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Biotinylated polypyrrole films: an easy electrochemical approach for the reagentless immobilization of bacteria on electrode surfaces

S. Da Silva[a], L. Grosjean[b], N. Ternan[c], P. Mailley[b], T. Livache[b], S. Cosnier[a,*]

[a] *Laboratoire d'Electrochimie Organique et de Photochimie Redox, URA UMR CNRS 5630, Université Joseph Fourier Grenoble 1, BP53 38041 Grenoble, Cedex 9, France*

[b] *Groupe CREAB, DRFMC/SI3M/EMSI, CEA de Grenoble, 17 Rue des Martyrs 38054 Grenoble, Cedex 9, France*

[c] *Microbial Biotechnology Research Group, School of Biological and Environmental Sciences, University of Ulster, Cromore Road, Coleraine, Co. Londonderry, BT52 1SA, Northern Ireland*

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Abstract

Biotinylated bacteria were immobilized onto biotin/avidin modified electrode surfaces. Firstly, an electrospotting deposition method, followed by fluorescence microscopy, showed that bacteria were specifically grafted onto a gold surface. Fluorescence intensity versus the quantity of bacteria deposited on the surface was correlated, allowing determination of the microbial saturation point. Secondly, biotinylated bacteria were immobilized onto a glassy carbon macro-electrode in order to assess immobilized bacterial denitrification activity. During a 7-day trial, the modified electrode completely denitrified 5 mM nitrate, with a rate of 1.66 mM/day over the first 3 days. When the same electrode was placed in fresh nitrate solution, the denitrification rate dropped to 0.80 mM/day. Crucially, the immobilized bacteria did not become detached from the electrode during the study.

Keywords: Biotinylated polypyrrole films; Reagentless immobilization; Electrode surfaces

1. Introduction

Deposition of biological macromolecules with controlled spatial resolution and retention of their activity is the subject of increasing research efforts owing to the potential application in the field of electrochemical biosensors, biochips and bioreactors. Among conventional immobilization methods, electro-generation of organic polymers is one of the few methods that allow the reproducible functionalization of conductive surfaces, of complex geometry, with a precise spatial resolution [1–4]. In particular, the combination of electrochemical addressing with the avidin–biotin immobilization technique provides a method for biomolecule immobilization that is highly compatible with biological function [5–8].

The avidin–biotin strategy presents a variety of specific advantages over other immobilization techniques. In particular, the extremely specific and high affinity interactions between biotin and the glycoprotein avidin (association constant $Ka = 10^{15}\ M^{-1}$) lead to strong associations similar to the formation of covalent binding [9]. In addition, the avidin–biotin immobilization method generally maintains biomolecule activity more successfully than other regularly used methods. Furthermore, avidin provides a passivation layer over the transducer surface that prevents further nonspecific adsorption of proteins on the surface [10]. In contrast with conventional grafting or affinity binding, this step-by-step approach can also be applied to the elaboration of assemblies containing multilayers of biological molecules [11–13]. Immobilization of bacteria with a view to denitrification of contaminating nitrate concentrations in ground water is one potential application of these techniques. Nitrate is a well-known environmental contaminant, largely encountered in ground waters, stream water and the food industry, which is relatively non-toxic to the adult but can be fatal to infants under six months of age. Since the USA and European Community health institutions have established, respectively, maximum contaminant nitrate levels of 10 and 50 mg/l for drinking water, the elimination of nitrate has become a significant objective of much environmental

* Corresponding author. Tel.: +33-4-76-51-49-98; fax: +33-4-76-51-42-67.
E-mail address: Serge.Cosnier@ujf-grenoble.fr (S. Cosnier).

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research. Biological denitrification is an important alternative which removes nitrate from drinking water sources. However, the process implies the presence of bacterial biomass in the treatment system: care must be taken to ensure that such biomass does not enter the final treated water product.

In this communication, we describe a new electro-chemical method for the immobilization of bacteria which combines the advantages of affinity interactions with those of the electrochemical addressing of polymer films.

