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Immobilization of *E. coli* bacteria in three-dimensional matrices for ISFET biosensor design

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Abstract

In recent years, cell-based biosensors (CBBs) have been very useful in biomedicine, food industry, environmental monitoring and pharmaceutical screening. They constitute an economical substitute for enzymatic biosensors, but cell immobilization remains a limitation in this technology. To investigate into the potential applications of cell-based biosensors, we describe an electrochemical system based on a microbial biosensor using an *Escherichia coli* K-12 derivative as a primary transducer to detect biologically active agents. pH variations were recorded by an ion-sensitive field effect transistor (ISFET) sensor on bacteria immobilized in agarose gels. The ISFET device was directly introduced in 100 ml of this mixture or in a miniaturized system using a dialysis membrane that contains 1 ml of the same mixture. The bacterial activity could be detected for several days. The extracellular acidification rate (ECAR) was analyzed with or without the addition of a culture medium or an antibiotic solution. At first, the microorganisms acidified their micro-environment and then they alkalinized it. These two phases were attributed to an apparent substrate preference of bacteria. Cell treatment with an inhibitor or an activator of their metabolism was then monitored and streptomycin effect was tested.

Keywords: Biosensor; ISFETs; Bacteria; Immobilization; Agarose gel; pH

1. Introduction

The great demand for the identification and determination of different toxic substrates in various media, e.g. milk, blood, urine, water supply and wastewater has led to the development of a variety of analytical tools. Among these, biosensors have been met with a considerable interest, mainly because of the inherent selectivity of the used biological recognition element. Specific enzymes are most frequently used as selective elements in biosensors and corresponding devices are excellent tools for the determination of a given metabolite even in the presence of other metabolites [1].

In order to improve the biosensors' response, living cells are suggested instead of purified enzyme. In fact, considered as complex biochemical plants, these cells grow, reproduce and communicate with the environment using biochemical and biophysical processes. Treated with drugs or toxic substances, they provide more information than enzyme-based biosensors and could be greatly useful for basic research and various biomedical and biotechnological applications [2]. This is why, in recent years, the development of whole-cell biosensors has been met with an increasing interest [3–6]. Whole cells have actually the possibility of converting complex substrates using specific metabolic pathways [7–9]. Unlike enzyme-based

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biosensors, whole-cell biosensors can be used to monitor typical parameters, such as toxicity [10–13], biological oxygen demand, nitrification inhibitors [14], xenobiotic compounds [15] or heavy metals [16]. Thus, they provide convenient early warnings or environmental screening capabilities that would otherwise require either a large number of specific sensors or time-consuming laboratory analyses [17]. Since the cell is a tightly regulated system, resulting biosensors give robust and reproducible signals [18]. Whole cell-based toxicity tests are then suitable for sensing applications that are inadequately carried out by conventional chemical analysis instruments, such as gas or liquid chromatographs and atomic absorption spectroscopy. In addition, living cells are continuously repairing their integrated enzyme activities and cascades. This is undoubtedly an advantage with respect to an improved long-term stability of reliable biological recognition elements [1].

In this respect, the utilization of whole cells as a source of intracellular enzymes has been shown to be a better alternative than purified enzymes in various industrial processes [19–21]. It avoids the lengthy and expensive operations of enzyme purification, preserves the enzyme in its natural environment and protects it from inactivation by external toxicants such as heavy metals. Whole cells also provide a multipurpose catalyst especially when the process requires the participation of a number of enzymes in sequence [9].

However, the ultimate step that limits the cell-based biosensor CBB technology is the cell immobilization on the transducer. Bacteria, for example, are mostly encapsulated in a supporting matrix that will ensure their immobilization during wash-out. These attached bacteria provide signal transduction and rapid communication with the environment [17].

Several successful results have been obtained with encapsulated bacteria in soft gels such as agarose [22], polyacrylamide or calcium and strontium alginates [23,24]. Nevertheless, these sensors have the known shortcomings of soft hydrogel supports, namely biodegradation susceptibility and diffusion limitation due to film thickness or low physical deformation resistance.

