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# Hydrogel Matrix Grafted Impedimetric Aptasensor for the Detection of Diclofenac

*G. S. Kassahun<sup>a</sup>, S. Griveau<sup>a</sup>, S. Juillard<sup>a</sup>, J. Champavert<sup>a</sup>, A.  
Ringuedé<sup>c</sup>, B. Bresson<sup>b</sup>, Y. Tran<sup>\*b</sup>, F. Bedioui<sup>a</sup>, C. Slim<sup>\*a</sup>*

<sup>a</sup> Institute of Chemistry for life and health sciences (iCLeHS),  
PSL Research University, CNRS, Chimie ParisTech, 11 rue Pierre  
et Marie Curie, 75231 Paris Cedex 05, France

<sup>b</sup> Soft Matter Engineering and Science, PSL Research University,  
UMR 7615 CNRS, ESPCI, 10 rue Vauquelin, F-75231 Paris Cedex 05,  
France

<sup>c</sup> Institut de Recherche de Chimie de Paris (IRCP), PSL Research  
University, CNRS, Chimie ParisTech, 11 rue Pierre et Marie  
Curie, 75231 Paris Cedex 05, France

Corresponding Authors:

[cyrine.slim@chimieparistech.psl.eu](mailto:cyrine.slim@chimieparistech.psl.eu)

yvette.tran@espci.fr

## **ABSTRACT**

Driven by the growing concern about the release of the untreated emerging pollutants and the need for determining small amount of these pollutants present in the environment, novel biosensors dedicated to molecular recognition are developed. We have designed biosensors using a novel class of grafted polymers, surface-attached hydrogel thin films on conductive transducer as a biocompatible matrix for biomolecule immobilization. We showed that they can be dedicated to the molecular recognition of Diclofenac (DCL). The immobilization of the aptamer onto surface-attached hydrogel thin films by covalent attachment provides a biodegradable shelter, providing the aptamer excellent environments to preserve its active and functional structure while allowing the detection of DCL. The grafting of the aptamer is obtained using the formation of amide bonds *via* the activation of carboxylic acid groups of the poly (acrylic acid) (PAA) hydrogel thin film. For improved sensitivity and higher stability of the sensor, a high density of immobilized aptamer is enabled. The aptamer modified electrode was then incubated with DCL solutions at different concentrations. The performances of the aptasensor

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2  
3 were investigated by Electrochemical Impedance Spectroscopy (EIS).  
4  
5 The change in charge-transfer resistance ( $R_{ct}$ ) was found to be  
6  
7 linear with DCL concentration in the 30 pM – 1  $\mu$ M range. The  
8  
9 detection limit was calculated to be 0.02 nM. The improvement of  
10  
11 the limit of detection can be mainly attributed to the three-  
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13 dimensional environment of the hydrogel matrix which improves the  
14  
15 grafting density of aptamer and the affinity of the aptamer to  
16  
17 DCL.  
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23 **Keywords:** Aptamer; Electrochemical Biosensors; Impedimetric  
24 aptasensors; Diclofenac; Emerging pollutants; Hydrogel  
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## INTRODUCTION

The detection of drug residues as contaminants in water and food is of high importance in the fields of human health and environment. One of the commonly used drugs is Diclofenac (DCL), or 2-(2-((2,6-dichlorophenyl)amino) phenyl)acetic acid, marketed with the trade name Voltaren®. This drug is a nonsteroidal anti-inflammatory drug (NSAID) prescribed for its anti-inflammatory, analgesic, and antipyretic properties <sup>1</sup> to treat inflammatory joint diseases and mild to moderate pain <sup>3,4</sup>. Because of its frequent use, DCL residues have often been detected in freshwater environments <sup>5-7</sup>. DCL also belongs to the list of emerging pollutants (EP) because of the risk it may presents for health and environment <sup>8,9</sup>. There is currently a large list of EPs substances, as drugs (antibiotics, pharmaceuticals, hormones), products for daily use (detergents, disinfectants, antioxidants...) and products of industrial origin (flame retardants, nanoparticles) characterized by a wide distribution in the environment due to their massive daily use and persistence <sup>10,11</sup>. EPs are commonly present at trace concentrations and characterized by their long-term health effects, such as nervous system damage, toxicity or cancer appearance due to their accumulation and persistence, even at very low concentrations <sup>12</sup>. Many studies have described the potential effects of DCL on organisms when exposed to environmental

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3 levels ranging from a few ng L<sup>-1</sup> to tens of µg L<sup>-1</sup> in surface water  
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5 13,14,15 .  
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8 On the light of concerns about possible adverse effects of DCL on  
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10 organisms and on aquatic life, its essential to quantify this drug  
11  
12 in environmental waters, not only to assess accurately its  
13  
14 environmental risk and characterize the dangers due to its  
15  
16 environmental exposure but also to verify and improve the  
17  
18 efficiency of wastewater treatment processes <sup>16</sup>. Until now, its  
19  
20 detection essentially depend on conventional techniques, such as  
21  
22 HPLC <sup>17,18</sup> and GC/MS <sup>19</sup>. Although these analytic techniques are very  
23  
24 robust, they are expensive, time consuming, require experts and  
25  
26 are not portable in the field. Electrochemical methods are an  
27  
28 attractive alternative for the analysis of pharmaceutical,  
29  
30 biological and environmental compounds. They provide many benefits  
31  
32 over thermal, optical and piezoelectric detection including ease  
33  
34 of use, rapidity, cost-effectiveness, high sensitivity and  
35  
36 selectivity, ease of miniaturization, compatibility with novel  
37  
38 micro-fabrication technologies and robustness <sup>20-29</sup>.  
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41 Electrochemical reactions generally produce an electronic signal  
42  
43 directly, without the the need of expensive signal transduction  
44  
45 equipment.  
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51 Various electrochemical methods have been reported for the  
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53 determination of DCL such as amperometric <sup>30-33</sup> and potentiometric  
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55 techniques <sup>24, 34-36</sup>. Modified electrodes comprising Cu-doped  
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3 zeolite-expanded graphite-epoxy electrode <sup>37</sup>, graphene or carbon  
4  
5 nanotubes <sup>38,39</sup> have been also used. For improving the limit of  
6  
7 detection (LOD), biomolecules that are specific for the DCL have  
8  
9 been utilized by using antibodies also <sup>40,41</sup>.

11  
12 When it comes to electrochemical sensing, aptamer based biosensors  
13  
14 (aptasensors) are promising tools, which take advantages of the  
15  
16 use of aptamers as probes in order to detect specific targets <sup>42</sup>.  
17  
18 Aptamer are single-stranded DNA (ssDNA) or RNA molecules able to  
19  
20 bind target molecules with high affinity <sup>43</sup>. Because of their  
21  
22 specific binding abilities and many advantages over antibodies  
23  
24 including their higher stability, lower cost, specificity and  
25  
26 affinity <sup>44-47</sup>, their easy reliable production <sup>44,45</sup>, their easy  
27  
28 chemical modification offering diverse immobilization ways <sup>50</sup>,  
29  
30 their easy regenerability <sup>46,48</sup> and their storability <sup>46,49</sup>, they  
31  
32 provide a great opportunity to produce sensing surfaces for  
33  
34 effective and selective electrochemical detection of harmful small  
35  
36 molecules such as DCL and their real-time monitoring in the  
37  
38 environments <sup>51</sup>. Among the different electrochemical detection  
39  
40 techniques, electrochemical impedance spectroscopy (EIS) has  
41  
42 emerged as appealing strategy <sup>52,53</sup>.

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48 Studies on detection of DCL using aptasensors are rarely described  
49  
50 to our knowledge, and recently a very few EIS aptasensors have  
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52 been built based on the interfacial property <sup>29,54</sup>.  
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3 In this work, we have developed a novel aptasensor for DCL. To  
4 this end, a new class of surface-attached hydrogel thin films was  
5 grafted on conductive transducer and used as biocompatible matrix  
6 for aptamer immobilization, tuned for the conception of biosensor.  
7  
8 The polymeric network environment favors the stable immobilization  
9 of the aptamer and the selective affinity to the target and  
10 sensitive electrochemical detection, by providing the aptamer  
11 excellent environments to preserve its active and functional  
12 structure <sup>55</sup>. Surface-attached hydrogel thin films present then  
13 many advantages as immobilization matrix: they are stable, robust,  
14 multiscale and multifunctional materials, with different thickness  
15 ranges from a few nanometers to several micrometers. The  
16 deformability, permeability and porosity of the hydrogel films can  
17 also be easily and finely adjusted with the film thickness and the  
18 network crosslinks density.

