Fast fabrication of reusable polyethersulfone microbial biosensors through biocompatible phase separation

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Abstract

In biosensors fabrication, entrapment in polymeric matrices allows efficient immobilization of the biorecognition elements without compromising their structure and activity. When considering living cells, the biocompatibility of both the matrix and the polymerization procedure are additional critical factors. Bio-polymeric gels (e.g. alginate) are biocompatible and polymerize under mild conditions, but they have poor stability. Most synthetic polymers (e.g. PVA), on the other hand, present improved stability at the expense of complex protocols involving chemical/physical treatments that decrease their biological compatibility. In an attempt to explore new solutions to this problem we have developed a procedure for the immobilization of bacterial cells in polyethersulfone (PES) using phase separation. The technology has been tested successfully in the construction of a bacterial biosensor for toxicity assessment.

Biosensors were coated with a 300µm bacteria-containing PES membrane, using non-solvent induced phase separation (membrane thickness ≈ 300 µm). With this method, up to $2.3x10^6$ cells were immobilized in the electrode surface with an entrapment efficiency of 8.2%, without compromising cell integrity or viability. Biosensing was performed electrochemically through ferricyanide respirometry, with metabolically-active entrapped bacteria reducing ferricyanide in the presence of glucose. PES biosensors showed good stability and reusability during dry frozen storage for up to 1 month. The analytical performance of the sensors was assessed carrying out a toxicity assay in which 3,5-dichlorophenol (DCP) was used as a model toxic compound. The biosensor provided a concentration-dependent response to DCP with half-maximal effective concentration (EC_{50}) of 9.2 ppm, well in agreement with reported values. This entrapment methodology is susceptible of mass production and allows easy and repetitive production of robust and sensitive bacterial biosensors.

Keywords

Bacterial immobilization, phase separation, polyethersulfone, microbial biosensor, respirometry, toxicity assessment.

1. Introduction

In biosensors, the recognition element (i.e. cells, enzymes, antibodies, etc.) is immobilized in close physical contact with the transducer in order to ensure compactness, sensitivity and short response times [1]. Ideal bio-functionalization protocols allow stable and controlled immobilization of the recognition element, without compromising its integrity or activity [2]. Regarding biosensors based on living bacteria, the biocompatibility of the immobilization protocol represents an additional and critical factor to be considered [3,4]. Until now, the most popular fabrication procedure has been the entrapment in biocompatible polymeric matrices [5-8], such as polymeric hydrogels of natural origin and synthetic polymers. Bio-polymeric hydrogels (e.g. alginate, agar, agarose and chitosan, among others) are usually preferred since they require soft reaction conditions (room temperature, neutral pH) and use harmless chemical reagents [9,10], which do not compromise the stability and activity of the biological elements. However, their application is compromised by the lack of reversibility of these materials to dehydration (they do not recover the initial polymeric structure after being dehydrated) and their poor chemical and biological stability [9]. On the other hand, synthetic polymers such as polyvinylalcohol (PVA) [11-13], polyacrylamide [14] and polyurethane [15] improve the chemical and structural stability as well as mechanical strength [16] of the matrix, but they require the use of toxic reagents [17], chemical/UV-light cross-linking and/or thermal treatment [18], which may compromise the function of the immobilized molecules.

In this work, we explore the possibility of using polyethersulfone deposited by phase separation, for the entrapment of active and viable bacterial cells. Phase separation is

a simple and quick procedure for the preparation of polymeric porous membranes that combines biocompatibility (extraction may be performed with a water-based solution) and stability of the final membrane. The procedure consists in the removal of solvent from the polymer to induce the formation of a porous solid membrane. Solvent removal from the polymeric solution can be achieved with temperature, evaporation or addition of a non-solvent solution (usually water) in which the polymer is insoluble [19]. This last approach, also known as non-solvent induced phase separation (NIPS), has been already used in the development of biosensors by immobilization of enzymes and antibodies in PES porous membranes [20-24], but so far has not been reported for the entrapment of whole microorganisms.

In this paper we explore for the first time the immobilization of *E. coli* by entrapment in polyethersulfone membranes through NIPS. Immobilization is carried out on the surface of a screen-printed electrode in order to obtain a working microbial activity biosensor. The integrity, viability and biological activity of the cells after the entrapment process is evaluated. Bacterial activity in the PES membrane is tested using electrochemical measurements and the applicability of the resulting biosensor is evaluated in an electrochemical respirometry assay for general toxicity assessment [25-29]. Biosensor reusability, reproducibility and stability under different storage conditions are also evaluated.

