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# Potentiometric detection of ATP based on the transmembrane proton gradient generated by ATPase reconstituted on a gold electrode

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ABSTRACT

Adenosine triphosphate (ATP) is a key molecule as energy vector for living organisms, therefore its detection reveals the presence of microbial colonies. Environments where the existence of microbial pathogens suppose a health hazard can benefit from real time monitoring of such molecule. We report a potentiometric biosensor based on ATP-synthase from *Escherichia coli* reconstituted in a floating phospholipid bilayer over gold electrodes modified with a 4-aminothiophenol self-assembled monolayer. The use of a pH-dependent redox probe on the electrode surface allows a simple, specific and reliable on site determination of ATP concentration from 1 μM to 1 mM. The broad range ATP biosensor can offer an alternative way of measuring in a few minutes the presence of microbial contamination.



## 1. Introduction

Adenosine triphosphate (ATP) is the main biochemical energy vector in living organisms that supplies energy to many endergonic metabolic processes [1,2]. ATP is produced in living cells by the transmembrane enzyme $F_1F_o$-ATP-synthase, which catalyzes the thermodynamically uphill reaction of ADP phosphorylation using the proton electrochemical gradient across the membrane as driving force [3,4]. The enzyme also catalyzes the reverse reaction of ATP hydrolysis, thus it is also known as ATPase, for generating the proton-motive force necessary for certain functions, such us locomotion and nutrient uptake in microorganisms [4]. ATP synthesis or hydrolysis at the active site located in the hydrophilic $F_1$ part of the enzyme is coupled to the translocation of protons across the cellular membrane by the hydrophobic $F_o$ part [3–5].

ATP is a good indicator of cell metabolism. Thus, its direct, sensitive and reliable detection is desired in order to monitor the presence of pathological events [6–10] or to correlate with microbial contamination on surfaces, which is an important issue in food industry and in healthcare settings [11,12]. For the latter application niches it is crucial to develop selective biosensors with fast-response, broad detection range, portable and easy to handle. Many types of ATP biosensors have been reported in the literature, including aptamer, [7–9,13,14] ion-channel [15] and enzyme-based ones [6,10,16].

Currently there is a great interest in studying *in vitro* applications of transmembrane proteins reconstituted in biomimetic membranes. One of the major applications is for the development of highly specific and robust biosensors [17]. Electrochemical transduction is convenient for designing portable biosensors for *in situ* detection. It has the advantages of versatility, fast-response, sensitivity and simplicity [18,19]. A way to couple the presence of ATP in the medium to an electrochemical process is by reconstituting ATPase on a biomimetic membrane over an electrode. Furthermore, the presence of a lipid layer over the electrode can prevent fouling of its surface by the medium or the non-desired signals due to redox-active interferents. Naumann and co-workers have studied the incorporation of ATPases to lipid bilayers supported on electrodes. The aim was to correlate the proton translocation activity of the enzyme across the biomimetic membrane with electrochemical processes occurring at the electrode by the methods of square wave voltammetry, double potential-pulse chronoamperometry or electrochemical impedance spectroscopy [20–22]. ATP-hydrolysing electrodes have also been studied by incorporation of the

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$Na^+/K^+$-ATPase pump to lipidic liquid crystalline cubic phases, which is a suitable material for membrane-proteins immobilization onto carbon electrodes [23].

In a previous work we reported the reconstitution of the $F_1F_o$-ATPase from *E. coli* in a floating phospholipid bilayer (PhBL) formed over a gold electrode modified with a self-assembled monolayer of 4-aminothiophenol. It was shown that the immobilized ATPase was simultaneoulsy functional for ATP hydrolysis and the translocation of protons into the membrane/electrode interface [24]. In the present work we study the application of the ATPase-modified electrode as ATP biosensor using the 4-aminothiophenol monolayer on the gold electrode as redox probe of proton translocation by the ATPase to the PhBL/electrode interface.