Cell encapsulation in a dialysis membrane [25] and a glycerol–acryl vinyl acetate copolymer latex membrane or sol–gel derived silicates have also been reported [17,26]. Using covalent binding, bacteria immobilization with an antibody grafted to glass or to gold-coated glass substrates has been recently described [27]. An immobilization process based on the cross-linking of bifunctional reagents like glutaraldehyde has been successfully carried out in various supports. Among these, proteinic supports such as gelatine [28,29], albumin [30] and hen egg white [31,32] have been extensively used. Although this technique obviates some of the limitations due to covalent binding, the used chemical cross-linking reagents often affect the cell viability. So, the cross-linking technique remains useful in immobilizing non-viable cell preparations containing active intracellular enzymes whereas entrapment and adsorption techniques are recommended in case of viable cells [9].

The present work illustrates the applicability of immobilized bacteria in spontaneously-forming agarose gel prior to electrochemical measurements. A fast and consistent signal is produced and detected in relationship with metabolic activities of bacteria. Comparing this generated signal to that obtained by conventional methods, it demonstrated the reliability of the electrochemical measuring system and the advantages of whole cells immobilized in agarose gel.

In this article, we validate a bacterium-based sensor by studying the effects of physiological stress on the bioencapsulated *Escherichia coli* in agarose gel. The bacterial activity has been monitored on-line, using a complex silicon system as a microsensor (mainly chemical field effect transistors).

2. Experimental

2.1. Strains and culture

The *E. coli* strain PHL818 is an *ompR234* hyper-adherent derivative of the *E. coli* K-12 strain MG1655 [33]. PHL1273 results from the transformation of PHL818 with a derivative of the pPROBE-*gfp[LVA]* plasmid (Stratagene) containing the promoter of the *csgBA* operon upstream of the *gfp[LVA]* reporter gene (G. Jubelin and P. Lejeune, personal communication). In the culture conditions used in this work this plasmid confers a strong fluorescence to the bacteria which can be imaged in epi-fluorescence microscopy (Zeiss Axiover 40).

For maintenance, the bacteria were grown in Petri dishes containing the solid medium LB, which was composed of agar (15 g), bactotryptone (10 g), yeast extract (5 g) and NaCl (10 g) in 1 l of distilled–deionized water [34]. To ensure plasmid maintenance, the LB medium was mixed with kanamycin (100 μg ml^{-1}) and then stored at 4 °C until use. The same medium (without agar) was used for liquid cultures incubated in a shaken bath at 29 °C and 80 rpm for about 18 h (cells were in a stationary phase). The cells were harvested by centrifugation (5000 rpm, 10 min). The pellet was then suspended in a volume of fresh LB calculated to obtain a final concentration of cells equal to 2.8×10^8 cells ml^{-1}. Optical density measurements were conducted at 600 nm.

2.2. Cell immobilization into agarose gel

Agarose was dissolved in 50 ml of sterile water at 0.4% (w/v) in a micro-wave at 100 °C. This solution was cooled down to approximately 40–45 °C, and mixed in an Erlenmeyer flask containing 50 ml of *E. coli* suspension prepared as described above. The reference electrode and the ISFET device were then introduced into this hydrogel matrix of agarose where the cells were immobilized. Gel formation immediately started and the gel strength was increased as temperature cooled down. The soft gel contained approximately 1.4×10^8 cells ml^{-1}.