2. Methods

2.1 Chemicals and materials

Commercial polyethersulfone (PES) was obtained from BASF (BASF Ultrasons S3010, Frankfurt, Germany). Screen-printed carbon electrodes (DRP-110) with carbon working and counter electrodes and Ag pseudo-reference were purchased from Dropsens (Spain).

Potassium ferricyanide, 3,5-dichlorophenol, glucose were purchased from Panreac (Spain) and dimethyl sulfoxide (DMSO), γ-butyrolactone, ethyl-lactate, methyl-lactate and N,N-dimethylformamide (DMF) from Sigma-Aldrich (US). All chemicals were of analytical grade and all solutions were prepared using Milli-Q water.

2.2 Microorganisms

Escherichia coli ATCC 10536 was grown aerobically in 100 mL of Luria-Bertani medium (LB) broth for 18 hours at 37 ºC. Grown cultures were centrifuged at 10100 x g for 10 min and re-suspended in 2 mL of Milli-Q water, providing a final bacterial concentration of $5.6x10^9$ cells/mL. Viable numbers were determined using LB agar plates after suitable dilutions. Total cell numbers were determined by epifluorescence microscopy of samples filtered through 0.2 µm polycarbonate filters, stained with 4′,6-diamidino-2-phenylindole (DAPI) fluorescent stain and observed with a Zeiss AXIO Imager A1 fluorescence microscope.

2.3 Survival of microorganisms in selected organic solvents

Succesful immobilization of live microorganisms in matrices of organic polymers requires that the cells survive the contact with the solvent used to prepare the polymer solution. To study the effect of different solvents in bacterial viability, bacterial pellets from overnight cultures of *Escherichia coli* were re-suspended in 500 µL of solvent, i.e. DMSO, DMF, ethyl-lactate, methyl-lactate, γ-butyrolactone and phosphate buffer used as control, and incubated for 5 min, 30 min and 24 h. After incubation, bacterial suspensions were centrifuged (5 minutes at 10100xg) to remove the solvent. The pellet was resuspended in phosphate buffer and centrifuged again. The procedure was repeated three times to ensure that any remaining solvent had been removed from the sample. Total and viable bacterial numbers were then determined as detailed above.

2.4. Biosensor fabrication

Non-solvent induced phase separation (NIPS) methods involve three components, namely the polymer, the solvent and the non-solvent. Membrane preparation started with the dissolution of the polymer, i.e. PES, in DMSO. Specifically, PES was dissolved (12% w/v) in DMSO at room temperature and stirred until obtaining a homogenous solution. Five microliters of the resulting solution were deposited on the working element of a screen-printed electrode (Dropsens, DRP-110). Then, 100 µl of nonsolvent were deposited on top of the PES/DMSO solution to induce phase separation and the formation of the porous membrane on the working electrode. For the electrodes used for the physical and electrochemical characterization of the membranes, the nonsolvent was MilliQ water. In the case of the electrodes used in the toxicity assays, the repeatability and reproducibility assays, and the storage stability assays, the nonsolvent was MilliQ water containing $5.6x10^9$ cells.mL^{-1}. Finally, for the electrodes used to assess the effect of cell concentration on electrode output, the nonsolvent was MilliQ water containing variable concentrations of microorganisms (0, $5.6x10^8$, $2.8x10^9$ and $5.6x10^9$ cells·mL^{-1}).

2.5. Biosensor characterization

Profilometry and SEM imaging. A Leica DCM 3D dual core optical profilometer was used to study the thickness and surface roughness of the deposited membranes.

Images of the inner structure of the PES membrane were acquired by Field Emission Scanning Electron Microscope (FE-SEM; Zeiss Merlin, Germany) from membrane fragments after cryofracture with liquid nitrogen.

Electrochemical measurements. Carbon screen printed electrodes (SPE; Dropsens 110) integrating a carbon working-electrode (4mm of diameter), carbon counter-electrode and Ag pseudo-reference electrode were used throughout this work. Electrochemical measurements were performed using the µSTAT8000 multi-potentiostat (Dropsens, Spain) and Dropview 8400 software (Dropsens, Spain). Cyclic voltammetries were performed between -0.5 and 0.6 V using equimolar mixtures

ferricyanide/ferrocyanide (1 mM) and a scan rate of 0.05 $V.s^{-1}$. Chronoamperometries were carried out at 0.4 V during 90 s. Under the conditions used the measurements leveled out after 20 s and stable values were recorded at 40 s.

