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Towards pancreatic cancer diagnosis using EIS biochips

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Maria Serena Chiriacò,*[a] Elisabetta Primiceri,[a] Anna Grazia Monteduro,[a] Anna Bove,[a] Stefano Leporatti,[a] Michela Capello,[bc] Sammy Ferri-Borgogno,[bc] Ross Rinaldi,[a] Francesco Novelli[bc] and Giuseppe Maruccio*[a]

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal cancers in Europe and the 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 phosphorylated α-enolase (ENOA), which in combination with other standard markers can increase specificity in diagnosis of PDAC. In this context we realized a microfluidic platform with integrated EIS biosensors. 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 the EIS technique as a strong tool for quick, cost-saving and label-free analysis of serum samples. Biochip results are in agreement with those from traditional techniques, such as ELISA and western blot, but measurements are much more sensitive and specific, increasing the possibility of PDAC diagnosis. In addition this approach is faster and more reproducible compared to traditional techniques making the developed biochips ideal for a quick, cost-saving and label-free analysis of serum samples.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is characterized by high aggressiveness, rapid progression, invasiveness, and resistance to treatments. It represents one of the principal causes of cancer deaths both in Europe and the United States. The American Cancer Society estimated that in 2012 in the United States, about 43 920 new cases of pancreatic cancer (22 090 in men and 21 830 in women) would be diagnosed and about 37 390 people (18 850 men and 18 540 women) will die from pancreatic cancer.[1] One of the causes of such a high mortality comes from the absence of early symptoms and clinical-pathological markers.

This consideration emphasizes the need to improve the early diagnosis of PDAC but biomarkers for its detection or differentiation from chronic pancreatitis (CP) are lacking. At the moment CA19.9 is the only biomarker identified for PDAC even if several abnormalities (not only cancer) are frequently associated with increased serum concentrations of CA19.9.[2] α-Enolase (ENOA) is a metabolic enzyme involved in the synthesis of pyruvate. It also acts as a plasminogen receptor and thus mediates activation of plasmin and extracellular matrix degradation. In PDAC cells, ENOA is overexpressed and supports anaerobic proliferation, a very important feature in tumors bigger than 1 mm^3, where the slow angiogenesis causes hypoxic conditions. ENOA cellular localization is at the cell surface where it supports cancer invasion by promoting matrix digestion. In addition ENOA is characterized by specific post-translational modifications such as acetylation, methylation and phosphorylation. Both ENOA overexpression and its post-translational modifications could be used as markers with diagnostic and prognostic value in cancer.[3] As recently demonstrated, most PDAC patients produce autoantibodies against two Ser-419 phosphorylated isoforms of α-enolase ($ENOA^{1,2}$),[4–9] which are overexpressed in PDAC. Autoantibodies to phosphorylated $ENOA^{1,2}$ were found frequently in patients with normal CA19.9 levels and the combination of these two markers resulted in a diagnostic accuracy greater than 95% in differentiating PDAC patients from control subjects. Moreover, patients with autoantibodies against $ENOA^{1,2}$ appeared to have a more favourable clinical course with significantly longer progression-free survival.[8]

Application of biosensors in cancer diagnostics is very promising with respect to conventional methods. It allows the achievement of better performance in terms of speed, flexibility, automation and costs. In particular electrochemical impedance spectroscopy (EIS) is an emerging biosensing technique since the immobilization of molecules onto the electrodes alters the capacitance C_{dl} and interfacial electron

[a]*NNL Istituto Nanoscienze - CNR and Dipartimento di Matematica e Fisica "Ennio De Giorgi", Scuola Superiore ISUFI, Università del Salento, Via per Arnesano, I-73100 Lecce, Italy. E-mail: serena.chiriaco@unisalento.it; giuseppe.maruccio@unisalento.it*
[b]*Center for Experimental Research and Medical Studies (CeRMS), San Giovanni Battista University Hospital, Via Cherasco 15, 10126, Turin, Italy*
[c]*Department of Medicine and Experimental Oncology, University of Turin, Turin, Italy*

transfer resistance R_{et}. One of the advantages of this technique is that no labels are required allowing to save time and reduce costs (since labels are often expensive molecules and require additional steps and reactions).[10]

