Towards pancreatic cancer diagnosis by EIS biochips

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal cancer among Europe and United States. It has a very low 5 years-survival rate and its diagnosis is often late and imprecise due to the lack of specificity of currently used markers for PDAC. As previously demonstrated PDAC patients sera may contain autoantibodies towards phosphorilated α-enolase (ENOA), which in combination with other standard marker can increase specificity in diagnosis of PDAC. In this contest we realized a microfluidic platform with integrated EIS biosensors [1]. We achieved a specific antibodies detection by immobilizing onto electrodes peptides corresponding to a portion of ENOA. Phosphorylation of peptides was found to influence the recognition of antibodies in PDAC patients sera detected by the developed biochip thus validating EIS technique as a strong tool for quick, cost-saving and label-free analysis of serum samples.

Impedance biochips (Figure 1) were implemented by integrating two modules: (1) a PDMS (*Sylgard 184*) microfluidic module obtained by replica molding from a 100µm-high hard master made in SU-8 photoresist (Microchem) and (2) two sensing areas, both including an array of gold interdigitated microelectrodes with 10µm space and width, fabricated on glass substrates (2.5 cm x 2.5cm – Visionteck) by optical lithography, using a Karl Suss MJB3 mask aligner. Each area of detection is enclosed in a PDMS chamber with a total volume of 28 µL (7mm x 4 mm x 100 µm height) connected with its own inlet and outlet microchannels. The whole device was assembled by a step of oxygen plasma exposure of PDMS (20 minutes at 0.5 mbar) and a rapid cleaning of the glass with the electrodes in piranha solution. These kinds of microfluidic chips have been already demonstrated to be able to detect cellular behavior in response to a chemical stimulus [2, 3], cell migration [4] and bio-recognition events between immobilized antibodies and related antigens in aqueous solution [5].

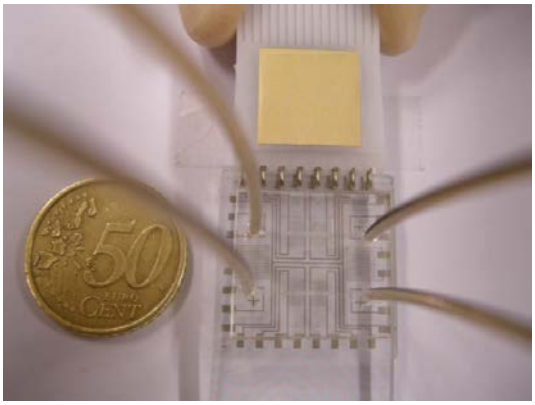

Figure 1. Biochip layout with interdigitated electrodes, temperature sensors and heaters, PDMS microfluidic components (chambers with microchannels), inlet and outlet tubes.

Here we developed a biochip in which two sensing areas were functionalized (i) the first one with a phosphorylated ENOA-derived peptide which can capture natural antibodies directed against Ser-419 phosphorylated residue; (ii) the second one with ENOA unphosphorylated peptide as a control (inset of Figure 2a). All steps of functionalization were realized on chip thanks to a microfluidic module made of PDMS, in which microchannels and chambers for reaction allow the complete handling of fluids and reagents (Figure 1). Bare electrodes exhibited very low impedance values with R_{et} around 500 Ω, while we observed an increase in the electron transfer resistance (Nyquist diagram diameter) as a consequence of the binding of molecules on chip after each functionalization step (and after the biorecognition of antibodies from serum samples) suggesting a proper immobilization of molecules. In particular, as

first step we incubated electrodes with a 3mM β-mercaptoethanol solution recording an increase in impedance values up to about 2 kΩ (black curve in Figure 2a) with respect to bare electrode measurements. Then, we immobilized the peptides (200nM) onto the thiol-functionalized electrodes: specifically in one chamber there was the unphosphorylated peptide (C-^{412}RIEEELGSKAKF^{423}), while in the second chamber electrodes were functionalized with the Ser-419 phosphopeptide (C-^{413}RIEEELGS$_p$KAKF^{423}) of ENOA. The two corresponding impedance curves were quite similar, both with $R_{ET} \approx$ 13 kΩ and only minor differences within standard deviation on similar devices (blue and red curve in Figure 2a). At this stage the biochips were ready for screening on serum samples. This study was carried out by incubating biochips with PDAC ENOA$^{1,2+}$ sera, PDAC ENOA$^{1,2-}$ and HS sera in both the analysis chamber and the control chamber containing respectively phosphorylated and unphosphorylated peptide-modified electrodes. As shown in Figure 2a (brown curve), the presence of antibodies anti-phosphoenolase in PDAC serum, can be easily detected since it leads to a strong increase in the R_{et} values (now around 70 kΩ) as compared to former values with only phosphopeptides on electrodes. As expected R_{ET} is much lower in spectra derived from the control chamber corresponding to incubation of PDAC ENOA$^{1,2+}$ sera on unphosphorylated peptide layer (cyan curve Figure 2a).

