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Continuous flow micro-cell for electrochemical addressing of engineered bio-molecules

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Abstract

A micro-cell consisting of a planar array of Au working electrodes, covered by a glass reservoir (600 nl capacity), connected with perpendicular capillaries and provided with an Ag/AgCl wire reference electrode (RE) has been manufactured. Addressed on-chip immobilization and sensing of biomolecules was demonstrated by the immobilization of histidine (HIS) tagged alkaline phosphatase (AP) under continuous flow (10 μl/min) conditions on the base of electrochemically deposited multilayer (EDM) of cysteamine modified by nitrilotriacetic acid (NTA). The presented method allows the immobilization of different biomolecules on the ready-to-use reservoir covered chip without needs of manual biomolecule deposition. Further on, the detection of analytes can be performed using the electrochemical measurements. Since the methods of biomolecule deposition and sensing are both electrochemical, this allows simplifying the production technology using cheaper detection instruments compared to prevailing present-day optical detection modes.
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Keywords: Electrochemical micro-cell; Continuous flow; Addressed immobilization; Protein chip technology; Nickel-NTA

1. Introduction

The recent technology of biochips is mainly based on non-specific immobilization of biomolecules (e.g. nanolitre spotting on preactivated surfaces) and optical detection methods (e.g. fluorescence) [1–11]. Such systems, although already available on market, face a serious limitation concerning their further miniaturization down to the submicron scale. Moreover, the concept of the lab-on-chip based on the above described technology brings limitations which will not fulfil the demands for cheap and sensitive instrument with high degree of operational use and portability [12]. The solution could be found in hybrid technology based on well-introduced nanolithography [13–15], 3D thick film technologies, self-assembling techniques [16–18] and various modes of electrochemical detection.

The key task still not satisfactorily solved is the method of locally specific immobilization of biomolecules on the electrode microarray. The use of nanopipeting as well as other recently developed methods (inject printing, laser beam assisted patterning, etc.) [19–21] brings serious technology complications during the production of the enclosed microchamber system since these biomolecules has to be deposited prior to enclosement of the chip. This can result in the lost of activity, orientation and local specificity of the immobilized species.

The concept of electrochemical biochip system brings many advantages and potentials: (i) the electrochemical methods are highly sensitive; (ii) they can be used for detection of enzymatic redox reactions as well as for hybridization

Abbreviations: AP, alkaline phosphatase; CYS, cysteamine; CA, chronoamperometry; CV, cyclic voltammetry; DPV, differential pulse voltammetry; EDM, electrochemically deposited multilayer; GA, glutaraldehyde; HIS, histidine; Ni-NTA, nickel-nitrilotriacetic acid chelator; PCB, printed circuit board; PB, phosphate buffer 0.1M (pH 7.0); RE, reference electrode (Ag/AgCl); SAM, self-assembled monolayer; WE, working electrode

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of DNA; (iii) the miniaturization of the conductive parts (electrodes) is already well developed and the solution lies in the field of nanolithography; (iv) the electronic circuits for signal and data processing can be miniaturized and can create the part of lab-on-chip instrument itself; (v) there is a plenty of electrochemical methods which can be used on the same electrode; (vi) electrochemical methods are useful not just for detection of the biomolecule presence or its activity on electrode, but also for locally directed immobilization of biomolecule species on it.

An original deposition procedure, that uses chronoamperometry (CA), was developed in our laboratory [22–27] obtaining oriented and reversible immobilisation of HIS_{6x}-tag engineered proteins and their on-chip purification from bacterial crude extracts. This method started with the electrochemical deposition of multilayers of CYS on Au or Pt surfaces and further chemical synthesis of a spacer with a Ni-NTA end.

Recently, we have designed and manufactured the micro-cell which consists of a micro-array of Au electrodes lithographically patterned on a silicon chip covered by a micro-machined glass reservoir [23–28] provided with vertical connections to capillaries [29] that allow an external pumped continuous flow of the analyte.

