Chapter 5

A Novel Three-Dimensional Biosensor Based on Aluminum Oxide: Application for Early-Stage Detection of Human Interleukin-10

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Abstract

Immunosensors based on electrolyte-oxide-semiconductors (EOS) have been extensively researched over the last few decades. By electrochemical impedance spectroscopy (EIS) the specific molecular biorecognition of the antibody–antigen (Ab–Ag) can be detected providing an alternative quantitative system to immunoassay techniques. The electrochemical variations from a fabricated immunosensor can provide quantitative values for the analyte of interest at reduced costs and analysis time. In this context, a novel EOS substrate based on aluminum oxide (Al_2O_3) grown by atomic layer deposition on silicon was applied. The interaction between recombinant human (rh) interleukin-10 (IL-10) with the corresponding monoclonal antibody (mAb) for early cytokine detection of an anti-inflammatory response due to left ventricular assisted device implantation was studied. For this purpose, a 3D biosensor was composed of multi-walled carbon nanotubes with carboxylic acid functionalities (multi-walled carbon nanotubes–COOH) to increase the surface area for the range of human IL-10 detection. These were activated with *N*-hydroxysuccinimide and *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride for the immobilization of the anti-human IL-10 mAb. First, the interaction between the Ab and Ag was observed by fluorescence patterning to ensure that the biorecognition event was achievable. Then, EIS is explained for the quantification of commercial human IL-10 on this capacitance-based EOS macroimmuno-FET sensor.

Key words Immunosensor, Aluminum oxide, Interleukin-10, Silane, Carbon nanotubes, Fluorescence, Electrochemical impedance spectroscopy

1 Introduction

Biosensors are widely regarded with interest due to the potential that they can be applied as an alternative to immunoassay techniques (e.g., enzyme-linked immunosorbent assay (ELISA)) when utilized within clinical diagnostics. Biosensors based on electrical measurement are devices that employ biochemical molecular recognition for desired selectivity with a specific biomarker of interest,

Ivana Vancurova (ed.), *Cytokine Bioassays: Methods and Protocols*, Methods in Molecular Biology, vol. 1172,
DOI 10.1007/978-1-4939-0928-5_5,

e.g., the antibody–antigen (Ab–Ag) complex. The bioreceptor (Ab) is incorporated onto the transducer, and the biosensitive material is converted into a quantitative signal that can be measured in the form of an electrochemical response. One of these techniques is known as electrochemical impedance spectroscopy (EIS) where upon biorecognition a change can be observed in the interfacial charge, resistance, capacitance, mass, and thickness at the electrode surface. These biosensors require no or little sample preparation [1–5], though nonspecific binding (NSB) is an issue. There are no discriminatory processes that are capable of differentiating between the measured signal from specific and nonspecific interactions [6]. Therefore, the surface of the biotransducer has to be developed for sensitivity towards only the binding of the desired interaction whilst retaining its high specificity against NSB.

Over the past few decades, biosensor fabrication has been based upon the semiconducting properties of silicon with thermally grown silicon dioxide (SiO_2) and silicon nitride (Si_3N_4) being most favorable, when used as transistor gates within field effect transistors (FETs) [7–11]. In today's climate, thickness reduction of SiO_2 complementary metal oxide semiconductor (CMOS) devices has introduced a fundamental challenge to downscaling as high gate oxide leakage becomes apparent when the quality of the SiO_2 layer is jeopardized. Capacitance can be enhanced by increasing the dielectric constant (k), and, therefore, many materials have been considered as potential alternatives for gate-k materials instead of SiO_2. These metal oxides can present the necessary capacitance due to their physical thickness and, thus, a reduction of the gate leakage current [12]. Previously, we have demonstrated the application of a novel immunosensor based on hafnium oxide (HfO_2) that was functionalized with an aldehyde-silane ((11-(triethoxysilyl) undecanal (TESUD)) for the direct immobilization of the anti-human interleukin-10 (IL-10) Ab. This was followed by the EIS detection of human IL-10. This biosensor was sensitive between 0.1 pg/mL and 20 pg/mL, respectively [13]. Therefore, to improve the sensitivity of a biosensor, carbon nanotubes (CNTs) have been intensively researched due to their unique properties and promising applications in nanotechnology. This interest reflects upon their extraordinary chemical, electrical, mechanical, and structural properties [14, 15]. For electrochemical sensors, CNTs are advantageous due to their large surface area that can produce high sensitivity, improved chemical stability, and good biocompatibility for electrochemical biosensors that require electron transfer of redox proteins and enzymes [16].

To ensure the functionality of a biosensor, fluorescent imaging is a rapid tool for analyzing biolayers, which ensures that detection can be made by formulating biorecognition processes due to the high affinity an Ab has for its corresponding Ag. The soft-lithographical technique, micro-contact printing (μCP), facilitates

the printing of the required pattern by applying a structured poly(dimethylsiloxane) (PDMS) stamp [17]. The functionality of the immobilized receptor by the fluorescent pattern is, therefore, firstly required before EIS measurements can be undertaken.

