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Integrated microfluidic platform for the electrochemical detection of breast cancer markers in patient serum samples

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A microsystem integrating electrochemical detection for the simultaneous detection of protein markers of breast cancer is reported. The microfluidic platform was realized by high precision milling of polycarbonate sheets and features two well distinguishable sections: a detection zone incorporating the electrode arrays and the fluid storage part. The detection area is divided into separate microfluidic chambers addressing selected electrodes for the measurement of samples and calibrators. The fluidic storage part of the platform consists of five reservoirs to store the reagents and sample, which are interfaced by septa. These reservoirs have the appropriate volume to run a single assay per cartridge and are manually filled. The liquids from the reservoirs are actuated by applying a positive air pressure (*i.e. via* a programmable syringe pump) through the septa and are driven to the detection zone *via* two turning valves. The application of the realised platform in the individual and simultaneous electrochemical detection of proteic cancer markers with very low detection limits are demonstrated. The microsystem has also been validated using real patient serum samples and excellent correlation with ELISA results obtained.

Introduction

Recent advances in the fabrication of microfluidic platforms initiated during the late 90s have facilitated the realisation of micro total analysis systems as they enable the miniaturization, integration and automation of (bio)chemical assays.[1] These platforms combine a series of fluidic unit operations (transport, metering, mixing, incubation, detection, *etc*.)[2] within a device with interconnected elements, preferably in a single packaged support manufactured using cheap polymeric materials.[3]

The integration of miniaturised fluidic handling and delivery systems with chemical and biochemical sensors provides applied scientists with powerful tools for in-field measurements away from central laboratories.[4,5] Amongst the various classes of elements able to transduce a chemical or biochemical event into a measurable signal, electrochemical platforms undoubtedly present the most promising advantages. Electrodes of all type, sizes and geometries can easily be integrated within a microfluidic platform and provide excellent sensitivity and versatility in comparison to other transduction techniques based on, for example, optical or mass sensing.[6] Furthermore, the associated electronics used to drive the electrochemical detection and signal processing can also be easily miniaturised and integrated onto the same platform by carefully designing application specific integrated circuits.[7]

There are some examples in the literature of electrochemical immunoassays on microchip platforms. Strategies for electrical decoupling have been implemented for conventional capillary electrophoresis with electrochemical detection (CE-EC) that avoids the inherent difficulties in isolating both separation and detection fields, allowing the integration of CE-EC in a photo-ablated polymer microdevice with electroosmotic driven flow.[8] Wang *et al.* have reported on a microfluidic device integrating pre-column reactions of alkaline phosphatase-labeled antibody (anti-mouse IgG) with the antigen (mouse IgG), followed by electrophoretic separation of the free antibody and antibody–antigen complex and amperometric postcolumn detection.[9] On-chip separation of free and bound antigens has also been achieved based on differences in isoelectric point using a multi-channeled column coated with a cation-exchange resin.[10] In this case, off-chip detection was based on the amperometric response of a ferrocene-labeled antibody which avoids the use of enzymatic substrates enabling the detection of histamine released in whole blood within 2 min.

A more advanced microfluidic device was developed by Ko and co-workers based on a poly(dimethyl siloxane) (PDMS) substrate molded by polymer casting bonded to a poly-(methylmethacrylate) (PMMA) bottom substrate fabricated by hot embossing.[11] Two inlet ports and an air vent are opened through the PDMS top substrate and the fluidic control was implemented by designing the microfluidic channels with various geometries of width, depth, and shape. The system was used for the amperometric detection of model analytes such as streptavidin and anti-ferritin antibodies carried out on gold electrodes integrated onto the PMMA bottom substrate. Alternatively, the

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screen printing technique can also be used to pattern the electrodes on the microfluidic platform.[12]