In this paper, we aim to demonstrate the use of electrochemical continuous flow micro-cell for obtaining the addressed immobilization on one single electrode element of the micro-array. The demonstration was performed by immobilization of AP with an $(HIS)_6$-tag. CYS EDM and chemical synthesis of the spacer were performed on one single Au element of the micro-array. Afterwards the protein was addressed and immobilized on the Ni-NTA spacer group on that specific planar gold working electrode (Au WE), in few minutes and under flow condition. The presence of the AP on the electrode was verified by detecting its activity upon ascorbate-2P.

2. Materials and methods

2.1. Planar array of gold electrodes and microcell

The micro-cell is presented in Fig. 1 (front and top view) and consists of a planar array of Au electrodes on a silicon chip covered by a borofloat® glass reservoir provided with perpendicular adaptors to capillary tubes having and outer diameter of 330 μm. The WEs have a diameter of 70 μm; the four CE have a diameter of 140 μm; they have been patterned by means of optical lithography on layers obtained by evaporation or chemical vapor deposition techniques. The chip size is 12 mm × 13 mm. The four CEs have been short-circuited and symmetrically placed in the peripheral part of the μ-chip. The glass reservoir (600 nl capacity, 0.15 mm depth) has been manufactured by means of wet etching through a lithographically patterned Au/Cr mask.

The capillaries connections were built out of silicone adaptors with cylindrical and conical shape, the larger of them being fixed by means of a particle free silicone based adhesive (see Fig. 2). The adhesive polymerizes in situ becoming an opaque pellicle. Special care has been taken in order to determine the optimal quantity that should be applied on the surfaces in contact avoiding both an incomplete bonding and the entry of the glue in the opening area.

The adaptors have been cut to the appropriate length (1.5 mm, respectively, 5 mm). An in-house built tool of mini-lathe type has been used in order to obtain an appropriate

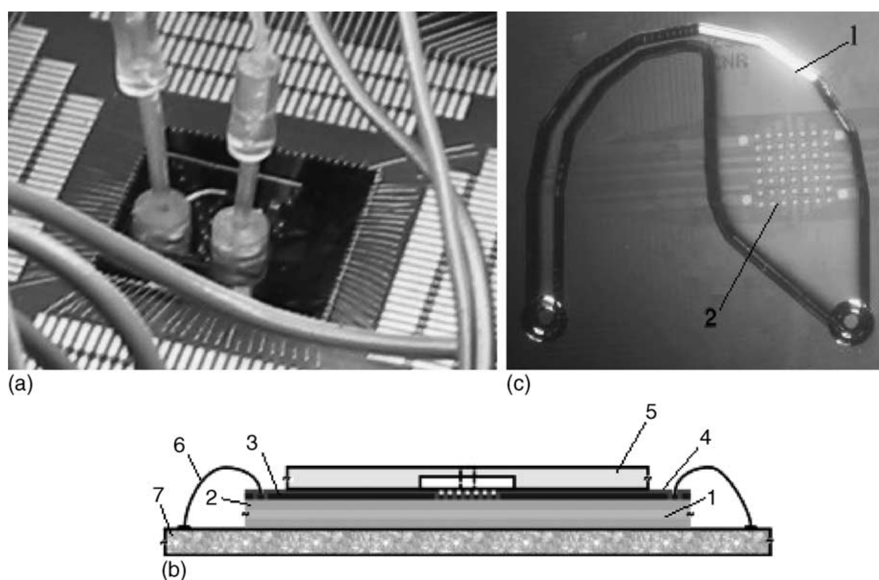


Fig. 1. (a) Continuous flow μ-chamber with vertical capillary connections. (1, glass reservoir; 2, inlet capillary; 3, oulet capillary; 4, aluminum wire connections to the PCB); (b) schematic cross view of the continuous flow micro-chamber (1, Si; 2, SiO_2; 3, Au; 4, silicon nitride; 5, glass; 6, Al wire; 7, printed circuit board); (c) top view of the glass reservoir (1) bonded over the metallic electrodes array (2).