2. Experimental section

2.1. Reagents

The biotinylated functionalized pyrrole was synthesized following the procedure described previously [14]. Avidin was purchased from Sigma (France). Streptavidin–Phycoerythrin conjugate was purchased from Molecular Probes. All other reagents were of analytical grade and were used without further purification.

2.2. Biotinylation of bacteria

Paracoccus pantotrophus GB17^T was provided by the Microbial Biotechnology Research Group at The University of Ulster. Cells, freshly grown in medium containing 50 mM acetate and 20 mM nitrate [15] were harvested by centrifugation and washed three times in ice-cold Phosphate Buffered Saline (PBS pH 8.0) and finally suspended in PBS at a concentration of approximately 2.5×10^7 cells/ml (A650 = 0.03). Sulfo-NHS-LC-Biotin (0.5 mg, Pierce, Rockford, IL, USA) was added to the bacterial suspension (1 ml) and the coupling reaction allowed standing at room temperature for 30 min. The biotinylated cells thus obtained were washed with ice-cold PBS to remove residual biotin. Bacterial denitrification activity following biotinylation was assayed by incubation of bacteria (25 mg/ml) in 10 mM phosphate buffer (pH 7.5) containing 50 mM sodium acetate and 5 mM potassium nitrate. Nitrate removal was followed at 30 °C by potentiometry using an ion selective electrode purchased from Detect/ONTM. Biotinylation resulted in a 16% decrease in denitrification rate compared to a non-biotinylated sample of cells from the same batch (N. Ternan, unpublished results).

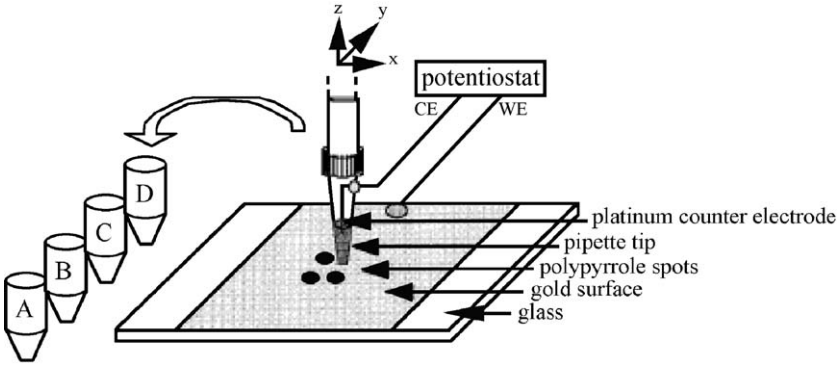
2.3. Polymerization procedure

Electrochemical directed polymerization by electrospotting was carried out on a gold layer deposited on a glass slide through the use of a 200-μl pipette tip as the electrochemical cell. Electrical contact was established at the tip by inserting a platinum wire used as a counter electrode. The tip was filled with 20 μl of an acetonitrile solution containing 0.1 M of $LiClO_4$ and 0.2 M of biotinylated functionalized pyrrole (BFP) monomer. The tip was then applied to a precise location on the gold layer used as the working electrode. This electrochemical system was connected to an EGG Princeton Applied Research model 283 potentiostat and to an 8300 Schlumberger X/Y recorder. Prior to use, this simplified electrochemical cell (two electrodes) was calibrated with a saturated calomel reference electrode. The polypyrrole film was synthesized by electrocopolymerization on the gold layer (working electrode) by a 2-V electrochemical pulse for (unless specified). During the pulse, the synthesis charge was recorded (Scheme 1).