Ferricyanide respirometry. In ferricyanide respirometry a preincubation is carried out in which oxygen is substituted by ferricyanide as the final electron acceptor for bacterial respiratory metabolism [25-29]. Importantly, only living and metabolically active cells reduced ferricyanide to ferrocyanide and thus, the level of ferricyanide reduction can be used to determine the level of microbial activity. The assay was performed preincubating PES biosensors with 1mM ferricyanide solutions containing 0.2% glucose as the carbon source. Different preincubation times were tested (0, 30, 60 or 90 min) but 60 minutes was chosen to carry out most of the experiments. Ferrocyanide accumulation was determined as the stable current value recorded after 40 s of chronoamperometry at 0.4 V (vs Ag/Ag Cl). Biosensors containing dead bacteria (killed by addition of 4% glutaraldehyde) or live bacteria without carbon source were used as negative controls.

3. Results

3.1 Biocompatibility of the solvents used in the phase-separation process.

To analyze the biocompatibility of several of the solvents available to dissolve PES we exposed *E. coli* cells to each of the solvents, as described in Methods, and checked their viability at different times. The results of the analysis are presented in Table 1. Cell viability was affected by the solvents, but remained relatively high (64-76%) after a 5 min exposure. After 30 minutes of contact ethyl-lactate and methil-lactate had the highest survival (36 and 32%) followed by DMSO (18%), DMF (11%) and γ-butyrolactone (9%). When contact between cells and solvent was extended to 24 h, viability decreased below $8.3x10^{-9}$ % in all cases, a reduction of more than 10 orders of magnitude from the initial value that indicates that virtually all the organisms present in the sample had been killed. Between the solvents tested, DMSO was chosen because

it allowed high survival at short times while providing the highest solubility for the type of PES used in our experiments**.**

3.2 Characterization of PES-coated electrodes

Screen-printed carbon electrodes coated with porous PES membranes were obtained by non-solvent induced phase separation following the procedure described in the Methods section. The resulting membranes, were analyzed by profilometry. The results, displayed in Figure 1a show the existence of a circular structure with an elevated central area which tapers towards the exterior. Maximum thickness at the center was 300 µm. Membranes were cross-sectioned and visualized by SEM in order to analyze their inner structure (Figure 1b). The porous structure of the PES matrix changed with depth. Close to the surface (first 100µm), the membrane presented a homogeneous microporous structure. Beyond this surface layer, the bulk of the membrane presented much larger pores showing a sponge-like structure. This can be attributed to variations in the solvent exchange kinetics, much faster in the surface than inside the PES matrix [30,31].

The electrochemical behaviour of the PES-coated electrodes was studied by cyclic voltammetry. Electrodes were covered with 100 µL of an equimolar solution of ferricyanide and ferrocyanide (1 mM) and voltammetric measurements were performed after 5 min of incubation to ensure diffusional equilibrium. Recorded voltammograms are presented in Figure 1c. PES-coated electrodes showed a 60 % reduction of anodic and cathodic current magnitudes with respect to naked electrodes, as expected in membrane-modified electrodes.

3.3 Biosensor fabrication and characterization

Bacterial entrapment in PES membranes was evaluated using DMSO as solvent and water suspensions of *E. coli* as non-solvent. The underlying mechanism consisted of the replacement of DMSO by water and the concomitant entrapment of cells inside the

PES matrix (Figure 2). Membranes prepared using suspensions of *E. coli* containing $5.6x10^9$ cells per mL^{-1} (figure 3a) are compared to control PES membranes obtained by NIPS using water without bacteria as non-solvent (figure 3b). In the first case, rod-shaped *E. coli* cells were clearly observed in the interstitial spaces between the membrane pores, confirming bacterial entrapment.

Entrapment efficiency. Entrapment efficiency was determined by dissolving PES membranes in 100 µL of DMSO, staining with DAPI and counting bacterial cells by epifluorescence microscopy. An average of $2.3\pm0,1x10^6$ cells were recovered from PES membranes. Taking into account that the membranes had a volume of 5 µL, this corresponds to a concentration of $4.6x10^8$ cells.mL^{-1} in the membrane and to an entrapment efficiency of 8.2%.

Biosensor response as a function of preincubation time and number of entrapped cells. To assess the effect of preincubation time on the response of the sensor, we performed ferricyanide-respirometry measurements of PES membranes containing $5.6x10^8$ cells.mL^{-1} preincubated in the presence of 1 mM ferricyanide and 0.2 % glucose during 0, 30, 60 and 90 minutes. The results of the experiment, have been represented in Fig. 4a. As a rule, controls containing live cells without carbon source displayed detectable currents albeit very low. However, PES membranes containing live bacteria and incubated in the presence of glucose and ferricyanide displayed high current values that increased with the length of preincubation period, up to 60 minutes. Since extending preincubation from 60 to 90 minutes only provided a marginal increase in current, we decided to use 60 minutes preincubations for all future assays.