Here, we present a novel substrate of Al_2O_3 where the biorecognition process of human IL-10 on the immobilized multiwalled carbon nanotubes (MWCNTs) was first of all characterized by fluorescence patterning using μCP. After ensuring that the biorecognition process worked, an immuno-FET was fabricated and analyzed using EIS to evaluate this novel biosensor for early-stage detection of human IL-10 inflammation for left ventricular assisted device (LVAD) recipients.

2 Materials

1. Anti-human IL-10 monoclonal antibody (R&D Systems): Dilute the antibody to 10 μg/mL in PBS.
2. Recombinant human IL-10 protein (R&D Systems): Dilute to 25 μg/mL in PBS.
3. Carboxyfluorescein-conjugated anti-human IL-10 monoclonal antibody (R&D Systems): Dilute to 2.5 μg/mL in PBS.
4. Carboxylic acid-functionalized MWCNT solution (DROPSENS).
5. PDMS (Sylgard 184).
6. Piranha solution: Mix three volumes of H_2SO_4 and one volume of H_2O_2.
7. Base piranha solution: Mix three volumes of NH_4OH and one volume of H_2O_2.
8. Octadecyltrichlorosilane (OTS) solution: 160 μL of OTS and 10 mL of CCl_4 in 40 mL of heptane.
9. 3-(Aminopropyl)triethoxysilane (APTES) 1 % solution: Mix 50 μL of APTES and 4,950 μL of DI water.
10. MWCNT–COOH mixture: Disperse 0.17 μL from 0.25 mg/mL MWCNT in 183 μL of DI H_2O containing sodium 5.77 mg (0.1 M) dodecyl sulphate (SDS). This produces a final volume of 200 μL.
11. 0.8 M Solution of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC).
12. *N*-hydroxysuccinimide (NHS) solution: Prepare 0.2 M solution of NHS (2.30 mg) and 0.8 M solution of EDC (12.42 mg) in PBS, and then add together to produce a final volume of 200 μL.
13. 0.1 % Ethanolamine (ETA): Mix 1 μL of ETA in 999 μL of PBS in Eppendorf tube; centrifuge.

14. Sulphuric acid (H_2SO_4).
15. Ammonium hydroxide (NH_4OH).
16. Hydrogen peroxide (H_2O_2).
17. Carbon tetrachloride (CCl_4).
18. Heptane.
19. Ethanol.
20. Acetone.
21. UV/Ozone cleaner (BioForce).

3 Methods

3.1 Preparation of Al_2O_3

The macro-FET structures were developed at the Centro Nacional de Microelectrónica (CNM) in Barcelona using 100 mm diameter p-type silicon wafers (<100>), oriented and with a resistivity 4–40 Ω cm. After standard cleaning, the high-*k* dielectric is deposited by atomic layer deposition (ALD) (Cambridge NanoTech Savannah 200 ALD system). The system uses deionized water (DI H_2O) as the oxygen precursor, together with trimethylaluminum (TMA) for Al_2O_3 deposition and N_2 as the carrier/purging gas. Deposition of the Al_2O_3 layer is carried out at a temperature of 200 °C and at a base pressure of 300 mTorr using 90 ALD cycles. A first estimation of the deposited Al_2O_3 layer thickness is carried out by means of ellipsometry, obtaining a thickness of 10.8 nm having fixed the refractive index to 1.64.

Finally, a 500 nm thick aluminum (Al) layer is deposited on the back of the wafers for electrically contacting the silicon substrate (Fig. 1).

3.2 Biorecognition by Micro-contact Printing

3.2.1 Preparation of Micro-contact Printing Tools

1. Fabricate a positive 10 μm² square patterned silicon mold. Define microstructures into the surface of the silicon to produce a master with positive superficial structures. Here, a silicon wafer (<100> orientation) with a photoresist is patterned using a mask containing the required squared structures by established photolithography techniques. Etch the surface of the photoresist using deep reactive ion etching (DRIE) (601 DRIE, Alcatel) to the required depth. The process for the etching conditions is optimized to machine whole-silicon wafers [18].

