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Multiple biomarkers biosensor with just-in-time functionalization: Application to prostate cancer detection

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ABSTRACT

We present a novel lab-on-a-chip (LOC) device for the simultaneous detection of multiple biomarkers using simple voltage measurements. The biosensor functionalization is performed *in-situ*, immediately before its use, facilitating reagents storage and massive devices fabrication. Sensitivity, limit of detection (LOD) and limit of quantification (LOQ) are tunable depending on the in-chip flown sample volumes. As a proof-of-concept, the system has been tested and adjusted to quantify two proteins found in blood that are susceptible to be used combined, as a screening tool, to diagnose prostate cancer (PCa): prostate-specific antigen (PSA) and spondin-2 (SPON2). This combination of biomarkers has been reported to be more specific for PCa diagnostics than the currently accepted but rather controversial PSA indicator. The range of detection for PSA and SPON2 could be adjusted to the clinically relevant range of 1 to 10 ng/ml. The system was tested for specificity to the evaluated biomarkers. This multiplex system can be modified and adapted to detect a larger quantity of biomarkers, or different ones, of relevance to other specific diseases.

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1. Introduction

Successful cancer treatment is strongly correlated with early diagnosing the pathology. Cancer-related biomarkers provide key information on the disease progression but are usually present at ultra-low levels during early stages. The cost, the sensitivity, the accuracy and the turn-around times are often limiting factors of biomarkers *in vitro* tests which otherwise could be massively used as cancer monitoring tools in point-of-care (POC) setups. These devices would be a desirable alternative to current clinical lab methods while bringing further portability and usability. Lab-on-a-chip (LOC) systems provide an opportunity to cover this demand. These devices could be suitable to detect cancer at curable stages or to continuously monitor its progression being usable at the bedside or in primary health care (Wang, 2006). Due to their short turn-around times they could also facilitate personalized treatment and a tighter surveillance when a recurrence is presented (Soper et al., 2006).

Prostate Cancer (PCa) is the second most diagnosed cancer on men worldwide (Dean and Lou, 2013). In USA, the American Cancer Society (ACS) estimated more than 238,000 new cases as for 2013, being PCa the most diagnosed cancer type in men (Siegel et al., 2013). In Europe, PCa is the third most common cause of death by cancer (Malvezzi et al., 2013). Prostate Specific Antigen (PSA) is a serine protease produced by the prostate epithelium that is used in oncology as an unspecific biomarker for prostate cancer (PCa) pre-screening (Balk et al., 2003). Levels of PSA in serum lower than 4 ng/ml had been used as a reference in healthy men (El-Galley et al., 1995). However, the use of PSA test as a screening tool in diagnosing prostate cancer is controversial. The Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO), in the United States, claimed that PSA test plus a digital rectal examination has no mortality benefit (Andriole et al., 2009). In contrast, the European Randomize Study of Screening for Prostate Cancer (ERSPC), in Europe, says that the PSA screening had reduced the mortality in a 20% (Schröder, 2011). Therefore, novel biomarkers, and combinations of them, are being studied to diagnose, stage and treat PCa. These aim to improve the detection of the disease and to contribute developing more personalized treatments.

Spondin-2 (SPON2) is a relatively novel biomarker that seems to have the ability to avoid some of the drawbacks of PSA tests.

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SPON2 is an extracellular matrix protein that belongs to the F-Spondin family. It is expressed in cancerous and non-cancerous cells, plays a key role in the initiation of the immune response and is a recognition molecule for microbial pathogens. Qian et al. tested different cutoff values for this novel biomarker (3, 6, 8, 10, 15 and 20 ng/ml). However, they concluded that the best cutoff value, between healthy patients and patients with PCa, when showing PSA concentration levels under 10 ng/ml, was 8 ng/ml for SPON2. In that case sensitivity was 100% and specificity 84.6%, associated with positive and negative values of 97.2% and 100%, respectively