## 2. Materials and methods

### 2.1. Chemicals

NaOH 99%, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid 99% (HEPES), 4-aminothiophenol 97%, adenosine 5′-triphosphate disodium salt hydrate BioXtra, ≥99% (ATP), chloroform absolute grade, methanol absolute grade, ethanol absolute grade, $H_2NaPO_4$ 99% and $Na_2HPO_4$ 99% were purchased from Sigma-Aldrich. The Bio-Beads SM-2 adsorbents used were from Bio-Rad, and sulfuric acid 96% and hydrogen peroxide 33% were supplied by Panreac. The MicroPolish alumina suspensions were obtained from Buehler. All solutions were prepared with Mili-Q grade water (18.2 MΩ, Millipore).

### 2.2. Enzyme

*E. coli* ATP-synthase was isolated as described by Gutiérrez-Sanz et al. [24]. The concentration of the enzyme was determined with RC DC™ Protein Assay kit (BioRad) based on the Lowry protocol [25].

### 2.3. Preparation of liposomes and proteoliposomes

Two types of liposomes and proteoliposomes with different mixes of lipids were made. One of them consisted in 90% L-α-phosphatidylcholine from chicken egg (Egg PC, Avanti Polar Lipids) and 10% L-α-phosphatidic acid from chicken egg (Egg PA, Avanti Polar Lipids). The other mix contained 82% Egg PC, 9% Egg PA and 9% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-5000] ammonium salt (DSPE-PEG5000, Avanti Polar Lipids). Both types of liposomes and proteoliposomes were prepared following an already published protocol [24].

### 2.4. Preparation of microbial samples

Microbial samples were prepared by growing gram-negative *E. coli* CECT 516 cells in Nutrient Broth (NB: 5 g/L beef extract, 10 g/L peptone, 5 g/L NaCl, pH 7.0 ± 0.2) at 37 °C under agitation. The *E. coli* bacterial cells concentration was correlated with the optical density ($A_{600nm}$) of the culture. Cultures of $10^3$, $10^6$ and $10^8$ cell/mL in NB were used to extract ATP by $HClO_4$ treatment [26]. To that end, 1.2 ml of culture medium were added to 0.3 ml of ice-cold 30% (w/v) $HClO_4$. After 10 min the samples were mixed in a vortex and, subsequently, centrifuged at 11,300 × g for 5 min. The samples were frozen until the electrochemical measurements were done, for which their pH was readjusted to 7.6.

### 2.5. Preparation of Au/PhBL/ATPase electrodes

Disk electrodes of polycrystalline gold and 0.5 cm diameter (Pine Instruments) were first immersed in a freshly prepared "*piranha solution*" consisting of 3 volumes of sulfuric acid 96% and 1 volume of hydrogen peroxide 33% during 15 min, rinsed after with MilliQ water and then polished successively with alumina suspensions of 1, 0.3 and 0.05 μm particle diameter. To remove alumina residues the electrodes were sonicated during 20 min in a solution of EtOH:$H_2O$ 1:1. Afterwards the electrode surfaces were cleaned electrochemically by cyclic voltammetry (CV). First 15 cycles between 0 V and −1.5 V at 200 mV/s scan rate in 0.5 M NaOH were performed, followed by 20 cycles between 0 V and +1.5 V at 200 mV/s scan rate in 0.5 M sulfuric acid. The clean bare gold electrodes were modified with a thiol self-assembled monolayer (SAM) by overnight incubation in a solution of 1 mM 4-aminothiophenol in ethanol at room temperature. Finally, the electrodes were rinsed carefully with ethanol and water and incubated overnight at 4 °C with a drop of 40 μL of 0.4 mg/mL proteoliposomes deposited onto the gold surface.

### 2.6. Electrochemical measurements

An Autolab PGSTAT30 potentiostat controlled by Nova 2.1.1 software (Eco Chemie) was used for the electrochemical experiments. The three electrodes cell comprised a BAS Ag/AgCl (3 M NaCl) reference electrode (+200 mV vs. NHE), a platinum wire as counter electrode and the working electrode. All redox potentials are given relative to the used reference electrode. Differential pulse voltammetries (DPV) were performed between 80 mV and 230 mV with a step potential of 0.75 mV, a modulation amplitude of 25 mV, a modulation time of 0.05 s and an interval time of 0.5 s. For improved signal stability and reproducibility, a potential of −300 mV was applied during 3 min before each DPV measured. All electrochemical measurements were carried out in 20 ml of 0.1 M HEPES buffer pH 7.6 at 37 °C.