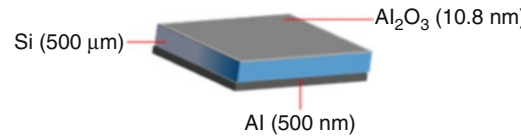

Fig. 1 Schematic illustrations of the macro-FET fabricated at the CNM, Barcelona

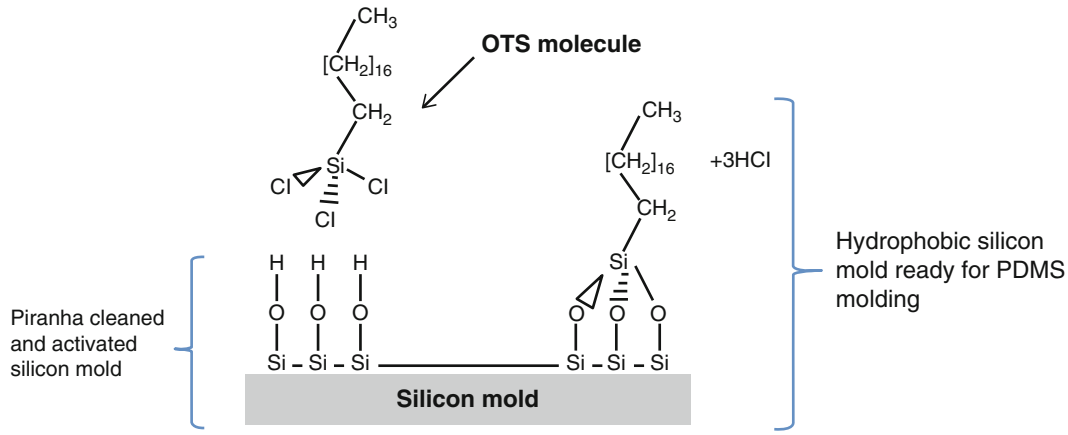

Fig. 2 Hydrophobic monolayer to enable PDMS peeling from the silicon master mold

2. Silanize the mold to ensure that PDMS replicas can be easily demolded by creating a hydrophobic monolayer onto the silicon surface (Fig. 2). To do this, clean and activate the silicon mold in freshly prepared piranha solution. Rinse the mold comprehensively with DI H_2O and dried with nitrogen. (Attention: Piranha solution is highly corrosive and a powerful oxidizer. Wear suitable laboratory clothing and activate within a fume cupboard.)
3. Incubate the silicon mold in OTS solution for 20 min at room temperature (RT) [19]. Here, the OTS molecules are chemically adsorbed onto the silicon surface.
4. Rinse the mold in heptane, and place it in the oven at 100 °C for 1 h. This will silanize the OTS molecules to the silicon substrate.
5. Rinse the mold again in heptane to ensure that all non-bound OTS is removed from the surface.
6. The silicon mold is now hydrophobic (*see* **Note 1**) and can be applied to produce a polymeric replica.

3.2.2 Soft Lithography and Replica Molding

1. Prepare PDMS by weighing a 10:1 (w/w) mix of the elastomer:cross-linking agent within a plastic and disposable container.
2. Mix the polymer thoroughly with a plastic stirrer, and ensure that the two mixtures have comprehensively mixed.
3. Degas the PDMS by desiccation to remove all air bubbles that are generated by mixing.
4. Place the silanized silicon mold with its structures top side up within a small petri dish (60 × 15 mm).
5. Pour the PDMS mixture on top of the silicon mold at a depth of 3–5 mm, and degas once again until all air bubbles have been removed.

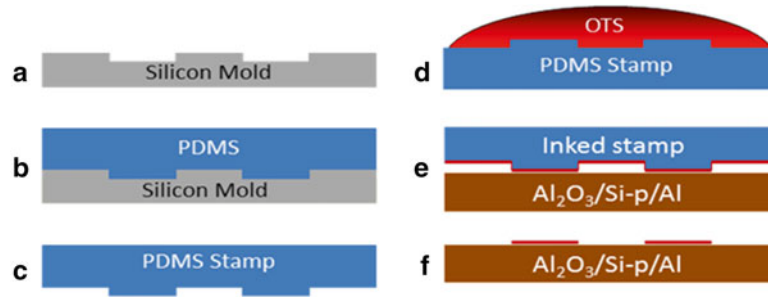

Fig. 3 Scheme for the fabrication of a structured PDMS stamp by soft lithography: (**a**) Silanized silicon master mold, (**b**) PDMS (10:1 w/w) poured onto the silicon mold, (**c**) cured PDMS stamp, (**d**) inking of the stamp with physically adsorbed OTS molecules, (**e**) μCP of the OTS from the structured PDMS stamp onto the activated Al_2O_3 substrate, and (**f**) transferred OTS pattern onto the Al_2O_3 substrate

6. Place the petri dish containing the PDMS and silicon mold into a preheated oven at 90 °C for 1 h. This enables the PDMS to polymerize and cure.
7. After hardening, remove the PDMS from the petri dish using a scalpel and separate the structured PDMS from the silicon mold using a scalpel and tweezers (*see* **Notes 2** and **3**).
8. This produces a PDMS stamp that now obtains negative features (*see* **Note 4**).

3.2.3 Preparation of Al_2O_3

1. Clean the bare Al_2O_3 substrates by sonication in ethanol for 10 min, followed by thorough rinsing in DI H_2O. Dry the substrate with nitrogen.
2. Activate the surface of the Al_2O_3 substrate using a UV/Ozone cleaner for 20 min, followed by 15 min in base piranha solution at RT (*see* **Note 5**).
3. Rinse the substrate thoroughly in DI H_2O and dry with nitrogen. (Attention: Base piranha is highly corrosive and a powerful oxidizer. Wear suitable laboratory clothing, and activate within a fume cupboard.)

3.2.4 Micro-contact Printing

1. Clean the negative 10 μm square patterned PDMS stamp by sonication in heptane for 1 min (*see* **Note 6**). Afterwards, dry the PDMS stamp with nitrogen.
2. Immerse the PDMS stamp in 5 mM OTS solution made up in heptane for 1 min (*see* **Note 7**). Here, the stamp adsorbs the OTS molecules.
3. Dry the PDMS stamp with nitrogen, and micro-contact print the OTS onto the hydroxyl activated Al_2O_3 substrate. Maintain printing for 10 s. This will produce a negative pattern of OTS on the Al_2O_3 surface (Fig. 3) (*see* **Note 8**).