Cancer is one of the main causes of mortality worldwide, the cure for which has not yet been achieved and early diagnosis is critical to avoid a fatal outcome. Certain proteins can signal the presence or recurrence of cancer although only a few serum markers are truly specific for a disease in a defined organ. In cancer, the multi-factorial nature of oncogenesis and the heterogeneity in oncogenic pathways make it unlikely that a single biomarker will detect all cancers with high specificity and sensitivity. Multiplex analysis of panels of markers has thus the potential to provide information about patient prognosis and response to therapy that cannot be obtained by analysis of single markers. Hence, quantitative identification of biomarkers in a mixture without separation and at clinically relevant concentrations is a crucial requirement for the development of more effective and simpler diagnostic devices. Arrayed tests enable the use of pattern recognition approaches to assess disease changes, which are important in disease diagnostics and monitoring.[13]

In the present paper, we report on the development of a packaged amperometric immunosensor for the detection of cancer markers in real samples based on monolayers of a carboxylic acid terminated dithiol[14,15] supporting a monoclonal capture antibody that recognizes the target antigen and is detected by an in-house prepared peroxidase–antibody reporter conjugate. The microfluidic platform has been engineered to contain two separated areas: a detection zone incorporating an array of 16 working electrodes and a fluid storage section. The detection zone is divided into separate microfluidic chambers addressing selected electrodes for the measurement of samples and calibrators. The fluidic storage part of the platform consists of five reservoirs to store the reagents and sample, which are interfaced by septa and actuated by applying a positive air pressure *via* a programmable syringe pump. In comparison with other existing microfluidic methods such as CE-based microchips,[16] this device has the advantage of integrating a series of microfluidic operations in a single platform, namely, reagent storage and transport, metering, incubation and detection. In addition, the design has been conceived in such a way that if the fluidic operations are fully automated, the assay only requires the end-user intervention of application of sample, highlighting the simplicity of its operation.

For this purpose, we have chosen a panel of clinically relevant tumour biomarkers, including the carcinoembryonic antigen (CEA), cancer antigen 15-3 (CA15-3) and prostate specific antigen (PSA) as model systems. CEA is present in about 95% of all colon tumours and 50% of breast tumours, and is also associated with ovarian carcinoma, lung cancer and others.[17] Therefore, it can either be part of a panel of cancer markers for different cancers or, more importantly, can also be used as an independent prognostic factor. The normal CEA levels in healthy adults lie in the range 3–5 ng mL^{-1}, although some benign diseases can increase these levels up to 10 ng mL^{-1}.[18] Cancer antigen 15-3 is a protein that is produced by normal breast cells. In many patients with cancerous breast tumors, there is an increased production of CA15-3, which is shed by the tumor cells and enters the bloodstream, making it useful as a tumor marker to follow the course of the cancer.[19] CA15-3 levels above the clinically relevant threshold (30 U mL^{-1}) are present in about 10% of women with early localized breast cancer and in about 70% of those with metastatic breast cancer.[20] CA15-3 may also be elevated in healthy people and in individuals with other cancers, conditions, or diseases, such as colorectal cancer, lung cancer, cirrhosis, hepatitis, and benign breast disease. Finally, PSA is a 30 kDa single-chain glycoprotein expressed predominantly by human prostate. It is usually present in the blood at low concentrations (<4 ng mL^{-1}) in healthy males and because of the correlation of PSA concentration with tumour volume and tissue specificity, its use as a tumour marker for prostate cancer has flourished over the past decade.[21,22]

Experimental

Reagents

Dithiol 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide ester (DT2-NHS) was purchased from SensoPath Technologies (Bozeman, MT). Stock solutions (1 mM) were prepared in acetonitrile, purged with argon and kept at −20 °C when not in use. Carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) were purchased from Scipac Ltd (Kent, UK). Monoclonal anti-CEA, anti-CA15-3 and anti-PSA antibodies were kindly provided by Fujirebio Diagnostics AB (Göteborg, Sweden). The affinity column Freezyme® Conjugation purification kit and *N*-succinimidyl-*S*-acetylthioacetate (SATA) were purchased from Pierce.