### 2.7. AFM measurements

The goldcoated glass slides employed in the AFM measurements were purchased from Arrandee Metal GmbH. After cleaning in piranha solution, the gold substrates were flame-annealed to produce large and well-ordered and large Au(1 1 1) terraces. Once cooled, they were incubated overnight for modification with a 4-aminothiophenol SAM as described above. After thorough rinsing with ethanol and MilliQ water, the gold substrate was incubated overnight in a solution of proteoliposomes (0.4 mg/mL in phosphate buffer pH 5.5) at 4 °C. Before any further experiments the gold surface was rinsed with HEPES buffer at pH 7.6.

Tapping mode AFM images were acquired with an Atomic Force Microscope from Agilent Technologies 5500 with the substrate immersed in 0.1 M HEPES buffer solution at pH 7.6. All images were recorded at room temperature employing rectangular silicon nitride cantilevers with a nominal spring constant of 0.72 $Nm^{-1}$ and nominal resonant frequency of 70 kHz (Olympus OMCL-RC). Data acquisition and analysis were performed using PicoView 1.3 (Agilent Technologies) and WSxM 5.0 Develop 8.0 (NanoTech), respectively.

## 3. Results and discussion

### 3.1. Preparation and characterization of ATPase-modified gold electrodes

Two types of proteoliposomes containing *E. coli* $F_1F_0$ ATPase were prepared for its reconstitution over Au electrodes modified with a SAM of 4-aminothiophenol. In previous reports we have shown that this surface is appropriate for the fusion of proteoliposomes containing membrane enzymes such as hydrogenase,

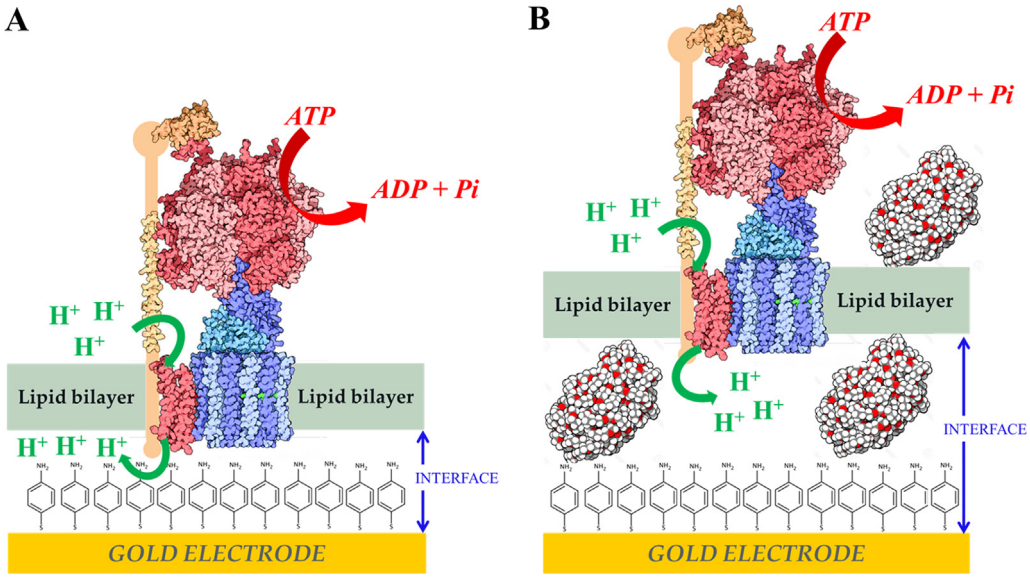
complex I or ATPase, leading to formation of floating PhBLs [24,27,28]. For the present work, we have compared the results obtained with the phospholipid mix used previously for functional reconstitution of *E. coli* $F_1F_o$-ATPase, which was active for both ATP hydrolysis and proton translocation across the biomimetic membrane [24], with a mix that contained in addition a phospholipid derivatized with poly(ethylene glycol) of molecular weight 5,000 (DSPE-PEG5000). The purpose of including the latter phospholipid was to increase the volume of the aqueous interface between the PhBL and the electrode surface due to the presence of the bulky hydrophilic polymer [29–31], as shown in Schematic 1. It was expected that increasing the hydrophilic boundary between the electrode and the PhBL would increase the amount of protons that could be translocated from the bulk electrolyte across the biomimetic membrane due to the ATPase activity.