Here we report the development of a label-free EIS biosensor enabling a highly sensitive detection of antibodies of anti-phosphorylated ENOA. As we have already shown for other applications,[11–13] the integration of a PDMS microfluidic platform for sample handling allows achieving very good reproducibility and a reduction in sample handling and costs. The biochips include two reaction chambers corresponding to two sensing areas with 4 electrodes employed to perform repeated experiments at the same time and for statistical analysis. The electrodes are functionalized with synthetic phosphopeptide from ENOA[8] (or peptide without phosphorylation as a control) to detect autoantibodies in human sera. The comparison of data obtained through the chips with traditional enzyme-linked immunosorbent assay (ELISA) highlights EIS as a more sensitive and specific technique to detect anti-phosphorylated ENOA autoantibodies.

Experimental section

Sera specimens

The study was conducted with ethical approval from the Ethical Committees of the Department of Internal Medicine, University of Turin, San Giovanni Battista Hospital, Turin (authorization No. 0058870). Serum samples were isolated from venous blood at time of diagnosis with the informed consent of patients and healthy donors and stored at −80 °C until use. De-identified numeric specimen codes were used to protect the identity of the individuals. Diagnosis of PDAC was always confirmed by histological or cytological analysis. The reactivity of three pools of sera from 10 PDAC patients able to recognize ENOA^{1,2} isoforms (hereafter referred to as ENOA$^{1,2+}$), 10 ENOA$^{1,2-}$ PDAC patients and 10 healthy subjects (HS) was compared. The ability of these sera to recognize ENOA^{1,2} was previously assessed by two dimensional electrophoresis western blot.

ENOA related peptides

In order to detect autoantibodies to phosphorylated ENOA^{1,2} in patient sera, we used two different synthetic peptides previously identified by LC-MS/MS analysis[9] having the same sequence of 13 amino acids and differing only for the presence of a phosphorylated serine in position 419 (C-^{412}RIEEELGSKAKF^{423} and C-^{412}RIEEELGSpKAKF^{423}, Primm, Milan, Italy), where the N-terminal cysteine was added to allow peptide immobilization. For ELISA experiments, the same peptides were N-terminal conjugated with ovalbumin (OVA) to enable the coating with the solid phase (Fig. 1a).

ELISA test

(OVA)-conjugated phosphorylated and unphosphorylated ENOA-derived peptides were used to capture autoantibodies to phosphorylated ENOA. Briefly, each peptide was coated (1 μg ml^{-1} in PBS) in 96-well microplates overnight at room temperature, followed by blocking with PBS containing 4% bovine serum albumin for 90 min at room temperature. ENOA$^{1,2+}$, ENOA$^{1,2-}$ and HS sera (working dilution 1 : 100) were pre-incubated with 2.5% OVA at 37 °C for 30 min and then added to the coated wells for 2 h at room temperature. After washing, microplates were incubated with HRP-conjugated rabbit anti-human IgG (dilution 1 : 1000, Santa Cruz Biotechnology, Santa Cruz, California, USA) for 1 h at room temperature and TMB one solution (Promega, Madison, WI, USA) was added to each well. The reaction was stopped by 2 N HCl solution and the optical density (OD) value was measured at 450 nm. All samples were assayed in triplicate and the results were the mean values of the reading.

