Development of a monoclonal antibody based potentiometric biosensor for terbuthylazine detection

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Abstract

In the present paper a new potentiometric biosensor for terbuthylazine (TBA, a herbicide widely utilized in agriculture) suitable for this determination in water samples is shown. As previously described, monoclonal antibody (mAb) showing high specificity for the analyte was utilized as biological element to perform the biosensor assay. A light addressable potentiometric sensor (LAPS), measuring NH_3 ions concentration in term of pH variations by a urease conjugated, was chosen as transducer element. In this system a competitive reaction occurs between herbicide TBA and a TBA–protein-conjugated coat on a cover slip to bind a specific mAb urease labeled. The bind of the specific mAb involves NH_3 production in an inversely proportional way to TBA concentration in the analyzed sample. Using TBA solution as a standard, a calibration curve was constructed according to a conventional immunoassay which showed a linear measurement ranging 1.5–10 µg/l. Further improvements are in progress to optimize the biosensor assay on the strictly analytical aspects.
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1. Introduction

The availability of rapid and reliable methods for "in field" determination of herbicides, pesticides and other organic micro-pollutants in water samples, is an increasing need for environmental monitoring. Gas chromatographic (GC) and high pressure liquid chromatography (HPLC) methods are generally utilized, due to their high detection sensibility and selectivity. However, GC and HPLC analyses are time and labor consuming and needs sample pre-treatment and pre-concentration procedures. Immunoassays and biosensors are becoming a recognized alternative to conventional analytical techniques for the detection of trace amounts of analyte substances.

Recently, biosensors based on the use of monoclonal or polyclonal antibodies have seen a great development in the field of organic micro-pollutants analytical determination [1] and specifically in the s-triazines analyses [2,3]. Among biosensors for s-triazines monitoring, optical [4] or potentiometric [5] devices were described by different authors. The present work describes the development of a potentiometric biosensor for terbuthylazine (TBA, a herbicide widely utilized in agriculture). This technology was previously described for application on potentiometric biosensor for *Escherichia coli* monitoring [6,7]. The novelty of this biosensor is the utilization of an highly specific monoclonal antibody (mAb) in a potentiometric sensor utilising light addressable potentiometric sensor (LAPS) technology. This anti-TBA mAb (P6A7) [8] was already utilised for the development of different biosensors, such as optical sensors [9]. In our case the resulting biosensor showed a very high specificity for TBA and poor rate of cross-reaction for other s-triazines. This could be a great advantage in the different application— for example, on table water analysis—where other triazines can be present. The absence of cross-reactivity obtained with this biosensor, the possibility of on-line measurement, the absence of sample pre-treatment, can really put it in competition with other conventional systems such as HPLC and ELISA.

2. Experimental

2.1. The transducer

The transducer consists of a structure made of n-type silicon, silicon dioxide and silicon nitride: it is placed into a measuring chamber (top of Fig. 1) and put in contact with an electrolyte. The silicon structure acts like a working electrode: the typical electrochemical cell has been obtained by
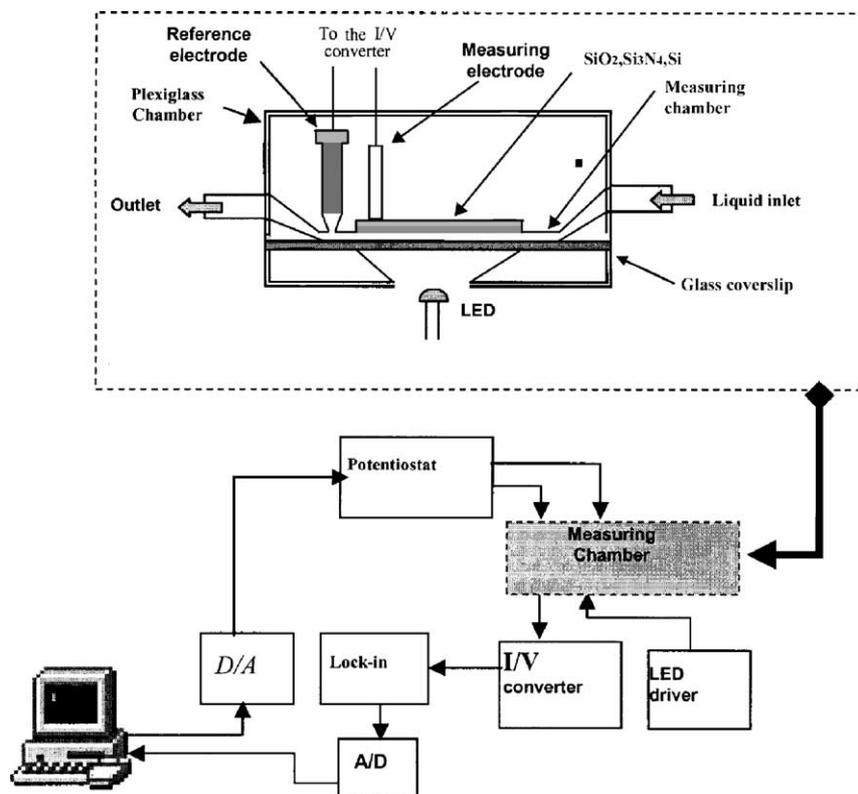
Fig. 1. PAB system based on LAPS.