Preparation of antibody–HRP conjugates

One milligram of the corresponding monoclonal antibody was dissolved in 0.1 M MES, 0.15 M NaCl, pH 7.2, to obtain a final volume of 1500 μL. A 10 μL aliquot of a solution containing 1 mg of SATA was then dissolved in 100 μL of dried DMSO and added to the antibody solution and the mixture was gently stirred for 30 minutes at room temperature, protected from light. A deacetylation solution (100 μL) consisting of 0.5 M hydroxylamine hydrochloride in 0.15 mM NaCl, pH 7.2, was added to the SATA–antibody mixture and allowed to react for 2 hours at room temperature, again protected from light. Subsequently, 1 mg of maleimide activated HRP was added and the solution was incubated for 90 minutes at 37 °C. To deactivate the maleimide groups and avoid further non-desired reactions, an aliquot of 2-mercaptoethanol was added to the solution to a final concentration of 0.15 mM and stirring continued for 15 minutes.

Purification of the resulting conjugate was carried out with the metal chelate affinity chromatography Freezyme® conjugation purification kit. Finally, the pure conjugate was concentrated by passing through a molecular weight cut-off filter of 50 kDa at 7500 rpm for 5–10 minutes and the conjugates were washed with Milli-Q water.

Fabrication of the electrode arrays and microfluidic cell

The lithographic process employed to fabricate the electrode arrays was achieved as follows (Scheme 1): a circular borofloat glass wafer ($\phi = 12$ cm) was fully covered with 5 μm of LOR5214 lift-off resist, later UV patterned to reveal the reference electrode areas and corresponding connections. Ten nanometres of

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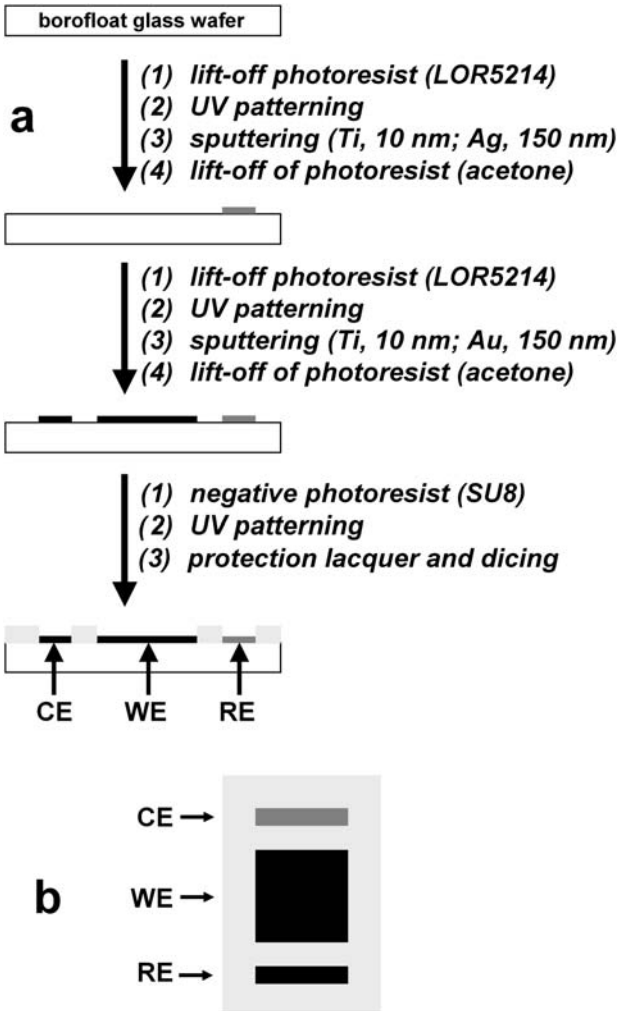
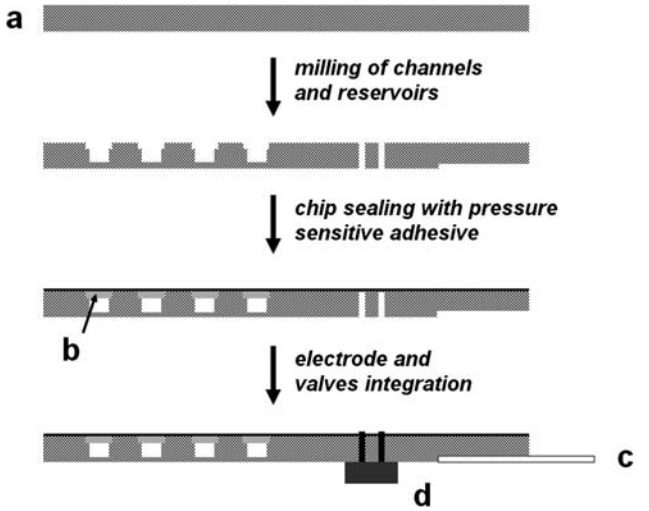