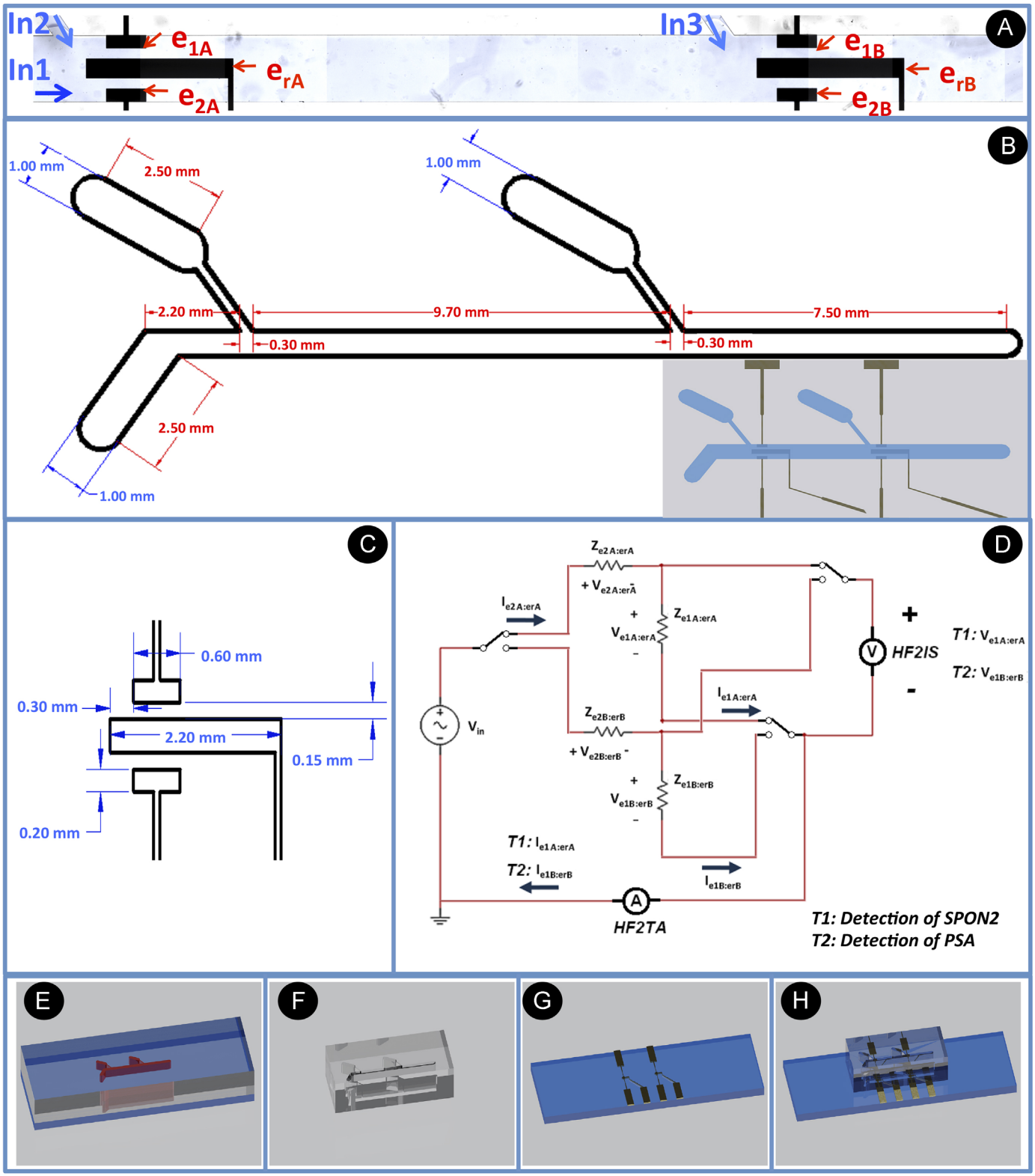

Fig. 1. Layout of the microfluidic device. (A) Microscopic image of the device. (B) Dimensions of the microchannels. (C) Dimensions of the serial microelectrodes (both sets have the same dimensions). (D) Electrical schematics of the impedance and voltage measurements. (E) The device was microfabricated and replicated using PDMS cast molding on top of a SU-8 mold, (F) PDMS replicas, (G) Gold microelectrodes fabricated on glass, (h) Plasma-bonded devices.

(Qian et al., 2012). Lucarelli et al. concluded that the levels of SPON2 were significantly higher in comparison with patients with no malignancy evidence, consistently with the data reported by Qian et al. (Lucarelli et al., 2013).

There had been several attempts to detect multiple PCa biomarkers with the aim of developing PoC devices. These are generally present in serum, urine or tissue. A multiplex suspension array technology with microbeads was developed to detect simultaneously PSA, prostate stem cell antigen, prostatic acid phosphatase and prostate-specific membrane antigen; the biomarkers levels were quantified by fluorescent intensities showing no significant cross-talk among biomarkers (Liu et al., 2013). Another approach was the development of a piezoelectric biosensor, two ceramic resonators were connected in parallel for the multiplexed detection of PSA and α-fetoprotein, showing high sensitivity and fast detection (Su et al., 2013). Approaches combining biosensors and microfluidics have also been tried. An electrochemiluminescence immunoarray with a microfluidic module was developed for the detection of PSA along with IL-6. The test used a ultrasensitive approach based on silica nanoparticles (Sardesai et al., 2013). However, the main issues of these devices are that they use a label to improve the sensitivity of the system. That characteristic increases the complexity of the test as well as its cost. Also, they don't have a tuning mechanism that can be adapted for different applications.

To the best knowledge of the authors, our work is the first attempt to detect simultaneously and in real-time PSA and SPON2, for an early diagnose of PCa. The aim of our work was to develop a low-cost label-free multiplexed biosensor exploiting microfluidics allowing *in-situ* self-functionalization. The sensitivity can be adjustable and the device can be used for detection of different biomarkers. The functionalization takes place *in situ* and selectively, just before the sensing, keeping the sensing area dry and inactive until the test starts, and conserving the functionality of the device (Parra-Cabrera et al., 2012). Also, the reagents and chips are stored separately and used before testing, without the need of overnight functionalization process or complex fabrication methods. The monitoring of the signal is performed on real time and changes in impedance modulus are equivalently recorded by using an alternative, and simpler, voltage measurement. We have tuned and characterized the system sensitivity to different concentrations of PSA and SPON2. Potential cross-sensing issues have also been explored by modifying SPON2 levels and keeping a fix concentration of PSA.

2. Materials and methods

2.1. Device design and fabrication

The biosensing device consists mainly of two sets of gold electrodes (e_{1A}, e_{2A}, e_{rA} and e_{1B}, e_{2B}, e_{rB}, in Fig. 1) placed in series in a main fluidic microchannel (In_1, in Fig. 1) and defining the two voltage-based biosensors. The overall LOC incorporates also two lateral microchannels (In_2 and In_3, in Fig. 1), which serve to perform the *in situ* functionalization and sensing protocols.

The device was fabricated using photolithographic and cast molding techniques through three main steps: microchannel molding (Fig. 1E–F) sensing electrodes fabrication (Fig. 1G), LOC bonding and assembly (Fig. 1H).

A mold was fabricated for the microfluidic structures by selectively exposing and developing SU-8 10 (MicroChem) after spinning it on top of a glass substrate (Deltalab). The microchannels were then cast-molded on poly dimethylsiloxane (PDMS, Sylgard®184, Dow Corning). Electrodes were evaporated and patterned on top of a clean glass substrate using a standard lift-off process based on AZ5214E photoresist (AZ Electronic Materials). After punching the inlets and outlets (Harris Uni-Core, ted Pella) on the PDMS part, the microchannels were plasma bonded to the microelectrodes slide. A more detailed description of the fabrication techniques can be found on Parra-Cabrera et al. (2012).

2.2. Hydrodynamic characterization

The fluid dynamics and width of the co-flow was characterized with the equivalent of the Ohm's law (Stiles et al., 2005). Simulations supporting the design, prior to the experimental characterization and verification, were performed on COMSOL multiphysics (COMSOL Inc, Palo Alto, CA, USA). Results were later replicated experimentally by controlling the input flows on the real device.

Two solutions were used, an un-dyed and a red-dyed phosphate buffered saline solutions (PBS, Sigma Aldrich, P4417). Both liquids could be observed by optical inspection. In the experimental set-up a flow was maintained in the main channel input In_1 (10 μl/min) while the flows on the lateral inlets (In_2 and In_3) were tuned according to the biosensor protocol phase or process characterization. The overall biomarkers quantification protocol had mainly three processes associated to different inlet flows: (a) reference electrodes (e_{2A}, e_{rA}, e_{2B} and e_{rB}) selective blocking and sensing electrodes (e_{1A} and e_{1B}) selective functionalization, $In_2 = 1/3 * In_1$ and $In_3 = 0$ μl/min; (b) e_{1A} and e_{1B} selective and simultaneous functionalization, $In_2 = In_3 = 1/3 * In_1$ μl/min; and (c) Biomarkers detection, $In_2 = In_3 = 0$ μl/min.