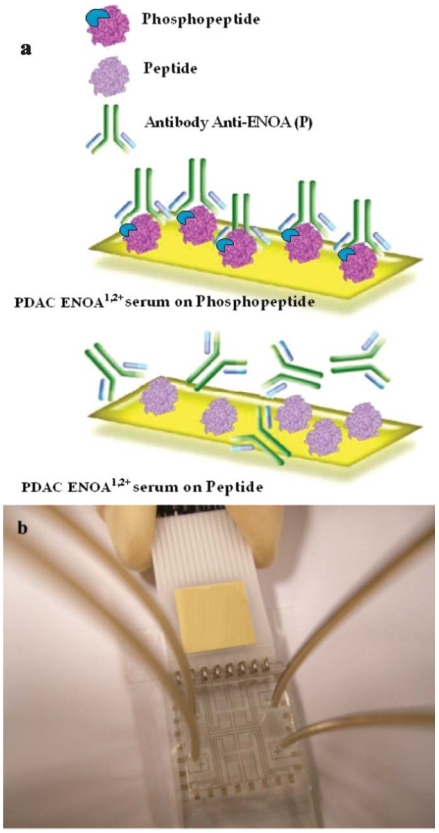

Fig. 1 Setup employed for on chip analysis of serum samples. **(a)** Schematic representation of bio-recognizing events between antibodies in serum from PDAC ENOA$^{1,2+}$ patients and peptides immobilized on gold electrodes. In the upper part, the recognition of autoantibodies by phosphopeptides is shown, while there is no binding in the absence of phosphorylation at Serine 419 as shown in the lower part of the figure. **(b)** Assembled biochip with microfluidic and electrochemical components. Gold interdigitated electrodes for impedance spectroscopy measurements are located within a PDMS chamber with microchannels connected to inlet and outlet tubes for fluids handling on chip.

Biochip structure and fabrication

Impedance biochips (Fig. 1b) were implemented by integrating two modules: (1) a PDMS (*Sylgard 184*) microfluidic module obtained by replica molding from a 100 μm-high hard master made in SU-8 photoresist (Microchem) and (2) two sensing areas, both including an array of gold interdigitated micro-

electrodes with 10 μm space and width, fabricated on glass substrates (2.5 cm × 2.5 cm 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 2.8 μL (7 mm × 4 mm × 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 min 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,[12,14] cell migration[13] and bio-recognition events between immobilized antibodies and related antigens in aqueous solution.[11] Here a biochip to detect directly antibodies from serum samples for early diagnosis of PDAC is demonstrated.

Biochip surface functionalization

To enable detection of serum anti-phosphorylated ENOA antibodies, gold electrodes have been opportunely modified. First of all a partial self assembled monolayer of β-mercaptoethanol (Sigma) has been immobilized by incubating electrodes for 2 h in a 3 mM ethanolic solution containing the thiols which on the gold surface act as spacers to avoid complete coverage of the electrodes by the peptides successively immobilized. Indeed the next step consists of incubating the electrodes for 2 h in a PBS solution of peptides (200 nM) in order to allow their attachment on the exposed gold areas by exploiting the presence of a terminal residue of cysteine with a –SH terminal group able to bind directly on gold. This functionalization procedure was employed for both phospho- and native peptides.

Serum analysis on chip

Once the biosensing peptide layer was immobilized on the electrodes, serum samples at a dilution of 1 : 100 in PBS were incubated into the PDMS chamber for 1 h. Devices were then washed with PBS through the microfluidic channels to reduce any possible unspecific deposition of serum constituents. Successively, devices were filled with PBS, a redox couple of potassium hexacyanoferrate(II/III) $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1 : 1) (Sigma Aldrich) was added at a final concentration of 10 mM and impedance spectroscopy measurements were performed in the frequency range between 0.1 and 10^6 Hz using an impedance analyzer (AutolabPGSTAT30, Eco Chemie) and a sinusoidal AC voltage with a 15 mV RMS amplitude. Pools of sera coming from both PDAC and healthy patients were investigated, the last ones as a control and to evaluate the unspecific adsorption of serum components on functionalized electrodes.