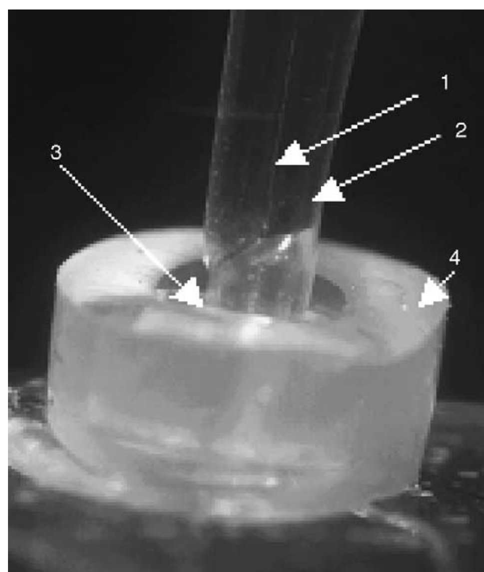


Fig. 2. Capillary tube (1) connected to the reservoir opening by means of the conical (2,3) and cylindrical (4) adaptors (stereo micro-photo) (outer capillary diameter = 0.33 mm; inner diameter of the conical adaptor = 0.35 mm; outer diameter of the conical adaptor = 0.9 mm).

quality of the cut surfaces. The good flatness of these surfaces insures the perpendicularity between the adaptor axis and the reservoir surface. The silicone cylindrical adaptor has to be centered over the reservoir opening allowing the capillary tube to pass through the opening and enter the reservoir chamber. For centering the cylindrical adaptor an in-house developed coaxial device has been used. The top view and the transversal section of the opening are presented in Fig. 3. The maximum and minimum diameters are, respectively, 650 μm (*D*) and 350 μm (*d*). The outer diameter of the

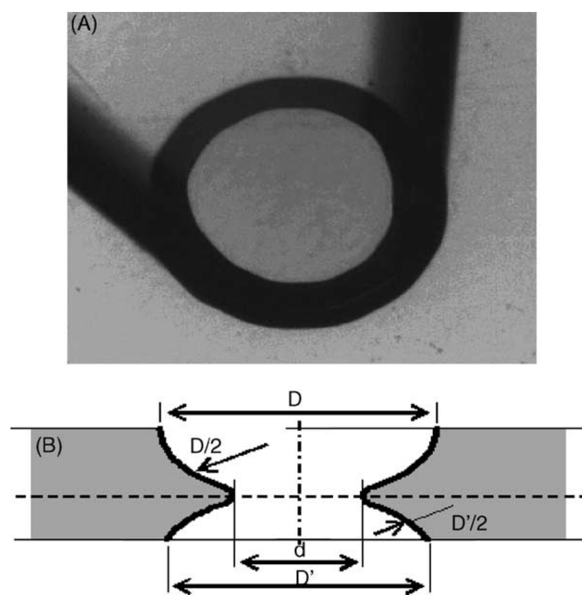


Fig. 3. Top view of the reservoir opening (upper surface focusing) (stereo microphoto) (Panel A). Schematic transversal view of the opening: $D = 0.65$ mm; $d = 0.45$ mm; $D' = 0.6$ mm (Panel B).

cylindrical silicone adaptor is 2400 μm and its inner diameter is 850 μm. The conical adaptor (which has a minimum inner diameter of 335 μm and an outer diameter of 600–950 μm) acts as a buffer between the capillary tube and the cylindrical fixed adaptor due to the elasticity of both adaptors. Misalignments of the cylindrical adaptor up to 100 μm are compensated by the elasticity of the adaptors still allowing the capillary tube to enter the reservoir chamber under the opening.The glass reservoir with adaptors has been bonded over the microarray-chip by means of an UV in situ polymerized Loctite™ product. The obtained micro-cell (see Fig. 1a and b) has been mounted on the PCB and the Al wires have been soldered. The Kelvin type electrical connections to the amperometric unit are obtained *via* Al wires soldered on a standard RS™-PCB(4) (6 cm × 6 cm) and *via* optimized plugs mounted on an in-house built adapting board.