The devices used to control the microfluidic flow at the inlets and to perform the fluid dynamics characterization were two syringe pumps: a KDS-101, from Kd Scientific, and a PHD 2000, from Harvard Apparatus. For the practical measurements and image acquisition, an inverted microscope from Olympus (IX71) with an integrated CCD camera was employed (results are presented on ESI file under fluid dynamics).

2.3. Multiple functionalization in-situ protocol

The establishment of an efficient functionalization protocol was critical to our biosensor. Laminar co-flow was used to perform an on-chip selective surface bio-functionalization of LOC-integrated multiple sensors. Final protocol consisted of:

i. Selective blocking of the electrodes e_{2A}, e_{rA}, e_{2B} and e_{rB} (Fig. 2A). A solution of 11-Mercaptoundecyl tetra(ethylene glycol) (PEG-Thiol, Sigma Aldrich 674508) in ethanol, at a concentration of 10 mg/ml, was introduced through the main channel (In_1) at a flow rate of 6 μl/min, while PBS was introduced at 2.5 μl/min, through In_2, and at 1 μl/min, through In_3. This process lasted 30 min.
ii. Selective deposition of a biotin-thiols self-assembled monolayer over e_{1A} and e_{1B} (Fig. 2B). A solution of biotinylated alkyl thiols with a polyethylene glycol linker (biotin-thiol), prepared as reported by Prats-Alfonso et al. (2006), and dissolved in ethanol at a concentration of 1 mg/ml, was introduced at 6 μl/min through In_2 during 60 min. Simultaneously, PBS buffer was kept flowing through In_1 at 20 μl/min.
iii. Formation of a Streptavidin layer on top of the biotin layer (Fig. 2C). A solution of streptavidin (Streptavidin from Streptomyces avidinii, SAV, Sigma Aldrich 85878) was introduced through the lateral channel (In_2) at 6 μl/min, during 60 min) while PBS was kept flowing into In_1 at 20 μl/min.
iv. Selective and simultaneous deposition of biotinylated specific antibodies over the SAV layers of e_{1A} and e_{1B} (Fig. 2D). An anti-Prostate Specific Antigen antibody (APSA, Abcam ab77310) 100 μg/ml solution was flown at 3 μl/min through In_3, during

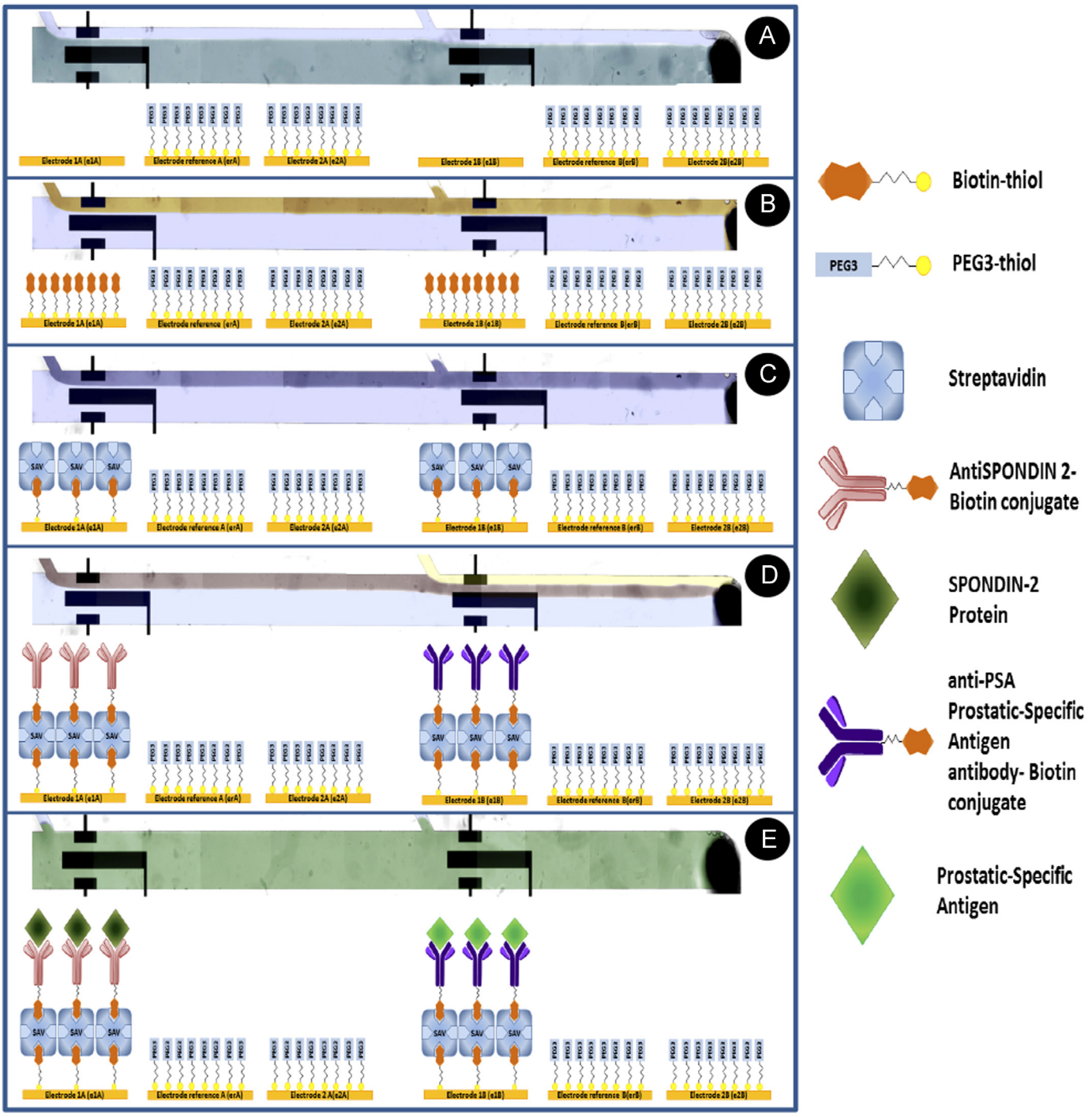

Fig. 2. Protocol for the multiplexed fluidic detection of Spondin-2 and PSA. (A) Selective blocking with a PEG-thiol solution, (B) Electrodes surface modified with biotin-thiol, (C) Addition of a layer of streptavidin, (D) Simultaneous deposition of a layer biotinylated antibodies. (E) Detection of Spondin-2 and PSA. Microchannels images are represented in false color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

90 min, while simultaneously introducing a 100 μg/ml solution of Spondin-2 antibody (SPON2/M spondin/Mindin antibody, antibodies-online ABIN1389055) at 3 μl/min through In_2, and PBS at 10 μl/min through In_1.

v. PBS rinsing after the last step.