19  
20  
21 The synthesis of reproducible and reliable hydrogels films was  
22 produced using a simple and versatile approach, the Cross-Linking  
23 And Grafting process (CLAG). It consists of using thiol-ene click  
24 chemistry to preform functionalized polymers. As described by  
25 Chollet *et al.* <sup>56</sup> the advantages of the synthesis of the preformed  
26 reactive polymers chains consist of avoiding the constraint of  
27 working under a controlled atmosphere due to the oxygen inhibition  
28 of radical polymerization. This versatile approach allows then  
29 easy adjustment of the chemical properties (platform for covalent  
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3 immobilization) and physical properties (size and architecture)  
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5 hydrogel films.  
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## EXPERIMENTAL SECTION

### Chemicals

Poly(acrylic acid) (PAA) ( $M_w \sim 250$  kg/mol, 35 wt% solution in water), allylamine, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 1,4-dithioerythritol (DTE), diclofenac (DCL), potassium ferricyanide [ $K_3Fe(CN)_6$ ], potassium ferrocyanide [ $K_4Fe(CN)_6$ ], and potassium/sodium phosphate monobasic/dibasic were purchased from Sigma-Aldrich and were used as received. Chloroform, methanol, formic acid solvents were also purchased from Sigma-Aldrich.

The aptamer used for sensing (amine terminated fluorescently labeled DCL aptamer (75 bases)) had the 5'-/5AmMC6/ATA CCA GCT TAT TCA ATT GCA ACG TGG CGG TCA GTC AGC GGG TGG TGG GTT CGG TCC AGA TAG TAA GTG CAA TCT/36-FAM/-3' sequence and was purchased from Integrated DNA technologies.

The aptamer solution was prepared using a phosphate buffer solution (0.1 M, pH 7.4). The working aptamer solutions were prepared from a 100  $\mu$ M aptamer stock solution by diluting to the desired final concentration. The aptamer stock solution was prepared and stored at  $-20$  °C.

Other chemicals used were of analytical grade and have been used as supplied. Deionized water (18.2 M $\Omega$ /cm) purified using a Pure Labflex system (Elga Water, Veolia, France) was used throughout the experiment for aqueous solution preparation.

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3 For the determination of DCL in water sample, a stock solution of  
4 100 mM DCL was daily prepared, and was then diluted at different  
5 concentrations using 200  $\mu$ L binding washing buffer (BWB) (10 mM  
6 NaCl, 0.5 mM KCl, 0.2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub> and 5% ethanol in 2  
7 mM Tris-HCl at pH 7.5).

## 16 **Characterization of the biosensor**

### 18 **EIS measurements**

19 We used a conventional three-electrode cell. A Saturated Calomel  
20 reference Electrode (SCE), a platinum wire as counter electrode  
21 and a modified gold working electrode. A Bio-Logic instrument (Bio-  
22 Logic SP-300) equipped with EC-Lab software was used to carry out  
23 all the cyclic voltammograms and EIS measurements. EIS  
24 measurements were performed with a potential equal to 0 V (relative  
25 to the OCP), over the frequency range of 10 kHz to 1 mHz with an  
26 oscillation potential of 10 mV. Cyclic voltammograms and impedance  
27 measurements were carried out in the presence of a mixture of  
28 K<sub>3</sub>[Fe(CN)<sub>6</sub>]/K<sub>4</sub>[Fe(CN)<sub>6</sub>] at 5 mM, in PBS (0.1 M at pH 7.4).  
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### 44 **Atomic Force Microscopy**

45 The gold electrode and the PAA hydrogel functionalized electrodes  
46 were also characterized by AFM. A Bruker ICON microscope controlled  
47 by a Nanoscope V was used for the acquisition of all the images.  
48 The thickness of the gold electrode and the PAA hydrogel is  
49 determined at the edge of the pattern. The height images of the  
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3 gold surface and the films of hydrogel in air were obtained in  
4 tapping mode at 300 kHz with a standard tip of stiffness 40 N/m.  
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6 The half width of the height distribution histogram extracted from  
7 the images defines the roughness. The imaging conditions for very  
8 soft gel in water have been discussed in details by Li *et al.* <sup>57</sup>.  
9  
10 As very soft matter is probed, a tip with a lower stiffness is  
11 preferred if the adhesion is not preponderant. In our case, a  
12 compromise is chosen with a typical contact tip with a stiffness  
13 of 0.2 N/m.  
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16  
17 The Peak Force mode is preferred to probe hydrogel films in water  
18 when imaging soft matter in water. In this mode, the probe and the  
19 sample are intermittently brought into contact for a short period  
20 and can be considered as a succession of approach-retract curves  
21 performed at high frequency (1 kHz) and limited by a maximum set  
22 force which can be finely tuned. This mode allows to reduce the  
23 lateral and shear forces during the scanning. A cantilever of  
24 stiffness of 0.7 N/m, with a typical Force Set Point of 1nN was  
25 used to obtain the Peak Forces images in water. The amplitude of  
26 the tapping was about 200 nm. The size of the images is 1  $\mu\text{m}$ .  
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#### 46 **Other characterization techniques**

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48 ATR-FTIR was performed in Agilent Cary 660. The water contact angle  
49 measurements of the different stages of electrode fabrication was  
50 obtained by dropping a drop of water and using the KRUSS drop  
51 shape analyzer with the Sessile Drop method.  
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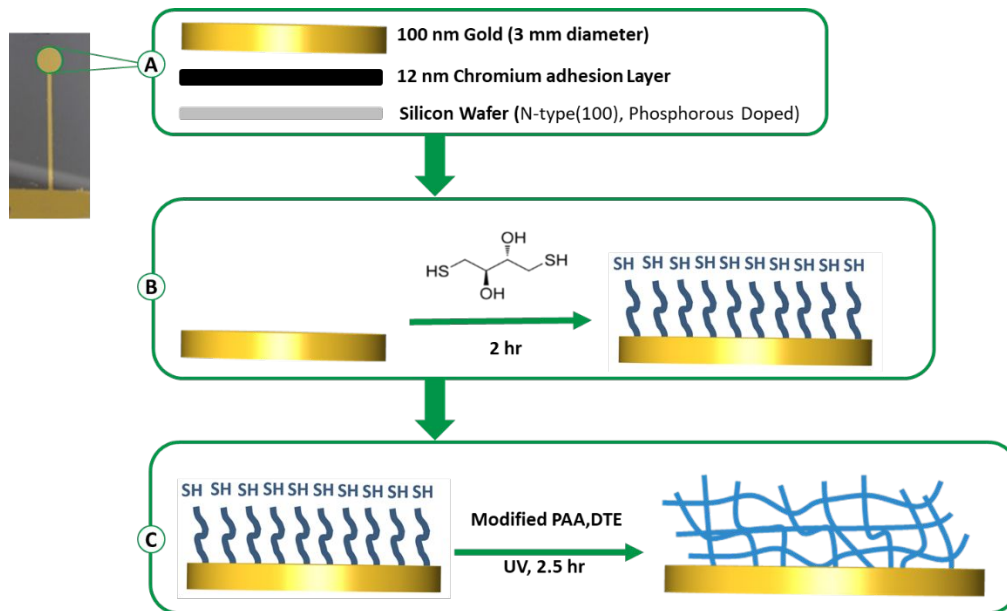
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3 The secondary electron imaging of the different stages of  
4 construction was done using FEI Thermo Fischer Magellan SEM  
5 equipped with a FEG source.  
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### 10 **Target Detection**

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12 Detection of DCL were carried out by incubating the aptasensor in  
13 200  $\mu$ L BWB solution containing DCL for 50 min at room temperature.  
14  
15 After detection, the non-bound molecules of DCL was removed by  
16 washing three times the electrode with BWB solution.  
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### 21 **Construction of the aptasensor**

22  
23 The subsequent steps of manufacturing the aptamer based  
24 electrochemical sensor undergone in this study are illustrated in  
25 Scheme 1. It consists in preparing the gold electrode with positive  
26 lithography (A) (see Figure S1, supporting information), modifying  
27 the gold electrode with dithiol molecules (B) and simultaneously  
28 CLAG ene-functionalized PAA by thiol-ene click reaction to form  
29 hydrogel surface-attached matrix (C). The steps are detailed  
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Scheme 1: Different steps of aptasensors construction ; (A) gold electrode made by positive photolithography; (B) thiol modification of the gold electrode; (C) hydrogel grafting using thiol-ene click chemistry.