2.2. The electrochemical measurement using micro-cell

The current intensity on the WE was registered with a multiplexed (48 channels) Autolab™ PGSTAT 10 potentiostat. The RE is an Ag/AgCl wire fitted in a Y-shaped piping at the outlet capillary. This set-up allowed the electrochemical measurements as CV and CA. The flow rate of 10 μl/min was obtained by means of an external peristaltic pump Gilson™ MiniPulse 3.

2.3. Deposition of the EDM layer of cysteamine and synthesis of NTA chelator

Here we have followed method already described [23–28]. Briefly, the deposition of EDM of CYS was obtained by applying the potential +850 mV versus Ag/AgCl on WE nr.4 (10 mM cysteamine in 25 mM PB, pH 7.5). The synthesis of the Ni-NTA chelator [27] followed the common procedure: (i) GA 12.5% (v/V) in PB for 1 h, (ii) N_α-N_α-bi (carboxymethyl)-L-lysine hydrate (NTA) 5% (w/V) in PB for 1 h, (iii) $NiSO_4$ 1% (w/V) in distilled water for 15 min.

3. Results and discussion

3.1. Manufacturing of array and outer connections of the micro-cell

The previously manufactured micro-reactor (a planar array of 7 × 7 metallic electrodes covered by a borofloat® glass reservoir with a chip size of 12 mm × 13 mm and around 0.6 μl volume), provided with input and output openings, has been coupled to fused-silica capillaries of 220–400 μm outer diameter. The perpendicular coupling of these capillaries on the micro-reactor plane has been approached. The capillaries have been inserted in the reservoir's openings and leaned by means of silicone adaptors with cylindrical and, respectively, conical shape.

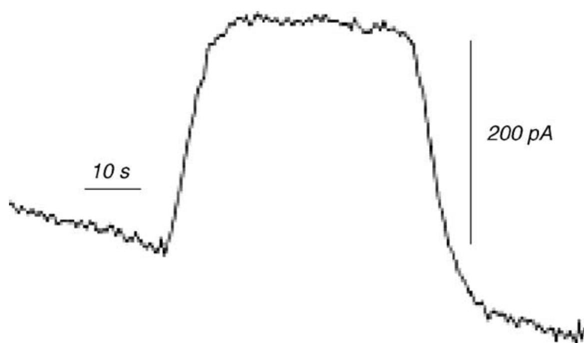


Fig. 4. Continuous flow chronoamperometry of a 1 mM H_2O_2; electrode test. Potential applied on the working electrode no.4 $E = 600$ mV; 25 mM PB solution (pH 7.5, 100 mM KCl).

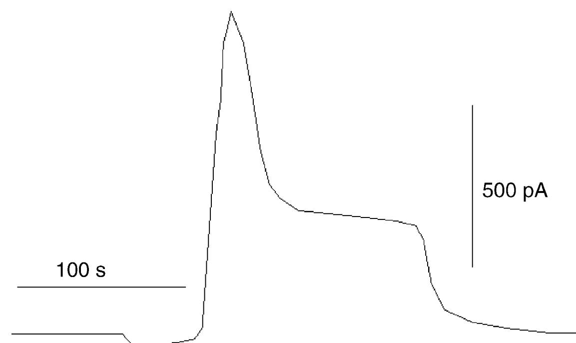


Fig. 5. Continuous flow chronoamperometry during the electrochemicall deposition of cysteamine multilayer (10 mM in PB) on WE no. 4 ($E = 850$ mV).

The cylindrical silicone-rubber adaptor was glued in situ on the micro-reactor surface by means of a silicone-based adhesive. The conical silicone adaptor acted as a buffer between the capillary tube and the cylindrical fixed adaptor. The elasticity of the adaptors allowed the capillary tube to pass through the opening and enter the below reservoir chamber.