3.2.5 Amino Functionalities and Multi-walled Carbon Nanotubes Immobilization

1. After μCP, immerse the Al_2O_3 substrate in APTES solution for 45 min (*see* **Note 7**).
2. Afterwards, gently rinse the Al_2O_3 substrate in DI H_2O and dry by nitrogen.
3. Place the Al_2O_3 substrate into a preheated oven at 100 °C for 1 h and the monolayer will silanize by the double-silanization process.
4. Prepare MWCNT and NHS/EDS solutions.
5. Mix 200 μL of MWCNT solution and 200 μL of NHS/EDC solution to formulate the active EDC/NHS ester groups on the MWCNTs. This produces a final volume of 400 μL of MWCNT/NHS/EDS solution.
6. Immerse Al_2O_3 in MWCNT/NHS/EDC solution for 2 h at RT, and follow this by rinsing in PBS to remove all non-bound MWCNTs (*see* **Note 9**).
7. This produces a 0.11 μg/mL concentration of functionalized MWCNTs.

3.2.6 Biorecognition of Human IL-10

1. Micropipette diluted anti-human IL-10 antibody onto the surface of the Al_2O_3-MWCNTs and incubate for 1 h at 4 °C. Afterwards, rinse the substrate with PBS (*see* **Note 10**).
2. Micropipette the diluted human IL-10 protein onto the surface of the Al_2O_3-MWCNTs-anti-IL-10 and incubate for 1 h at 4 °C. Then, rinse the substrate with PBS (*see* **Note 10**).
3. Micropipette the diluted carboxyfluorescein-conjugated anti-human IL-10 antibody onto the surface and incubate for 1 h at 4 °C. Finally, rinse the substrate with PBS and place in a darkened container.
4. Analyze the substrate for fluorescence using a fluorescence microscopy. For fluorescein, the excitation is made with a 470 (±40) nm band-pass filter and the fluorescence is observed with a 525 (±50) nm band-pass filter [20]. The schematic for the complete blocking, functionalization, and biodetection steps for human IL-10 can be observed in Fig. 4.
5. In Fig. 5, the fluorescence image of the biodetection for human IL-10 is shown with the positive squared regions fluorescing due to the biorecognition of human IL-10. The darkened negative regions are consistent to the blocking of the surface with hydrophobic OTS molecules.
6. After ensuring the functionality of the biorecognition event with human IL-10, the Al_2O_3 substrate can now be prepared to formulate a macro-immunoFET for the detection of human IL-10 by EIS measurements.

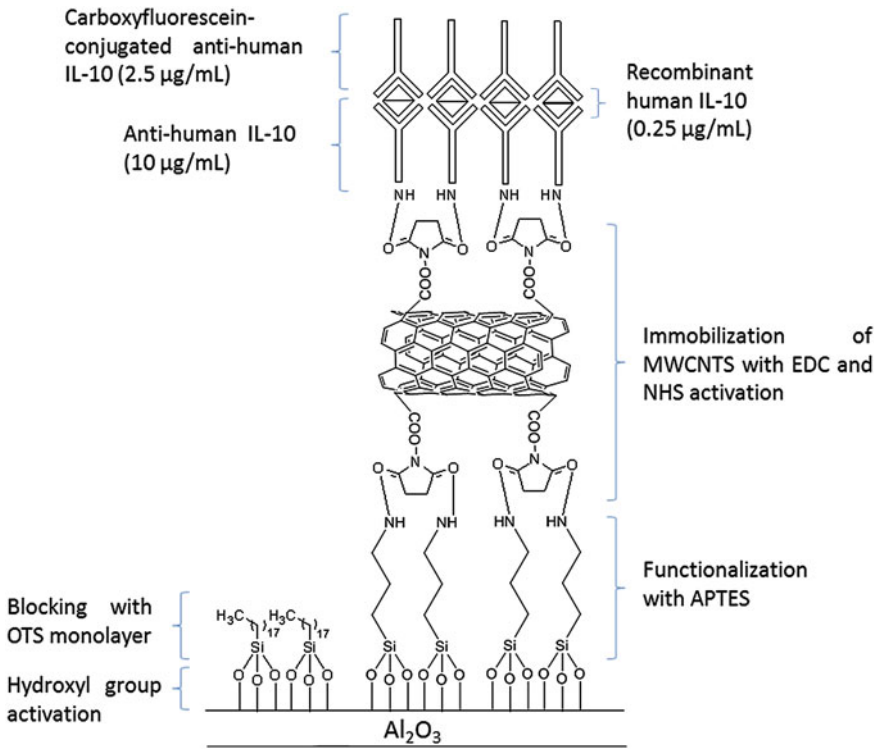

Fig. 4 Al_2O_3 oxidation followed by μCP of OTS to block the surface using a positive PDMS (10 μm) stamp. The positive regions were functionalized with APTES for the immobilization of MWCNTs with NHS and EDC activation. Then, the anti-human IL-10 was immobilized onto the MWCNT followed by the biorecognition with the recombinant human IL-10 and the carboxyfluorescein-conjugated anti-human IL-10 antibody