introducing a reference electrode and a controlling one. By applying a potential V between the controlling electrode and the bulk of the semiconductor it is possible to force the semiconductor into an enhanced and then a depleted mode (for majority carriers); in the same time, by addressing the back side of the silicon chip with an infrared light emitting diode modulated by an alternating driving signal, a current of photo-generated hole–electron pairs is induced in the circuit. The photocurrent I is the parameter measured, usually as an rms value. The insulator layer is pH-sensitive owing to the proton binding capacity of its surface groups (essentially $Si-O^-$ and $Si-NH_2^+$) over a large pH range (2–12), with a theoretical Nernstian response: so, if the electrolyte changes its pH value, the resulting plot of the measured rms current I vs. the applied potential V shift towards higher or lower values of V with increasing or decreasing pH, respectively. Consequently, pH measurement is possible simply by measuring the displacement between the curve of a reference pH solution and the curve of an unknown solution, once the displacement for V per pH unit has been defined for the device. It should be noted that changes in pH can be due to changes in buffer solution, to enzymatic reactions or to cell alterations, allowing the monitoring of a wide range of biophysical events.

The complete system (named potentiometric alternating biosensing system, PAB) is shown in the bottom of the Fig. 1: the apparatus is computer controlled in order to obtain "real time" data acquisition and recording [10,11].

2.2. The biosensor

The Galvani-potential difference, revealed by the PAB system, is generated by the assay whose principle is schematically described in Fig. 1. Changes (i.e. increase) in the pH value are due to the production of NH_3 by an urease-antibody-conjugate which reacts in a competitive assay to free antigen or to the coated form. Urea is enzymatically converted to ammonia in inversely proportional way and proportionately to the amount of TBA present in the sample. The sensitivity recorded is 59 mV per pH units.

2.3. Antibody source

The murine hybridoma clone (P6A7) secreting a monoclonal IgG2a P6A7 TBA specific (mAb P6A7) as well as the TBA derivative [4-chloro-6(isopropylamino)1,3,5-triazine-2-(6-amino) caproic (atrazine-caproic acid)] [12] were produced by Dr T. Giersch and Prof. B. Hock University of Munich and kindly provided.

Mass production of mAb P6A7 for PAB system was obtained from a roller bottle device (MINIPERM). This device is made up of two connected modules that is a production module (a culture chamber of 40 ml volume, in which the cells are inoculated) and a nutrient module (a chamber of 450 ml volume) to be filled with cell culture medium, renewed after 7 days. Secreted mAbs are retained into the small volume of the production module. According

to operator manual, 7×10^7 viable cells of hybridoma clone were injected into the Miniperm production module and 1 mg ml^{-1} was collected after each 15 day production cycle (about 35 mg in the 35 ml final supernatant volume). Although the total amount of mAbs produced by Miniperm is lower, the smaller supernatant volume makes the successive purification procedures easier.

P6A7 mAb was purified from the supernatant by protein A affinity chromatography column, after sulfate ammonium precipitation and coupled to urease.