2.3. Miniaturization of the system

From an agarose solution prepared as above, an aliquot of 500 μl was allowed to cool down to approximately 40–45 °C in a pipette tip before mixing it to 500 μl of the *E. coli* culture. The mixture was immediately pipetted into a dialysis membrane (2 cm length, 2.5 cm diameter, 12–14 kD porosity) and the ISFET device was introduced into the gel before cooling. After

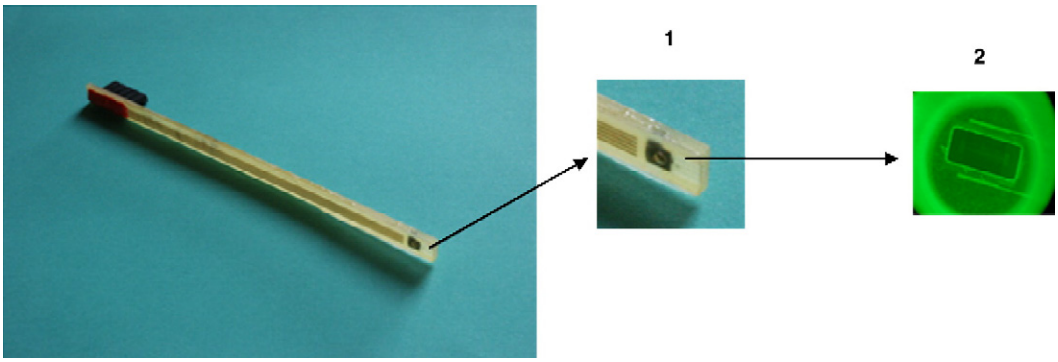

Fig. 1. Optical image of the top view of an ISFET device. 1 — the Si_3O_2/SiN_4 pH-sensitive gate, 2 — enlarged Si_3O_2/SiN_4 pH-sensitive gate.

gel solidification, the reference electrode and the dialysis membrane containing the bacteria and the ISFET were placed beneath the surface of 100 ml of culture medium (LB1/2: LB medium diluted twice) with or without antibiotics.

2.4. The ISFET device

Fig. 1 shows the top view of an ISFET device and its sensitive part that was purchased from CNM Barcelone (Spain). ISFETs are metal-oxide-semiconductor field effect transistors (MOSFET) with the gate connection separated from the chip in the form of a reference electrode. In the case of pH-sensitive ISFETs, the generated electrical signal depends on the surface potential of the gate insulator. This signal is caused by pH variations that occur in the micro-environment of the ISFET's sensitive part.

Chemical measurements were performed by *I–V* experiments, the gate-source voltage (V_{GS}) variations being monitored for constant drain-source current (I_{DS}) and drain-source voltage (V_{DS}) (I_{DS}=100 μA–V_{DS}=500 mV).

To calibrate the ISFET device sensitivity, different standard buffer solutions (pH=3.00, 4.00, 7.00, 10.00 and 12.00) were used. Its stability performance was studied by monitoring the voltage V_{GS} variations during the addition of hydrochloric acid (HCl, 0.5 M) or sodium hydroxide (NaOH, 0.5 M) to tris (hydroxymethyl)-aminomethane buffer solution (0.1 M).

2.5. Electrochemical measurements

With an ISFET device, as a working electrode, and a KCl reference electrode, potentiometric measurements were conducted on the microoganisms immobilized in the agarose gel.

Acidic products of energy metabolism acidify cellular environments and the ISFET device measures the rate of proton excretion very close to the sensitive surface of the ISFET device as well as the global extracellular acidification. The potential, measured at room temperature, is related to the micro-environment pH. In fact, the more the pH solution decreases the more the gate voltage V_{GS} increases.

In order to control the proton diffusion from the inside of the dialysis membrane to the outside, conventional pH measurements were reported at the same time as those measured by the ISFET device. The results were analyzed and compared according to measurement sites.

3. Results and discussion

3.1. Chemical characterization of the ISFET device

The good functioning of our ISFET chemical transducer has been demonstrated by the H^+ ion detection in different pH solutions (Fig. 2). A quasi-Nernstian pH response has been obtained when pH varies from 3 to 12. Its sensitivity has been finally estimated at 50 mV/pH. This result is in accordance with those reported in the literature [35–39]. The dynamic potentiometric response of the ISFETs was studied by adding known aliquots of acid and base to a 25 ml of 0.1 M tris (hydroxymethyl)-aminomethane with an initial pH 9.4. Every 5 min, 400 μl of 0.5 M HCl were added to decrease pH. After that, the same aliquots of 0.5 M NaOH were added to increase it. Fig. 3 shows a very stable electrochemical response of the ISFET versus time. Comparable sensitivity and stability have been recently proved by Castallarnau et al. [40].