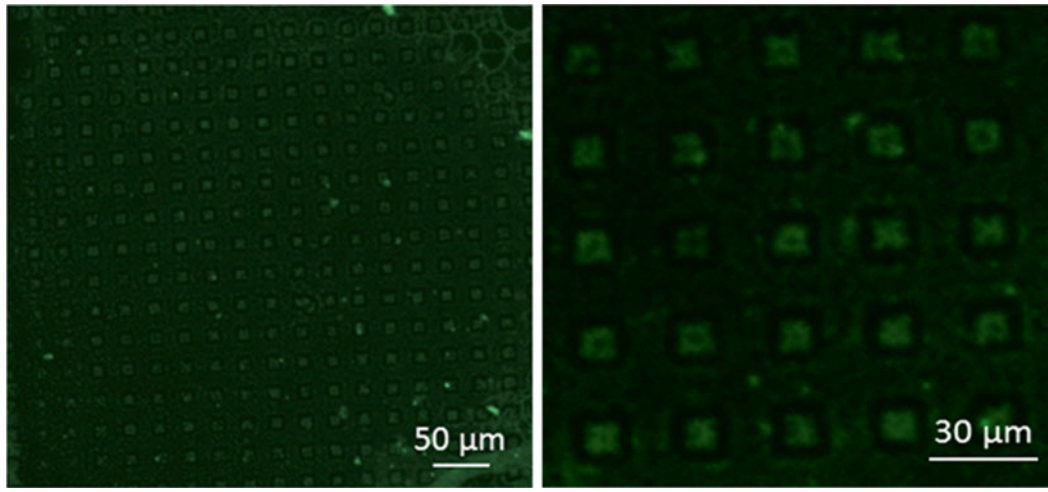

Fig. 5 Fluorescence microscope image of the Ab–Ag–Ab biorecognition for the detection of human IL-10 with positive square patterns demonstrating the detection of human IL-10 using a fluorescent microscope (Zeiss Axio Scope.A1, France)

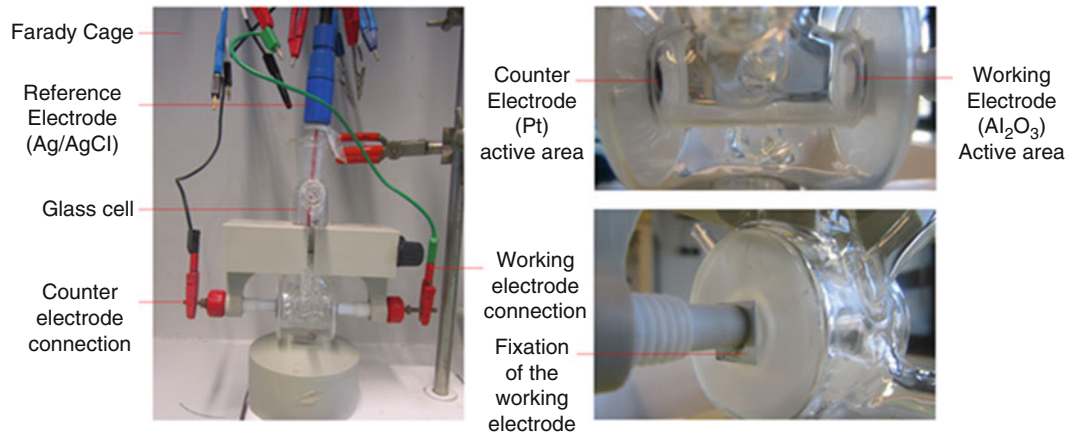

Fig. 6 Impedance setup for the detection of human IL-10. All measurements were made inside a Faraday cage using a specialized glass cell with the working electrode (Al_2O_3), counter electrode (Pt), and reference electrode (Ag/AgCl)

3.3 Electrochemical Impedance Spectroscopy

1. The setup for EIS measurements can be observed in Fig. 6. Here, the Al_2O_3 substrate is connected (after all immobilization steps) to the active area for the working electrode (WE).
2. A platinum (Pt) plate electrode is used as the counter electrode (CE). Clean the CE by sonication in acetone for 10 min, rinse thoroughly with DI H_2O, and dry with nitrogen. Connect the Pt electrode to the perpendicular active area towards the WE.
3. A calomel reference electrode (RE) made from silver/silver chloride (Ag/AgCl) is then inserted through the top to complete the three-electrode system.
4. Here, the electrolyte solution is freshly prepared PBS at pH 7.4. Fill the glass cell with PBS to ~8 mL.
5. To reduce the signal:noise ratio, carry out all EIS measurements inside a Faraday cage.

3.3.1 Calculation of the Monolayer of APTES by Electrochemical Impedance Spectroscopy

1. The percentage coverage of the amino silane can be calculated by the estimation

$$\theta = 1 - \frac{R_{before\ silanization}}{R_{after\ silanization}}$$

where R is the calculated resistance value.

2. Therefore, clean a bare Al_2O_3 substrate by sonication in ethanol for 10 min, followed by thorough rinsing in DI H_2O.
3. An impedance measurement of the bare Al_2O_3 substrate is made by the connection shown in Fig. 6. Apply with the following conditions (potential, amplitude, and frequency at −2 V, 200 mV, and 200 kHz to 100 mHz, respectively (56 s/scan)) (*see* **Note 11**).