It is important to highlight that reagents use could be reduced by setting lower fluidic flows or, more interestingly, by reducing microfluidic channel dimensions. Also, by substituting some of the stages, which donot need to be selective, in a classical way, by stopping the fluid and performing incubation. An optimization in this sense has not been considered for this proof of concept and the effort has been set to obtain stable and repeatable results with a well-defined protocol.

2.4. Biomarkers detection and quantification

In order to adjust the detection of both biomarkers and to study the cross talk between proteins in our device several solutions were prepared and flown through channel In_1 at 6 μl/min during 60 min (Fig. 2E). Impedance and voltage measurements were performed simultaneously on real time for characterization,

comparison and verification purposes. Sensitivity, limit of detection (LOD) and limit of quantification (LOQ) (Armbruster and Pry, 2008) were calculated for both, impedance and voltage – based measurements. After a calibration curve was obtained, these parameters were calculated by linear regression analysis (Shrivastava and Gupta, 2011).

PBS-based solutions with different concentrations of PSA (PSA, Abcam ab78528), in particular 1 ng/ml, 5 ng/ml, 10 ng/ml and 50 ng/ml, as well as with different concentrations of SPON2 (Spondin 2 (SPON2) protein (GST tag), antibodies-online ABIN1321223), in particular 1 ng/ml, 5 ng/ml and 10 ng/ml, were used for the characterization of the biosensor. A new chip was used for each experiment. Measurements were also done with human plasma containing a controlled concentration of PSA (5 ng/ml).

Solutions containing a fixed amount of PSA, 5 ng/ml, and variable amounts of SPON2, 1 ng/ml, 5 ng/ml, and 10 ng/ml, were used to evaluate biosensing selectivity. An AC input voltage (V_{in}) was applied between e_2 and e_1. A 0.1 V input voltage at a frequency of 1 kHz was chosen after a complete characterization of the frequency spectrum.

2.5. Impedance and voltage measurements

A diagram of the performed electrical measurements is presented on Fig. 1D. Each electrode has originally impedance due to its geometry, physical configuration, interaction with the contacted media and material. The impedance is changed by functionalization as molecules attach to the electrode surface. By applying a fixed and controlled voltage between electrodes, we can measure the current, and impedance, as electrodes surface evolve in time, relating this quantity with the concentration of a specific deposited biomolecule. Therefore, each functionalization or blocking step modifies the relative impedance.

The differential voltage of the functionalized electrode ($V_{e1:er}$) was measured directly with an impedance spectroscope (HF2IS by Zurich Instruments) while a transimpedance current amplifier (HF2TA by Zurich Instruments) gave us the current flowing across the circuit ($I_{e1:e2}$). Using Ohm's Law, and given a fixed V_{in}, we calculated the value of the functionalized ($|Z_{e1:er}|$) and the blocked ($|Z_{e2:er}|$) impedances.

Each biosensor of our system behaves basically as a voltage divider. An input voltage (V_{in}) produces an output voltage on each pair of electrodes that is a fraction of V_{in}, proportional to the electrodes and in-between fluid impedance. However, since we had two sets of electrodes, we had to control and multiplex input and output from both sets of biosensors. The measurements started by applying V_{in} and measuring the output of to the first set of electrodes (e_{1A}, e_{2A} and e_{rA}). After 500 milliseconds, with the help of an analog switch (DG333 from Vishay/Siliconix), V_{in} was applied to the second set of electrodes (e_{1B}, e_{2B} and e_{rB}) and the process was subsequently repeated (Fig. 1A). The analysis was performed *in situ* and in real-time using a custom-made Matlab program (The MathWorks Inc., Natick, MA, 2000) (Parra-Cabrera et al., 2012).

The use of an alternative measurement of voltage for sensing purposes due to the voltage divider electrodes disposition is supported by the definition of the factor α and the evaluation of $\Delta\alpha$, as a substitute to $\Delta|Z|$, (Parra-Cabrera et al., 2012). See the ESI for further information.

Results for simple voltage measurements (through α) and impedance modulus were compared to characterize their equivalence and suitability as biosensing principle in our system.

3. Results and discussion

3.1. Determination of the adjustability in the detection range for PSA

A t-student test was performed to compare the impedance module changes ($\Delta|Z_{e1:er}|$) values for 1 ng/ml, 5 ng/ml, 10 ng/ml and 50 ng/ml, PSA concentrations, at specific experimental running-times: 10, 20, 30, 40, 50 and 60 min (Fig. 3A). Very significant differences ($p < 0.01$) were found when comparing the impedance modulus results obtained at a fixed running-time for the PSA concentration solution pairs 1 ng/ml and 5 ng/ml or 5 ng/ml and 10 ng/ml. A similar result was obtained when comparing the outcomes of 10 ng/ml to 50 ng/ml solutions for experimental running-times kept under 30 min. Oppositely, at longer analyte exposition times, no significant differences were found ($p > 0.1$) between these higher concentration solutions. This result indicated a point of inflection where the sensor surface was saturated at lower concentrations when flowing larger samples, or equivalently when increasing sample flowing times.

When sensitivity in the linear region was studied (Fig. 3A) for the same specific sample flowing times, values ranged from 22 to 51 Ω ng/ml in the 10–60 min range (equivalent to the 60 μl–360 μl sample volume range). These values reflected the adjustability of the sensitivity and detection range limits depending on the amount of sample flown through the system. Sensitivity, LOD and LOQ of the system were later studied and a fitting analysis was done in order to characterize the adjustability of these parameters (Fig. 3B–D).