3.2. Immobilization of bacteria in an agarose-gel preparation

CBBs performance criteria are not only dependent on the molecular recognition element, but also on its immobilization. Some progress has been made in improving stability by optimizing the immobilization conditions.

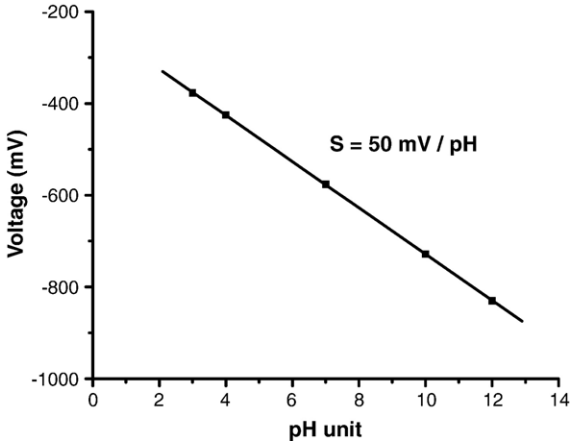

Fig. 2. ISFET device calibration: ISFET response indicated by Gate-source voltage V_{GS} in function with different pH solutions.

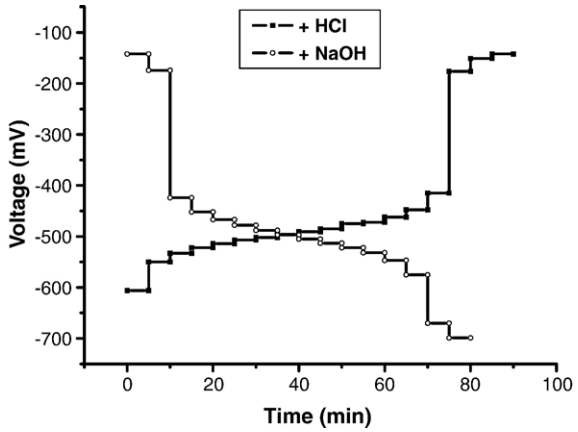

Fig. 3. ISFET dynamic response curves due to pH variations of tris buffer solution (0.1 M). The solution pH was initially reduced by addition of 0.5 M HCl and than decreased by 0.5 M NaOH.

Using agarose gel, the luminous microorganisms could live more than 1 week. This was controlled by culture of the bacteria in a new medium one week later. Bacterial growth was observed and fluorescence persisted. Therefore, it could be assumed that with this three-dimensional immobilization system, the biosensor's stability would be suitable.

The bacteria were confined and immobilized in the interstices of the agarose gel while keeping their physiological functions and viability (Fig. 4). However, the bacteria were not homogenously distributed in the agarose gel, probably as a result of nutrient diffusion. It is, in fact, well documented that agarose gels, which consist mainly of water, have a solid structure based on the formation of linear polysaccharide chains bridged together by hydrogen bonds. These bridges lead to a pentagonal porous structure revealed by scanning electron microscope studies on agarose gels [41]. These pores are large enough to allow the diffusion of the ionic or molecular constituents of the medium to microorganisms. Measurements of solute concentration gradients indicate that transport through a porous medium such as an agarose gel is dominated by diffusion and not by convection. Wolfaardt et al. [41] showed a linear relationship between fluorescein concentration, considered as a biological conservative tracer, and fluorescence intensity with an effective diffusion coefficient $D_e = 4.7 \times 10^{-10}$ m^2 s^{-1}. These findings might explain the concentration gradients of nutrients and therefore the degree of agarose-gel surface colonisation.