4. After the measurement, clean the same Al_2O_3 substrate in ethanol and activate using UV/Ozone cleaner for 20 min, followed by 15 min in base piranha at RT. Then, rinse the substrate thoroughly in DI H_2O and dry with nitrogen.
5. Directly afterwards, immerse the Al_2O_3 substrate in 1 % APTES solution for 45 min at RT.
6. Then, gently rinse the substrate with DI H_2O and place into a preheated oven at 100 °C for 1 h. This allows the monolayer to silanize onto the Al_2O_3 surface.
7. Connect the Al_2O_3-APTES substrate to the glass cell, and apply the same conditions as the bare Al_2O_3 substrate. This produces a Nyquist plot for the functionalized Al_2O_3 surface (frequency: 200 kHz to 10 mHz (5 min 2 s/scan)) (*see* **Note 12**).
8. After the formation of the two plots for the Al_2O_3 (bare) and Al_2O_3-APTES-functionalized surfaces, fit the curves to extract the component values using an equivalent circuit that was chosen on the interfaces seen in the Nyquist plot of the semicircles. Normalize the impedance data with the fitting software installed from the instrument provider. Here, we modeled with EC-Lab V10.18 modeling software (Bio-Logic Science Instrument, France).
9. Fit with the randomize + simplex method with randomization stopped on 10,000 iterations and the fit stopped on 5,000 iterations.
10. Due to the different observable interfaces, we applied two different equivalent circuits to normalize the data (Fig. 7, inset) (*see* **Note 13**). The polarization resistance value for bare Al_2O_3 was measured at 9,950 kΩ ($X^2 = 0.0436$), and the Al_2O_3-APTES functionalized was 27,380 kΩ ($X^2 = 0.0465$) (Fig. 7); therefore, the silane monolayer was equivalent to 64 %.

3.3.2 Biodetection by Electrochemical Impedance Spectroscopy

1. Activate the Al_2O_3 substrate as described in Subheading 3.2.3.
2. Afterwards, functionalize the macro-FET by all the steps described in Subheading 3.2.5 (note: no μCP is made here).
3. Connect the Al_2O_3-MWCNTs substrate (WE) and the Pt plate electrode (CE) to the glass cell as shown in Fig. 6.
4. Turn the glass cell onto its side (*see* **Note 14**), and micropipette the diluted anti-human IL-10 antibody onto the WE active area of the Al_2O_3-MWCNTs. Incubate for 2 h at 4 °C (*see* **Note 15**).
5. After this time, rinse the glass cell thoroughly with PBS to ensure that the excess anti-human IL-10 antibody has been removed (*see* **Note 16**).
6. Block the surface of the MWCNTs with the mAb in 0.1 % ETA solution for 20 min. This deactivates all binding sites and prevents NSB from occurring.

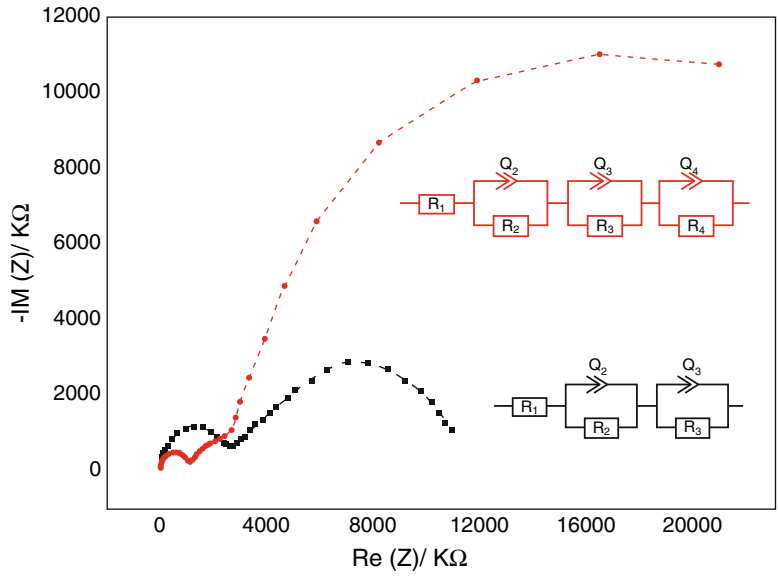

Fig. 7 Nyquist plot of the bare Al_2O_3 (*black square*) and the Al_2O_3-APTES (*red circle*) functionalized substrate with a 64 % of the silane monolayer on the Al_2O_3 surface. The inset shows the applied equivalent circuits to normalize the Nyquist plots

7. Fill the glass cell with PBS (~8 mL), and make all electrical connections (WE, RE, and CE) to the potentiostat. Keep all conditions (potential (−2 V), amplitude (−200 mV), and frequency (200 kHz to 600 mHz) (23 s/scan)) constant to those applied in Subheading 3.3 (*see* **Note 11**).
8. By impedance, firstly, measure the anti-human IL-10 mAb. (This is already immobilized on the Al_2O_3 substrate.)
9. After stabilization of the semicircle, stop the scan and disconnect all electrical connections.
10. Then, follow with the detection of the human IL-10. Starting with the weakest concentration, inject 50 μL of the human IL-10 directly on top of the Al_2O_3 active area. Incubate for 30 min at 4 °C (*see* **Notes 14–16**).
11. After the incubation period, rinse the glass cell with PBS and refill the cell with fresh PBS. Continue the impedance measurements until the stability of each human IL-10 concentration is observable.
12. Continue with the EIS measurements with increased concentrations of human IL-10 until the saturation of the immunosensor can be observed. This is achieved by superimposing the Nyquist plots together. The resulting Nyquist plot can be seen in Fig. 8.
13. After the formation of the Nyquist plots, normalize the impedance data with the software provided from the instrument provider. Here, we applied EC-Lab V10.18 modeling software.