In-house built devices have been manufactured for cutting and aligning the silicone adaptors. The capillaries have been further connected to an external pump and the continuous flow of 10 μl/min was performed. Experimental electrical connections to the signal, power and measuring unit, have been developed (by using standard PCB, Al wires, adapting board, mini-plugs, connectors), in order to allow the individual addressing of the electrodes. A Ag/AgCl RE was inserted in the output capillary.

3.2. Electrochemical addressing of alkaline phosphatase

After establishing the electric and fluidic connections of the micro-cell, PB solution of KCl 0.1 M (pH 7.0) was bubbled for 15 min with N_2 prior to start and during all the measurements. The functioning of the electrode has been checked by performing CA of a 1 mM of H_2O_2, applying

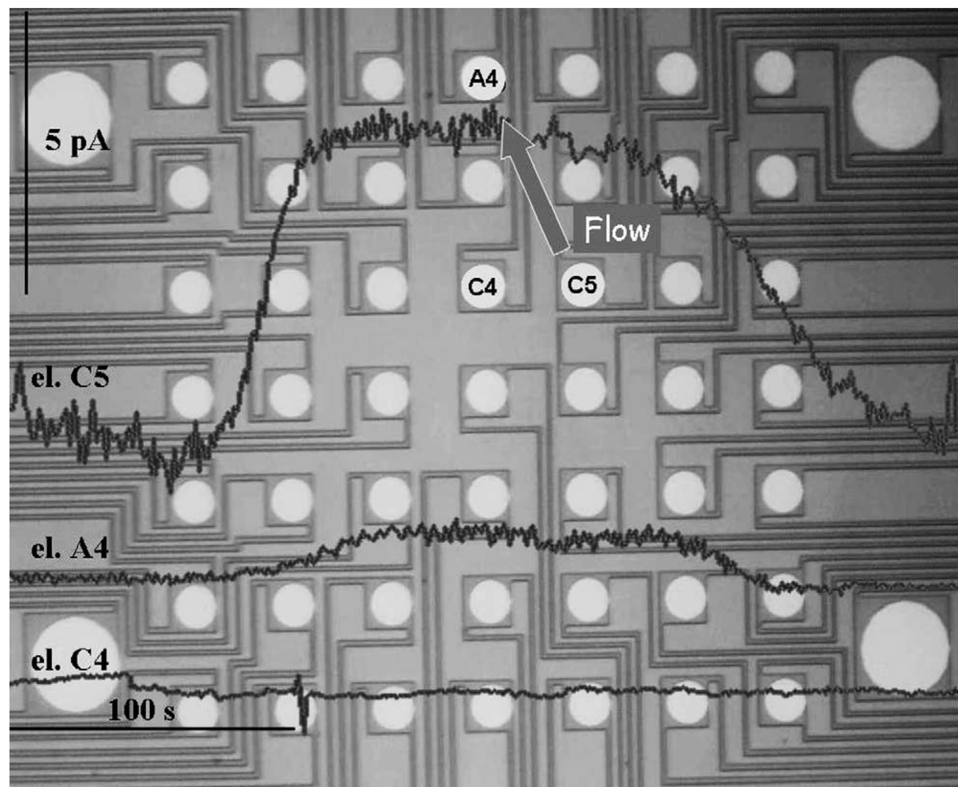
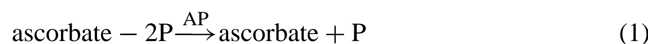


Fig. 6. Signals due to 1 mM ascorbate-2P, the substrate of AP, from WE n.4 (electrochemically deposed AP), WE n.3 (bare electrode) and WE n.16 (bare electrode, down the flow). Picture of the μ-array in the background shows the relative positions of WEs and the direction of the flow stream.

600 mV on the WE nr.4 versus the RE. The measured current is presented in Fig. 4. Further on, the deposition of CYS multilayer was done as described in Section 2.3. The oxidation current measured during the deposition of CYS EDM is presented in Fig. 5.

A steady state is attained after the first layer deposition. The current decreases when buffer solution replaces CYS solution. Following the deposition of EDM CYS layer, the synthesis of Ni-NTA chelator [27] was performed according to a step by step chemical synthesis (see Section 2.3).