Scheme 1 (a) Fabrication process for the electrode array; (b) top view of the electrodes arrangement: CE: counter electrode.

titanium, used as a glass/metal adhesion promoter, were sputtered followed by a 150 nm thick silver layer. The patterned substrate was fully coated a second time with the same lift-off resist and UV patterned to reveal working and counter electrode areas and their corresponding connections, and finally coated with a 10 nm thick titanium layer followed by a 150 nm thick gold layer. The remaining photoresist was finally removed with acetone, revealing the planar gold and silver patterns. Since the connection lines between the electrodes and their connection points have to cross the fluidic channels, an insulation of the chip was required. Thus, an SU8 negative photoresist was applied to the whole surface of the chip and was only partially removed at the electrode and connection areas. This ensured that only electrode would be later exposed to the sample fluids. In order to extend storage lifetime by reducing contamination of the electrode surfaces, the patterned wafers were coated with a 5 μm protection layer of AZ6632, which can be easily removed by rinsing the arrays with acetone, isopropanol and water immediately prior to use.

The microfluidic cell was fabricated in a polycarbonate substrate and diced to have the format of a microscope slide



Scheme 2 Fabrication process for the microfluidic platform: (a) polycarbonate block; (b) septa; (c) electrode array; and (d) valves.

(75.5 × 25.5 × 5 mm, Scheme 2). The channels (1 mm depth), reservoirs and valve holes were realised by high-precision milling and sealed with a layer of a pressure sensitive adhesive. Valve bodies were fabricated by injection moulding and integrated on the chip by means of a metallic holder.

Electrochemical measurements

The working electrodes were spotted with 5 μL of a stock solution of DT2-NHS for 3 hours followed by rinsing with copious amounts of acetonitrile. The capture antibodies were covalently immobilized on NHS-activated SAM by spotting with 5 μL of a 0.5 mg mL^{-1} solution of antibody in 10 mM acetate buffer, pH 5.0, for one hour at 37 °C. The remaining NHS active ester sites were blocked with 1.0 M ethanolamine, pH 8.5, for 30 minutes at 37 °C. The modified arrays were then assembled in the microfluidic cells by means of a double-sided adhesive gasket.

Antigens were diluted in phosphate buffer, pH 7.2, to the desired concentration, while real serum samples were applied without any dilution for 2 minutes at room temperature. After rinsing with PBS, a 1 μg mL^{-1} aliquot of the prepared HRP-labelled antibody was added and left to incubate to form a sandwich immunocomplex for 2 minutes. The amperometric measurements were carried out by first recording the background response at −0.2 V in PBS followed by injection of a mixture of 1 mM hydroquinone/1 mM H_2O_2 in PBS, pH 6.

Results and discussion

Microfluidic platform design and fabrication

The microfluidic platform was realized by high precision milling of polycarbonate sheets, which offers flexibility and rapid turn over of the desired designs. It contains two well distinguishable sections: a detection zone incorporating the electrode arrays and the fluid storage part (Fig. 1).

The detection zone is divided into separate microfluidic chambers addressing selected electrodes for the measurement of samples and calibrators depending on the assay to be realised.