In order to assess the effect of the number of organisms immobilized on the response of the sensor, membranes were prepared containing different amounts of entrapped cells ($2.3x10^5$, $1.15x10^6$ and $2.3x10^6$ cells/membrane). The results, plotted in Fig. 4b indicate the existence of a linear relationship between the number of organisms and

the signal obtained. In our particular case, decreasing cell numbers below 10^5 cells per sensor provided currents in the nA range that fell below the measuring range of our equipment. Increasing the number of cells per membrane potentially could increase the response of the sensor but this was not possible in practice because the microbial suspension used in the membrane fabrication had already been concentrated 100x from a fully grown culture (see Methods section 2.2).

Stability of cell entrapment. In order to evaluate the stability of cell entrapment, *E.coli*-PES membranes were incubated in phosphate buffer at 37 ºC in a shaking incubator at 100 rpm. Under these conditions, in the absence of nutrients, cell growth was arrested, and we expected mechanical shaking to remove cells loosely attached to the membrane. The number of entrapped cells was again determined, after 2, 5 and 7 days, by dissolving the membrane in DMSO, and performing microscopy counts of DAPI stained samples. The results have been plotted in Figure 5a. As shown, the total number of cells in the PES membrane remained almost constant along the seven days of the experiment. This result demonstrates that cells were stably trapped within the membrane.

Sensor repeatability. Repeatability was determined by analyzing the metabolic response of 20 independent sensors by ferricyanide respirometry. The sensors were preincubated for 60 minutes in the presence of glucose and ferrycianide as described in Methods. After that, the sensors were subject to chronoamperometry and the stable current after 40 seconds was recorded. The whole procedure was repeated three times for each sensor. The results have been plotted in figure 5b. An average current of 5.4±0.3 μA was obtained, with a coefficient of variation of 5.5% well in the range of the values usually found in sensors based in biological processes.

Stability during storage. Finally, in order to establish the stability of the sensors when stored at different temperatures, a number of them was allowed to dry at 25 ºC for 1 hour and was stored at 25, 4 and -80ºC for a period of 30 days. The sensors (4 for each temperature) were taken out of storage at different times, and their metabolic

response was determined by ferricyanide respirometry. At the end of the measurements, the sensors were rinsed, allowed to dry for one hour at 25 ºC and stored again. As can be seen in Fig. 5c, when stored at -80ºC the biosensors preserved their metabolic response during the 4 weeks tested. Storage at 4 and at 25 ºC, however, was less successful. Although the sensors maintained most of their activity during a three day storage at these temperatures, their activity decreased to less than 50% in 6 days and was virtually zero after 9 days of storage. According to these results, freezing the biosensor is the only option that guarantees their stability and activity during mid term storage.

3.5 Toxicity assessment with PES biosensors

In order to assess the analytical performance of the PES-bacteria biosensors we applied them to the detection of toxicity in water samples using 3,5-dichlorophenol (DCP) as a model toxic agent. Screen printed electrodes containing live bacteria entrapped in PES were exposed for 30 min to different DCP solutions at concentrations ranging from 0.1 to 50 ppm. After that, the activity level of the sensors was monitored by ferricyanide respirometry as described in Methods.

Biosensor response was expressed in terms of inhibition percentage (I%) referred to sensors unexposed to the toxic agent. A concentration-response curve was obtained by plotting I% against DCP concentration, which is presented in Figure 6. The biosensor response was concentration-dependent, from negligible inhibition at 0.2 ppm to 100% at 25 ppm of DCP. A half-maximal effective concentration (EC50) of 9.2 ppm was obtained, in agreement with the values reported for this toxic (ranging from 7 to 16.48 ppm) using other cell-based assays [32-36]. The results validate the suitability of non-solvent induced phase separation entrapment of bacteria in polyethersulfone matrices as a tool for the development of highly stable and reproducible microbial biosensors.

