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A biosensor for *Escherichia coli* based on a potentiometric alternating biosensing (PAB) transducer

C. Ercole[a,*], M. Del Gallo[a], M. Pantalone[a], S. Santucci[b], L. Mosiello[c], C. Laconi[c], A. Lepidi[a]

[a]*Department of Basic and Applied Biology, University of L'Aquila, 67010 L'Aquila, Italy*
[b]*Department of Physics and Unità INFM, University of L'Aquila, 67010 L'Aquila, Italy*
[c]*ENEA—Casaccia, via Anguillarese 301, 00060 Rome, Italy*

Abstract

In the present paper, we describe a biosensor for the detection of bacterial contamination in drinking water based on an immunoassay test. The biological component was made by a rabbit polyclonal antibody obtained utilising an environmental strain of *Escherichia coli*, DH5α. This strain, showed a capsular protein pattern typical of this species. Cells present in water samples were detected by a complete potentiometric alternating biosensor (PAB). The PAB system consists of a measuring chamber, acquisition and driving electronics and the appropriate software: the apparatus is computer controlled in order to obtain on-line acquisitions and recording of data. The transducer principle is based on a light addressable potentiometric sensor (LAPS) technology which, in our case, reveals the production of NH_3 by a urease–*E. coli* antibody conjugate. The proposed system appears to be very sensitive and fast, in comparison with conventional methods: concentrations of 10 cells per ml were detected in an assay time of ca. 1.5 h. No signals were detected from other potential water polluting bacteria, such as *Pseudomonas marina* and *Sphaerotilus natans* and also from bacteria such as *Klebsiella oxytoca* phyletically related to *E. coli*.

Keywords: Biosensor; *E. coli*; Water pollution; Light addressable potentiometric sensor (LAPS); Immunoassay

1. Introduction

The rapid, sensitive and reliable screening for bacterial contamination in drinking water is fundamental for the prevention of infections. The routinary detection methods for microorganisms are based on colony forming unit (CFU) count and require selective culture, biochemical and serological characterisations. Although, bacterial detection by these methods is sensitive and selective, days to weeks are needed to get a result. Besides, these methods are costly and time consuming.

Despite the progress achieved in recent years, there is still no practical sensor for microbial detection which can satisfy market requirements, such as short analysis time, cost-effective instrumentation, high sensitivity and aptitude to on-line monitoring of industrial processes and environmental surveying [1].

Immunoassays are known to be competitive with conventional analytical techniques because they can detect trace amount of analyte substances in a short time and at lower cost. Gerhing et al. [4] describe a system based on a sensor utilising LAPS for the detection of *Escherichia coli*, directed towards the detection of pathogen strains on food. The system, utilising polyclonal antibodies, was able to detect about 10^3 cells per ml.

The present work describes the properties of a biosensor for the determination of *E. coli* cell number in water samples. The potentiometric alternating biosensing (PAB) system utilised is based on a transducing element (light addressable potentiometric sensor (LAPS)) [2–4] detecting pH variations due to NH_3 production by an urease–*E. coli* antibody-conjugate.

2. Experimental

2.1. The transducer

The transducer consists of a heterostructure made of silicon n-type, silicon dioxide and silicon nitride positioned into a measuring chamber (top of Fig. 1). 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

* Corresponding author. Tel.: +39-862-433-282; fax: +39-862-433-273.
E-mail address: claudia.ercole@aquila.infn.it (C. Ercole).

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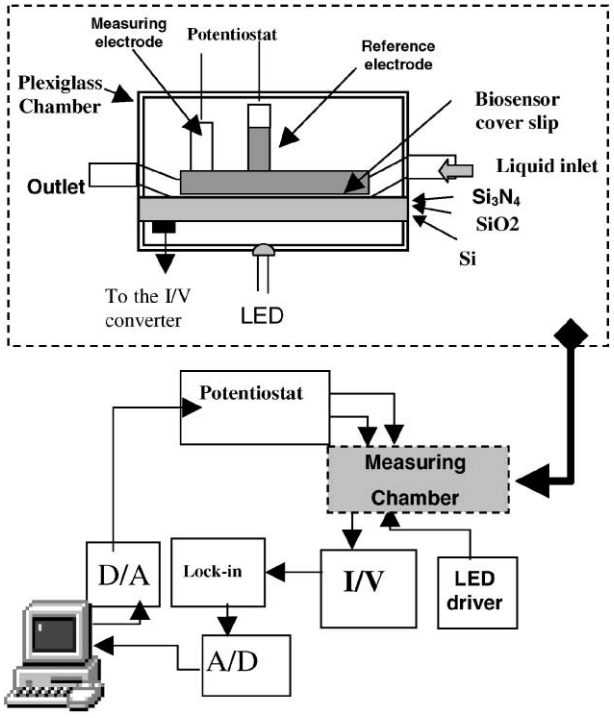