Because nutrients diffusion is known to be more significant in a solution than in a porous medium, the ISFET measurements were conducted on a soft gel (0.2% agarose) and on a sterile water solution. A 1% NaCl solution was added to ensure the conduction of both media. In order to compare the response of the ISFET device to the proton in a porous medium to that in a water solution, potential variations versus time were performed while a 40 ml of HCl solution (10^{-3} M) was injected. The results, reported in Fig. 5, clearly indicated that without agarose gel, the injected protons reached the ISFET sensitive element more rapidly than with the gel preparation. The slope was in fact higher (slope ≈ 16 mV/min) in the first experience than in the second one (slope ≈ 5 mV/min).

3.3. Bioprocess monitoring with an electrochemical sensor system

The electrochemical sensor system has proved very suitable for use as a bioactivity monitor in bacteria and could be of great interest in bioprocess control. In our work, we first compared the metabolic activities of bacteria via pH variations simultaneously detected by ISFETs and conventional pH measurements on immobilized bacteria in an agarose-gel preparation.

The recorded ISFET signal illustrated on Fig. 6 showed that the bacterial metabolism was characterized by two phases. The first one was attributed to a micro-environment acidification. Here, the bacteria acidify their micro-environment because of acidic products of carbohydrate energy metabolism. This pH decrease was explained, as in [42], by the oxidation and/or the fermentation of carbohydrates. Since this carbohydrate source decreases and there are only peptones in the culture medium, a second phase corresponding to an alkalization of the micro-environment appears. This result confirms those showing that the biological breakdown of peptones leads to NH_3 release and induces a pH increase [37,43,44]. This apparent substrate

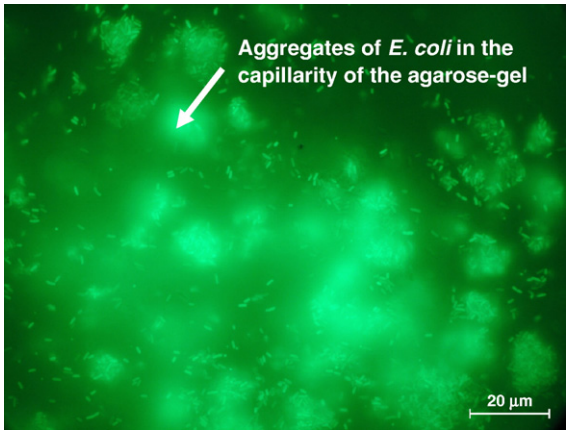

Fig. 4. PHL 1273 immobilized in the capillarity of the agarose gel (×100). An example of bacteria aggregates is shown by an arrow.

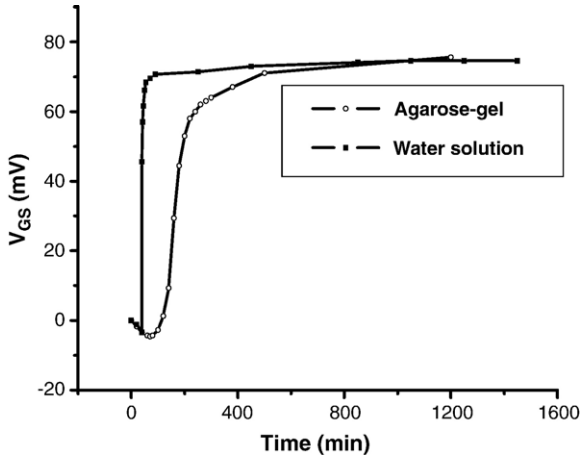

Fig. 5. Comparison between HCl diffusion into a liquid medium and that into a soft gel.

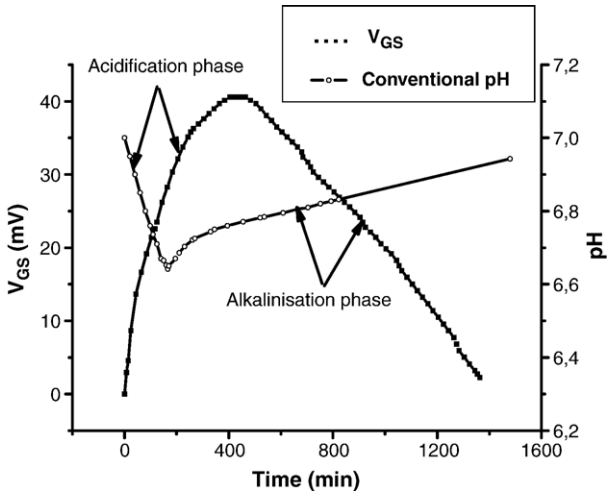

Fig. 6. Comparison between ISFET and conventional pH measurements during *E. coli* metabolism in agarose gel without a dialysis membrane.