### Preparation and thiol-functionalization of gold electrode transducer

First, the transducer was prepared, as a thin layer gold electrode deposited on silicon wafer. To this end, evaporated gold films were obtained using N-type phosphorous doped silicon wafer with 100 orientations (resistivity: 1-20 ohm.cm) as substrate . To promote adhesion and cleanliness, the silicon wafers were cleaned by oxygen plasma prior to coating. A positive photolithography process was used to prepare the thin layers by thermal evaporation of ~100 nm gold onto a ~12 nm chromium adhesion layer.

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3 The gold thin film working electrode, with a diameter of 3 mm,  
4 were subsequently cleaned in 50 mM KOH and 25 % H<sub>2</sub>O<sub>2</sub> for 30 minutes.  
5  
6 The electrodes were then characterized by cyclic voltammetry at 50  
7  
8 mV/s in K<sub>3</sub>[Fe(CN)<sub>6</sub>] aqueous solution to check the reversibility of  
9  
10 redox couple and compare experimental to theoretical peak currents  
11  
12 (see Figure S2, supporting information).  
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16 The functionalization of the activated gold surfaces with thiol  
17  
18 self-assembled monolayers was carry out using a solution of 1 mM  
19  
20 dithioerythritol (DTE) in chloroform for 2 hours. The thiol-  
21  
22 modified surfaces were then rinsed and sonicated in chloroform and  
23  
24 dried with nitrogen flow.  
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### 30 **Hydrogels grafting on gold electrodes**

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32 Poly(acrylic acid) hydrogel chains were randomly functionalized  
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34 with ene-groups at their carboxylic acid sites. Allylamine was  
35  
36 graft onto polymer chains using a peptide reaction in the presence  
37  
38 of EDC as the dehydration agent and NHS as the addition agent to  
39  
40 increase yields and decrease side reactions <sup>58</sup>. Briefly, a 20 wt %  
41  
42 solution of PAA in Milli-Q water at a pH of 4.5 was mixed with NHS  
43  
44 and EDS for 2 hours at 60 °C. Allylamine was then added (the molar  
45  
46 ratio AA/allylamine/EDC/NHS being set equal to 10/1/1.5/1.5) and  
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48 the pH was adjusted to 10 before the reaction was allowed to  
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50 proceed for 16 hours at 60°C. After 3 days of dialysis in 0.1 M  
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3 NaCl solution and 3 days of dialysis in Milli-Q water, the polymer  
4  
5 was finally recovered through freeze-drying  
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7 PAA hydrogel thin films were synthesized on the electrode surface  
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9 by simultaneously cross-linking and grafting preformed polymer  
10  
11 chains through thiol-ene click chemistry strategy as reported by  
12  
13 Chollet *et al.* <sup>56</sup> and Li *et al.* <sup>57</sup>. More precisely, the ene-  
14  
15 functionalized PAA polymer (0.5 wt %) was dissolved in the methanol  
16  
17 and formic acid mixture 7:3 (V/V) and the solution was stirred for  
18  
19 one night. The coatings were carry out by spin-coating of the ene-  
20  
21 functionalized polymer solution on thiol-functionalized gold  
22  
23 electrodes, with the addition of dithiol cross-linkers at 3000 rpm  
24  
25 for 30 seconds.  
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30 Aluminum foil were used as a mask for patterning. The CLAG were  
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32 simultaneously obtained by exposing the assembly to UV irradiation  
33  
34 at 254 nm for 2.5 hours. The electrode was then rinsed in methanol  
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36 using ultrasonic bath for 1 minute and then dried with N<sub>2</sub> flow. We  
37  
38 obtained homogeneous hydrogel thin films with well-controlled  
39  
40 thickness with a dry thickness being about 57 nm.  
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#### 44 **Immobilization of aptamer on hydrogel-modified transducer**

45

46 The covalent grafting of the amino-labeled aptamer solution on the  
47  
48 resulting Poly(acrylic acid) hydrogel modified electrode was  
49  
50 performed by using *in situ* two-step attachment method. The modified  
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52 electrode was incubated for 30 minutes at 20°C in phosphate buffer  
53  
54 of pH 6.2 containing 50 mM EDC and 100 mM NHS. The electrode was  
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3 subsequently rinsed with phosphate buffer (pH 7.4) and then  
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5 incubated with 10  $\mu\text{M}$  aptamer in phosphate buffer solution of pH  
6  
7 7.4 for 1 h at 20°C. Before grafting, the aptamer solution was  
8  
9 heated at 75°C for 5 min. Once the grafting of the aptamer have  
10  
11 been done, the electrode was washed with a phosphate buffer  
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13 solution to remove the unreacted aptamer.  
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## 19 **RESULTS AND DISCUSSION**

### 21 **Topography and thickness of electrode-attached hydrogel films**

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23 For the different stages of electrode construction, contact angle  
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25 measurements were performed to evaluate the presence of the  
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27 different layers put on and hence (see figure 1), the same is  
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29 confirmed by analyzing the materials hydrophobicity and/or  
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31 hydrophilicity. Though the water contact angle (WCA) value shows  
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33 relatively a hydrophobic surface for the silicon wafer, after the  
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35 plasma cleaning the wafer exhibited dramatic differences in the  
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37 contact angle indicating the surface change from a hydrophobic  
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39 state to hydrophilic. The WCA of the gold ( $\sim 62^\circ$ ) indicated that  
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41 the material does not have a hydrophilic character.  
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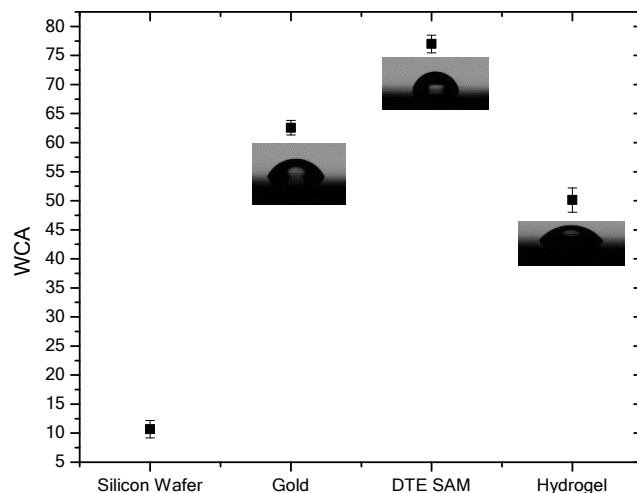


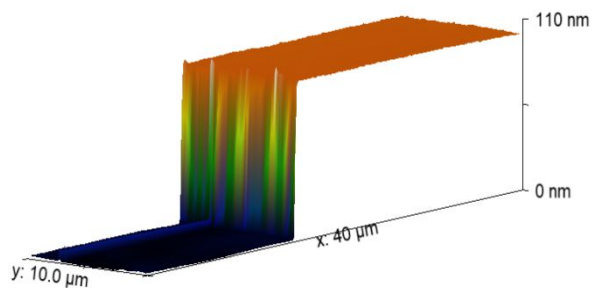
Figure 1. Water contact angle of the different stages of electrode fabrication

The WCA on the DTE SAM exhibited distinctly higher contact angles ( $\sim 75^\circ$ ) compared to that of the clean gold electrode, evidencing the modification of gold surface properties. WCA measurements of SAMs is reported to have a parity (or odd-even) vs chain-length relationship<sup>59,60</sup>.

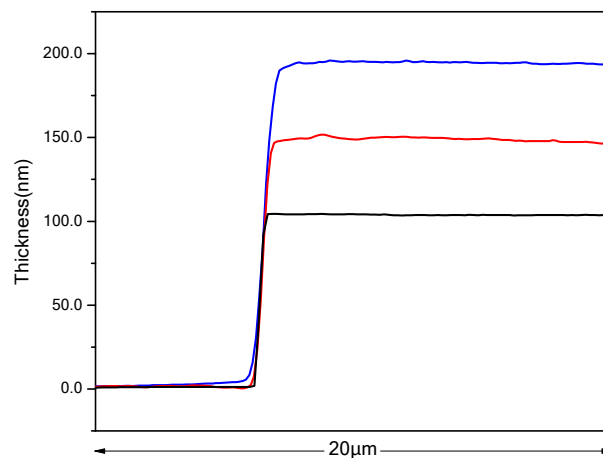
After the formation of PAA hydrogel on thiol-modified gold electrode, the water contact angle is around  $50^\circ$ . Accounting for the homogeneity and smoothness of the thin film, the contact angle expresses the presence of polymeric polyelectrolyte surface and this result is consistent with a study described for the water contact angle of hydrophilic and negatively charged hydrogel at constant grafting density<sup>61</sup>.