2.4. Preparation of conjugates and standard solution for PAB assay

An amount of 25 μmol of TBA derivative [4-chloro-6(isopropylamino)1,3,5-triazine-2-(6-amino) caproic (atrazine-caproic acid)] were mixed with 100 μmol of *N*-hydroxysuccinimide (NHS) and 200 μmol of *N,N′*-dicycloexylcarbodiimide (DCC) (Aldrich, Milan, Italy) in 0.2 ml of anhydrous *N,N′* dimethylformamide (Sigma, St. Louis, MO, USA) and allowed to react under constant stirring, overnight at room temperature; 50 μl of this solution were then added to 1 ml of 0.05 M borate buffer, pH 8.0, containing 5 mg of bovine serum albumine (BSA) (Fluka, Buchs, Switzerland) and the reaction was incubated overnight at room temperature under constant stirring. TBA–BSA conjugated was then purified by dialysis or on G-10 Sephadex column (Pierce, Rockford, IL, USA) and as antigen coated on PAB system.

TBA standard solutions for competitive assay on PAB system were prepared as described followingly: TBA herbicide pure grade from Supelco (Bellefonte, PA, USA) (10 mg) was dissolved in a volumetric flask (10 ml) in absolute ethanol and different solutions were prepared by serial dilutions of the stock solutions to obtain the following herbicide concentrations: 0.01, 0.1, 0.5, 1,10, and 100 μg l^{-1}.

mAb P6A7 (TBA specific) was coupled to urease enzyme for PAB competitive assay following the periodate method [13]. 0.5 mg urease (Aldrich, Milan, Italy) was resuspend in 1.2 ml water. A total of 1.2 ml urease solution was incubated to 0.3 ml of 0.1 M Na-periodate suspended in phosphate buffer 10 mM, pH 7.0 for 20 min at room temperature; this urease solution was dialyzed vs. 1 mM sodium acetate overnight at 4 °C. 0.5 ml of antibody solution (1 mg ml^{-1}) were added to urease solution and the mixture was incubated at room temperature for 2 h and dialyzed in PBS. Final concentration of mAb P6A7-urease was 1 mg ml^{-1}.

2.5. Silanization and antigen immobilization procedures

The TBA–BSA conjugate was used as antigen and immobilized on a glass cover slip (1 cm × 1 cm) previously cleaned with hot sulfochromic mixture and successively silanized. The silanization was made by plunging the cover slip on a solution containing 5% aminopropyltriethoxysilane (APTES)

and 5% water in isopropanol at 60 °C for 1 h, followed by drying at 130 °C for 1 h. As APTES presents NH$_3$ groups, a bifunctional crosslinking agent like glutaraldehyde was used to create covalent bonds with the amino-groups of the proteic part of the TBA–BSA conjugate; the cover slip was immersed in a 5% glutaraldehyde:water solution at room temperature for 30 min and then a TBA–BSA solution (40 μg ml^{-1}) was laid on the cover slip and kept overnight at room temperature.

The cover slip was rinsed with a PBS solution to remove unbound TBA–BSA and incubated for 1.5 h on a 0.1% casein solution in PBS in order to block the unlinked sites so avoiding non-specific linking of the antigen.

2.6. Competitive assay on PAB system

To characterize TBA concentration using the PAB system, we developed an indirect competitive immunoassay (Fig. 2): standard solutions containing free TBA were preincubated with mAb P6A7-urease conjugated, properly diluted in PBS. An amount of 500 μl of each solution were then incubated for 1 h at room temperature on a cover slip coated with TBA–BSA, as previously described. After removal of the unbound antibody by several washing steps with PBS, the cover slip was introduced inside the measuring chamber of the PAB system and the substrate solution (urea-50 mM/PBS 1×) was introduced by a peristaltic pump, at a flow rate at 200 μl min^{-1}. Finally the enzymatic activity was automatically calculated from the signals acquired during flow off periods of about 5 min.