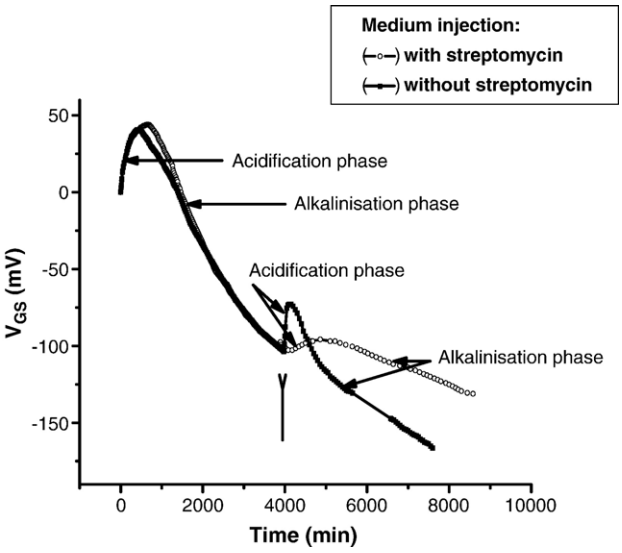

Fig. 7. *E. coli* strain response to addition of culture medium with or without streptomycin (20 μg/ml) during the alkalization phase. The injection time is indicated by an arrow. In each case, acidification and alkalization phases are indicated by arrows.

preference clearly led to differences in the quantity and quality of organic carbon substrates in the subsurface of the gel preparation. That is why, in the first stage, the increase in the proton excretion was more rapid (slope ≈ +2.7 mV/min) than its decrease during the alkalization stage (slope ≈ −0.28 mV/min).

These findings corroborate the low ratios of aminopeptidase to β-glucosidase activity observed in attached and free-living aquifer bacteria [45]. So, the microorganisms could have a higher polysaccharides hydrolysis rate than that of polypeptides.

While conventional pH measurements show that acidification step lasts for 3 h 30 min after the onset of incubation, the ISFET monitoring indicates a duration of 7 h for this step. Therefore, the pH variation was very weak and seems to increase very slowly (Fig. 6). The ISFET technique could be considered as a more sensitive and a more rapid device than direct pH measurements.

Exposing the *E. coli* PHL 1273 to a new-culture medium with or without antibiotics is a typical and suitable procedure for cell activity monitoring and toxicity screening. Antibiotics are, in fact, known to inhibit protein synthesis and bind to S12 protein of 30S ribosomal subunit, causing misreading or the inhibition of the initiation step. During the alkalization phase, the addition of 20 ml of the culture medium (LB 1/2, v/v) immediately led to the reappearance of the acidification stage. Two hours later, the alkalization phase was again observed. However, the injection of 20 ml of the culture medium (LB 1/2, v/v) associated with streptomycin (100 μg ml^{-1}) induced a decrease of about 85% in the acidification phase as well as a slight alkalinization (Fig. 7). This could be explained by the death of most of the bacteria.

Fundamental advances in whole cell-based sensors and microsystems have only established the acidification phase which is nonetheless considered as a significant indicator of the global cellular metabolism [42,46–48]. Our results, however, confirm that the bacterial metabolism follows two phases detected by the ISFET measurements. Furthermore, the three-dimensional immobilization of bacteria in agarose gel represents a cell-based biosensor able to reveal the presence of an inhibitor or an activator of the bacterial metabolism.