Results and discussion

To implement dedicated devices for on-chip PDAC screening on serum samples, we optimized a microfluidic platform with an impedimetric sensing technology. In this respect, Electrochemical Impedance Spectroscopy (EIS) is a powerful technique because of the possibility to functionalize electrodes with sensing biomolecules to enable highly sensitive detection of biological events. In AC spectra in the form of Nyquist diagrams (Z_{im} as a function of Z_{re}), at high frequencies a semicircular shape is clearly identifiable due to faradic electron-transfer at the electrode solid interface while at low frequencies the obtained spectrum is related to the diffusion process of transport of redox species to the electrode surface through the electrolyte solution. The charge flow which gives information about the surface chemistry of electrodes can be simplified to an equivalent (Randles) circuit where the interfacial layer at the working electrode is represented by an electron transfer resistance R_{et} in parallel combination with a capacitance C_{dl} accounting for the electrical double layer at the interface, and a series Warburg impedance Z_w describing depletion of the redox species at the interface. Additionally a series resistance R_s accounts for the uncompensated solution resistance. The component R_{et} is very sensitive to electrode modifications, thus allowing detection of bio-recognition events with high sensitivity.

Specifically 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 (Fig. 1a).

To obtain biochips, we realized all steps of functionalization 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 (Fig. 1b). Bare electrodes exhibited a 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 the first step we incubated electrodes with a 3 mM β-mercaptoethanol solution recording an increase in impedance values up to about 2 kΩ (black curve in Fig. 2a) with respect to bare electrode measurements. Then, we immobilized the peptides (200 nM) 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-^{412}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 a standard deviation on similar devices (blue and red curves in Fig. 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 (Fig. 1a).

As shown in Fig. 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

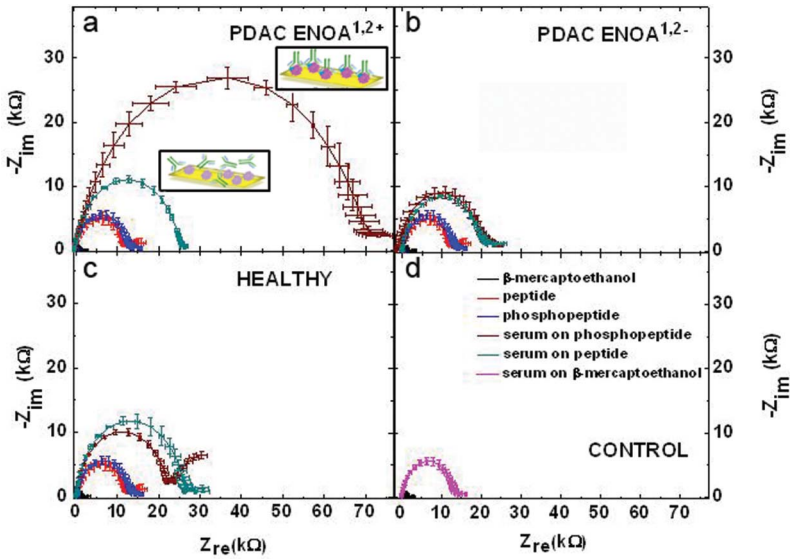

Fig. 2 Electrochemical Impedance Spectroscopy analysis on chip. **(a)** Results from PDAC ENOA$^{1,2+}$ sera: impedance Nyquist spectra at different steps of electrode functionalization (black, red and blue curves) and after incubation with positive serum. The electron transfer resistance increases significantly 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 peptides are employed as capture probes (cyan curve). **(b)** Results from PDAC ENOA$^{1,2-}$ 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 ENOA$^{1,2-}$ sera and for incubation on both phosphopeptide and unphosphorylated peptide. **(d)** Control: negative control set up by incubating human serum on the 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.

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 the unphosphorylated peptide layer (cyan curve Fig. 2a).

The increasing of the 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 Fig. 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 at around 14 kΩ (Fig. 2d).