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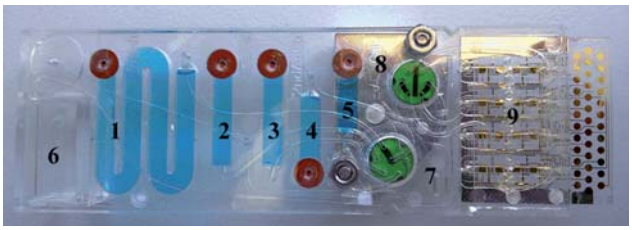

Fig. 1 Microfluidic platform components: (1) washing buffer reservoir, (2) substrate reservoir, (3) secondary antibody reservoir, (4) sample reservoir, (5) calibrator reservoir, (6) waste reservoir, (7) valve A, (8) valve B, and (9) detection zone with integrated electrode array.

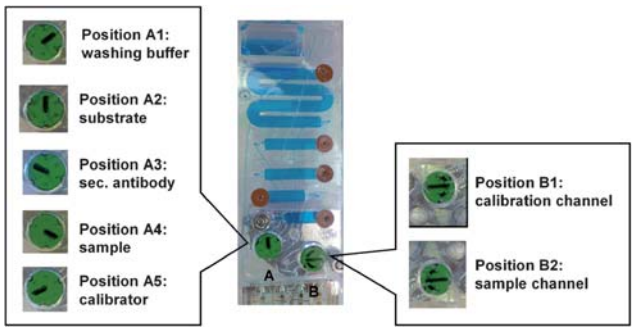

Fig. 2 Valve positions in the microfluidic platform.

The electrode arrays were fabricated using photolithographic deposition technologies in order to realize three-electrode cells comprising of gold counter and working electrodes as well as silver reference electrode. They consist of 16 independently addressable gold working electrodes (1×1 mm^2) in a 4×4 arrangement placed between a silver pseudo reference (0.2×1 mm^2) and a gold counter electrode of the same size in order to create 16 planar electrochemical cells. The working principle is similar to any classical three-electrode cell in which the potential at the working electrode is applied *versus* the reference electrode, most commonly made of silver, and the counter electrode (made of an inert material, in this case gold) serves to close the electrochemical circuit. All reference and counter electrodes were short circuited at their connection points to realise an array of 16 working electrodes surrounded by common reference and counter electrodes. The array also features an equalized length to the connection pads from the working electrodes to the connector in order to avoid differences in electrical resistance between these two points. The electrode array is integrated on the fluidic chip using a double-sided medical grade adhesive foil of 50 μm thickness previously laser machined to generate microchannel structures of 1 mm width and is connected *via* pogopin connectors to an external multichannel potentiostat.

The fluidic storage part of the platform consists of five reservoirs to store the reagents and sample, which are interfaced by rubber septa plugged into the injection ports. These reservoirs have the appropriate volume to run a single assay per cartridge and are manually filled. A waste reservoir is placed at the top left corner of the chip to collect the liquids from the different reservoirs during the assay.

Operation of the microfluidic platform

The liquids from the reservoirs are actuated by applying a positive air pressure (*i.e. via* a programmable syringe pump) through the septa and are driven to the detection zone *via* two turning valves (Fig. 2). Valve A has 6 positions and serves to select one of the five reservoirs from which the liquid is pumped or a venting position used to empty the microfluidic channels and connected directly with a waste reservoir. The second valve selects to which chamber in the detection zone the selected liquid will be driven, *i.e.* calibrator or sample chambers and has two positions. For example, the combination of positions A1–B1 will drive the washing buffer to the calibrator channel. No liquid cross-contamination is observed due to the efficiency of the sealing of the valves, in spite of the apparent complexity of the fluidic design. However, care should be taken to avoid the use of very high air pressure to drive the liquids to avoid the generation of air bubbles inside the channels.

Fig. 3 shows a flow diagram of the sandwich assay protocol employed in this work for the detection of proteic cancer markers. The first step is a rehydration of the sensor surface, followed by the successive incubations of sample and HRP-labelled secondary antibody for a given period of time (see next section). Detection is carried out amperometrically after injection of the substrate mixture. Although the integrated microfluidic platform has been devised to be disposable, the chip can be disassembled following measurement and most parts can be re-used.