3.4. Miniaturisation of the system and validation of an antibiotic effect

In order to reduce the measurement volume from 100 ml to 1 ml, 500 μl of agarose (0.4%, w/v) were mixed with 500 μl of *E. coli* suspension and introduced into a dialysis membrane. Then the membrane was put in a 100 ml culture medium (LB 1/2) with or without different concentrations of streptomycin.

Fig. 8 shows voltage versus time when the ISFET measurements are carried out both with and without a dialysis membrane. Two phases (acidification and alkalinization) were observed as previously described. Kinetically, they are more noticeable with a dialysis membrane than without, particularly during the alkalinization stage. Thus, with a miniaturized system, the ISFET's detections have an apparent greater sensitivity probably due to

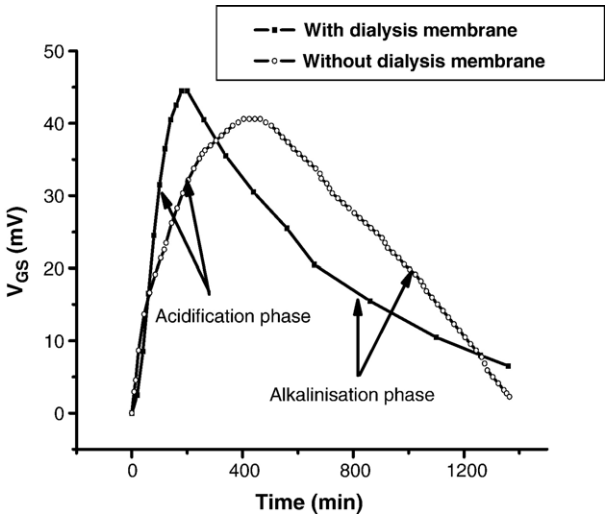

Fig. 8. Comparison between the ISFET measurements conducted with or without a dialysis membrane.

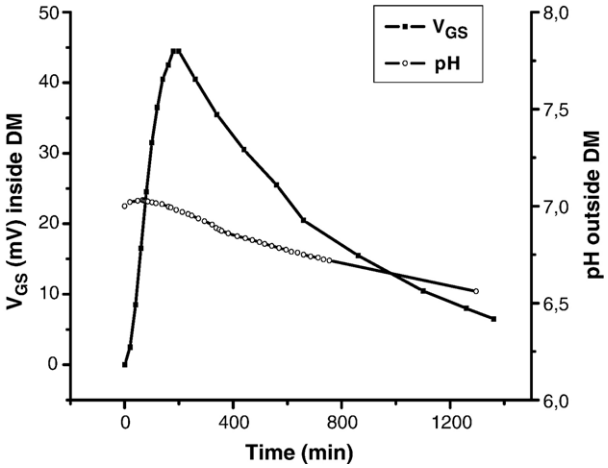

Fig. 9. Agarose-gel immobilized *E. coli* in a dialysis membrane. Comparison between the ISFET responses inside the dialysis membrane and conventional pH measurements outside the membrane.

the diffusion process being limited by the dialysis membrane. Such a process is based on the transport of ionic constituents (the proton and the nutrients) from the inside to the outside of the membrane (in the direction of their concentration gradient).

To confirm or invalidate this diffusion mechanism, the ISFET measurements inside the dialysis membrane were compared to the pH values recorded simultaneously outside the membrane (Fig. 9). The conventional pH evaluation seemed less sensitive than the ISFET monitoring especially in detecting the acidification phase. During alkalization, a slight pH decrease was observed. It reached 4% 11 h after the experience had started. This may be explained by the transport of the proton from the agarose-gel preparation inside the dialysis membrane to the culture medium outside the membrane. When the ISFET and the conventional pH measurements were simultaneously conducted outside the dialysis membrane, an almost constant pH was observed whereas the voltage V_{GS} varied greatly indicating a pH decrease outside the dialysis membrane. This result confirmed H^+ diffusion process from the inside to the outside and showed a