4. Discussion

In this work we demonstrate how microorganisms can be successfully entrapped in polyethersulfone membranes, produced by non-solvent induced phase separation, in order to obtain a microbial biosensor. We chose DMF as the reference standard solvent, together with four of the compounds proposed by Figoli et al. (2014) as non-toxic solvents for green NIPS membrane preparation (DMSO, γ-butyrolactone, ethyl-lactate and methyl-lactate) [37]. Despite being non-toxic, the biocompatibility of these solvents was in general low, killing virtually all the microorganisms present in the sample after a 24 h exposure. In general, non-toxic status is granted on the basis of toxicity or carcinogenicity tests carried out using low concentrations that mimic environmentally relevant values. In the case of microorganisms, however, resistance of microorganisms to full contact with organic solvents is related to the polarity of the solvent, represented as the logarithm of the partition coefficient of the solvent in a equimolar mixture of n-octanol and water (log P_{ow}) [38]. Log P_{ow} values in the range 1 to 4 are toxic to microorganisms at very low concentrations while solvents with log P_{ow} values lower than 1 display toxicity but only when their concentration is very high [39]. The solvents used in our work (DMSO, DMF, γ-butyrolactone, methyl-lactate and ethyl-lactate) have log P_{ow} values of -1.1, -0.85, 0.06, 0.165 and 0.15 (Sigma, Safety Data Sheets), all of them lower than 1. According to this and despite their reported biological safety, use of these solvents undiluted, as occurs during NIPS, should have a negative effect on cell viability which is what we observed. Fortunately for our work, the lethality of the products decreased when reducing duration of the exposure to 30 min and 5 min, indicating that we could use them with little loss of microbial viability, as far as contact was kept as short as possible.

The entrapment efficiency after phase separation (8.2%) was relatively low when compared to alginate-based methods, with entrapment efficiencies between 54 and 80% [40,41], or to calcium-induced protein gels that provide entrapment efficiencies of

96% [42]. Despite the lower entrapment efficiency, the use of a high concentration of cells in the non-solvent phase allowed an effective final concentration of $4.6x10^8$ cells.mL^{-1} of membrane, enough to produce a robust signal in the sensing process.

Regarding stability during storage, our results indicate similar behaviour when sensors were kept at 4 or at 25 ^oC. In both cases the response of the sensors decreased steadily until becoming inoperative after 9 days. Storage stability data at 4 ^oC reported by different authors using dfferent immobilization matrices indicate much higher rates of survival. Thus, biosensors containing microorganisms entrapped in protein matrices crosslinked with glutaraldehyde were able to keep high activity levels (100%, 74% and 78%) after 20, 15 and 18 days of wet storage at 4 ^oC [43-45]. Similar data have been reported for microorganisms adsorbed to glass fiber paper, collagen fibers or polyaniline (70%, 95% and 90% of activity retained after 120 days, 15 days and 7 days of dry storage at 4 ^oC) [46-48]. During frozen storage, our sensors kept 100% of their initial activity during the 30 days tested, despite repeated thaw/freeze cycles during intermediate measurements. The results suggest that the sensors could be produced and stored frozen until needed, providing at least 24 hours of stable operation after thawing.

5. Conclusions

Cell entrapment inside porous PES membranes by NIPS is here demonstrated and employed for the development of a PES-based microbial biosensor for general toxicity assessment. Screen printed electrodes have been coated with a 300 µm layer of polyethersulfone containing $2.3x10^6$ cells per electrode. Entrapment efficiency is high (8.2%) and cell integrity and activity are maintained during the process. The procedure yelds reproducible (CV 5.4%) and robust sensors that can be repeatedly used without loss of activity when subject to dry frozen storage without additional treatments.

Entrapped bacteria remain active and reduce ferricyanide to ferrocyanide in the presence of glucose, a process that can be detected electrochemically. When exposed

to a toxic, microorganisms loose activity, ferrocyanide production stops and the electrochemical signal decreases. In order to evaluate their performance in a real application the sensors have been tested against DCP as a fast and simple method for the detection of toxicity. The biosensor provides a concentration-dependent response with an EC_{50} of 9.2 ppm for DCP, completely comparable to the values reported for the same compound using cell based assays.

Succesful immobilization of microorganisms in organic polymers susceptible of dry storage opens the door to the development of robust and durable sensors with potential use in industrial and environmental applications.

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Figure captions

Figure 1. a) Optical profilometry results of the observations carried out on polyetersulfone-coated screen-printed electrode obtained. Maximum thickness at the center of the electrode is around 300 µm. **b)** Scanning electron microscopy cross-section of the PES membrane deposited on top of the electrode. **c)** Cyclic voltammograms of equimolar mixtures ferricyanide/ferrocyanide (1mM) with and without polyetersulfone membrane.

Figure 2. Entrapment of microbial cells on the biosensor surface by nonsolvent induced phase separation of a solution of polyethersulfone in DMSO. A water suspension of *E. coli* was used as a non solvent. During the non-solvent induced phase separation, the DMSO is substituted by the non-solvent, so that the membrane is formed with microorganisms trapped inside it.

Figure 3. a) Cross-section image of a PES membrane obtained by Scanning Electron Microscopy. **b)** Image of a cross-sectioned PES membrane with microorganisms entrapped during phase separation.