The AFM images shown in Fig. 1 confirmed that both types of proteoliposomes fused on the SAM thiol-modified Au surface and formed a continuous PhBL. Fig. 1A shows the image of a bare gold electrode, whereas Fig. 1B shows the surface of a Au/PhBL/ATPase electrode. The presence of PhBL smooths the underlying roughness of the gold surface, as was shown in a previous work [24]. The 5–10 nm high protrusions observed on top of the flat gold regions (Fig. S1A-C, Supp. Inf.) correspond to the large $F_1$ soluble domains of the ATPase [24]. Fig. 1C and S12D-E (Supp. Inf.) show that the surface of the Au/PEG-PhBL/ATPase substrate is more heterogeneous and presents additional larger 15–20 nm protrusions (Fig. S1E), which could correspond to PEG5000 aggregates extending away from the upper leaflet of the PhBL.

We have previously shown that the 4-aminothiophenol SAM on the Au electrode surface can be used as redox probe of generation of a proton gradient across the floating PhBL [32]. This detection method is based on electrochemical oxidation and subsequent dimerization of 4-aminothiophenol groups on the surface for obtaining quinone diimines (Schematic S1, Supp Inf.), which have a 2 $e^-$/2 $H^+$ redox process [33]. Therefore, the redox potential of this process is pH-dependent and measuring the shifts of the peak potential by DPV allows sensitive monitoring of local pH changes in the electrode/PhBL interface [32]. Fig. 2 shows the cyclic voltammograms recorded for the generation of the redox probe at a gold electrode modified with the 4-aminothiophenol SAM and reconstituted ATPase in a PEG-PhBL. In the first cycles the oxidation of SAM thiols at potentials higher than 0.3 V is clearly observed, while the quasi-reversible redox process of the redox probe at approximately 0.18 V is gradually enhanced as the number of scans is increased to 15.

### 3.2. Potentiometric detection of ATP

Once the pH-sensitive redox probe was generated on the electrode surface, DPV measurements were done before and after addition of ATP into the electrolyte solution. The DPVs were scanned in the oxidative direction, thus a preconditioning potential of −0.3 V was applied during 3 min before the start of the measurement in order to have all the quinone diimine groups on the electrode surface in the reduced state. In this way a higher sensitivity and reproducibility of results was obtained. Fig. 3 shows the time evolution of the DPVs measured for an Au/PEG-PhBL/ATPase after the addition of 300 μM ATP. It can be clearly observed that the peak potential corresponding to the oxidation of the redox probe gradually shifted towards higher potentials, which indicates that the pH at the electrode/PEG-PhBL interface was decreasing during the experiment [32]. This result is in agreement with reconstituted ATPase hydrolysing ATP from the bulk solution while translocating protons across the biomimetic membrane as depicted in Schematic 1B. Fig. 4 compares the effect of ATP addition to ATPase-modified electrodes with PhBL that either contained PEG5000 spacer or not. In the absence of DSPE-PEG5000 a higher initial shift of the DPV peak potential was measured, but after the first minute the rate of potential change decreased and finally stopped after 90 min (Fig. 4a). This result suggests that the ATPase activity of proton translocation towards the PhBL/electrode interface was diminishing with time. Another possible explanation may be that a steady state was reached due to the reconstituted enzyme catalysing also the reverse reaction of ATP synthesis and translocation towards the bulk solution [5]. When the biomimetic membrane contained DSPE-PEG5000, the rate of peak potential shift was more regular (Fig. 4b), reaching higher values than with the Au/PhBL/ATPase electrode after 60 min. Control experiments were performed either with an Au/PEG-PhBL/ATPase electrode in absence of ATP (Fig. 4d) or with an electrode only modified with the redox probe (Au-SAM) in presence of ATP (Fig. 4c). In both control measurements, the shifts of the DPV peak potential during 120 min were either negligible or much smaller than for the ATPase-modified electrodes in presence of ATP. The small initial shift