Experiments were also performed in order to evaluate specific binding between antigen TBA–BSA coated on a cover slip and the specific mAb: P6A7-urease conjugated at different concentrations was incubated on a cover slip coated with TBA–BSA and treated as described for the competitive assay. As blank an urease solution was utilized on a slide coated with TBA–BSA.
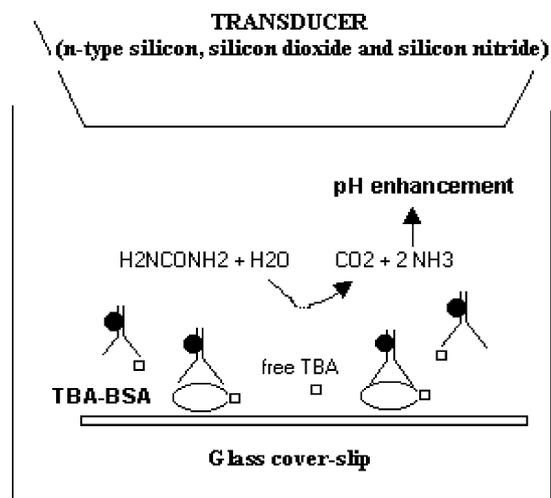

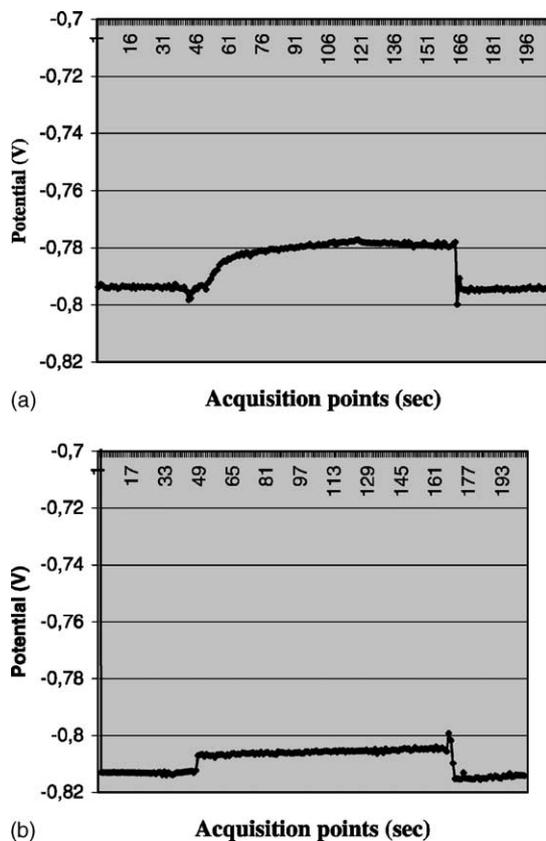Fig. 2. Competitive immunoassay for TBA detection in PAB system.

(a)

(b)

Fig. 3. PAB assay for standard TBA concentration (a) 0.01 ppb; (b) 10 ppb.

3. Results and discussion

A solution containing the mAb P6A7 $(5 \, \mu g \, ml^{-1})$—specific for the recognition of the herbicide TBA—and conjugate to the enzyme urease was preincubated in PBS with different concentration of free TBA. These solutions were then incubated on TBA–BSA coated cover slips and tested by the PAB system.

In the Figs. 3 and 4 the crude graphs of the acquisition points from the biosensor are shown after elaboration of the software associated to the PAB ("Cassiopea Light" from Technobiochip srl, Marciana Marina, Livorno, Italy).

Fig. 3 shows two examples of acquisition curves of two concentrations—0.01 ppb (Fig. 3a) and 10 ppb (Fig. 3b) of free TBA—of a standard solution: the voltage intensities were measured during each run for about 200 s. For each concentration, 201 flex points were acquired, each one of them being the shift value in volt of the respective sigmoide curve (IV curve). The first 40 point of each curve (Fig. 3a and b) are the acquisition points after urea flowing in the cell; then, the potential increase is measured after flow stop, which lead to NH_3 accumulation and, consequently, proportional pH increase. As in any competitive assay the signal linearly decreased by increasing the TBA concentration. Fig. 4 shows the results collected in the PAB assays for the P6A7-urease direct binding (Fig. 4a) on antigen-coated slide and for the blank obtained using the enzyme urease at a concentration of $5 \, \mu g \, ml^{-1}$ (Fig. 4b).