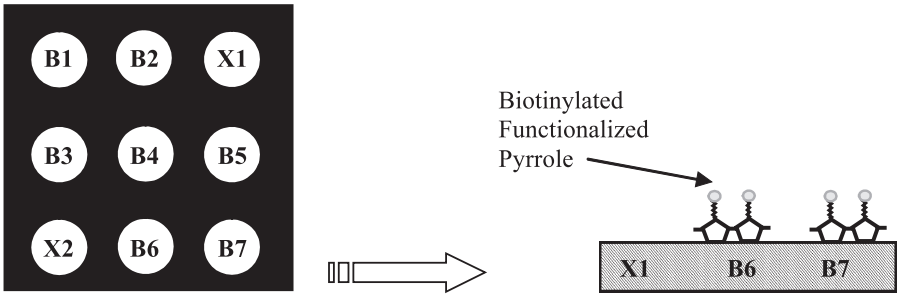
Polymerizations on glassy carbon surfaces (diameter of 5 mm) were performed following the previously described method of Cosnier et al. [14].

2.4. Immobilization procedure

Slides coated with polymerized biotinylated functionalized pyrrole were rinsed in PBS. Then, 40 μl avidin solution (0.5 mg/ml) was deposited onto the surface, incubated for 30 min at room temperature (25 °C). Slides were rinsed with PBS and then saturated by a solution of PBS containing 1% Bovine Serum Albumin. Biotinylated bacteria were then added to the surface and incubated at room temperature for



Scheme 1. Scheme of electrospotting procedure.



Scheme 2. Map of the electrospotting slide.

30 min to allow coupling. Slides were finally rinsed thoroughly to remove unattached bacteria. Glassy carbon surfaces were treated in the same way except that the saturation step of BSA deposition was not carried out.

2.5. Fluorescence measurements

The resulting BFP surfaces were exposed by a solution of Streptavidin–Phycoerythrin (0.1 mg/ml) over 15 min in darkness. Substrates were then visualized by fluorescence microscopy (Olympus BX60) at 488-nm excitation and 578-nm emission wavelengths.

3. Results and discussions

3.1. Bacterial immobilization survey

Experiments were performed using the electrospotting procedure and fluorescence measurements. Polymerizations of the BFP on a glass slide covered by a gold layer were carried out following the Scheme 2. Fig. 1 depicts a typical polymerization chronoamperogram, while Table 1 reports the electrical charges consumed by the electropolymerization process.

The average charge value utilized to polymerize BFP for each spot was 5.4 μC. The low standard deviation value obtained (0.4 μC) for the polymerization charges indicated that, critically, the spot generation method was fully reproducible.

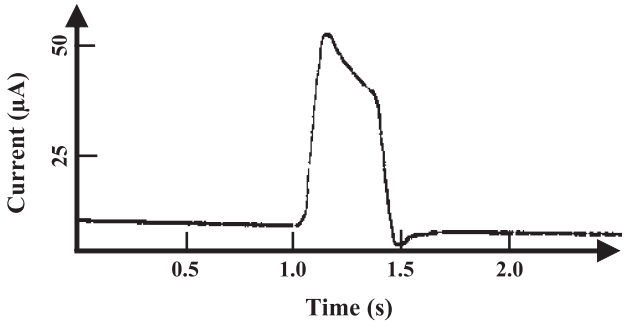


Fig. 1. Chronoamperogram of the BFP electropolymerization by electrospotting.

The obtained spots generally present a typical thickness of 10 nm making these polymeric matrices 2D like anchoring surfaces [7]. Following the immobilization step, 40 μl of a biotinylated bacteria solution (2.5 mg/ml) was deposited on the slide. The slide was then rinsed, labeled by Streptavidin–Phycoerythrin and observed by fluorescence microscopy (Fig. 2). Seven luminescent spots were observed, with positions perfectly matching Scheme 2. The biotinylated bacteria were fixed specifically to the biotinylated pyrrole alone, with no traces of nonspecific bacterial attachment over the rest of the slide.

The substrate luminescence was transduced in an array of pixels by the CCD camera. For each spot, we have measured in a grey level scale the relative fluorescence intensity of each pixel contained in the spot image. The standard deviation of fluorescence did not exceed 11%. Bacterial biotinylation has been designed to obtain an average derivatization of approximately 4.93×10^{10} biotin molecules/cell (20 pg biotin/cells, based upon a molecular weight for biotin of 244 g/mol^{-1}). However, such a figure is statistical and the theoretical number of attached biotin molecules/cell could not be approached. It is possible that an inhomogeneous fluorescence response could be generated due to a modulation of the anchored number of fluorophore conjugates on the cell shell. However, the recorded fluorescence images obtained were relatively homogenous and the spot's signals were monitored as the average intensity of the overall surface.