Figure 4. a) Stable chronoamperometry readings from *E. coli* containing PES membranes preincubated in the presence of Fe^{3+} for a period of 0, 30, 60 and 90 min. Metabolically active cells with glucose (●), without glucose (○) and glutaraldehyde-killed control (▲). **b)** Stable chronoamperometry readings from PES membranes containing different concentrations of *E. coli*, after a 60 min. preincubation in the presence of Fe^{3+} and glucose.

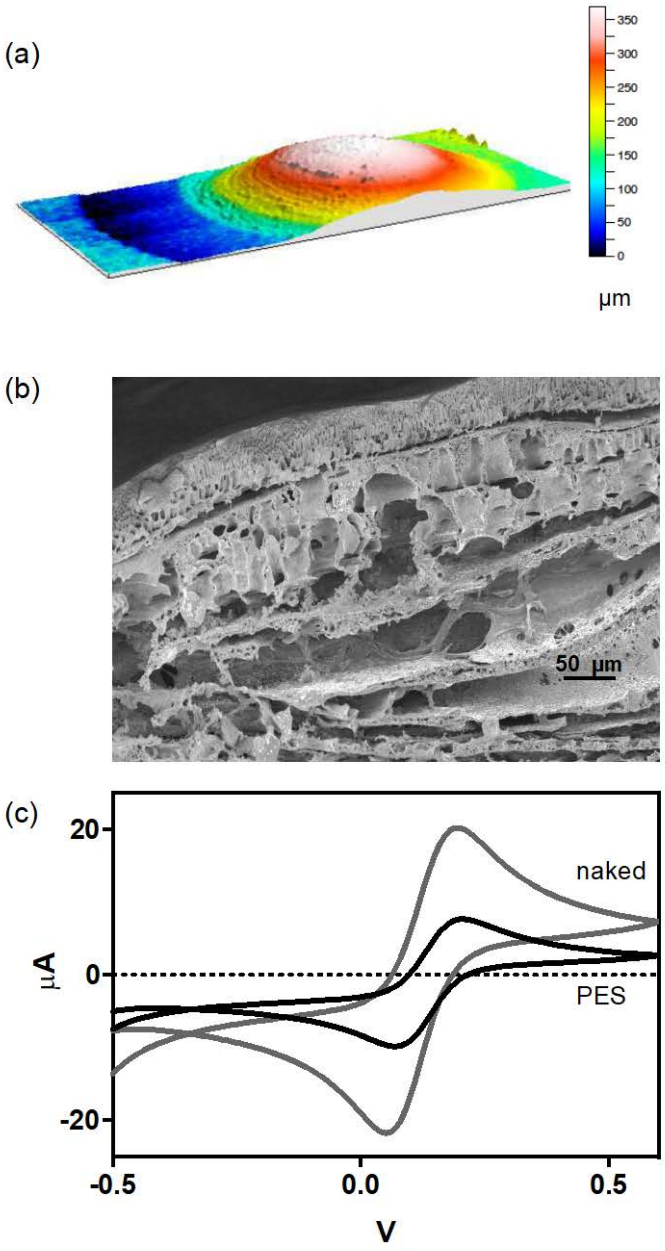
Figure 5. a) Variation of the number of organisms entrapped in PES membranes as a function of the time submerged in phosphate buffer with shaking. Numbers do not decrease significantly after one week indicating that entrapment is stable and cells are not easily removed from the PES matrix. **b)** Stable chronoamperometry readings from 20 different *E. coli* containing PES sensors after a 60 min. preincubation in the presence of Fe^{3+} and glucose. Repeatibility is very high with an average reading of 5.4 µA and a coefficient of variation of 5.4%. **c)** PES microbial biosensor stability determined by repeated measurements of the same sensors dried and stored at different temperatures (4ºC, 25ºC and -80ºC) over 30 days.

Figure 6. Application of the microbial-PES biosensor to toxicity determination using 3,5-DCP as a model compound. Percentage inhibition has been plotted as a function of 3,5-DCP concentration. The results indicate an EC_{50} of 9.2 ppm well in the range of previously reported values.

Table 1. Study of the biocompatibility of organic solvents at different microorganism-solvent contact times (5 and 30 minutes, 24 hours).