A calibration curve was obtained in a competitive assay, by utilizing Abs-coated cover slip and mAb P6A7 specific anti-TBA mAb in our PAB system. TBA standard solutions in the range from 0.01 to $100 \, \mu g \, l^{-1}$ were analyzed with a biosensor dipstick as well as a control. Potential values (ΔV) relative to the TBA standard solutions are fitted with a four-parameter logistic function using a commercial software package (ORIGIN™) in order to elaborate the calibration standard curve, Fig. 5.

At this stage, this proposed potentiometric biosensor for TBA, based on LAPS technology seems to be a fast assay for TBA monitoring in water samples suitable for environmental applications.

Environmental samples could be analyzed without any preliminary enrichment or clean-up treatment, the method is relatively low time consuming, easy to perform and quite inexpensive. All the positive samples can be successively analyzed in laboratory according to an analytical standard method, in order to confirm the presence of the analytes and obtain officially acceptable data. Further improvements are needed in order to optimize the system on the strictly analytical aspects (increasing precision and accuracy of the method and lowering the detection limit).

At present the detection limit for TBA is suitable for environmental monitoring purposes, but it is still too high for drinking water analysis.
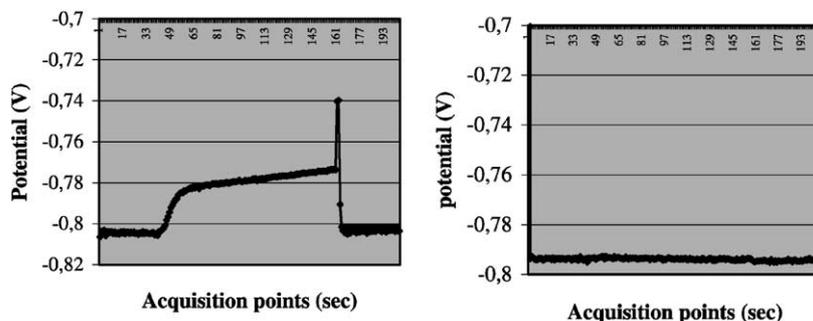

Fig. 4. Direct binding of mAb P6A7-urease $5 \, \mu g \, ml^{-1}$ conjugate (a); blank binding of urease $5 \, \mu g \, ml^{-1}$ (b) on TBA–BSA in PAB assay.
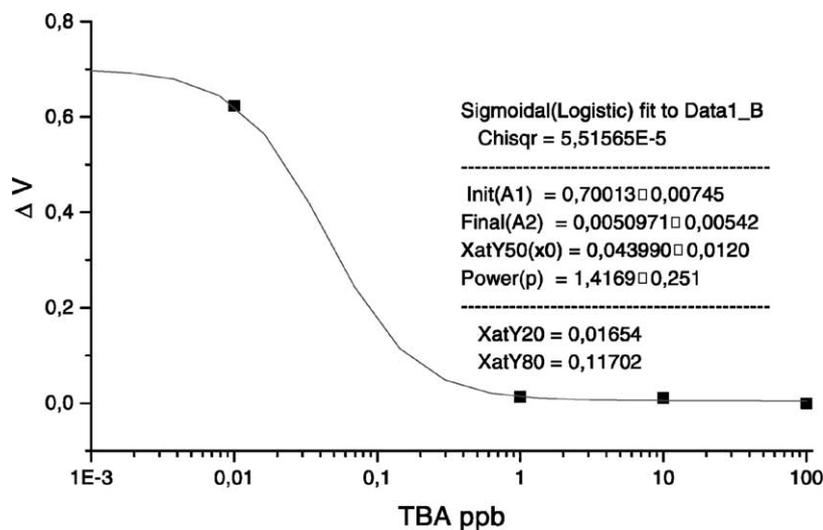
Fig. 5. TBA standard curve.

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References

[1] E. Mallat, D. Barcelo, C. Barzen, G. Gauglitz, R. Abuknesha, Immunosensors for pesticide determination in natural waters, Trends Anal. Chem. 3 (2001) 124–132.

[2] B. Hock, A. Dankwardt, K. Kramer, A. Marx, Immunochemical techniques: antibody production for pesticide analysis: a review, Anal. Chim. Acta 311 (1995) 393–405.