**Schematic 1.** ATPase reconstitution in a floating PhBL (A) or PhBL-PEG (B) over Au electrodes modified with a 4-aminothiophenol SAM.

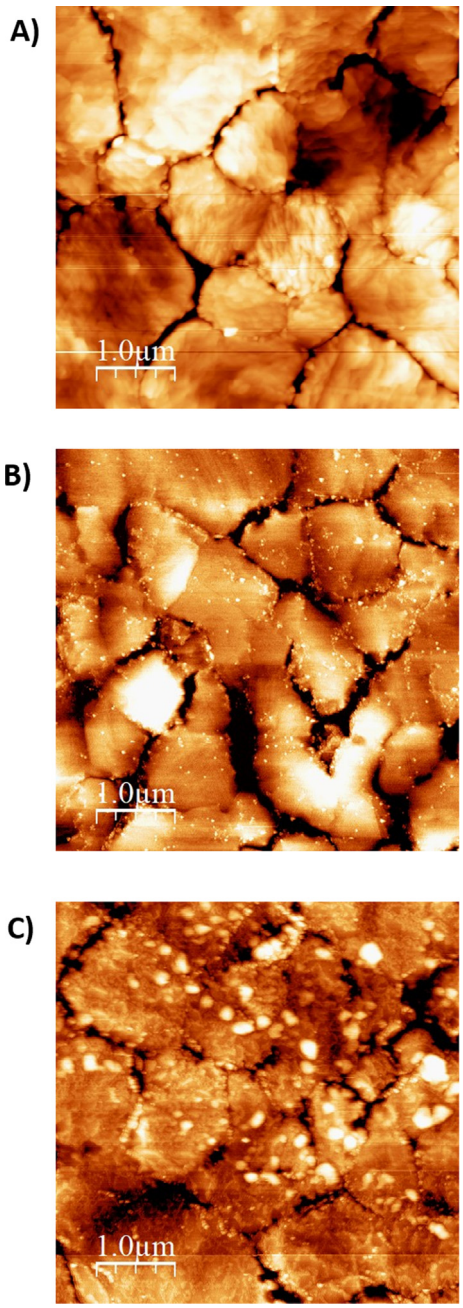



**Fig. 1.** Surface topography of the modified Au(1 1 1) gold substrates. **A**FM topography images of bare Au (A), Au/PhBL/ATPase (B) and Au/PEG-PhBL/ATPase (C).

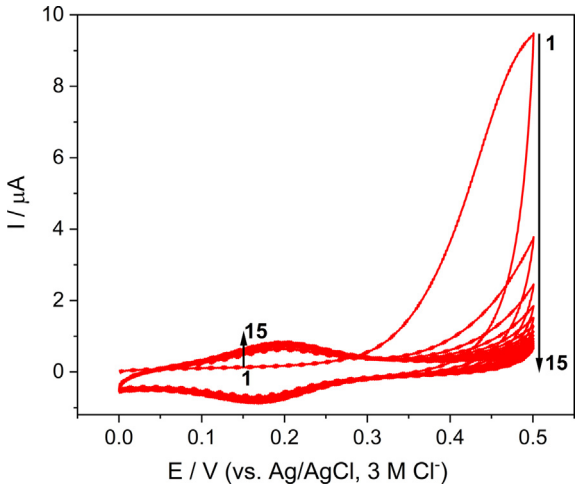



**Fig. 2.** Electrosynthesis of the pH-dependent redox probe at the surface of a Au/PEG-PhBL/ATPase electrode by 15 successive scans of cyclic voltammetry in 0.1 M HEPES buffer pH 7.6 at 37° C. The scan rate was 0.2 V/s.