Fig. 1. Block diagram of the transducer system. On top a detailed drawing of the measuring chamber is reported with the sensing electrode made of silicon, silicon dioxide and silicon nitride. In the bottom, the signal measurements performed by a synchronous demodulation techniques and the conditioning electronics blocks are shown.

pH range (2–12), with a theoretical Nernstenian response (if hysteresis and drift phenomena are not considered). Redox potential measurements are obtained when the solution to be analysed reacts with the biosensor immobilised on the cover slip in contact with the silicon structure. Redox pairs produced, change the silicon nitride potential towards a value stated by the Nernst equation. This process is the result of the initial differences in electron affinities of the two phases in contact (Si_3N_4 layer and the solution) producing an electron exchange between the silicon and the solution which continues up to the equilibrium.

When the sinusoidally modulated infrared LED illuminates the back side of the silicon structure, it produces an alternating photocurrent with a characteristic (*I*,*V*) shape [3] changing according to the concentration of the reacting analyte. The PAB system's response is implemented by a lock-in technique using a modulated pulsed light from an infrared LED. The light is focused on the back of the silicon substrate and the potentiometric signal in-phase from the metallic electrode is recovered. In the bottom of the Fig. 1, a block diagram of our conditioning and acquisition electronic is shown. The apparatus is computer controlled in order to obtain on-line data acquisition and recording.

2.2. The biosensor

The redox potential revealed by the transducer system is generated by the biosensor whose principle is schematically

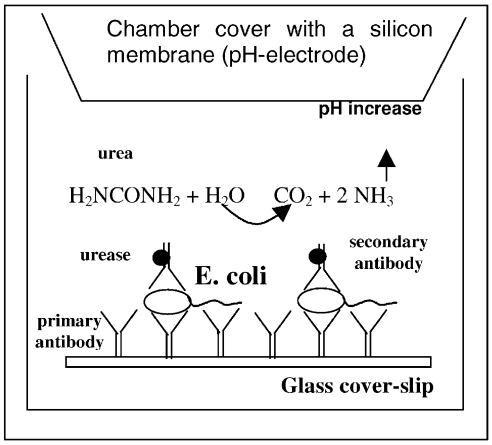

Fig. 2. Biological component of the sensor. The primary anti-*E. coli* antibody is coated on a silanised glass cover slip. *E. coli* present in the sample link to the antibody and the secondary urease-bound antibody is fluxed and then washed with PBS. At the end urea is fluxed and the reaction is recorded.

described in Fig. 2. The change in the redox potential is due to the production of NH_3 by a urease–*E. coli* antibody-conjugate linked with the *E. coli* cells present in the water. Urea is enzymatically converted to ammonia proportionately to the amount of *E. coli* present in the sample. The sensitivity recorded is 59 mV per pH units.

2.3. Bacterial culture and sampling procedures

An environmental *E. coli* strain, DH5α, was utilised to produce rabbit polyclonal antibodies. It was chosen out of several *E. coli* strains, environmental, enteric and enteropathogenic, after capsule isolation and SDS-page protein analysis, because of its band pattern typical of this genus.

The strain was grown in LB broth at 30 °C in a rotary shaker (New Brunswick G25) for 1 day at 200 rpm. For solid media, 2% agar was added. Bacterial cells were removed from liquid culture by centrifugation at 4000 × *g* at 4 °C for 20 min, washed twice and resuspended in phosphate buffer saline (PBS). The concentration was adjusted to 10^8 cells per ml. Cells were attenuated by heating at 60 °C for 30 min (three cycles). Cell attenuation was estimated by viable cell counts. Capsule presence also was estimated by a contrast dyeing [5]. Aliquots of fresh culture were stored at −20 °C for up to 4 months, while solid cultures were stored at +4 °C for up to 1 month.

The PAB system was also utilised for the analysis of environmental water samples. Water samples were aseptically collected on sterile bottles and kept at 4 °C. Sampling was done in the following sites: Aterno River, L'Aquila upstream and downstream of the municipal sewage treatment plant, Bracciano Lake, Rome, and a see water from the beach of Ladispoli, Rome. Samples were analysed at the same time by conventional microbiological methods (FM disks [7]), ELISA (the same antibody utilised for the biosensor) and by the biosensor. The sample collected from the

Lake of Bracciano was also spiked with an *E. coli* cells suspension, 1 ml in PBS per litre, to a concentration of 1×10^3 cells per ml.

