamer immobilized on 4-CP-modified SPCE. The scanned surface was $2000 \times 2000 \mu\text{m}^2$ with step size $x=y=40 \mu\text{m}/\text{point}$, $z = 10 \mu\text{m}$; $E_{\text{UME}} = 0.5 \text{ V vs Ag/AgCl}$; $E_{\text{SPCE}} = -0.3 \text{ V vs Ag/AgCl}$

As discussed herein, we used SECM to characterize the anti-TET aptamer immobilization on a SPCE by analyzing 3D images (x-y-current). SECM surface imagery technique allowed us to evaluate the distribution of this electroactive aptamer on a functionalized electrode by observing the apparition of intense spots on the scanned area. These spots correspond to an increase in the current recorded at UME tip, generated thanks to the aptamer oxidation in absence of additional redox probes. These results were validated by a similar immobilization of a non-electroactive aptamer, having the same nucleotides as the previous one, but distributed randomly [10]. Based on the results obtained after incubation with tetracycline, SECM imagery could be considered as a possible technique for detecting this antibiotic by the anti-TET aptamer immobilized on the electrode surface. These preliminary results pave the way for the use of SECM as a detection technique for electrochemical aptasensors.

Experimental

The following chemicals were purchased from Sigma-Aldrich (France): sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), sodium chloride (NaCl), magnesium chloride (MgCl_2), potassium chloride (KCl), calcium chloride (CaCl_2), sulfuric acid (98%), sodium nitrite (NaNO_2), N-hydroxysuccinimide (NHS), N-(3-dimethyl amino propyl)-N'-ethylcarbodiimide hydrochloride (EDC), 2-(N-morpholino)ethanesulfonic acid (MES), 4-amino benzoic acid (4-ABA), ethanolamine, Trizma[®] hydrochloride and Tween 20. All solutions were prepared in deionized Milli-Q water (Millipore, Bedford, MA, USA). Anti-TET aptamer was a 76-mer oligonucleotide with the following sequence: 5'-NH₂-CGT ACG GAA TTC GCT AGC CCC CCG GCA GGC CAC GGC TTG GGT TGG TCC CAC TGC GCG TGG ATC CGA GCT CCA CGT G-3', as described in [9]. A 76-mer aptamer (random aptamer) which has the same proportion of each base than the anti-TET aptamer was also tested to investigate the redox signal: 5'-NH₂-AAC GTT TCA GCG CAG GCG CTC ACG GTT GCG GTT GGT CCG CGT GGA TCC GAG CTC CAC GTC CGC ACG GCC GCA CTG C-3'. The oligonucleotides were purchased from Microsynth (Switzerland). Aptamer solutions were prepared in a 10 mM phosphate buffer pH7.4 containing KCl (10 mM) and MgCl₂ (5 mM). SPCE electrodes were fabricated using a DEK248 screen-printing system as previously described [10]. Prior to modification, the SPCE were electrochemically pre-treated by applying five cyclic voltammetric scans from 1.0 to -1.5 V vs Ag/AgCl in a sulfuric acid solution (H_2SO_4 0.5 M) containing 0.1 M KCl. A diazonium salt was synthesized *in situ* by mixing 2 mM NaNO_2 with 2 mM of 4-ABA in 1 mL of 0.5 M HCl [8, 10]. The mixture was left to react for 5 min at room temperature, and electrochemical generation of surface 4-carboxyphenyl (4-CP) was performed by linear sweep voltammetry, with potential scanning from 0.6 to -0.8 V vs Ag/AgCl [8, 10]. The carboxylic groups of 4-CP-modified SPCE were then activated by incubating the working electrode for 60 min in 5 mL of 100 mM MES buffer containing 100 mM of EDC and 25mM of NHS. After washing the electrode surface with deionized water, 20 μL of 1 μM

aptamer solution were drop-casted onto the activated surface and allowed to react for 4 h under humid atmosphere. The electrodes were then rinsed with distilled water to remove unbound aptamers. Modified SPCE were finally incubated with 50 μ L of 1M ethanolamine solution for 3 hours in order to block unreacted succinimide groups. The buffer used as electrolyte in all measurements is a binding buffer for aptamer/TET recognition, based on Tris-HCl (20 mM, pH 7.6) containing NaCl (100 mM), MgCl₂ (2 mM), KCl (5 mM), CaCl₂(1 mM) and Tween 20 (0.02 %).

All SECM measurements were performed with a 25 μ m diameter gold UME electrode and using a Uniscan M370 bipotentiostat. A three-electrode setup was employed consisting in the gold UME as working electrode, carbon and Ag/AgCl from (SPE) electrodes as counter and reference electrodes respectively. The UME was polarized at 0.5 V vs Ag/AgCl, corresponding to the oxidation potential of anti-TET aptamer, while the working SPCE was polarized at -0.3 V vs Ag/AgCl, to ensure that the immobilized aptamer was present in its reduced form on the substrate. Preliminary experiments based on approach curves in a feedback mode allowed the determination of an optimal UME-substrate distance of 10 μ m. For surface electrochemical characterization, a Substrate Generation / Tip Collection mode (SG/TC) was used.

Acknowledgements

Ahmad Alawad sincerely thanks Erasmus Mundus Peace 2 Programme and Programme national d'aide à l'Accueil en Urgence des Scientifiques en Exil (PAUSE, Collège de France) for their kind help in financing his doctoral work.

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