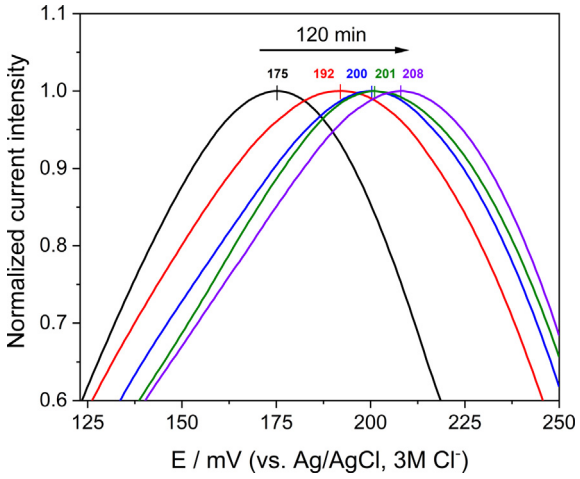



**Fig. 3.** Differential pulse voltammograms of the oxidation of the pH-dependent redox probe at the surface of a Au/PEG-PhBL/ATPase electrode measured in 0.1 M HEPES buffer pH 7.6 before (black line) and 30 min (red line), 60 min (blue line), 90 min (green line) and 120 min (purple line) after the addition of 300 μM ATP. The temperature was 37 °C. The current intensity values are normalized against the peak currents. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measured for the control electrode without the biomimetic membrane is caused by a slight acidification of the electrolyte after addition of a high concentration of ATP. The small shifts variation with time of the blank measurements obtained with a Au/PEG-PhBL/ATPase electrode, which look almost periodic, could reflect the influence of fluctuations of the biomimetic membrane (it is negatively charged in the hydrophilic part) on the redox potential of the surface-attached redox probe.

These measurements shown in Fig. 4 clearly indicate that the significant peak potential increase of the redox probe with time can be correlated with the reconstituted ATPase activity. The presence of PEG5000 between the PhBL and the electrode leads to a more stable ATP hydrolysis activity, which is compatible with a thicker aqueous phase in the Au/PhBL interface that allows the accumulation of more membrane-translocated protons [29,30]. In consequence, more reliable DPV measurements of the redox probe were obtained with the Au/PEG-PhBL/ATPase electrode. Thus all further experiments reported were performed with electrodes modified with PEG-containing proteoliposomes or liposomes.

The correlation between the electrochemical response of the redox probe and the ATP concentration in the 1 μM −10 mM range was studied for several Au/PEG-PhBL/ATPase electrodes equally prepared. For each electrode the DPV peak potential of the redox probe was measured after 5, 10 and 20 min of ATP addition (Fig. 5). Although a much larger potentiometric change was measured after longer times of analyte addition (Fig. 4), a compromise between time response and transduction signal magnitude was contemplated for studying the effect of ATP concentration. At all measured times the average peak potential shift increased with the amount of ATP added to the electrolyte. The negative control measurements with Au/PEG-PhBL electrodes yielded a lower electrochemical response in presence of ATP, especially at shorter

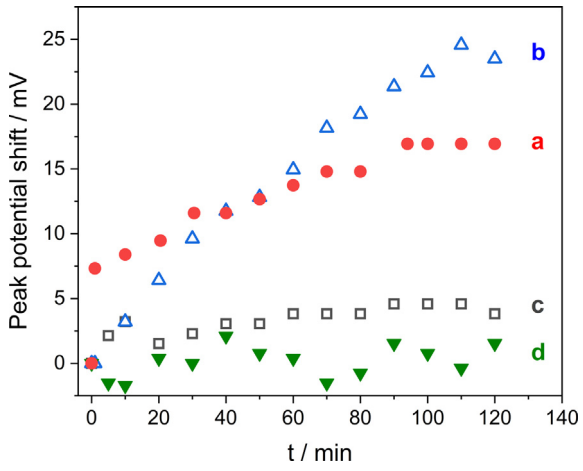



**Fig. 4.** Evolution of the DPV potential peak after addition of 150 μM ATP for (a) Au/PhBL/ATPase (solid red circles), (b) Au/PEG-PhBL/ATPase (open blue triangles) and (c) Au-SAM electrodes (open black squares). (d) The closed green triangles correspond to the control measurement for Au/PEG-PhBL/ATPase electrode in absence of ATP in solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