The purified $(HIS)_6$-AP was immobilized on the surface of Au-CYS-Ni-NTA modified micro-electrodes in flow conditions (flow rate = 10 μl/min). Amperometric measurements of AP activity onto the WEs were done in the μ-flow-cell, continuously fed with buffer. Fig. 6 reports the experimental results that proved the specific deposition of $(HIS)_6$-tag AP on WE nr.4 in a micro-array with our original procedure based on electrochemically addressed CYS and organic synthesis of a Ni-NTA end. Only one WE (nr.4) was polarised at 0.85 V versus Ag/AgCl RE and 1 mM CYS has been loaded into the micro-chamber under continuous flow (10 μl/min) for 5 min. After GA (12.5%), 10 mM N_α-N_α-bi (carboxymethyl)-L-lysine hydrate and washing treatments with PB, performed under continuous flow at the same flow rate, the $(HIS)_6$-AP has been loaded into the micro-chamber for 5 min. The expected immobilization of this protein by Ni-NTA chelator only on the WE nr.4 has been verified using the substrate of AP, i.e. ascorbate-2P. The following reaction, catalysed by AP, gives ascorbate anion as a product, which can be chrono-amperometrically detected at 400 mV:



$$\text{ascorbate} - 2\text{P} \xrightarrow{\text{AP}} \text{ascorbate} + \text{P} \qquad (1)$$

Fig. 6 reports the activity of $(HIS)_6$-AP only on the WE nr.4 while the WE nr.3 did not give any signal due to ascorbate anion. This means that AP activity is not present or not detectable on all the other WEs in the micro-array. The small signal observed on WE nr.16, which is positioned down the flow stream, is due to the ascorbate anion produced on WE nr.4. When the direction of flow stream was reversed, the signal on WE nr.16 was eliminated contrary to WE nr.4.

4. Conclusions

A technical solution for micro-fluidic interfacing an on-chip microreactor with external sources has been designed, experimentally obtained and tested. Electrochemical deposition of CYS layers on Au WE surface at 0.85 V versus RE was applied to the micro-array assembled in the micro-flow cell. Immobilization of biosensing molecules and electrochemical analysis were performed in flow condition. The previously stated theoretical possibility to address biomolecules on a single Au electrode in a micro-array [26] by applying an electrochemical potential, has been experimentally proved. Unspecific binding of biomolecules on not treated WEs was not detected.

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Biographies

Jan Maly received his MSc degree in general biology and chemistry (2000) from the University of Jan Evangelista Purkynje. Recently, he is finishing his PhD on Faculty of Biology at the University of South Bohemia. His current fields of interest are the studies of the structure and functional properties of protein monolayers, development of new immobilization methods of biomolecules and application of conductive polymers for mediator-less electron transport from photosynthetic proteins.

Michaela Ilie received her PhD degree in technical sciences/optoelectronics (1999) from the University "Politehnica" of Bucharest. She is a senior research fellow of the same university (LAPI-group) recently granted by ENEA-Casaccia as international fellow in 2004-2005 (biosensors group). Her main area of expertise regards lithographic techniques for micromachining of silicon, polymers or glass. Her current field of interest is application of micromachining techniques in order to obtain microanalytical devices for biosensors (micro-array of electrodes, reservoirs and microchannels and their fluidic connections to the outside world, low temperature bonding techniques).

Vittorio Foglietti received his Laurea in Physics in 1982 and is now principal researcher in the staff of IFN-CNR and leader of the MEMS group. His current field of interest is the development of micromachining technologies (mainly plasma assisted etching and deposition) for sensors and transducers applications.

Roberto Pilloton obtained his PhD degree in chemistry in 1986. He is experienced in electrochemical biosensors and analytical chemistry and has been involved in research in biosensor field since 1983. His expertise is in enzyme immobilization techniques, electrochemical detection, flow systems, thick film technologies and environmental, clinical and food analysis.