Results verified that sensitivity could be improved using larger volumes of samples, or longer sample flowing times. LOD and LOQ improved in a similar way. However, these two parameters seem to reach a plateau in our system, when used for PSA detection at 6 μl/min flow rate, for detection times higher than 30 min. Larger sample flowing times, or sample volumes, had no significant effect ($p > 0.05$) on LOD and LOQ. When compared to 10 min detection (60 μl sample volume), sample flowing times over 30 min (180 μl sample volume) improved these two parameters up to 1.8 and 1.9 times respectively.

These results using PSA as biomarker showed a reasonable detection time, or sample volume, showing sensitivity and LOD values similar to ELISA tests, for the medical range of interest. The detection time would be 30 min. However, in order to improve sensitivity we chose to use the 60 min sample flow setup. This biosensor with these parameters would saturate at near 10 ng/ml concentration, providing a sensitivity of 51.1 Ω ng/ml, and a LOD of 118 pg/ml. It is important to notice that the PSA study working principle of the overall biosensor could be extrapolated to different biomarkers depending on the application of interest.

3.2. Characterization of voltage based sensing mechanism for detection of a single prostate cancer biomarker: PSA

Once the sample volumes and flows were set for our device to behave in the medically relevant domain for PSA detection, our proposed novel system to substitute impedance measurements through a simpler voltage detection mechanism was tested.

The system input voltage (V_0) was fixed at 100 mV amplitude at 1 kHz and $V_{e1:er}$ was monitored allowing real-time α factor calculation. We measured the impedance modules and calculated factor α, after the deposition of the PSA antibody ($|Z_{e1:er}(APSA)|$, α (APSA)) and after the detection of this protein ($|Z_{e1:er}(PSA)|$, α (PSA)). Increments ($\Delta|Z_{e1:er}|$, $\Delta\alpha$) were calculated and plotted (Fig. 4A–B).

A linear tendency for concentrations between 1 ng/ml and 10 ng/ml, for impedance and factor α differential measurements was obtained as expected from previous results.

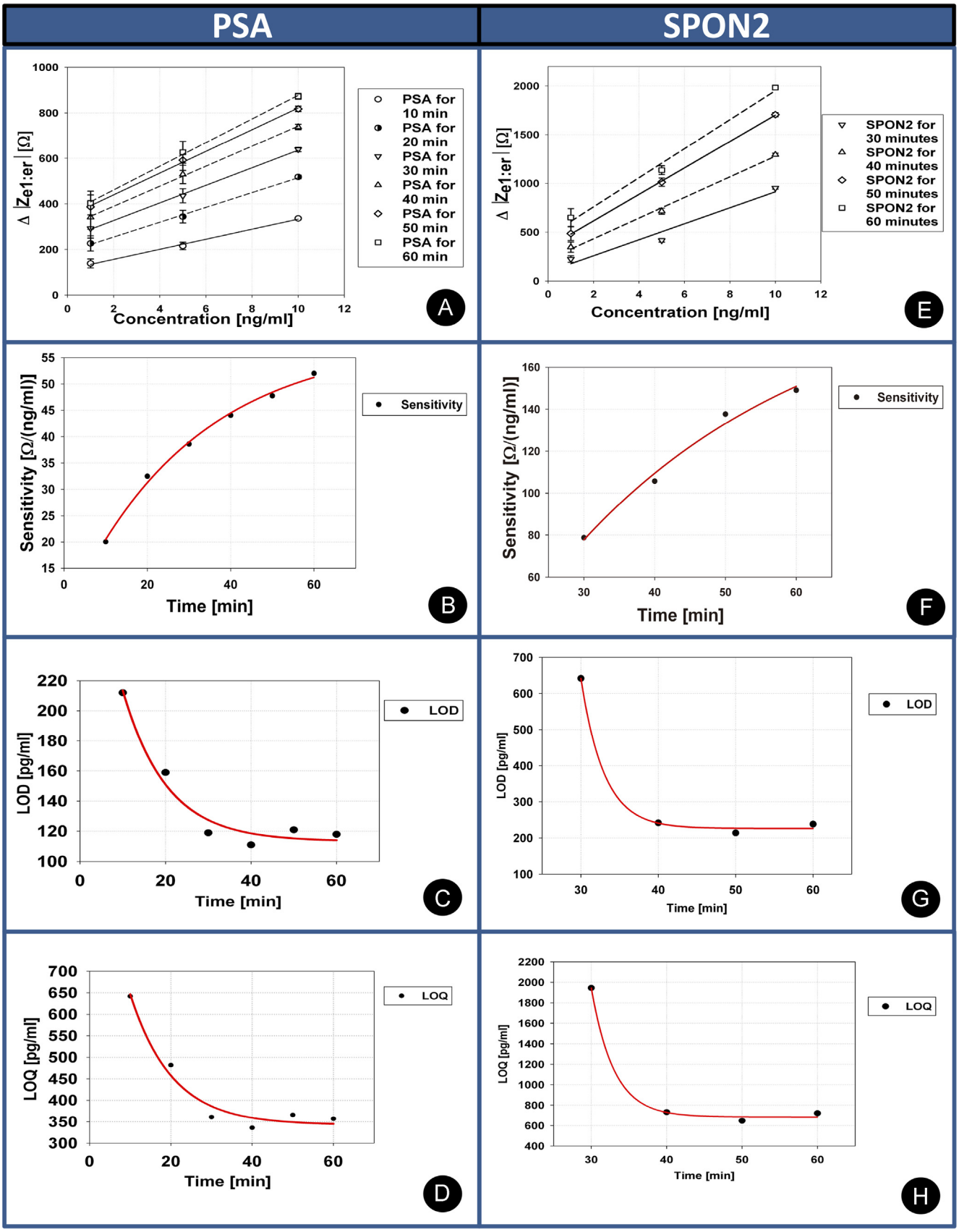

Fig. 3. Adjustability of the sensing parameters of our device. (A) Sensitivity adjustment of the microfluidic system for PSA. Linear behavior of the fitting in the PSA concentrations. (B) Fitting of the PSA sensitivity with the experimental data for different detection times. (C) Exponential fitting for PSA LOD with experimental data and different detection times. (D) Exponential fitting for PSA LOQ with experimental data and different detection times. (E) Sensitivity adjustment of the microfluidic system for SPON2. Fitting in the SPON2 concentrations. (F) Linear fitting of the SPON2 sensitivity with the experimental data for different detection times. (G) Exponential fitting for SPON2 LOD with experimental data and different detection times. (H) Exponential fitting for SPON2 LOQ with experimental data and different detection times.