On-chip optimisation of assay time

Incubation times are one of the bottlenecks in assay development since it not only influences the overall assay time but also the analytical performance of the immunosensor and the time required for the complete reaction between the target and the immobilized biorecognition probe.[15] For the optimisation of the incubation times, electrode arrays modified with the capture antibody were integrated in the microsystem and the target and reporter conjugate were injected into the system and incubated for variable times. Fig. 4 shows the influence of incubation time on the amperometric response of the system in the presence of 100 ng mL^{-1} CEA. As can be seen, the optimum combination of incubation times was 2 minutes for each sample and reporter conjugate. These very fast incubation times contrast greatly with those commonly used in ELISA (30–60 minutes) and, as has been previously shown, highlight the importance of the use of microfluidic systems in a speeding-up of analytical processes.[23,24] A similar result was obtained with the other markers, allowing the possibility of a fast multiplexed assay.

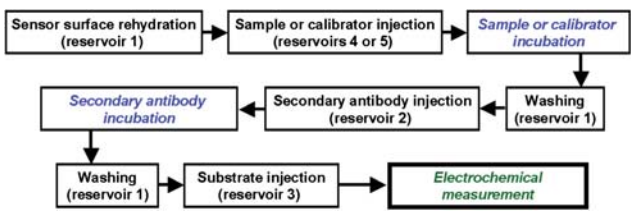

Fig. 3 Assay structure indicating the reservoirs employed in each step.

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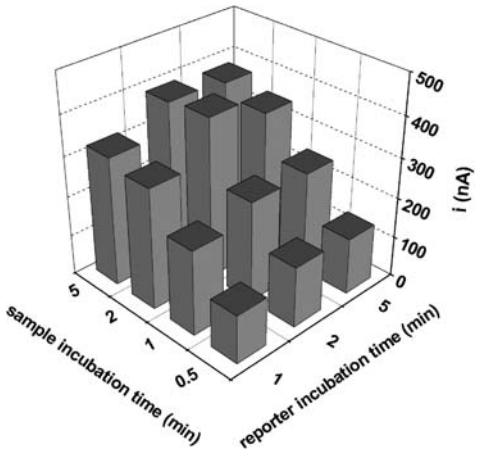

Fig. 4 Amperometric responses obtained for the detection of CEA at different sample and reported antibody conjugation times.

Proof of principle of individual detection of protein markers

The approach for multiplexed electrochemical detection consists in the immobilisation of several marker probes on an electrode array, each electrode being functionalised with a particular probe selective to the desired target. The immobilization of antibodies against CEA, CA15-3 and PSA was achieved *via* crosslinking to a bipodal dithiol chemisorbed on gold electrodes.[14]

Multiplexed detection requires that the sensor is, in the first place, able to detect each individual marker individually with good sensitivity. Fig. 5 shows the calibration curves obtained for each marker. The amperomeric response increased linearly with antigen concentration in the studied range with a LOD = 0.2 ng mL^{-1} for CEA, 5.2 U mL^{-1} for CA15-3 and 2 ng mL^{-1} for PSA. All these values are well below the commonly accepted concentration thresholds used in clinical diagnosis for these markers,[17,19,21] which demonstrates the sensitivity of the developed electrochemical assays. The markedly higher currents obtained with PSA is due to its lower molecular weight (34 kDa) as compared with CEA and CA15-3 (200 and 450 kDa, respectively), which increases the number of captured antigen molecules on the surface and thus generates a higher electrochemical signal after interaction with the labelled reporter conjugate.

Reproducibility studies

In order to assess the reproducibility of the on-chip electrochemical detection of cancer markers using the developed microfluidic cell, electrode arrays were modified with the usual surface chemistry and the same concentration of CEA was applied on all electrodes. Fig. 6 shows the amperometric responses obtained in the detection of 100 ng mL^{-1} of CEA. The average current value obtained was (391 ± 27) nA, with a residual standard deviation (RSD) of ~7% ($n = 16$). At lower CEA concentrations (10 ng mL^{-1}) the average signal was (89 ± 10) nA with RSD = 11% ($n = 16$), indicating an excellent degree of reproducibility of the microfluidic platform.