1  
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3 The thickness of both the gold and hydrogel layer grafted on a DTE  
4 SAM-modified gold substrate was determined using AFM measurements  
5 (Figure 2). It was found to be around 100 nm for the bare gold  
6 electrode and 57 nm for the PAA hydrogel. The dry thickness of the  
7 PAA hydrogel film is consistent with the value found by our group  
8 for surface-attached hydrogel films using spin-coated polymer  
9 solution with concentration of 0.5 wt % and molecular weight of  
10 250 kg mol<sup>-1</sup> <sup>56</sup>.

11  
12 The swelling ratio measured as the ratio of the swollen thickness  
13 of hydrogel films (measured in water) to the dry thickness  
14 (measured in air) was found to be 1.8. It was obtained for the  
15 hydrogel films fabricated with 2 % ene-reactive groups and 30 times  
16 the ene-reactive groups excess of dithioerythritol cross-linkers.  
17 This result is consistent with the study that states for thin films  
18 less than 150 nm, the swelling rate increases with the thickness  
19 of the film and it is independent of the thickness in the range of  
20 150 nm to a few micrometers <sup>56,57</sup>. This is related to the strong  
21 effect that the surface grafting has on the swelling of the films.  
22 Due to this constraint of surface attachment nanometric hydrogel  
23 films swell on average less than micrometric layers.  
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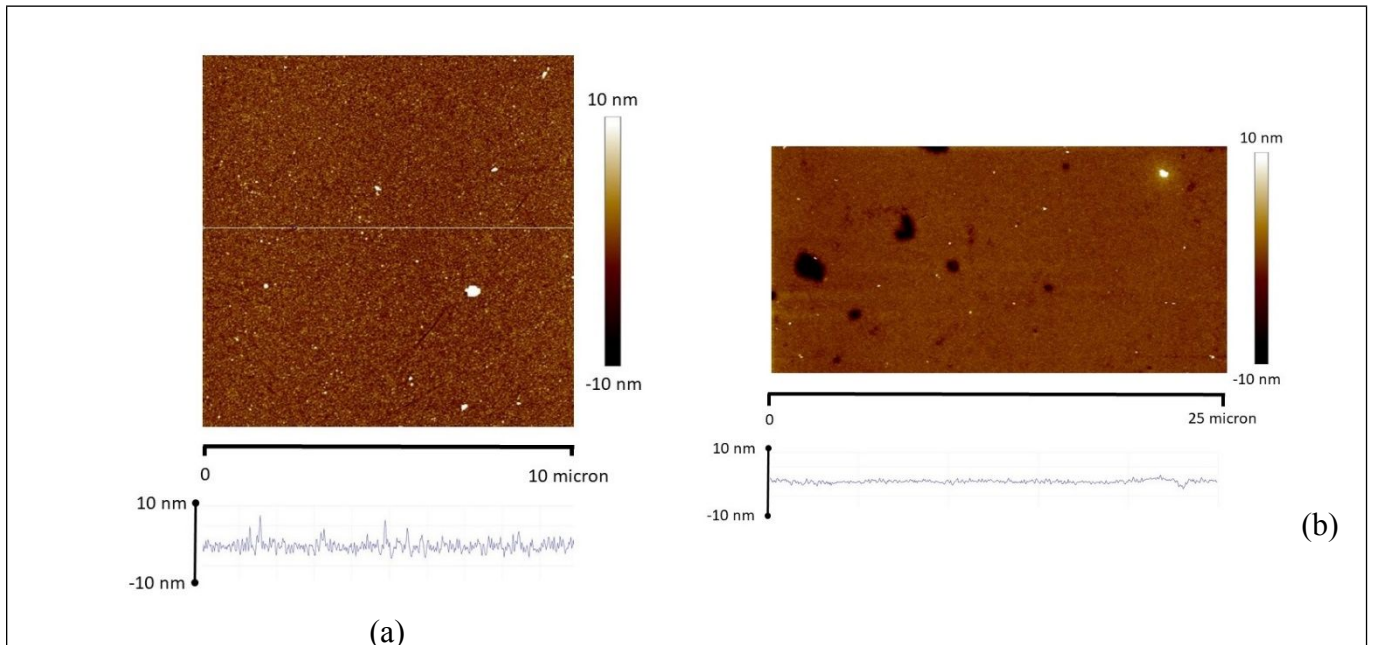
(a)



(b)

Figure 2. (a) Schematics of height profiles obtained by AFM for bare gold. (b) Height profiles of gold electrode (in black), PAA hydrogel film in air (red) and PAA hydrogel film in water (blue). Topographic images and height profiles of the bare gold surface, the surface-attached hydrogel film in air and the surface attached hydrogel immersed in water have been determined by AFM (Figure 3 and Figure S3 in the supporting information) and scanning electron microscopy (SEM) (Figure 4 and Figure S4 (Supporting Information)). The height image obtained from the evaporated gold surface shows a typical grainy structure (Figure 3a). To illustrate the roughness difference between the samples on Figure 3, a profile line is extracted for each height image and displayed beyond the image. The roughness of the gold surface can be extracted from the

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3 AFM images measuring the half width of the height histogram of the  
4 image. From the Figure 3 (a), a roughness of 4 nm is obtained.  
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7 Moreover, the same value ( $\sim 4$  nm) is obtained for both images (1  
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9  $\mu\text{m}$ - and 10  $\mu\text{m}$ -scales), proving that the roughness is independent  
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11 of the size of the sample and is homogeneous. In the general case,  
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13 the roughness strongly depends on the image size, but here, the  
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15 roughness strongly depends on the image size, but here, the  
16  
17 roughness of the sample is completely determined by the morphology  
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19 of the coated gold on the (plane) silicon wafer. It means that  
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21 increasing the image size does not reveal additional lower spatial  
22  
23 frequency. All in all, the large homogeneity of the bare gold  
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25 electrode at 10  $\mu\text{m}$ -scale and the granular structure at 1  $\mu\text{m}$ -scale  
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27 observed by AFM are also confirmed by SEM (Figure 4).  
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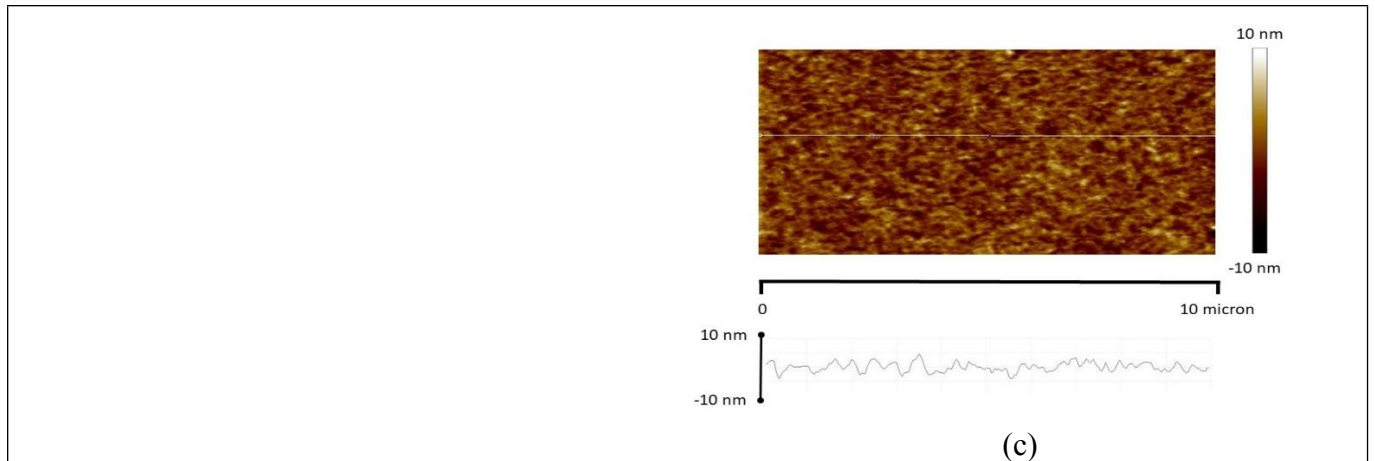


Figure 3. AFM tapping height images of the bare gold electrode surface (a), AFM tapping height images of the hydrogel film in air (b) and AFM height contact images of the hydrogel film immersed in water (c).