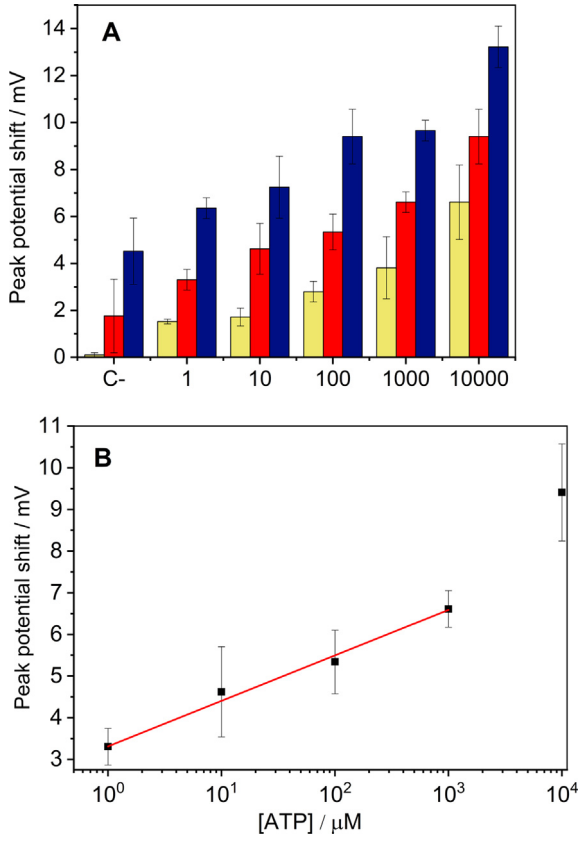



**Fig. 5.** DPV response of Au/PEG-PhBL/ATPase electrodes at different [ATP] values in 0.1 M HEPES buffer pH 7.6 at 37° C. A) Bars graphic representing the values measured after 5 (yellow), 10 (red) and 20 (dark blue) min after ATP addition. The negative control (C-) corresponds to electrodes without ATPase (Au/PEG-PhBL). The data represent average values from at least 3 different electrodes prepared in the same way. B) Calibration plot of the potentiometric ATP biosensor obtained from the 10 min measurements. The correlation coefficient ($r$) for the linear fitting in the 1–1000 μM range is 0.98. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

times (Fig. 5A). Although in some cases the standard deviation of measurements obtained with different electrodes was high, an ANOVA statistical test indicated that the differences measured at different ATP concentrations are significant (Tables S1 and S2, Supporting Information). Fig. 5B shows that the peak potential shift of the redox probe measured at t = 10 min increased logarithmically with ATP concentration in the 1 μM-1 mM range, whereas at 10 mM ATP the electrochemical response deviated from this dependence. Similar fits were obtained for the measurements at 5 and 20 min after ATP addition (Fig. S2, Supp Inf.). This logarithmic dependence of the electrochemical signal on the ATP concentration is the expected one if the local pH change at the Au/PhBL interface is due to the ATPase translocation activity with a constant thermodynamic $H^+$/ATP ratio [3]. Therefore, the developed Au/PEG-PhBL/ATPase electrode can be used as a specific potentiometric biosensor of ATP in a concentration range of 3 orders of magnitude.