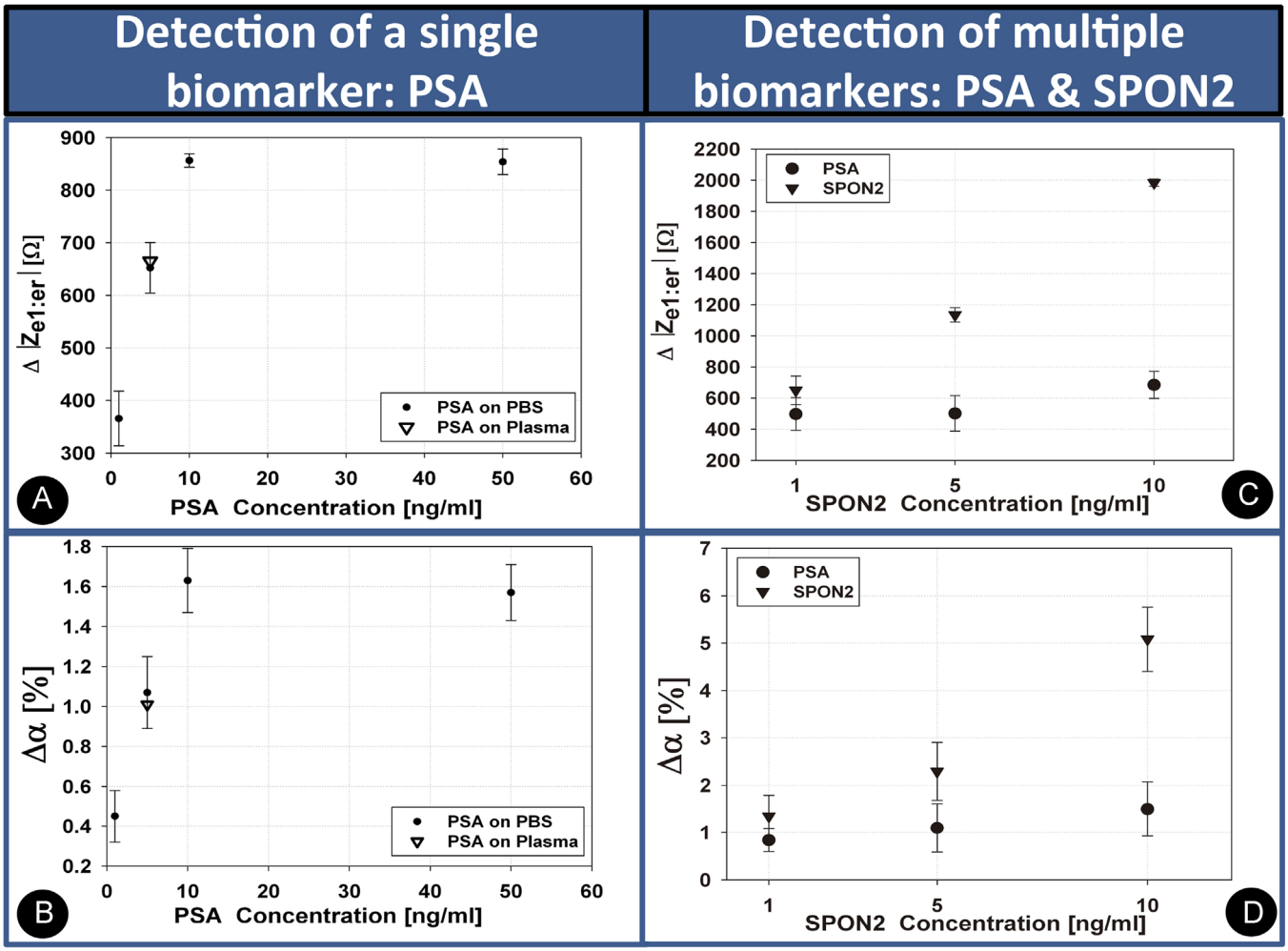

Fig. 4. Detection range of the microfluidic system for single and multiple detection of prostate cancer biomarkers. (A–B) Detection of PSA (round marks). The system was tested with human serum at a known concentration of 5 ng/ml (triangle mark). A linear tendency was observed between 1–10 ng/ml and a fitting analysis was performed. (A) Shows the results for the impedance modulus analysis, (B) Corresponds to the factor α calculations. (C–D) Detection of SPON2 (red dots) and PSA (black dots). A linear tendency was observed and a fitting analysis was performed. The PSA was kept at a fix concentration of 5 ng/ml to study the cross talk among biomarkers. (C) Shows the results for the impedance modulus analysis, (D) Corresponds to the factor α calculations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
LOD, LOQ and sensitivity for the different electrical measurements for PSA for a clinical relevant detection range of 1–10 ng/ml.

Measurement	LOD [pg/ml]	LOQ [pg/ml]	Sensitivity
$\Delta\|Z_1\|$	118	357	51.1 [Ω*(ng/ml)$^{-1}$]
$\Delta\alpha$	376	1100	0.13 [%*(ng/ml)$^{-1}$]

After processing all data we obtained the sensitivity, LOD and LOQ of our system for the detection of PSA with the different measurement methods (Table 1).

A t-student test was performed among $\Delta|Z_{e1:er}|$ and $\Delta\alpha$ values at different PSA concentrations: 1 ng/ml, 5 ng/ml, 10 ng/ml and 50 ng/ml. Very significant differences ($p < 0.01$) were found when comparing the $\Delta|Z_{e1:er}|$ results obtained for any of these solution pairs with PSA concentrations under 10 ng/ml. Results were similar for $\Delta\alpha$ values showing only a slightly lower significance ($p < 0.05$) for the 5 ng/ml and 10 ng/ml pair. Oppositely, when comparing the outcomes of 10 ng/ml and 50 ng/ml PSA solutions, using either $\Delta|Z_{e1:er}|$ or $\Delta\alpha$, no significant differences were found ($p > 0.05$). Thus the system was not sensitive to PSA concentrations over 10 ng/ml which is much higher than the cutoff clinical value of 4 ng/ml (El-Galley et al., 1995).