The AFM images of PAA hydrogel film in air (Figure 3b) also show that the roughness, estimated to 1 nm (half width of the height histogram of the image), is homogeneous. Even though the thickness of the hydrogel film (~ 57 nm) is much higher than the roughness, the surface of the hydrogel film replicates that of the gold electrode and follows strictly the same granular structure. The replication of the morphology of gold surface is clearly confirmed by SEM. In Figure 5, the SEM image displays the frontier between the hydrogel film (dark region) and the bare gold surface. The inset enables to highlight the granular structure on both surfaces of the gold electrode and the hydrogel film with a slightly lower topography contrast in the hydrogel region. The fine observation of the hydrogel region (upper part of the Figure 5) on the large

(50  $\mu\text{m}$ -) scale confirms the high planarity of the hydrogel surface. Finally, the hydrogel film immersed in water (Figure 3c) showed higher roughness ( $\sim 4$  nm) than that in air, as the gel swells (the swelling ratio is 1.8).

As a result, we show that the roughness of the hydrogel film surface is due to the replication of the granular morphology of the gold electrode. It is anyway much lower than the thickness of the layer, so that the hydrogel film can be considered as fully homogeneous and plane. From the measure of the dry thickness ( $h = 57$  nm) and the swelling ratio ( $S = 1.8$ ), we can calculate the concentrations of acrylic acid unit. The volume concentration  $C_V$  can be calculated by:  $C_V = \rho / (SM)$  where  $\rho$  is the density of poly(acrylic acid) and  $M$  is the molecular weight of acrylic acid unit. The surface concentration  $C_S$  is calculated by:  $C_S = (hx\rho) / M$ . We obtained  $C_V = 8 \cdot 10^{-3}$  mol/L and  $C_S = 0.82 \cdot 10^{-9}$  mol/m<sup>2</sup>.

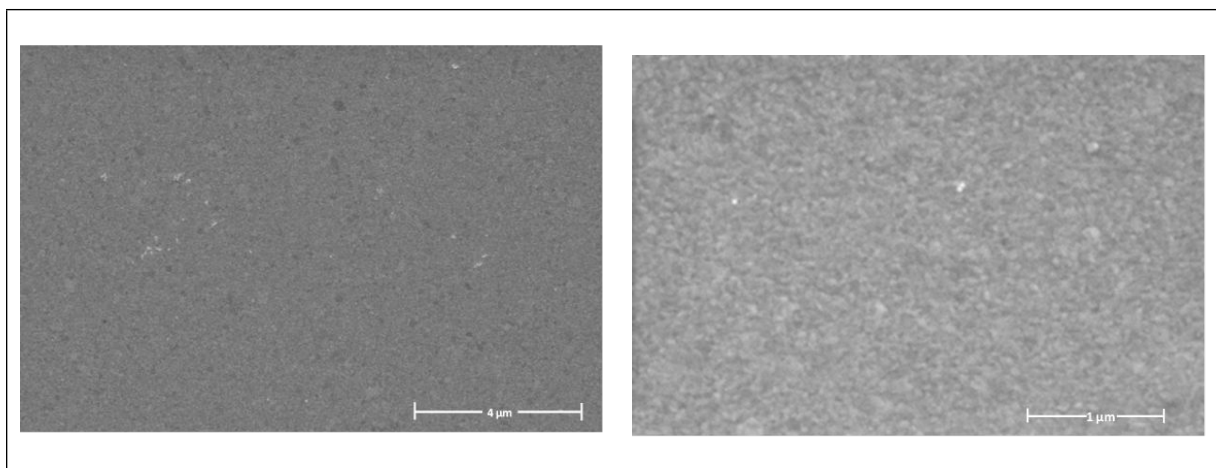


Figure 4. SEM images of the bare gold electrode surface at different scales

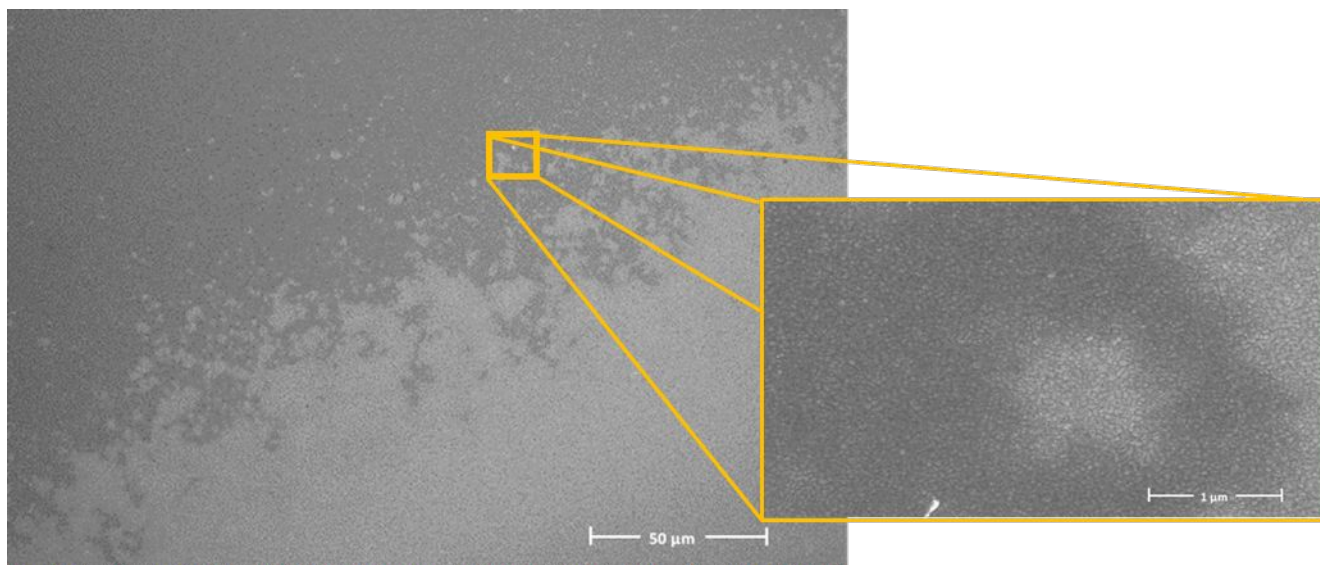


Figure 5. Surface morphology of hydrogel (dark area) and gold (white area) visualized using scanning electron microscope (SEM)

### **Characterization of the hydrogel-modified electrode**

Electrodes were also characterized by ATR-FTIR spectroscopy and electrochemistry, after cross-linking and grafting of PAA to evidence the presence of the hydrogel on the electrode surface. Figure 6 shows the ATR-FTIR spectra between 500 and 4000  $\text{cm}^{-1}$  of the gold electrode after thiol modification and the formation of PAA hydrogel. As the contact between the ATR-diamond waveguide and the solid silicon substrate cannot be reproducible for different samples, the absorbance is in arbitrary unit. This representation also enables to highlight the absorption bands. The spectrum (a) clearly demonstrates that the gold electrode is modified by thiol molecules. First, the covalent gold-sulfur bond is proven by the presence of the large band in the range of 500 to 750  $\text{cm}^{-1}$ , which



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3 can be attributed to the stretch mode of C-S bond. Second, the  
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5 vibration mode of alkyl thiol is observed at  $2917\text{ cm}^{-1}$ . Moreover,  
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7 the peaks at  $1017\text{ cm}^{-1}$  correspond to the O-H deformation mode. The  
8  
9 O-H stretching vibrations in the range of  $3100\text{--}3500\text{ cm}^{-1}$  can also  
10  
11 be clearly seen.  
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14 The FTIR-ATR spectrum (b) is obtained with the gold electrode  
15  
16 modified by the PAA hydrogel. It evidences the O-H stretching band  
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18 in  $3400\text{ cm}^{-1}$  region and the  $\text{CH}_2$  (asymmetric) stretching at  $2925$   
19  
20  $\text{cm}^{-1}$ . Also, the bands at  $1700\text{ cm}^{-1}$  are attributed to C=O stretching  
21  
22 band of PAA. Comparing spectra (a) and (b), it could be noticed  
23  
24 that the O-H stretching vibration in the range of  $3100 - 3500\text{ cm}^{-1}$   
25  
26 is different. The absorption band of the PAA hydrogel which is  
27  
28 characteristics of hygroscopic compound is broader due to the  
29  
30 presence of H-bonds (in comparison with the O-H stretching band of  
31  
32 thiol monolayer). The decrease of the O-H band at  $1017\text{ cm}^{-1}$  is due  
33  
34 to the arbitrary unit of the absorbance. Finally, it should be  
35  
36 noted that the presence of C-S band in PAA hydrogel spectrum is  
37  
38 not surprising as the thickness of the hydrogel film is much less  
39  
40 than the penetration depth of the evanescent wave of the ATR-  
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42 infrared beam (a few microns).  
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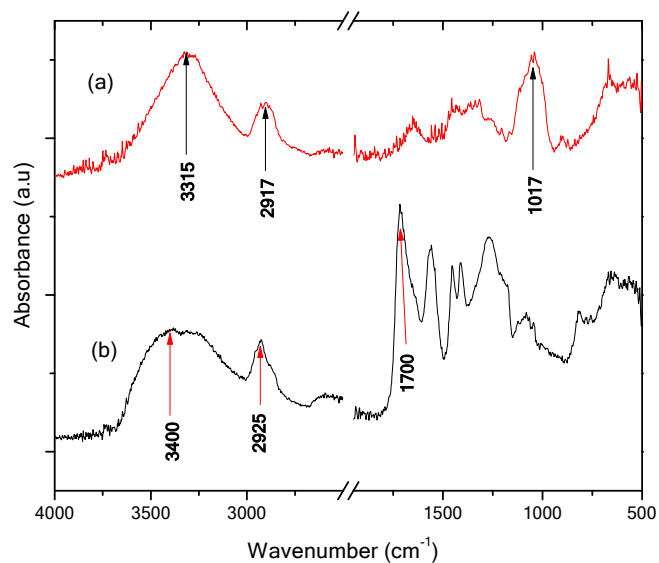