### 3.3. Application to microbial contamination detection

This broad range for quantitative determination of ATP by the Au/PEG-PhBL/ATPase electrode together with the detection limit of 1 μM (estimated from a response 3 times higher than the blank measurements standard deviation) are suitable for monitoring the presence of microbial contamination in different environments. In order to test whether the potentiometric response of the ATP biosensor could be correlated with the amount of microbial cells present in a sample, ATP was extracted from *E. coli* cultures at $10^3$, $10^6$ and $10^8$ cell/mL concentrations. DPV measurements were recorded after addition of 0.5 ml of each sample to the electrolyte solution. Fig. 6 shows the average potentiometric response of the ATP biosensor at different times after the sample additions. It can be clearly observed that the potentiometric response increased with time and with the concentration of *E. coli* cells from which the samples had been prepared. Therefore, the biosensor is able to detect the microbial ATP extracted. The control experiments done by addition of samples prepared from different bacterial concentrations to Au/PhBL electrodes resulted in significantly smaller potential shifts, especially at the shortest measured time of 5 min.

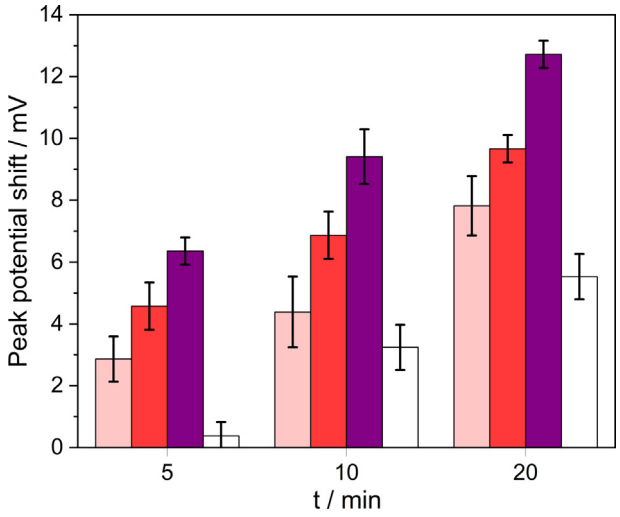



**Fig. 6.** Potentiometric response of the ATP biosensor at different times after 0.5 ml addition of samples prepared from $10^3$ cells/ml (pink), $10^6$ cells/ml (red) and $10^8$ cells/ml (purple) *E. coli* cultures. The white bars correspond to control measurements done with a Au/PEG-PhBL electrode after 0.5 ml addition of sample from $10^6$ cells/ml culture. Data represent average values of at least 3 different electrodes prepared in the same way. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As shown in Fig. 4d, the negative control measurement with no ATP nor microbial sample added did not give an appreciable change in the redox probe potentiometric response. These results confirm that the detection of bacterial contamination required specific ATP hydrolysis activity by the ATPase reconstituted on the electrode.

The conventional method for measuring ATP concentration is the bioluminescent assay based on luciferase activity [34]. Several studies have been done about the use of this assay for quantitative or semi-quantitative analysis of microbial contamination for on-site monitoring in food industry, water supply or healthcare settings [11,12,35]. Although very sensitive detection of total bacterial contamination has been reported with the bioluminescent assay, it required incubation times of 6–3 h for the measurement of samples with an initial *E. coli* titre of 3–1000 cell/mL, respectively [35]. With our ATP potentiometric biosensor the measurement time is much faster, requiring only 5 min for detection of extracted ATP in a 40-times diluted sample taken from an *E. coli* 1000 cell/mL culture. Furthermore, an innovative aspect of the developed potentiometric ATP biosensor is that the analyte recognition system, the reconstituted ATPase on the biomimetic membrane, is integrated with the transductor, which is the Au electrode modified with the pH-dependent redox probe.

## 4. Conclusions

Specific potentiometric detection of ATP is measured with ATPase from *E. coli* reconstituted in a floating phospholipid bilayer over a gold electrode modified with a 4-aminothiophenol SAM. The incorporation of PEG5000 as spacer between the electrode surface and the phospholipid bilayer allows a more reliable electrochemical response to the presence of ATP in the bulk electrolyte. The potentiometric biosensor can provide a quantified value of ATP concentration within 5–10 min ranging from 1 μM to 1 mM ATP. This broad range ATP biosensor can offer an alternative way of measuring in few minutes the existence of microbial contamination without needing big expensive laboratory facilities, although further studies will be required in industrial or healthcare settings in order to demonstrate the applied suitability of the method.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2020.107490.

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