LOD and LOQ, when evaluating PSA concentration, were around 3-fold for $\Delta\alpha$ when compared to $\Delta|Z_{e1:er}|$ measurements (Table 1). The parameter α is closely related to voltage changes in the electrodes ($V_{e1:er}$ and $V_{e2:er}$) and is affected by the behavior of the blocking. Variability among samples at the same PSA concentration was also higher when evaluating $\Delta\alpha$ instead of $\Delta|Z_{e1:er}|$. We hypothesized that these results could be due non-perfect PEGylation of the reference electrodes e_1 and e_r. However, equivalent behavior between the two methods was proven. The method opens the door to develop a cheap point-of-care device, based on a simple real-time voltage read-out strategy, supported by our electrodes configuration and system. Both LOD values are in the range of other microfluidic immunosensors for the detection of PSA that are between 10 pg/ml (Triroj et al., 2011) and 2 ng/ml (Fragoso et al., 2011)

Finally a test with real human serum was also performed for PSA. Three aliquots of the serum sample were first tested using three chips out of the shell that had their electrodes functionalized with anti-PSA and PEG *in situ*. No significant changes of $|Z_{e1:er}|$ or α values were monitored. Three other aliquots of the serum were then prepared to contain 5 ng/ml of PSA. The detected values after using three new chips for $\Delta|Z_{e1:er}|$ and $\Delta\alpha$ were in the range of the previously calibrated curves for this PSA concentration.

3.3. Determination of the adjustability in the detection range for Spondin-2

A statistical analysis was done using a *t*-student test. We compared the different concentrations of SPON2 (1, 5 and 10 ng/ml) at specific experiment running times (Fig. 3E). Significant differences ($p < 0.01$) were found in all the cases between all concentrations and times. The sensitivity, LOD and LOQ were plotted and characterized by a curve fitting (Fig. 2F–H).

When sensitivity was studied (Fig. 3F) for the same specific sample flowing times, values ranged from 79 to 149 Ω ng/ml in the 30–60 min range (equivalent to the 180 μl–360 μl sample volume range). These values reflected again, as in the case of PSA detection, the adjustability of the sensitivity and detection range limits depending on the amount of sample flown through the system.

LOD and LOQ improved also when larger samples or longer detection times were used. Again, at 6 μl/min flow rate, they reached a plateau when detection times longer than 40 min were used. When compared to 30 min detection, sample flowing times over 60 min improved these two parameters up to 2.6 and 2.8 times respectively.

In order to improve sensitivity and evaluate the performance of our sensor when acting in voltage measurement mode we chose to use the 60 min sample flow setup. This biosensor with these parameters would provide a sensitivity of 149 Ω ng/ml, and a LOD of 238 pg/ml, without saturating the biosensors.

3.4. Characterization of voltage based sensing mechanism for detection of multiple prostate cancer biomarkers: SPON2 and PSA

Following previous results, the sample volumes and flows could be easily set in our device to behave linearly in the medically relevant domain of SPON2 and PSA concentration detection (between 1 and 10 ng/ml).

Again, the system input voltage (V_{in}) was fixed at 100 mV amplitude at 1 kHz and $V_{e1A:erA}/V_{e1B:erB}$ were monitored allowing real-time α factor calculation. We measured the impedance modules and calculated factor α, after the deposition of the antibodies ($|Z_{e1A:erA}$(ASPON2)|, α(ASPON2), $|Z_{e1B:erB}$(APSA)|, α(APSA)) and after the detection of both proteins ($|Z_{e1A:erA}$(SPON2)|, α(SPON2), $|Z_{e1B:erB}$(PSA)|, α(PSA)). Increments ($\Delta|Z_{e1A:erA}|$, $\Delta\alpha_A$, $\Delta|Z_{e1B:erB}|$, $\Delta\alpha_B$) were calculated (Fig. 4C–D).

The detection range graph shows a linear tendency for concentrations between 1 ng/ml and 10 ng/ml, when measuring increments of impedance and factor α related to SPON2 concentrations. On the other hand, PSA was kept fixed at 5 ng/ml and no significant changes on the PSA related values ($\Delta|Z_{e1B:erB}|$, $\Delta\alpha_B$) were observed when comparing the different tested concentrations of SPON2 ($p > 0.1$), indicating robustness of the sensors to cross selectivity.

Table 2 summarizes the obtained results for sensitivity, LOD and LOQ, when evaluating SPON2 biomarker in our system.

A *t*-student test was performed among $\Delta|Z_{e1A:erA}|$ and $\Delta\alpha_A$ values at different SPON2 concentrations: 1 ng/ml, 5 ng/ml and 10 ng/ml. Similarly to what happened with PSA, very significant differences ($p < 0.01$) were found when comparing the $\Delta|Z_{e1A:erA}|$ results obtained for any of these solution pairs. Results were similar for $\Delta\alpha_A$ values showing only a slightly lower significance ($p < 0.05$) for the 1 ng/ml and 5 ng/ml pair. Oppositely, when comparing the outcomes of PSA concentrations with all the SPON2 tests, using either $\Delta|Z_{e1B:erB}|$ or $\Delta\alpha_B$, no significant differences were found ($p > 0.1$). We think that the selectivity of both antibodies avoid crosstalk among proteins.

Again, similarly to what was observed for PSA, LOD and LOQ, when evaluating SPON2 concentration, were around 3.5 times for $\Delta\alpha$ when compared to $\Delta|Z_{e1:er}|$ measurements (Table 2). However, equivalent behavior between the two methods was proven. The method opens the door to develop a cheap point-of-care device, for a multiple detection of biomarkers, based on a simple real-time voltage read-out strategy, supported by our electrodes configuration and system.

Table 2
LOD, LOQ and sensitivity for the $\Delta|Z_{e1A:erA}|$ and $\Delta\alpha_A$ parameters for SPON2.