Figure 6. FTIR-ATR spectra of thiolated gold electrode (a) and PAA hydrogel grafted gold electrode (b).

The PAA hydrogel modified electrode was also electrochemically analyzed by cyclic voltammetry. Figure 7 shows the voltammograms of  $\text{Fe}(\text{CN})_6^{3-/4-}$  of the gold electrode at different steps of the hydrogel film preparation: bare gold electrode, thiol monolayer and PAA hydrogel film.

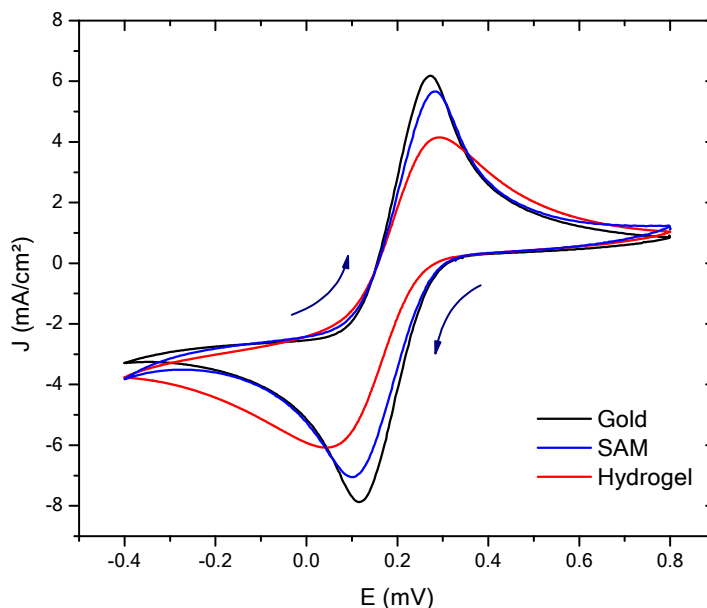


Figure 7. Characterization of the sensing interface by cyclic voltammetry at  $50 \text{ mV}\cdot\text{s}^{-1}$  in a solution of  $\text{KCl } 0.1 \text{ M}$  containing  $5 \cdot 10^{-2} \text{ M } \text{K}_4[\text{Fe}(\text{CN})_6]$  and  $\text{K}_3[\text{Fe}(\text{CN})_6]$  : bare gold electrode (black), thiol monolayer (blue), PAA hydrogel film (red).

The voltammogram of  $\text{Fe}(\text{CN})_6^{3-/4-}$  obtained with thiol-modified electrode is almost similar to that observed at bare gold electrode. This is attributed to the fact that the thiol molecules form a non-dense monolayer<sup>62</sup>. The introduction of terminal groups ( $-\text{SH}$ ,  $-\text{COOH}$ ,  $-\text{OH}$ ,  $-\text{NH}_2$ ) instead of the  $-\text{CH}_3$  group usually results in a decrease in self-assembled ordering<sup>63</sup>. Thus, the monolayer of DTE thiol small molecule on gold is too thin to effectively block electron transfer on a gold surface.

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3 At relatively high pH for which the PAA hydrogel is swollen and  
4 dissociated, when comes in contact with the negative redox probe,  
5  $\text{Fe}(\text{CN})_6^{3-/4-}$ , it causes electrostatic repulsion which will affect  
6 the interfacial electron-transfer rate of the probe and a decrease  
7 in peak current and increased separation potential in comparison  
8 to the bare gold and thiol-modified electrodes. Moreover, this can  
9 be explained with the study that describes that at low pH, polyacid  
10 brushes are fully protonated and adopt a conformation similar to  
11 that of neutral brushes <sup>64</sup>. At high pH, the polyacid brushes become  
12 dissociated and the brush adopt an extended polyelectrolyte brush  
13 conformation similar to that of the osmotic or Pincus regime <sup>65</sup>.  
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### 31 **Functionalization and Immobilization of the Aptamer**

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33 The covalent immobilization of the aptamer on the surface of the  
34 PAA hydrogel film grafted on the underlying electrode was achieved  
35 through two stages EDC/NHS chemistry. Figure 8 shows the ATR-FTIR  
36 spectra at different stages of the sensor construction: the PAA  
37 hydrogel, the activated PAA hydrogel and the grafting of the  
38 aptamer. The spectrum of the activated hydrogel shows three  
39 characteristic bands at 1815, 1790, and 1750  $\text{cm}^{-1}$  which are  
40 representative of the carbonyl stretch modes in the COO-NHS ester  
41 moiety. After aptamer immobilization, the anhydride and NHS ester  
42 peaks disappear and new amide bands appear around 1580  $\text{cm}^{-1}$  and  
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1660  $\text{cm}^{-1}$  (characteristics of amide I and amide II), thus confirming the grafting.

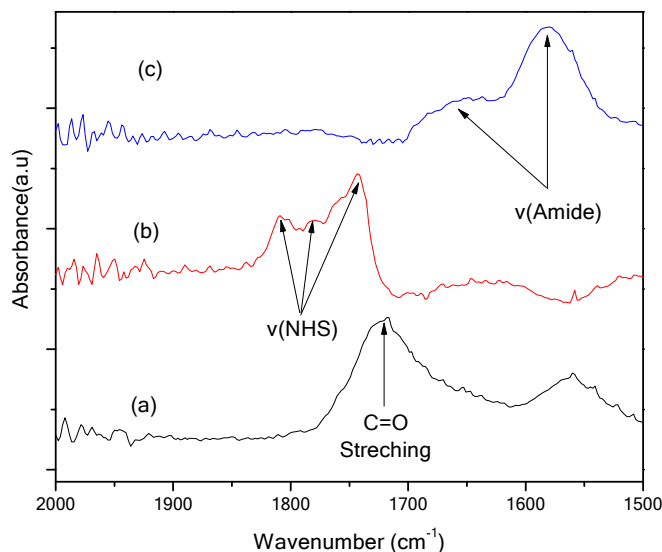


Figure 8. ATR-FTIR spectra of Au/PAA hydrogel (a), Au/activated PAA hydrogel (b) and Au/PAA hydrogel/grafted DCL aptamer (c).

Figure 9 shows the Nyquist diagrams obtained after each construction steps. The Nyquist plots of the electrodes include a semi-circle at the highest frequency range explored, and linear portion at the lowest frequencies. The semicircle is related to charge transfer resistance ( $R_{ct}$ ). The linear part is not affected by the aptasensors construction and is ascribed to diffusion processes. The variation in the  $R_{ct}$  value is explained by the blocking effect of the step-by-step modification procedure of the electrode. The double-layer capacitance ( $C_{dl}$ ) and  $R_{ct}$  are

parameters relating to the dielectric and insulating properties at the electrode/electrolyte interface. These parameters are affected by variations occurring on the electrode surfaces <sup>66</sup>.