Multiplexed electrochemical detection of cancer markers

To test the possibility of simultaneous detection of several protein markers, electrode arrays were modified with anti-CEA and anti-PSA in alternating electrode spots. An antigen mixture composed of CEA (50 ng mL^{-1}) and PSA (20 ng mL^{-1}) was then applied to the sensor surface and the response obtained in the 16 channels was measured amperometrically (Fig. 7). As can be seen, the signal obtained in the respective channels is very reproducible, with RSD lower than 8% for both CEA and PSA, and the ability to differentiate both signals and detect CEA and PSA simultaneously is clearly demonstrated.

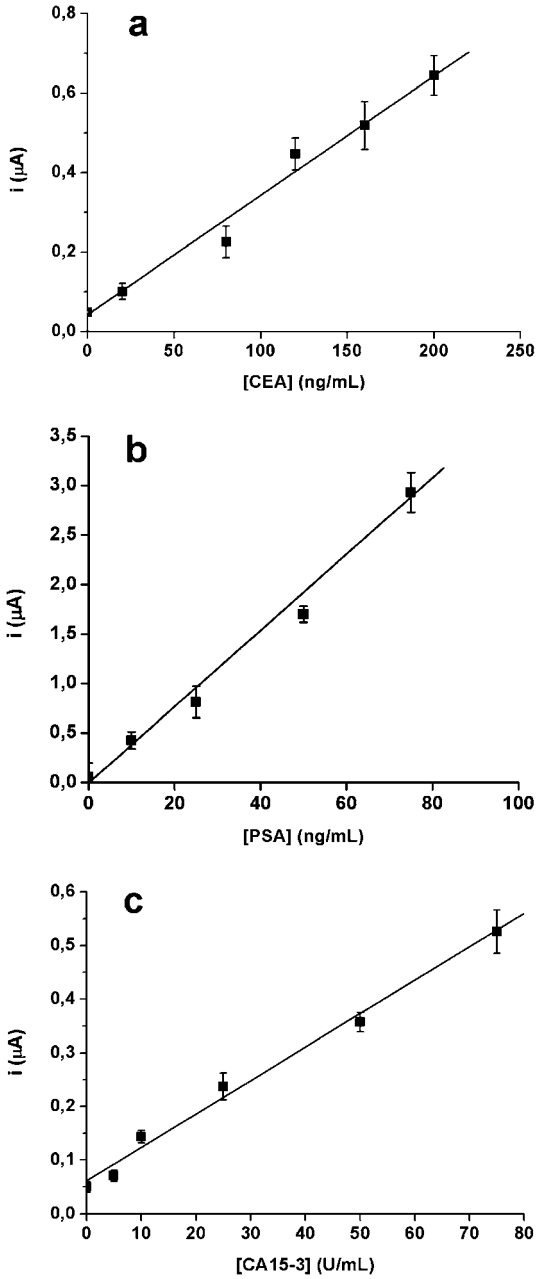

Fig. 5 Calibration plots obtained for the amperometric detection of CEA (a), PSA (b) and CA15-3 (c).

Real sample analysis

The developed immunosensor was applied to the detection of CEA in real samples using serum samples from cancer patients.

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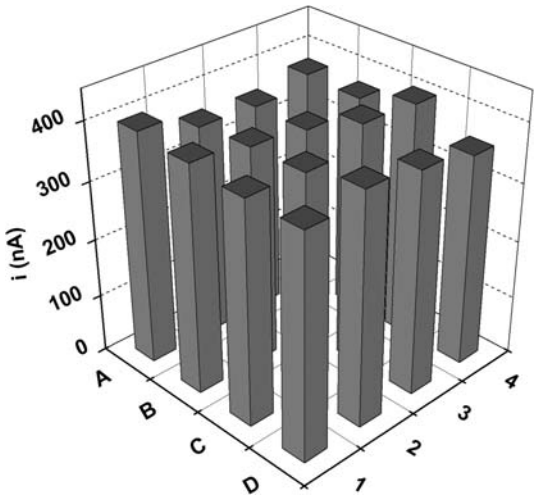

Fig. 6 Current values obtained in the amperometric detection of 100 ng mL^{-1} CEA using the microfluidic platform.