Measurement	LOD [pg/ml]	LOQ [pg/ml]	Sensitivity
$\Delta\|Z1\|$	238	721	149.1 [Ω*(ng/ml)$^{-1}$]
$\Delta\alpha$	856	2590	0.4206 [%*(ng/ml)$^{-1}$]

4. Conclusions

We have presented an easy to fabricate multiple-biomarkers biosensing device that combines simple electronics and microfluidics to overcome limitations regarding the commercialization of Point of care devices. The integrated electrodes are self-functionalized selectively *in-situ*, just before device operation, using the laminar co-flow principle. This facilitates reagents and antibodies long-term storage as well as massive device fabrication. Furthermore, this versatility could provide a solution to the need of universal customizable platforms on some particular applications such as biomarkers screening. We demonstrated that sensitivity; limits of detection and detection range; are tunable by modifying sample flow times and associated sample volumes during the detection operation. Our novel biosensor allows substitution of more complex impedance measurements by a much direct voltage monitoring method based on the utilization of three selectively functionalized microelectrodes. This is possible through a voltage divider arrangement and the definition of the α parameter. The overall biosensing system was set-up, tested and conceptually proven for the real case of PSA and SPON2 detection. Sensitivity, LOD, LOQ and detection ranges were adjusted to be on the clinically relevant limits for these biomarkers detection in PCa cases. The sensitivity, as well as the detection range, of this biosensor, can be adjusted for other given biomolecules or biomarkers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in

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References

Andriole, G.L., Crawford, E.D., Grubb, R.L., Buys, S.S., Chia, D., Church, T.R., Fouad, M. N., Gelmann, E.P., Kvale, P.A., Reding, D.J., Weissfeld, J.L., Yokochi, L.A., O'Brien, B., Clapp, J.D., Rathmell, J.M., Riley, T.L., Hayes, R.B., Kramer, B.S., Izmirlian, G., Miller, A.B., Pinsky, P.F., Prorok, P.C., Gohagan, J.K., Berg, C.D., 2009. Mortality results from a randomized prostate-cancer screening trial. N. Engl. J. Med. 360 (13), 1310–1319.

Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. Clin. Biochem. Rev. 29 (Suppl. 1), S49–S52.

Balk, S.P., Ko, Y.-J., Bubley, G.J., 2003. Biology of prostate-specific antigen. J. Clin. Oncol. 21 (2), 383–391.

Dean, M., Lou, H., 2013. Genetics and genomics of prostate cancer. Asian J. Androl. 15 (3), 309–313.

El-Galley, R.E.S., Petros, J.A., Sanders, W.H., Keane, T.E., Galloway, N.T.M., Cooner, W. H., Graham Jr., S.D., 1995. Normal range prostate-specific antigen versus age-specific prostate-specific antigen in screening prostate adenocarcinoma. Urology 46 (2), 200–204.

Fragoso, A., Latta, D., Laboria, N., von Germar, F., Hansen-Hagge, T.E., Kemmner, W., Gartner, C., Klemm, R., Drese, K.S., O'Sullivan, C.K., 2011. Integrated microfluidic platform for the electrochemical detection of breast cancer markers in patient serum samples. Lab Chip 11 (4), 625–631.

Liu, N., Liang, W., Ma, X., Li, X., Ning, B., Cheng, C., Ou, G., Wang, B., Zhang, J., Gao, Z., 2013. Simultaneous and combined detection of multiple tumor biomarkers for prostate cancer in human serum by suspension array technology. Biosens. Bioelectron. 47 (0), 92–98.

Lucarelli, G., Rutigliano, M., Bettocchi, C., Palazzo, S., Vavallo, A., Galleggiante, V., Trabucco, S., Di Clemente, D., Selvaggi, F.P., Battaglia, M., Ditonno, P., 2013. Spondin-2, a secreted extracellular matrix protein, is a novel diagnostic biomarker for prostate cancer. J. Urol. 190 (6), 2271–2277.

Malvezzi, M., Bertuccio, P., Levi, F., La Vecchia, C., Negri, E., 2013. European cancer mortality predictions for the year 2013. Ann. Oncol. 24 (3), 792–800.

Parra-Cabrera, C., Sporer, C., Rodriguez-Villareal, I., Rodriguez-Trujillo, R., Homs-Corbera, A., Samitier, J., 2012. Selective in situ functionalization of biosensors on LOC devices using laminar co-flow. Lab Chip 12 (20), 4143–4150.

Prats-Alfonso, E., García-Martín, F., Bayo, N., Cruz, L.J., Pla-Roca, M., Samitier, J., Errachid, A., Albericio, F., 2006. Facile solid-phase synthesis of biotinylated alkyl thiols. Tetrahedron 62 (29), 6876–6881.

Qian, X., Li, C., Pang, B., Xue, M., Wang, J., Zhou, J., 2012. Spondin-2 (SPON2), a more prostate-cancer-specific diagnostic biomarker. PLoS ONE 7 (5), e37225.

Sardesai, N., Kadimisetty, K., Faria, R., Rusling, J., 2013. A microfluidic electrochemiluminescent device for detecting cancer biomarker proteins. Anal. Bioanal. Chem. 405 (11), 3831–3838.

Schröder, F.H., 2011. Stratifying risk – the U.S. preventive services task force and prostate-cancer screening. N. Engl. J. Med. 365 (21), 1953–1955.

Shrivastava, A., Gupta, V.B., 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chron. Young Sci. 2 (1), 21.

Siegel, R., Naishadham, D., Jemal, A., 2013. Cancer statistics, 2013. CA: Cancer J. Clin. 63 (1), 11–30.

Soper, S.A., Brown, K., Ellington, A., Frazier, B., Garcia-Manero, G., Gau, V., Gutman, S.I., Hayes, D.F., Korte, B., Landers, J.L., Larson, D., Ligler, F., Majumdar, A., Mascini, M., Nolte, D., Rosenzweig, Z., Wang, J., Wilson, D., 2006. Point-of-care biosensor systems for cancer diagnostics/prognostics. Biosens. Bioelectron. 21 (10), 1932–1942.

Stiles, T., Fallon, R., Vestad, T., Oakey, J., Marr, D.W.M., Squier, J., Jimenez, R., 2005. Hydrodynamic focusing for vacuum-pumped microfluidics. Microfluid. Nanofluid. 1 (3), 280.

Su, L., Zou, L., Fong, C.-C., Wong, W.-L., Wei, F., Wong, K.-Y., Wu, R.S.S., Yang, M., 2013. Detection of cancer biomarkers by piezoelectric biosensor using PZT ceramic resonator as the transducer. Biosens. Bioelectron. 46 (0), 155–161.

Triroj, N., Jaroenapibal, P., Shi, H., Yeh, J.I., Beresford, R., 2011. Microfluidic chip-based nanoelectrode array as miniaturized biochemical sensing platform for prostate-specific antigen detection. Biosens. Bioelectron. 26 (6), 2927–2933.

Wang, J., 2006. Electrochemical biosensors: towards point-of-care cancer diagnostics. Biosens. Bioelectron. 21 (10), 1887–1892.