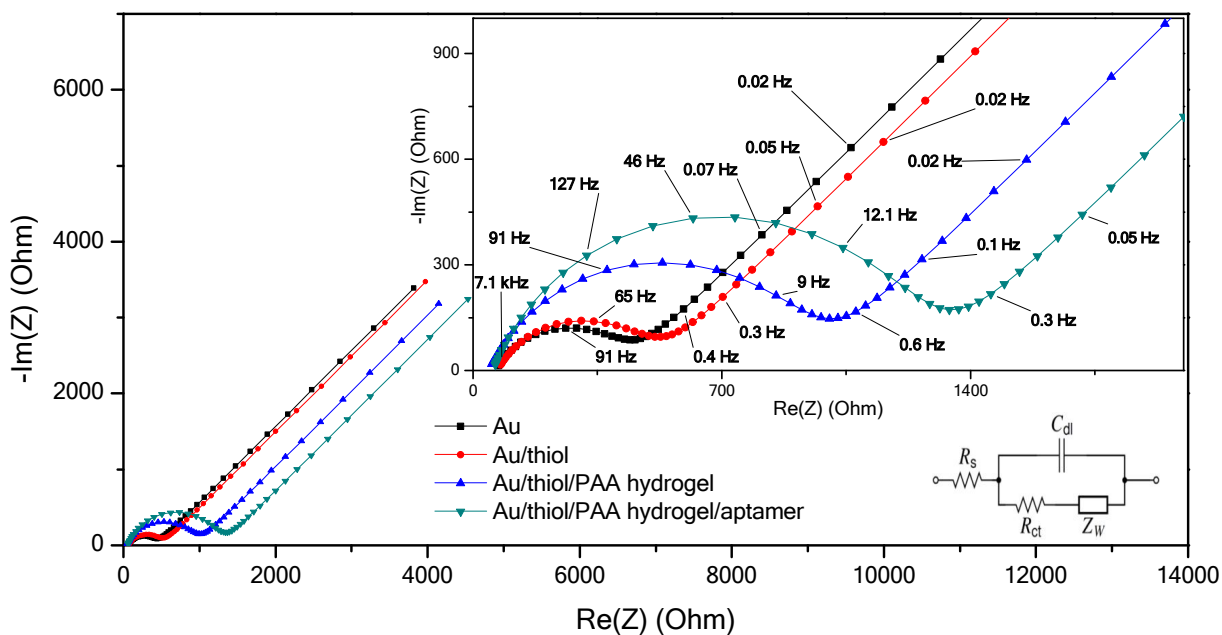


Figure 9. Nyquist plots of Au (■), Au/thiol (●), Au/thiol/PAA hydrogel (▲), Au/thiol/PAA hydrogel/aptamer (▼) electrodes in 0.1 M PBS containing 5 mM  $K_4[Fe(CN)_6]$  and  $K_3[Fe(CN)_6]$  in the frequency range of 10 kHz to 0.1 Hz.

When the gold electrode surface is modified using DTE layer, the  $R_{ct}$  value slightly increased (451 ohm). There is no very significant difference, in contrary to the insulator property of thiol self-assembled monolayer. This is due to the dithiol short chain used in this study as it has been discussed during cyclic voltammetry study. In contrast, when the electrode is grafted by PAA hydrogel film, because of repulsive interaction occurring

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3 between the negatively charged PAA hydrogel and anionic redox  
4 probe, the Rct value increased reaching 932 ohm. After  
5 immobilization of the aptamer on the electrode, a semi-circle with  
6 fairly large diameter was monitored. The observed increase in the  
7 value of Rct was attributed to the electron transfer hindrance by  
8 the negatively charged aptamer.  
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### 21 **Target Detection and Calibration modeling for DCL**

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23 The detection of DCL was carried out by incubating the aptamer-  
24 modified electrode with DCL solutions at different concentrations.  
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26 The performances of the aptasensor was investigated by recording  
27 its EIS responses in 0.1 M PBS containing 5 mM  $[\text{Fe}(\text{CN})_6]^{3-/4-}$   
28 solution.  
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35 As illustrated in Figure 10, when the DCL concentration increases  
36 Rct decreases. This can be attributed to the change in the spatial  
37 conformation of the aptamer in contact with higher concentrations  
38 of DCL. The results show that the negative charge density on the  
39 surface is lowered due to such conformational changes. Therefore  
40 there is a decrease in the interfacial charge transfer resistance  
41 to the negatively charged redox probe <sup>29</sup>.  
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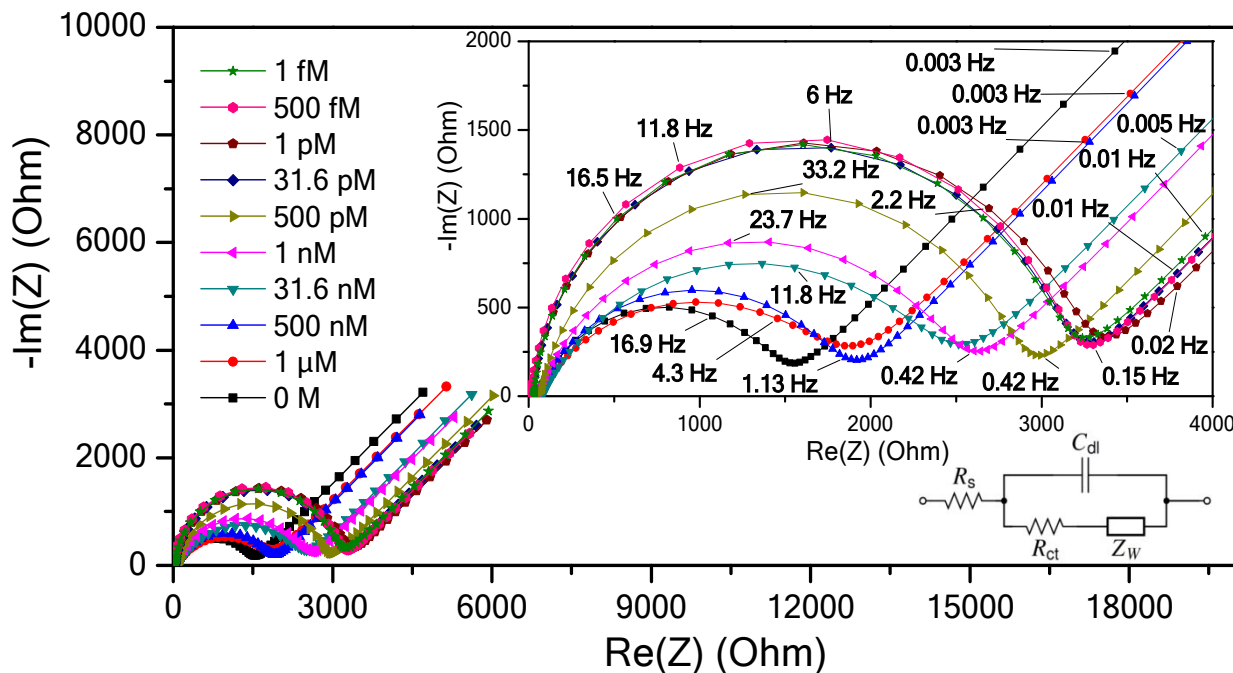


Figure 10. Nyquist plot obtained in 0.1 M PBS containing 5 mM  $\text{K}_4[\text{Fe}(\text{CN})_6]$  and  $\text{K}_3[\text{Fe}(\text{CN})_6]$  after treatment of the aptasensor with different concentrations of DCL in the frequency range of 100 kHz to 1 mHz.

The change in charge-transfer resistance (Fig. 10) was found to be linear with target concentration in the range of 30 pM to 1  $\mu\text{M}$ . A good linear relationship between  $R_{ct}$  and DCL concentration was obtained ( $R^2 = 0.9787$ ), according to the following equation:

$R_{ct} = -310.23 [\text{DCL}] - 99.637$ . The detection limit was calculated to be 0.02 nM from the intersection between the linear part and the non-sensitive linear region (Fig. 11).