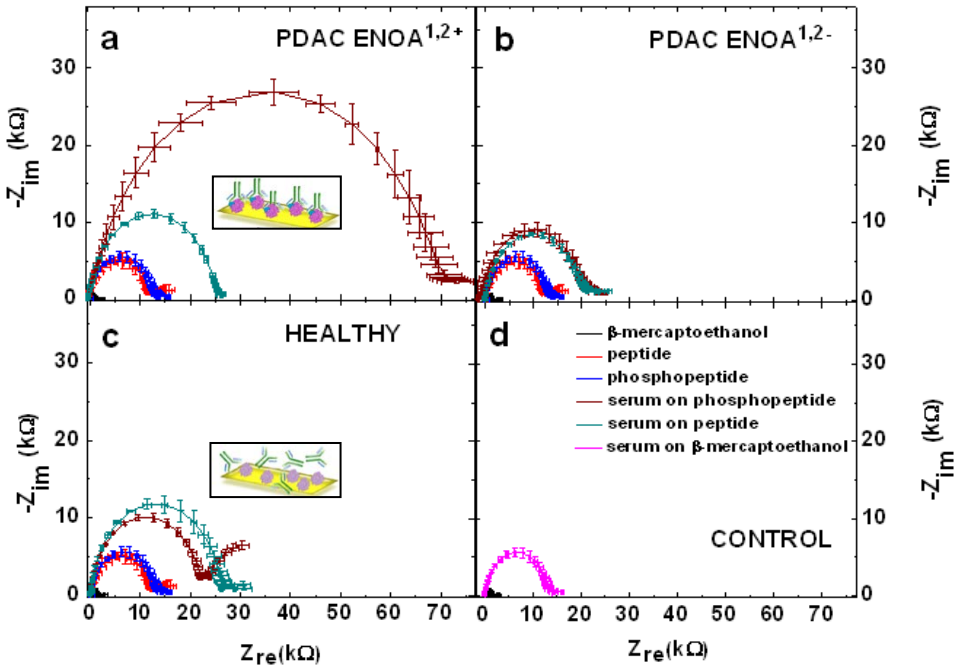

Fig. 2 *Electrochemical Impedance Spectroscopy analysis on chip.* ***(a)*** *Results from PDAC ENOA1,2+ sera: Impedance Nyquist spectra at different steps of electrode functionalization (black, red and blue curves) and after incubation with positive serum. The electron transfer resistance increases significantly for electrodes functionalized with phosphopeptides (brown curve), while only a small impedance increase (attributed to the unspecific adsorption of serum constituents on electrodes) is observed when unphosphorylated peptide are employed as capture probes (cyan curve).* ***(b)*** *Results from PDAC ENOA1,2- sera: in this case biorecognition events do not happen and this results in impedance values quite similar to those recorded for serum on unphosphorylated peptides.* ***(c)*** *Results from healthy sera: impedance spectra are quite similar to the case of PDAC ENOA1,2- sera and for incubation on both phosphopeptide and unphosphorylated peptide.* ***(d)*** *Control: negative control set up by incubating human serum on β-mercaptoethanol layer: only a small increase in impedance is observed. All curves are obtained on the basis of several experiments carried out on different biochips fabricated and functionalized in different days. Each plotted curve derives from the mean of at least five independent experiments and thus the error bars provide information on reproducibility of results. Each biochip has been used for a single experiment.*

The increasing of signal recorded for PDAC sera on unphosphorylated peptide is attributed to unspecific adsorption of serum components on functionalized electrodes. In order to better evaluate these contributions and demonstrate the diagnostic potential of Ser-419 phosphorylated ENOA functionalized biochips, similar experiments were repeated using sera from patients PDAC ENOA$^{1,2-}$ and HS. In both these cases, impedance spectra exhibited similar signals from both analysis and control chambers as shown in Figure 2b and 2c, where only small differences between brown and cyan curves can be observed, likely due to an intrinsic variability within serum samples. Also in these cases it is possible to observe an unspecific increase of impedance values due to serum constituents. To confirm this hypothesis, serum was incubated on the β-mercaptoethanol partial layer, to estimate the unspecific adsorption which was quantified in around 14kΩ (Figure 2d).Biochip results are in agreement with those from traditional techniques, such as ELISA and Western Blot, but measurements are faster, more reproducible and specific making the developed biochips ideal for a quick, cost-saving and label-free analysis of serum samples. Thus our approach could be applied in clinical field. In particular for PDAC early diagnosis, research is directed toward the identification of the presence of an altered molecular signature referable to the disease [6, 7]. For example testing even only two markers such as autoantibodies to phosphorylated ENOA^{1,2} together with CA19.9 results in a diagnostic accuracy greater than 95% in differentiating PDAC patients from control subjects. Thus, our biochips could be employed in combination with CA19.9 assays to provide an efficient PDAC diagnosis while the ability to discern between PDAC ENOA$^{1,2+}$ and PDAC ENOA$^{1,2-}$ patients has a further prognostic value in predicting the clinical course. These are both aspects of clear relevance for technological applications and are expected to contribute to move toward a more patient-centric, cost-effective and faster diagnosis, improving healthy ageing and positively impacting healthcare systems. In a near future, integration of multi-biomarker assays within the same biochip can be envisioned to provide more appropriate tests for early diagnosis, case management and treatment monitoring of patients.

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