The immobilization procedure was then repeated in the same way for five new slides with increasing amounts of bacteria spread onto the surface in the range 50 to 2500 μg/ml (in 40 μl). The resulting average fluorescence values versus the weight of bacteria spread on the surface is presented in Fig. 3. A linear relationship between fluorescence intensity and the amount of bacteria was obtained

Table 1
Values of the polymerization charge

Spots	Charge (μC)	Spots	Charge (μC)
b_1	6.00	b_5	5.41
b_2	5.80	x_2	No
x_1	No	b_6	5.18
b_3	4.99	b_7	5.13
b_4	5.41		

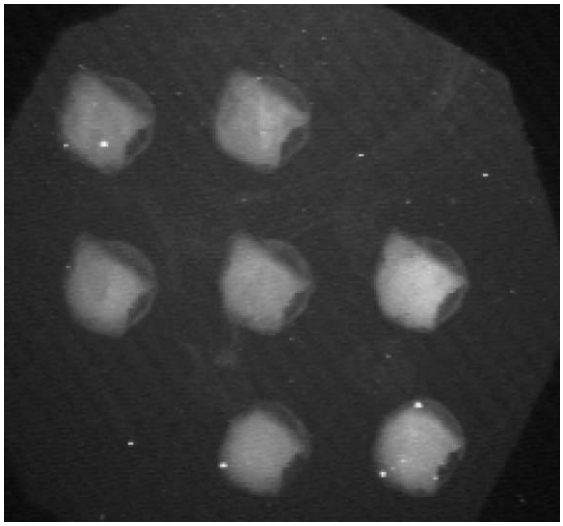


Fig. 2. Picture of a polypyrrole-biotin/avidin/biotinylated bacteria modified slide visualized by fluorescence microscopy (magnitude 1.25).

from 0 to 10 μg, with a correlation coefficient exceeding 0.98. Fluorescence intensity reaches a plateau for bacterial load values exceeding 10 μg/ml, most likely as a result of saturation of the spot surface by bacteria, leading to a monolayer of microorganisms. These results allowed determination of the optimal weight of bacteria to be used in the immobilization step.

3.2. Bacterial activity assay

A 5-mm diameter glassy carbon electrode was modified by BFP. Avidin coupling was effected by addition of 40 μl avidin solution (0.5 mg/ml) for 30 min at room temperature followed by thorough rinsing. Twenty microliters of a freshly grown biotinylated bacteria suspension (15 mg/ml) was then spread onto the surface and incubated for 30 min at room temperature. Finally, the electrode was thoroughly rinsed and immersed in 5 ml of 10 mM phosphate solution (pH 7.5) containing 50 mM of sodium acetate and 5 mM of potassium nitrate (Solution 1). The reaction temperature was 30 °C. The nitrate concentration was measured periodically throughout the experiment using a nitrate electrode. At the end of the experiment (3 days later), the nitrate concentration had been reduced to 0.2 mM, well below the EC limit. The electrode was then removed, rinsed and transferred to a new phosphate buffer solution containing 5 mM of potassium nitrate (Solution 2). Nitrate ions were added to Solution 1 to replenish the nitrate concentration up to 5 mM once more. The nitrate concentration in both fresh and replenished solutions was monitored and representative results are reported in Fig. 4.

The nitrate concentration of Solution 1 varied between 5 and 4 mM over the course of the experiment. It was difficult to assess this in terms of bacterial activity, as this variation could be accounted for by the imprecision of the nitrate probe. The probe was a potentiometric electrode for which the calibration curve was a plot of potential against decimal logarithm value of the nitrate concentration. As the electrode required regeneration before each measurement, as strongly advised by the manufacturer, a calibration curve had to be constructed for each reading. As the slopes of calibration curves tended to differ slightly, a non-negligible error occurred due to the logarithmic function. Thus, the obtained variation of nitrate concentration is not significant and we could conclude that there is no bacteria release into Solution 1 from the electrode during the first denitrification step. However, there was no ambiguity about the denitrification of the Solution 2, containing the modified electrode.