2.4. Preparation and purification of rabbit antisera

A New Zealand rabbit was given several small subcutaneous injections of an attenuated *E. coli* cells suspension in the Freund's complete adjuvant (FCA) for a total of 1×10^5 cells. The animal received two s.c. injections of 10^2–10^3 *E. coli* cells in FCA after 15 and 30 days and after 40 days it was bled under anaesthesia by a cut of the ascellary artery.

Immuneserum with high titre was cleared by centrifugation ($8000 \times g$, 15 min). Anti-*E. coli* polyclonal-IgG were purified from the serum by protein-A affinity-chromatography column, after ammonium sulphate precipitation. Measurements for quantity and purity of this antibody were done spectrophotometrically at 280 nm and the anti-*E. coli*-IgG were identified by ELISA immunoassay.

2.5. Preparation of immunochemicals

The anti-*E. coli* IgG was coupled to urease (Sigma–Aldrich) by a modified periodate coupling procedure [6] as follows: 0.5 mg urease was resuspend in 1.2 ml water. A total of 1.2 ml urease solution was added to 0.3 ml of 0.1 M Na-periodate suspended in phosphate buffer 10 mM—pH 7.00, and it was incubate at room temperature for 20 min.; the urease solution was dialysed versus 1 mM sodium acetate overnight at 4 °C; 0.5 ml of antibody solution (1 mg ml^{-1}) were added and the mixture was incubated at room temperature for 2 h and dialysed versus PBS. Final concentration of the anti-*E. coli*–urease was 1 mg ml^{-1}.

2.6. Immobilisation procedures

The primary antibody was immobilised on a glass cover slip (2 cm × 2 cm) previously cleaned with hot sulfochromic mixture and successively silanised. The silanisation was made by immersion 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 antibody; the cover slip was immersed in a 5% glutaraldehyde:water solution at room temperature for 30 min, and then an antibody solution (60 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 antibodies 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.

To detect the sensitivity of the PAB system, the cover slip was washed in PBS and kept horizontally for 15 min with 1 ml of *E. coli* solution in PBS at different concentrations (from 10 to 10^7 cells per ml), rinsed again with PBS and incubated with the secondary antibody (this last conjugated to urease at the concentration of 30 μg ml^{-1}) for 1 h.

Finally, the cover slip was introduced inside the measuring chamber (Fig. 2) of the PAB system, the substrate mixture (urea-50 mM) was introduced by a peristaltic pump, at flow rate of 200 μl min^{-1} and the enzymatic activity was automatically calculated from the signals acquired during flow off periods of about 5 min. For the application described here, a microvolume reaction chamber was utilised with a complete microchannel system near the sensitive area. A glass support was used, facing the transducer at a distance of about 300 μm, and an area of 75 mm^2 was delimited by a rubber spacer, yielding a volume of 20 μl (see Fig. 1).

Preliminary trials were also done to verify how to manage with the fluxing procedure needed for the on line use of the biosensor (data are not shown).

2.7. Acquisition curves

Instrument calibration is a prerequisite for the reliability of the data and it was made by measuring the potential difference between pH 7 and pH 8 standard titration solutions ($\Delta V_{out} = \Delta pH \times S$, where S is the sensitivity). Measurements of bacterial suspension were done utilising attenuated cells at different dilution, from 10 to 10^7 cells per ml for *E. coli* and 10^6 cells per ml for *Klebsiella oxytoca, Pseudomonas marina* and *Sphaerotilus natans*. Samples were prepared outside the measuring chamber. Cell suspensions in PBS (1 ml), and PBS without cell as a blank, were layered on the cover slip for 30 min. Unbound cells were thrice washed out with PBS and the second antibody was added, kept for 60 min and then washed with PBS. The cover slip was finally put in the measuring chamber, an urea/water solution 50 mM was introduced by a peristaltic pump at flow rate of 200 μl min^{-1} and the release of ammonia was read as pH variation. Blanks were treated with secondary antibody and urea both together and separately. The amount of released ammonia is a function of the number of cells present in the sample and bound to the cover slip. The pH variation due to ammonia release is therefore a measure of cell number.