	5 minutes		30 minutes		24 h	
	cfu/mL	% viability	cfu/mL	% viability	cfu/mL	% viability
DMSO	$9.2x10^{8}$	73.6±4.4	$2.0x10^{8}$	18.1±1.2	<0.1	$<8.3x10^{-9}$
DMF	$8.8x10^{8}$	70.4±2.1	$1.2x10^{8}$	10.9±3.2	<0.1	$<8.3x10^{-9}$
Ethyl-lactate	$8.0x10^{8}$	64.0±3.1	$3.9x10^{8}$	36.3±4.4	<0.1	$<8.3x10^{-9}$
Methyl-lactate	$9.5x10^{8}$	76.0±1.8	$3.4x10^{8}$	31.82±3.1	<0.1	$<8.3x10^{-9}$
γ-butyrolactone	$9.5x10^{8}$	76.0±2.4	$9.9x10^{7}$	9.0±1.8	<0.1	$<8.3x10^{-9}$
Control	$1.2x10^{9}$	100.0±0.8	$1.1x10^{9}$	91.6±0.9	$1.0x10^{9}$	89.1±1.1

Figure 1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350
300
250
200
150
100
50
0
µm
(b)
50 µm
(c)
20
0
-20
µA
naked
PES
-0.5
0.0
0.5
V

Figure 2

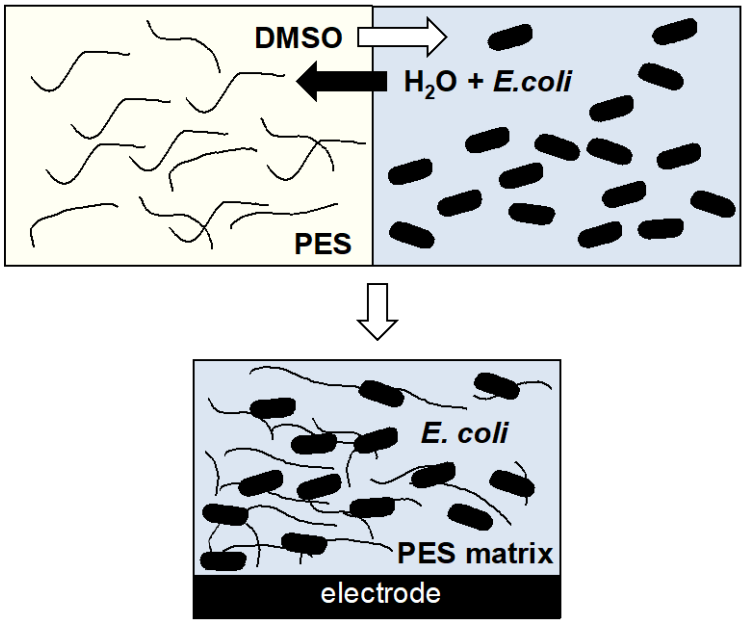

Figure 3

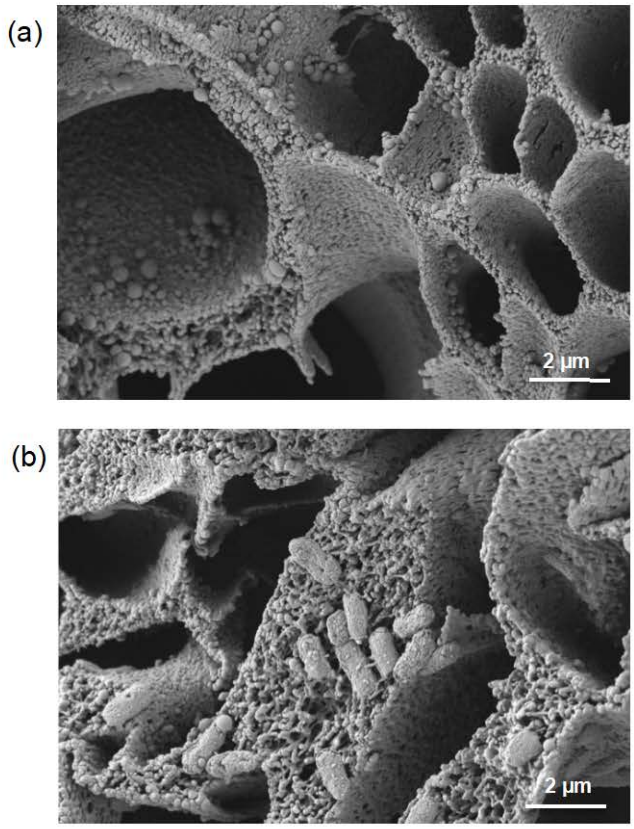

Figure 4.