[3] F.F. Bier, R. Jockers, R.D. Schmid, Integrated optical immunosensor for s-triazine determination, regeneration, calibration and limitation, Analyst (1994) 437–441.

[4] P. Orozlan, G.L. Duveneck, M. Ehrat, H.M. Widmer, Fiber-optic atrazine immunosensor, Sens. Actuators B 11 (1993) 301–305.

[5] F. Vianello, L. Signor, A. Pazzariello, M.L. Di Paolo, M. Scarpa, B. Hock, T. Giersch, A. Rigo, Continuos flow immunosensor for atrazine detection, Biosens. Bioelectron. 1 (1998) 45–53.

[6] C. Ercole, M. Del Gallo, M. Pantalone, S. Santucci, L. Mosiello, C. Laconi, A. Lepidi, A biosensor for *Escherichia coli* based on a potentiometric alternating (PAB) transducer, Sens. Actuators B 83 (2002) 48–52.

[7] C. Ercole, M. Del Gallo, L. Mosiello, S. Baccella, A. Lepidi, *Escherichia coli* detection in vegetable food by a potentiometric biosensor, Sens. Actuators B 91 (2003) 163–168.

[8] T. Giersch, A new monoclonal antibody for the sensitive detection of atrazine with immunoassay in microtiter plate and dipstick format, J. Agric. Food Chem. (1993) 1006–1011.

[9] A. Brecht, J. Piehler, G. Lang, G. Gauglitz, A direct immunosensor for atrazine, Anal. Chim. Acta 311 (1995) 289–300.

[10] M. Adami, M. Sartore, C. Nicolini, PAB: a newly designed potentiometric alternating biosensor system, Bioelectronics 10 (1995) 155–167.

[11] L. Piras, M. Adami, S. Fenu, M. Dovis, C. Nicolini, Immunoenzymatic application of a redox potential biosensor, Anal. Chim. Acta 335 (1996) 127–135.

[12] B. Hock, T. Giersch, K. Kramer, Monoclonal antibody-based enzyme immunoassays for the sensitive detection of s-triazines in water, Analysis 20 (1992) 29–33.

[13] E. Harlow, D. Lane (Eds.), Antibodies: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988, p. 342.

Biographies

Lucia Mosiello received her degree in biology in 1986 at University of Rome "La Sapienza", within the field of molecular biology: from 1986 to 1989 she enhanced her experience working as a fellow in private and public institutions (Sorin Biomedica and ENEA). She is working at ENEA (Italian National Agency for New Technologies, Energy and the Environment) since 1990 as researcher. She was visiting scientist at ITB of University of Stuttgart and at Institute of Botany of University of Munich in 1995 and became group leader at ENEA. Her expertise includes molecular and cell biology, new antibodies production technology, immunochemistry and biosensors development.

Cristina Laconi obtained her degree in biology 1998 at University of Rome, within the field of biochemistry. From 1998 to 2002 she worked in immunological projects on the production of new monoclonal antibodies and the development of potentiometric immunosensor and immunoassays for environmental application. she actually involved in a European project for the analysis of the effects on human nervous cell lines associated to mobile phones and she has a fellow at ENEA.

Maddalena Del Gallo is a full professor of Applied and Environmental Microbiology at the Faculty of Science of University of L'Aquila. Her research interests focus on plant associated microorganisms and on different theoretical, applied and biotechnological aspects of the Environmental Microbiology. She published more than 120 scientific articles and edited two books on plant-associated nitrogen-fixing bacteria and is coordinating international scientific projects.

Claudia Ercole received the Laurea degree in Biology at the University of L'Aquila in 1988. Since 1990 she is assistant professor of microbiology at the Department of Basic and Applied Biology, University of L'Aquila. Her research activities include the characterization of protein isolated from microorganisms involved in the oxido-reduction of manganese, the mechanisms of microbial precipitation of calcite and the development of biosensors. She has published more than 40 papers in various international journals.

Aldo Lepidi was graduated and trained to research in microbiology at the Scuola Superiore S. Anna, the science and technology section of the Scuola Normale in Pisa. He moved to the University of L'Aquila and became full professor of microbiology in 1986 and is actually the dean of the Faculty of Sciences of that University. The fields of interest concern the production and process improvement in industires using microbes and related topics.