The modified electrode was used for 1 week and reduced nitrate levels to below the EC limit for potable water in 6 days. The linked biotinylated bacteria were responsible for this denitrification since no nitrate removal occurred, in solution 1, in the absence of the electrode. Although the electrode denitrification activity dropped from an initial rate of 1.66 to 0.80 mM/day, total nitrate removal was achieved approximately 6 days. The surprising decrease in bacterial denitrifying activity in the electrode could possibly be explained by interference of avidin with *P. pantotrophus*

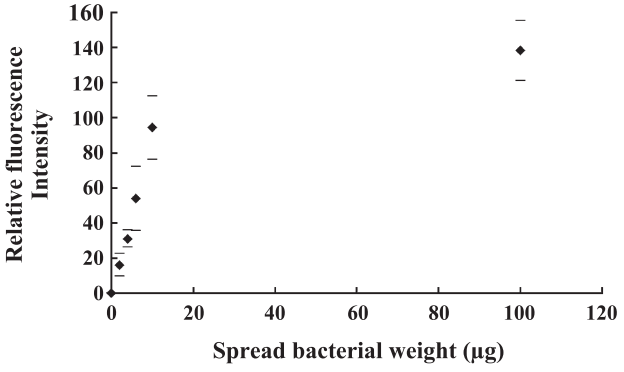


Fig. 3. Spot fluorescence intensity versus amount of bacteria spread on the spot.

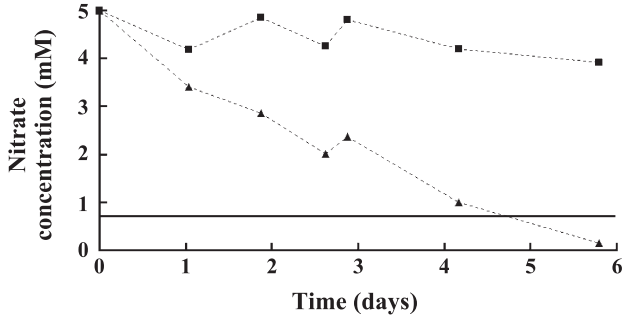


Fig. 4. Nitrate concentration evolution versus time of ▲: Solution 1 which had not contained modified electrode with bacteria over the measurement but was denitrified by it once time and ■: Solution 2 which contained the modified electrode bacteria witch denitrified Solution 1. Solutions contained initially a 10 mM phosphate solution pH 7.5 with 50 mM of sodium acetate and 5 mM of potassium nitrate. CE nitrate concentration tolerance in bold line.

physiology, as it was strongly linked to the BFP. However, to the best of the authors' knowledge, this is the first demonstration of the covalent immobilization of this kind of organism to an electrode. Following on from these proof of concept experiments, work is in progress to enhance the denitrification rate, by applying several layers of bacteria onto the entire electrode surface owing to the biotin–avidin interaction. This should ultimately allow the development of a larger scale 3D electrode for biodenitrification reactor purposes.

4. Conclusion

Biotinylated bacteria were grafted specifically onto electrode surfaces. Fluorescence microscopy studies allowed determination of the amount of bacteria required to saturate the electrode surfaces. Denitrification activity of biotinylated bacteria, immobilized onto a glassy carbon macroelectrode, was measured by following nitrate removal from solution. In spite of the strong linkage constraining bacterial cell division, denitrification occurred with a maximal initial rate of 1.66 mM/day; this value decreased to some 0.8 mM/day upon repeated treatment of nitrate containing solutions.

Furthermore, we have shown in this paper the reliability of this new anchoring method to modify electrode surfaces of different shapes and sizes. It appears, therefore, that biotinylated functionalized pyrrole appears suitable for bacterial immobilization, with retention of biological activity, on 3D electrode surfaces dedicated to bioreactor design.

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