3. Results

The concentration of bacterial cells in the analysed samples was read by the instrument as a pH variation proportional to signal response. The presence of *E. coli* cells induce a potential variation (Fig. 3) proportional to the cell number (Fig. 4). The system appears to be very sensitive: concentrations of 10 cells per ml are clearly detected over a blank of *E. coli*-free PBS. The calculated limit (intercept for $V = 0$ in Fig. 4) of the limit of detection of the instrument (LOD) appears to be 7.5 cells per ml. The background noise was not influential on the instrument sensitivity. No signals were

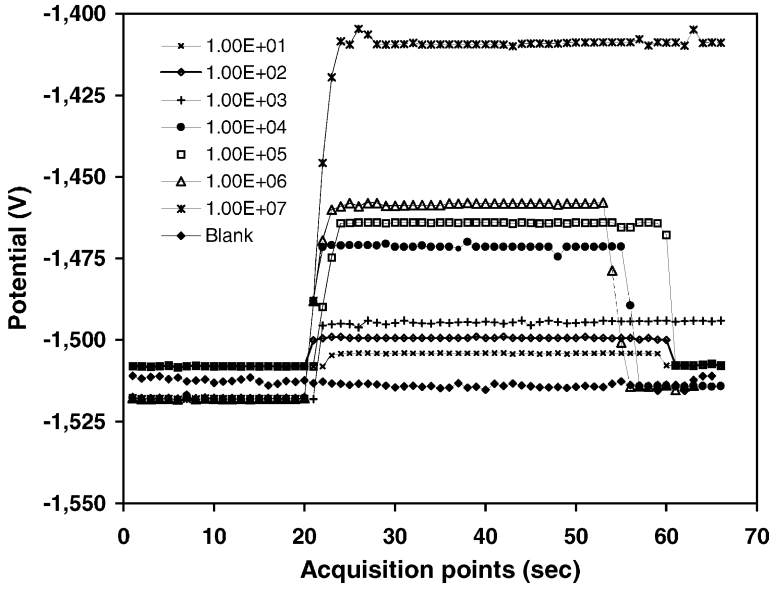

Fig. 3. Acquisition curves of different *E. coli* concentrations in the presence of polyclonal *E. coli* antibodies: the samples contained *E. coli* cells ranging from 10–10^7 ml^{-1}. Blank acquisition curve obtained by flowing urea on a cover slip treated as the samples but with cell-free is also reported.

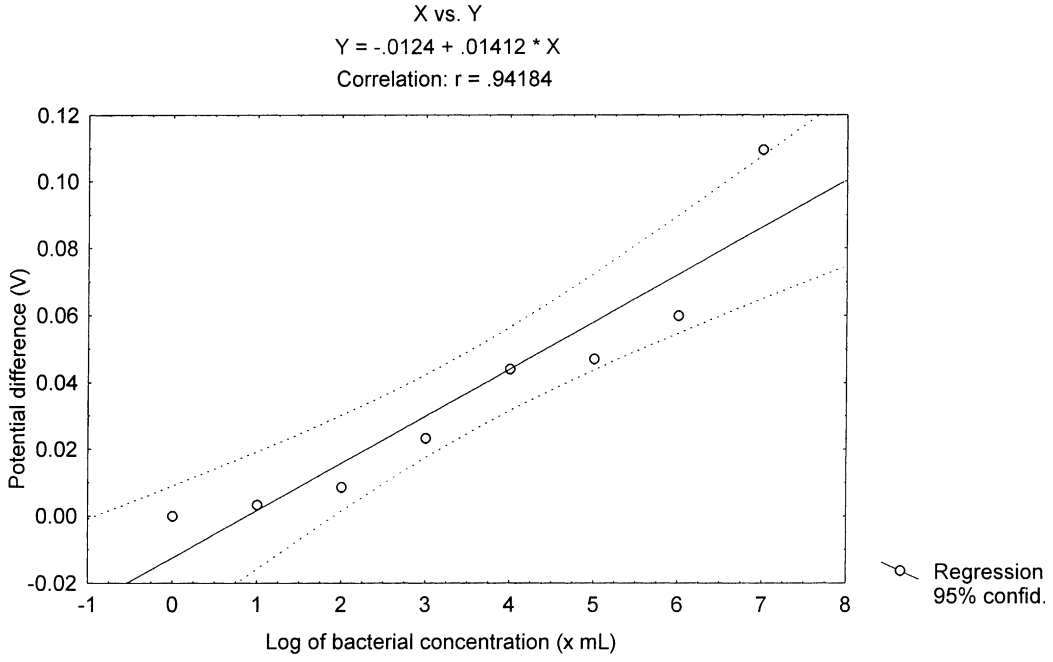

Fig. 4. Plot representing *E. coli* cell concentration vs. potential differences. The calculated LOD appears to be 7.5 bacteria per ml.

detected when bacteria other than *E. coli* were present and the same type of acquisition curves were given both by bacteria frequently polluting drinking water such as *P. marina* and *S. natans*, and by bacteria phyletically related to *E. coli* such as *K. oxytoca* (Figs. 5 and 6).