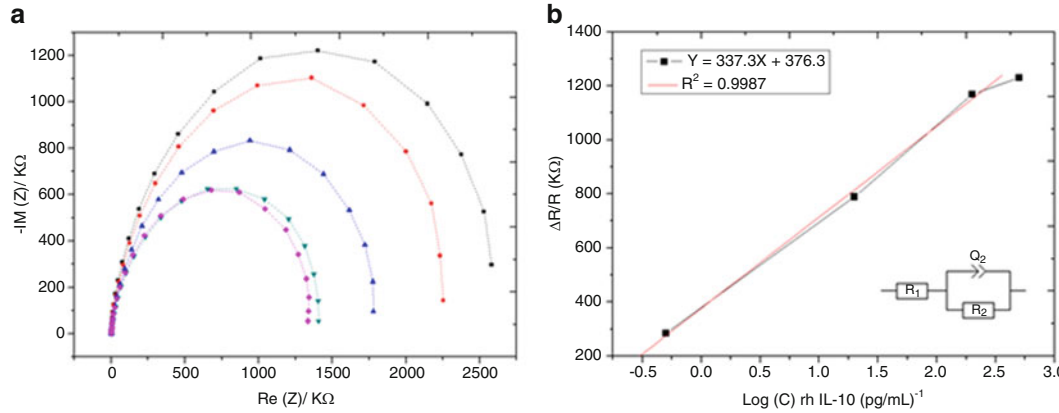

Fig. 8 (**a**) Nyquist impedance plot for the detection of human IL-10 using the following conditions: frequency between 200 kHz and 600 mHz, a sinus amplitude of 200 mV, and a polarization potential of −2 V. *Black square*, IL-10 mAb (10 μg/mL); *red circle*, rh IL-10 (0.5 pg/mL); *blue triangle*, rh IL-10 (20 pg/mL); *inverted green triangle*, rh IL-10 (200 pg/mL); and *pink diamond*, rh IL-10 (500 pg/mL). (**b**) Normalized curve by EIS detection of the logarithm of rh IL-10 with concentrations ranging from 0.5 to 500 pg/mL against the variation of the R_p calculated by $\Delta R/R$. Inset: Applied equivalent circuit for calculation of the fitting parameters

Table 1
Fitting parameters obtained from the equivalent circuit for the detection of human IL-10

Ag conc. (pg/mL)	R_1 (Ω)	Q_2 (nF·s$^{(a-1)}$)	R_2 (kΩ)	X^2
0	442.7 ± 0.2	9.9 ± 27.7 × 10^{-6}	2,797 ± 0.5 × 10^{-3}	0.0805
0.5	479.9 ± 0.2	10.5 ± 31.1 × 10^{-6}	2,312 ± 1.2 × 10^{-3}	0.0789
20	571.5 ± 0.2	11.6 ± 44.0 × 10^{-6}	1,808 ± 1.5 × 10^{-3}	0.0776
200	748.7 ± 0.2	13.2 ± 70.6 × 10^{-6}	1,429 ± 1.2 × 10^{-3}	0.1106
500	662.2 ± 0.2	12.4 ± 72.7 × 10^{-6}	1,367 ± 0.4 × 10^{-3}	0.0482

The R_2 values are the polarization resistance values used to normalize the data (*note*: 0 pg/mL is the mAb (10 μg/mL))

14. Apply an equivalent circuit to calculate the best fit of the data. The selection of the equivalent circuit is dependent upon the interfaces of the Nyquist plot to produce the smallest error (X^2) (*see* **Note 17**).
15. Here, we used $R_1 + Q_2/R_2$, where R_1 is the resistance of the electrolytic solution and Q_2 is the constant-phase element (CPE) and in parallel is R_2, which is the polarization resistance (R_p) and the value required to normalize the data (Table 1) (*see* **Note 18**).
16. For the extraction of the data, apply the equation $\Delta R/R$. The resistance for the real component decreases, and then Ab–Ag = resistance of the immunosensor. (The concentrations of human IL-10 are converted to logarithm values (Table 2).)

Table 2
Calculation of the logarithm values for human IL-10 concentrations and the plotted $\Delta R/R$ values shown in the normalized plot

Ag conc. (pg/mL)	Log conc. (pg/mL)	$\Delta R/R$ (kΩ)
0.5	−0.30	285
20	1.30	789
200	2.30	1,168
500	2.70	1,230

17. Plot these values onto a graph (Fig. 8b) to demonstrate the linearity for human IL-10 detection. Here, the sensitivity of the rh IL-10 detection was linear between 0.5 and 200 pg/mL at a linearity of $R^2 = 0.9987$. At 500 pg/mL the anti-human IL-10 antibodies on the immunosensor surface were saturated. This is observed by its nonlinearity towards the other human IL-10 concentrations that were measured.