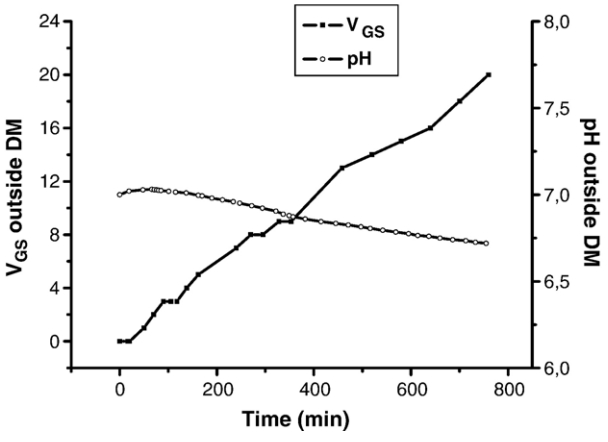

Fig. 10. Comparison between ISFET and pH measurements simultaneously conducted outside the dialysis membrane.

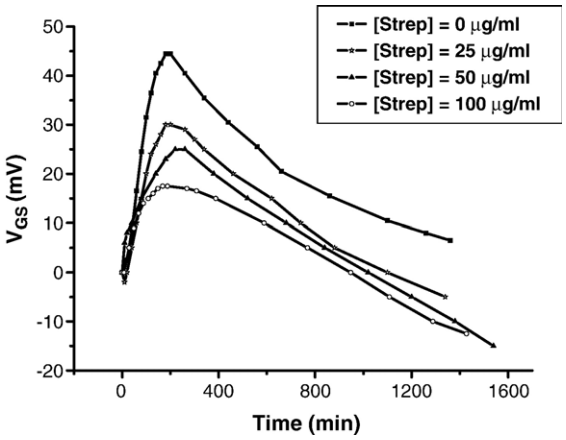

Fig. 11. Antibiotic effect at various concentrations on potential measurements during *E. coli* metabolism in a dialysis membrane.

better detection with the ISFET device than with conventional pH measurements (Fig. 10).

Because it is stable and offers possible perspectives in the study of cellular metabolic activities, this bacterial biosensor was tested with different bacterial preparations associated to variable antibiotic concentrations. Each experiment was carried out separately and repeated three times. The CBBs' responses showed that the potential variations versus time decreased when the streptomycin concentration increased (Fig. 11). An addition of streptomycin (100 μg ml^{-1}) to the culture medium reduced the acidification rate to 50%. At 200 min, the maximum acidification decreased with the antibiotic concentration. In fact, the higher the antibiotic concentration is the fewer the bacteria are which induces a lower acidification rate. This antibiotic effect on bacterial cells could be then considered as a model to test pH modifications by ISFET measurements. The same ISFET device behaviour has been recently observed during glucose catabolism by *E. coli* cells [40].

4. Conclusion

ISFET sensors sensitive to pH have been successfully adapted to the detection of *E. coli* bacterial activity using a three-dimensional immobilization in hydrogel matrix (agarose). Relying upon the ability of culturing bacteria for long periods while maintaining their physiological function, this reproducible immobilization system enables us to characterize the different activity phases of the tested bacterial strain.

By monitoring the ISFET gate-source voltage V_{GS} variations, our experiments demonstrated the presence of the two steps characterizing the bacterial activity (acidification and alkalization).

The presence of these two phases could be related to the bacterial metabolism of carbohydrates and peptones. This correlation could be better demonstrated using mutants with low ratios of aminopeptidase to β-galactosidase activity. The same technique could also involve one or several different strains. In fact, with different bacterial strains, sugar catabolism could be simultaneously assessed. When using a single strain, the concentration change of several ions induced by the modified

cellular metabolism could be easily and simultaneously detected. Although this multi-detection generally requires an adapted characterization, multi-ISFET cell-based biosensors and data processing, CBBs could have potentially great applications particularly in the biomedical and biotechnological fields. Thus, a better understanding of the dynamic cell behaviour might improve cell manipulations in the presence of drugs or toxic substances. In this study, the detection of an antibiotic effect on bacterial metabolism has validated these CBBs.

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