The PAB system was also utilised to analyse environmental water samples. These samples were analysed following the above mentioned procedure and the results obtained by the PAB were compared with the ones obtained by conventional microbiological methods and ELISA (the same antibody utilised for the biosensor). All analysed samples were found *E. coli*-free. We also spiked the Bracciano Lake water sample by adding 1 ml of *E. coli* cells, (1×10^3 ml^{-1}), to check for a possible background interference when treating environmental waters. No background interference was recorded as shown in Fig. 7.

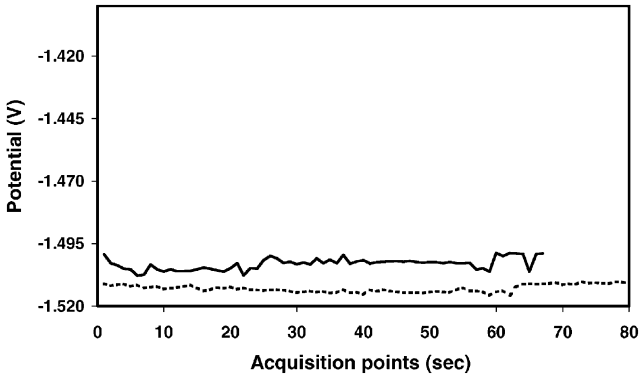

Fig. 5. Acquisition curves of *K. oxytoca* (top line) and *P. marina* (bottom line) in the presence of polyclonal *E. coli* antibodies. Cells were attenuated and diluted to 10^6 ml^{-1} for both *K. oxytoca* and *P. marina*.

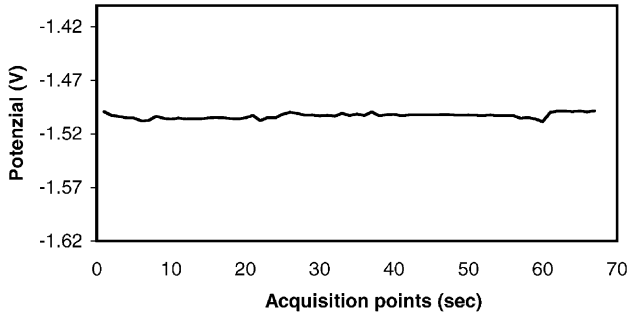

Fig. 6. Acquisition curves of *S. natans* in the presence of polyclonal *E. coli* antibodies. Cells were attenuated and diluted to 10^6 ml^{-1}.

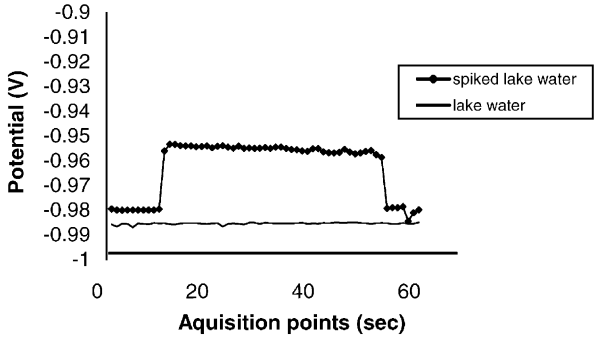

Fig. 7. Acquisition curves of water samples from the Lake of Bracciano (Rome) both untreated (bottom line), and spiked (top line) with 1×10^3 *E. coli* cells per ml.

4. Discussion

In this work, we demonstrate how an immunoassay-based PAB sensing system was able to specifically detect *E. coli* strains in water. The system can be connected to a signal-transducer in order to have a calibration curve giving the interrelationship between the registered answer and the number of *E. coli* cells present in the water.

This system is quite flexible, manageable and easy to construct utilising commercial electronic components and it can be further improved. At present, we are improving the system by improving LOD (sensitivity of the instrument below 10 cells per ml) and by developing a monoclonal antibody—to increase the reproducibility—and, in particular, by setting up regeneration procedures for the immunoaffinity layer to lower the costs and to speed up the reading of the samples. The possibility of measuring microbial pollution in lakes, rivers and ground water—being *E. coli* a typical indicator of foecal contamination—with a cheap, quick, compact and easy to use equipment, is attractive and should promote a larger diffusion of environmental monitoring.

Work is in progress to insert this biosensor system in a network monitoring city water distribution lines and to apply it to other water contaminants.

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