4 Notes

1. To ensure that the silicon mold for μCP has silanized and formed correctly, a droplet of water will provide a very high hydrophobic surface on the OTS monolayer of silicon. (Silicon is a hydrophilic material.)
2. Upon removal of the PDMS replica stamp from the silicon master mold, remove all excess PDMS from the petri dish leaving an easier and smaller dimension of the PDMS for removal. Plastic tweezers and a scalpel are easier to apply when peeling the two apart; however, pay attention not to scrape the silicon mold from the backside as this will remove the OTS monolayer.
3. Also, ensure that excess PDMS that has covered the backside of the silicon mold has been removed as the silicon mold can break upon trying to remove it from the PDMS stamp.
4. As the Al_2O_3 substrate is first of all blocked with OTS, a negative PDMS stamp is preferential to leave the positive regions available for the observation of the biorecognition event. Here, we applied square patterns though other designed relief patterns that can demonstrate blocking and biorecognition are just as useful.
5. For the activation of the Al_2O_3 substrate, base piranha was preferred due to its less aggressive nature compared to piranha solution (H_2SO_4:H_2O_2—3:1 v/v). A piranha solution is extremely exothermic; however, it will remove the metal oxide

and aluminum layer. Base piranha will also act in a similar fashion if it is heated up to 60 °C, and, here, NH_4OH at 4 °C was applied. The Al_2O_3 substrate was placed top side down at the liquid interface with air to prevent the aluminum contact from activating. Activation by oxygen plasma or hydrogen peroxide can also be applied to oxidize the surface.

6. Heptane will swell PDMS and alter the PDMS stamp structures on the top surface; therefore, cleaning of the PDMS stamp is recommended 10–15 min before incubation with the OTS solution.
7. Prepare the silane solution just before the transfer of the monolayer. The silane reagent will silanize quickly, and this reaction can be sped up at higher temperatures.
8. To reduce issues with swelling and nonconformal μCP, the PDMS stamp can be supported on a homogeneous glass substrate. By oxygen plasma treatment, both surfaces can be activated to produce strong chemical bonds (Si–O–Si). Here, the backside of the PDMS stamp (20 s) and glass (2 min) can be oxygen activated at 90 W. This bonding will be irreversible though prolonged periods in heptane (~30–1 h) will disrupt this bonding. By bonding the PDMS to glass, this will also help with future printing as the structured PDMS surface will be known.
9. For the functionalization of the Al_2O_3 substrate with MWCNTs this was made with a total volume of 400 μL. This was achievable using a small glass beaker. However, depending upon your glassware these volumes may need to be increased. If these are increased pay attention to the required mass of EDC as the chemical is a fine powder and an increased mass will be required.
10. For the mAb and human IL-10 concentrations, the solutions were previously made up in Eppendorf tubes and stored at −20 °C. Prior to analysis, these were allowed to defrost for 20–30 min. After defrosting, the solutions were mixed by centrifugation.
11. For our applied substrates, setup, and potentiostat these were our optimized conditions. Other ALD–Al_2O_3 substrates and applied instruments may observe different required conditions and, therefore, require optimization by the user.
12. For EIS measurements, the percentage coverage of the amino silane from the bare Al_2O_3 to Al_2O_3 functionalized with APTES will require a lower frequency range, and, therefore, an increased scan time will be observed with an increased resistance value.
13. For the choice of the equivalent circuit, this is dependent upon how many interfaces are seen, e.g., one, two, or three semicircles within the Nyquist plot. Therefore, the equivalent circuit has to be adapted to measure all parts of the observed Nyquist plot.

14. For each incubation step of the mAb to the human IL-10 concentrations, the glass cells were turned sideways and balanced on a glass beaker when placed at 4 °C. Due to the inlets on the glass cells, these were closed during the incubation step using parafilm.
15. The incubation of the anti-human mAb and the increasing human IL-10 concentrations was made at a fixed volume of 50 μL. By micropipette, the volume can be observed as the droplet falls within the concave active area of the WE. This ensures that the Al_2O_3 was in direct contact with the solutions, that a specific volume for each incubation step was maintained, and that the analysis remained consistent.
16. After incubation at 4 °C the glass cell and the connections condense with water molecules. Therefore, the cell and connections were dried with a stream of nitrogen. Leaving the cell for 5–10 min will also ensure that the glass cell returns to ambient temperature.
17. For the normalization of the fitting data ensure that X^2 is always small. This ensures a better fitting.
18. For the fitting, depending upon the form of the semicircle pure capacitive components cannot be applied as the semicircles appear compressed or elongated. Therefore, the imperfect capacitors of CPE will function on improving the fitting values.

Acknowledgements

We acknowledge the funding through the Ministère de L'enseignement Supérieur et de la Recherche (MESR) and from the SensorART project funded by the European Communities Seventh Framework Programme (FP7/2007–2013) under the grant agreement No. 248763, the InFuLOC project under grant agreement No 230749, the SMARTCANCERSENS project (FP7-PEOPLE-2012-IRSES) under the grant agreement No. 318053, and NATO project, SPS (NUKP.SFPP984173).

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