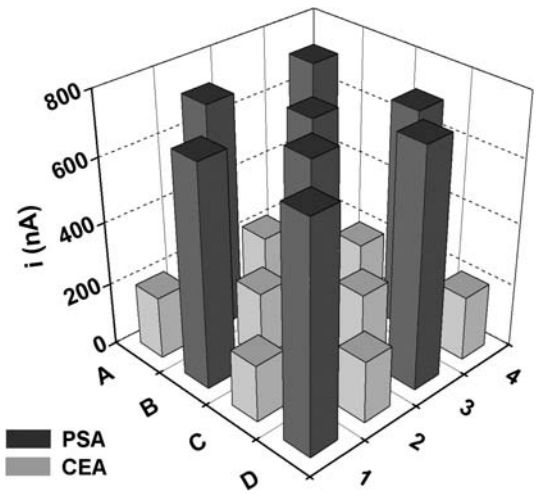

Fig. 7 Amperometric responses obtained for the simultaneous detection of CEA (50 ng mL^{-1}) and PSA (20 ng mL^{-1}) using the microfluidic platform.

Samples were taken and divided into two aliquots, one for ELISA and one for immunosensor detection. As can be seen from Table 1, samples 1–4 showed values lower than 10 ng mL^{-1} of CEA, which is the commonly accepted cut-off value used in clinical diagnosis.[17] These samples are from patients under cancer remission and can be thus considered as normal values. Therefore, the device can differentiate between normal and ill patients with an excellent correlation between the CEA levels obtained with the immunosensor and ELISA. Therefore, the microfluidic platform not only functions with high accuracy and sensitivity but also matches the performance of an established technique such as ELISA with the advantage of vastly reduced assay time and reagent consumption.

Table 1 CEA levels (in ng mL^{-1}, duplicate measurements) measured in serum samples with the developed immunosensor and ELISA

Sample	Immunosensor	ELISA
1	4.4 ± 1.7	3.6 ± 0.7
2	5.8 ± 1.1	6.9 ± 0.4
3	6.8 ± 1.4	5.9 ± 0.6
4	7.7 ± 0.7	8.9 ± 1.1
5	20.1 ± 2.9	22.5 ± 1.1
6	43.6 ± 2.7	41.5 ± 3.3

Conclusions

In this work, we demonstrate the versatility of a microsystem integrating electrochemical detection in the individual and simultaneous detection of breast cancer markers in real samples. The microfluidic platform was realized by high precision milling of polycarbonate sheets and features two well distinguishable sections: a detection zone incorporating the electrode arrays and the fluid storage part. The detection zone is divided into separate microfluidic chambers addressing selected electrodes for the measurement of samples and calibrators. The fluidic storage part of the platform consists of five reservoirs to store the reagents and sample, which are interfaced by septa. These reservoirs have the appropriate volume to run a single assay per cartridge and are manually filled. The liquids from the reservoirs are actuated by applying a positive air pressure (*i.e. via* a programmable syringe pump) through the septa and are driven to the detection zone *via* two turning valves. The application of realised platform in the individual and simultaneous electrochemical detection of proteic cancer markers with very low detection limits is demonstrated. The microsystem has also been validated using real patient serum samples and excellent correlation with ELISA results obtained.

This device has the advantage of integrating the microfluidic operations required to execute on-chip electrochemical immunoassays in a single platform—namely, reagent storage and transport, metering, incubation and detection. In addition, the design has been conceived in such a way that with fully automated fluidic operations, the assay only requires the introduction of microlitre amounts of sample by the end user, thus facilitating its operation and vastly reducing the reagent consumption and assay time.

The results presented here on the successful integration of fluidic storage of assay reagents with electrochemical detection are thus in the direction of achieving low cost disposable cartridges amenable for point of care applications. Advances in microfabrication and packaging necessarily need to meet with the specifications of stable reagents for storage[25] with the objective of achieving a fully disposable detection platform.

Acknowledgements

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