To validate biochip results the reactivity of PDAC and HS sera against Ser-419-phosphorylated ENOA was evaluated by ELISA (Fig. 3a and b). ENOA$^{1,2+}$ sera displayed a specific reactivity against the phosphopeptide that was significantly lower in ENOA$^{1,2-}$ ($p < 0.0001$) and HS sera ($p = 0.0008$). Conversely, reactivity against unphosphorylated peptide revealed non-significant differences between ENOA$^{1,2+}$, ENOA$^{1,2-}$ or HS sera (Fig. 3a). The data coming from both tests, normalized in a 0 to 1 scale, are compared and shown in Fig. 3b. Despite the ability of the ELISA approach to confirm that PDAC patients' autoantibodies to ENOA^{1,2} are directed to Ser-419-phosphorylated epitope, EIS stands out as a more specific and sensitive technique, especially avoiding the unspecific binding of sera to OVA-tagged peptides. In EIS

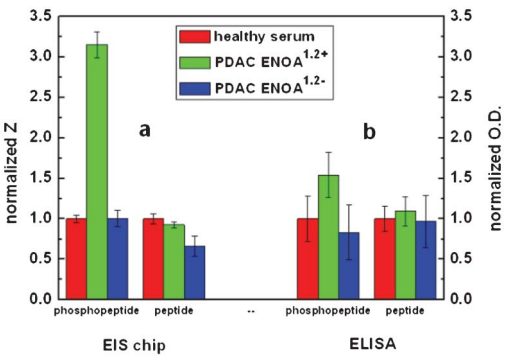

Fig. 3 Bar graphs related to EIS measurements and standard ELISA assay. Comparison between results from **(a)** impedance experiments on EIS chips and **(b)** the golden standard ELISA assays to test the reactivity of sera PDAC ENOA$^{1,2+}$, PDAC ENOA$^{1,2-}$ and HS sera with phosphorylated and unphosphorylated peptides derived from ENOA. All the data were normalized on the value of healthy serum to facilitate comparison between the different set of data.

experiments, increased sensitivity can be attributed to direct immobilization of cysteine-tagged peptides on gold electrodes allowing to achieve also a higher density of peptides, and a great accessibility to antibodies. On the other hand direct immobilization of peptides onto ELISA plates was not possible, so it was necessary to employ ovalbumin-conjugated peptides leading to a lower density and thus a lower sensitivity. These results on one side confirm the presence of antibodies against Ser-419-phosphorylated ENOA in the sera of PDAC patients, while on the other hand they represent a first step towards the fabrication of highly sensitive biochips to detect directly autoantibodies from serum samples for PDAC diagnosis. Our biochips have improved performance in terms of throughput, readout time and cost with respect to traditional approaches (estimated lab cost is around five Euros but it can be further reduced at an industrial scale).

Conclusions

Diagnostic biochips for clinical analysis and in particular for cancerous diseases investigation represent a very attractive research field and many R&D efforts are today directed in this direction where integration of smart platforms for automatic handling of biological fluids is a further challenge towards a saving-cost and label-free analysis which is the final goal. In this context we assessed the presence of autoantibodies against Ser-419-phosphorylated ENOA in sera originating from patients with pancreatic ductal adenocarcinoma by means of appositely developed biochips. This is relevant since ENOA overexpression, its phosphorylation and the presence of autoantibodies against this isoform can be used not only as a diagnostic tag but also as a marker with prognostic value[3] having $ENOA^{1,2+}$ patients a better clinical course with longer progression-free survival.

To reach this goal we realized a microfluidic platform with integrated EIS biosensors. The layout includes two PDMS reaction chambers with their own inlet and outlet microchannels, each of which contains a sensitive area composed of four couples of interdigitated electrodes for signal transduction. A specific antibodies detection is achieved by immobilizing peptides on electrodes and phosphorylation of peptides was found to influence the recognition of antibodies in PDAC patients' sera. 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 the 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.[8,15] 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 the move toward a more patient-centric, cost-effective and faster diagnosis, improving healthy ageing and positively impacting healthcare systems. In the 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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