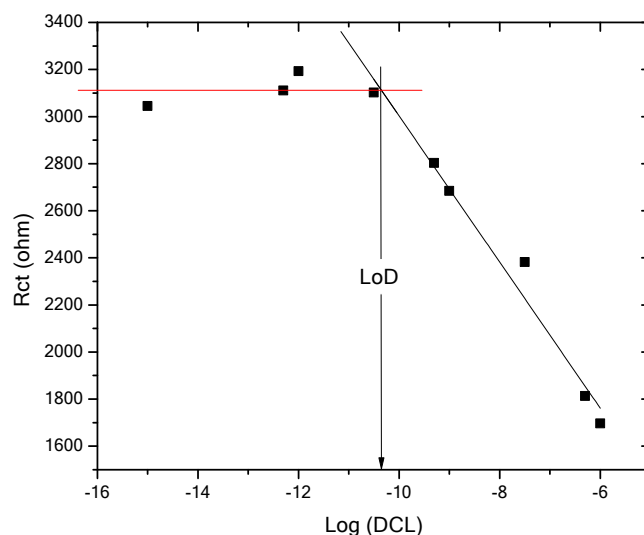


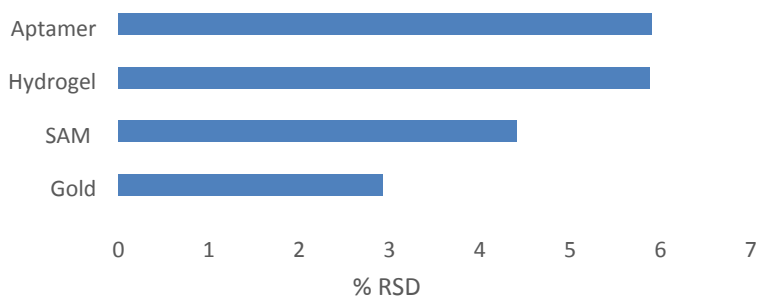
Figure 11. Calibration curve using Rct vs. Log DCL concentration

### Stability and Reproducibility Study

Once the response of the sensor to DCL demonstrated, the stability and reproducibility merits of the protocol developed were evaluated. The operational stability and performance of the developed aptasensor was assessed for a long-term storage at 4°C using five different electrodes modified with aptamers. The impedance response of the aptasensor was evaluated and it was observed that the aptasensor retained 95.6 % of its initial response after 7 days storage and 92.3 % after 14 days storage (detection of 500 pM of DCL), indicating a good stability.

Furthermore, in order to evaluate the reproducibility of the aptasensor, five aptasensors were treated with 500 pM DCL, and Rct was recorded in 0.1M PBS containing 5mM  $[\text{Fe}(\text{CN})_6]^{3-/4-}$ . The relative

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3 standard deviation (RSD) of their response was found to be 4.8 %.  
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5 This shows that the reproducibility of the aptasensor is quite  
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7 reliable. The reproducibility of the different stages of electrode  
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9 construction was also evaluated (Figure 12). The observed  
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11 variations can be explained by the several uncontrolled factors  
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13 that can affect the impedimetric results as the electrodes are  
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15 handmade.  
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30 Figure 12. Relative standard deviation of the recorded electron  
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32 transfer resistance ( $R_{ct}$ ) of different stages of electrode  
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34 construction in 0.1M PBS containing 5mM  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  for five  
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36 different aptasensors.  
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#### 41 **Comparative Data**

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44 As mentioned above, several electroanalytical methods have been  
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46 reported for the determination of DCL based on potentiometry,  
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48 differential pulse voltammetry, square wave voltammetry, linear  
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50 sweep voltammetry, photo electrochemical and electrochemical  
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52 impedance spectroscopy. The LOD reached with most of these  
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54 techniques are not lower than a few tens of nanomole  $\text{L}^{-1}$ .  
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3 Improvement of target detection at the transducing surface using  
4 biomolecules specific for DCL have been tried before and published  
5 using nucleic acid aptamers. In this regard, the latest improved  
6 LOD (in the nM range) achieved for detection of DCL using aptamers  
7 is the one demonstrated by Derikvand *et al.*<sup>54</sup>, using nanostructured  
8 electrodes. To the best of our knowledge, there are no reports on  
9 utilization of surface-attached hydrogel films for improved  
10 immobilization of aptamer and hence an increased detection of DCL  
11 using electrochemical methods, which is the focus of the present  
12 study. The figure of merits of different electrochemical methods  
13 for detection of DCL are summarized in Table 1, and compared with  
14 the previous reports for the determination of DCL. Our proposed  
15 method has obviously the best limit of detection and sensitivity  
16 for DCL analysis.  
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Detection method	Modifier	Linear range	LOD	Refs
Potentiometry	Membrane containing $\beta$ - cyclodextrin coupled with magnetite ferric oxide (CV-Fe(beta-CD))	10 $\mu$ M to 10 mM	11x10 <sup>3</sup> nM	36
Photoelectrochemical (PEC)	DCL Aptamer/Gold nanoparticles (Au NPs) and graphene-doped CdS (GR-CdS) (Au/GR-CdS/Aptamer)	1 to 150 nM	0.78 nM	67
Differential Pulse Voltammetry (DPV)	Functionalized multi-walled carbon nanotubes (f-MWCNTs) and gold-platinum bimetallic nanoparticles (Au-PtNPs) (Au-PtNPs/fMWCNTs/Au)	0.5 to 1000 $\mu$ M	300 nM	68
Electrochemical Impedance Spectroscopy (EIS)	DCL Aptamer immobilized on the surface of the glassy carbon electrode (GCE) (GCE/AHA/Aptamer)	0 - 5 $\mu$ M ; 10 $\mu$ M to 1 mM	270 nM	29
Differential Pulse Voltammetry (DPV)	A diclofenac (DCL) imprinted polymer	16.8 to 270 $\mu$ M	3.72x10 <sup>3</sup> nM	23
Electrochemical Impedance Spectroscopy (EIS)	Platinum nanoparticles (PtNPs) on carbon nanotubes (CNTs) functionalized with polyethyleneimine (PEI) (PtNPs/PEI/CNTs/Aptamer)	10 to 200 nM	2.7 nM	54

Linear Sweep Voltammetry (LSV)	A multi-walled carbon nanotubes (MWNTs)- dihexadecyl hydrogen phosphate (DHP) film-coated glassy carbon electrode (MWNTs-DHP/GCE )	0.17 to 2.5 $\mu$ M	80 nM	31
Potentiometry	Modified graphite pencil electrode (MGPE) with doped polypyrrole films (MGPE/PPy-DCL)	310 $\mu$ M to 11 mM	190 $\times$ 10 <sup>3</sup> nM	34
Differential Pulse Voltammetry (DPV)	Multiwalled carbon nanotube and ionic liquid modified carbon ceramic electrode (MWCNT-IL/CCE)	0.05 to 50 $\mu$ M	18 nM	38
Electrochemical Impedance Spectroscopy (EIS)	Aptamer immobilized on the Surface of hydrogel matrix modified gold electrode (Au/SAM/hydrogel/Aptamer)	30 pM to 1 $\mu$ M	0.02 nM	This work

Table1. Comparison between analytical performances of reported techniques for DCL detection.

## CONCLUSION

This study shows an electrochemical impedance spectroscopy biosensor for DCL detection with high sensitivity. Surface-attached hydrogel film with controlled architecture was fabricated as immobilization matrix and was finely characterized. The electrochemical properties of the biosensor in different media and its stability were then studied. The recognition of DCL by the aptamer-based electrode results in an electron transfer resistance change for the  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  as probed by the EIS technique. The addition of DCL shows a linear change in charge-transfer resistance with the target concentration. EIS aptasensor for DCL with nanomolar detection limit was evidenced. The stability and reproducibility of the biosensor were also proved. The aptasensor designed provides a platform for simple, selective and more importantly rapid detection of DCL. The sensing platforms can serve molecular recognition of different targets in the field of health, or in the field of environment. Furthermore, increased sensitivity and specificity can be achieved by incorporating nanostructured metallic materials like gold nanoparticles inside the hydrogel network.

## Corresponding Authors

\*Dr. Cyrine Slim (cyrine.slim@chimieparistech.psl.eu)

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3 \*Dr. Yvette Tran (yvette.tran@espci.fr)  
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### 6 **Author Contributions**

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8 The manuscript was written through contributions of all authors.  
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10 All authors have given approval to the final version of the  
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12 manuscript.  
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32 FTIR measurements.  
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39 Gennes (équipement d'excellence, "Investissements d'avenir",  
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41 program ANR-10-EQPX-0034).  
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### 44 ABBREVIATIONS

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46  
47 AFM, Atomic Force Microscopy; ATR-FTIR, Attenuated total reflectance-Fourier transform  
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49 infrared spectroscopy; BWB, Binding Washing Buffer; Cdl, double-layer capacitance; CLAG,  
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51 Cross-Linking And Grafting; CV, Cyclic Voltammetry; DCL, Diclofenac; DTE, 1,4-  
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53 dithioerythritol; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EIS, Electrochemical  
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55 Impedance Spectroscopy; GC, Gas chromatography; HPLC, High-performance liquid  
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3 chromatography; LOD, limit of detection; MS, Mass spectroscopy; NHS, N-hydroxysuccinimide;  
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5 PAA, Poly (acrylic acid); Rct, electron transfer resistance; SCE, Saturated Calomel Electrode;  
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8 SEM, Scanning Electron Microscopy; WCA, Water Contact Angle.  
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### 11 12 **Supporting Information**

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14 Fabrication steps of the Electrode using photolithography process,  
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16 cleaning procedure and surface characterization.  
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