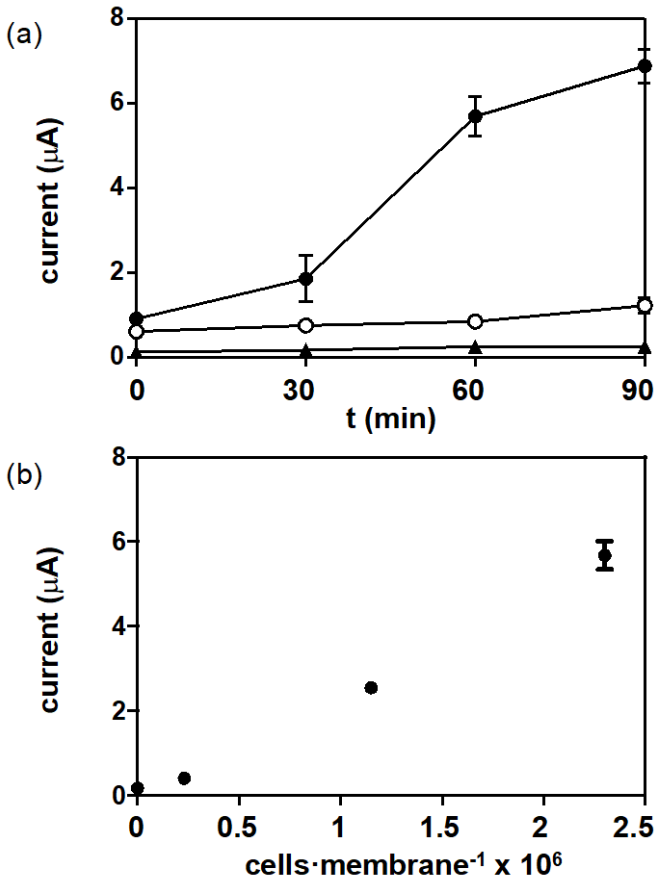
(a)
current (μA)
8
6
4
2
0
0
30
60
90
t (min)
(b)
current (μA)
8
6
4
2
0
0
0.5
1
1.5
2
2.5
cells·membrane-1 x 10^6

Figure 5.

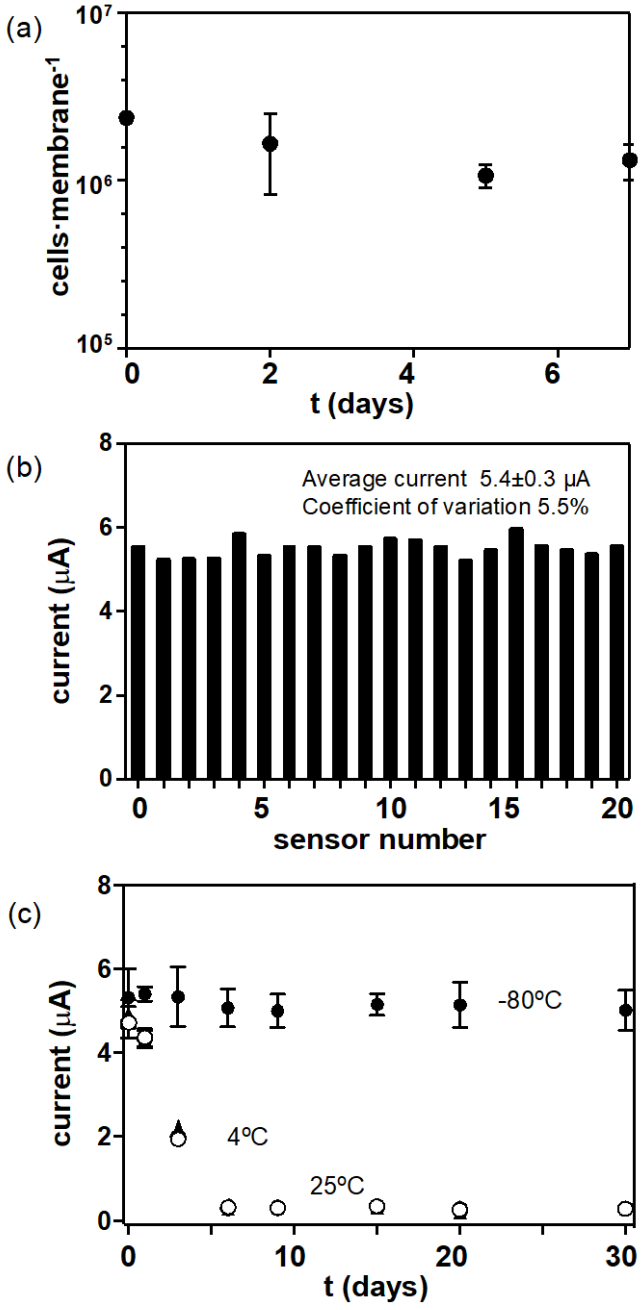
(a)
cells·membrane-1
10^7
10^6
10^5
0
2
4
6
t (days)
(b)
Average current 5.4±0.3 µA
Coefficient of variation 5.5%
current (µA)
8
6
4
2
0
0
5
10
15
20
sensor number
(c)
current (µA)
8
6
4
2
0
-80ºC
4ºC
25ºC
0
10
20
30
t (days)

Figure 6.