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Electrochemical nitrate biosensor based on poly(pyrrole–viologen) film–nitrate reductase–clay composite

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ABSTRACT

The immobilization of nitrate reductase (NR) was performed by entrapment in a laponite clay gel and cross-linking by glutaraldehyde. In presence of nitrate and methyl viologen, a catalytic current appeared at −0.60 V illustrating the enzymatic reduction of nitrate into nitrite via the reduced form of the freely diffusing methyl viologen. The electropolymerization of a water-soluble pyrrole viologen derivative within the interlamellar spaces and channels of the host clay matrix successfully carried out the electrical wiring of the entrapped NR. Rotating disk measurements led to the determination of kinetic constants, namely $k_2 = 10.7\ s^{-1}$ and $K_M = 7\ \mu M$. These parameters reflect the efficiency of the electro-enzymatic reduction of nitrate and the substrate affinity for the immobilized enzyme.

1. Introduction

Beside its use as fertilizers in agriculture, nitrate is the end oxidative product of nitric oxide and other nitrogen species and hence constitutes a well-known environmental contaminant largely encountered in ground and stream water. Owing to the significant impact of high nitrate concentrations on human environment (eutrophication of water surfaces, deterioration of water quality) and public health (formation of carcinogenic nitrosamines), the determination of nitrate is significant concerns. The commonly employed methods for nitrate determination include spectrophotometric, ion-selective chromatographic technics, and electrochemical methods such as polarographic, voltammetric and potentiometric determinations [1–5]. However, the centralized analytical systems, like spectrophotometric and chromatographic methods require extensive pre-treatments and are time consuming while the electrochemical methods based on ion-specific electrodes are faced to interferences and poor stability and even are not sufficiently specific.

On the other hand, some bioassays based on nitrate reductase that are multiredox center enzymes responsible to the biological conversion of nitrate to nitrite, have been developed. An attractive alternative thus consisted in the design of biosensors due to their simple use in complex sample and the possibility of fabricating fast portable biosensors. The majority of biosensors reported for the determination of aqueous nitrate concentrations are based on the immobilization of nitrate reductase (NR) [6–15]. Although a biosensor based on conductometric measurements has been recently developed [15], most of the biosensors involve a voltammetric or amperometric transduction based on the electrical wiring of the immobilized NR enzymes. Since the redox centers of NR are deeply embedded in the protein structure, preventing thus a direct electron transfer with the electrode, redox mediators such as methyl viologen were used as electron shuttle between the immobilized enzyme and the electrode surface. In particular, the NR entrapment was carried out by electropolymerization of polymers functionalized by viologen groups where the electron transport to enzymes is ensured by electron hopping between immobilized redox centers [7,8]. However, these enzyme electrodes were faced to the fragility of NR. The partial hydrophobic character of the host films, indeed, may alter the three-dimensional structure of the entrapped NR and hence diminish their biological activity. In order to improve the biocompatibility of organic polymers, the use of inorganic clay nanoparticles as hydrophilic additives was widely studied, in particular in combination with polypyrrole films. For the last two decades, there has been a growing interest in the design of these organic-inorganic composites [16]. The aqueous electrogeneration of electroactive polypyrrole or polyaniline in clay-modified electrodes was extensively investigated due to the electronic conductivity conferred to layered solids such as hectorite and montmorillonite [17,18]. These polymers also reinforced the mechanical stability of the clay and improved the stability of redox species incorporated into the inorganic coating thanks to the ion exchange property of clays.

In this context, a two-step procedure consisting first in the physical entrapment of NR in highly hydrophilic inorganic clay followed by the

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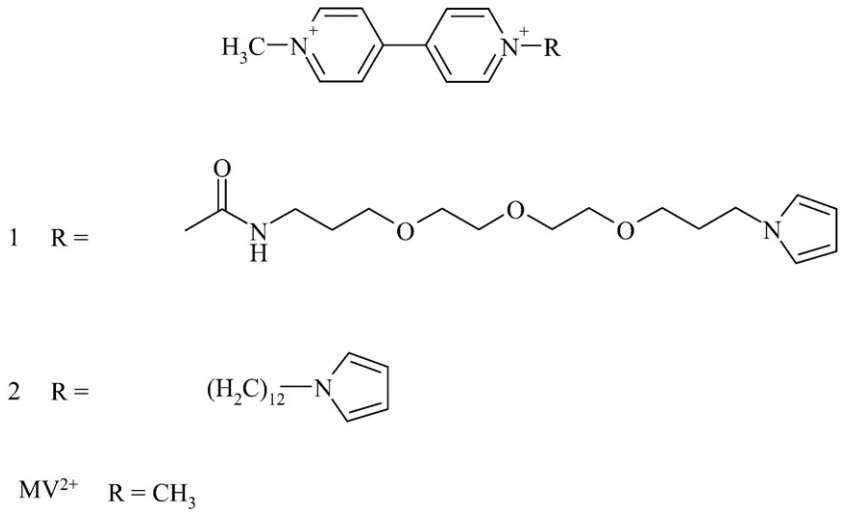

Scheme 1. Schematic representation of the viologen derivatives.

electropolymerization of a poly(pyrrole–viologen) film assuming the electrical wiring of the immobilized enzyme, is reported (Scheme 1). The kinetic parameters and turnover of the resulting wired NR were investigated by rotating disk electrode experiments.

2. Experimental section

2.1. Electrochemistry

Electrochemical experiments were performed with a conventional three-electrode system. The working electrodes were glassy carbon disk electrodes purchased from Radiometer analytical. An Ag/AgCl electrode was used as reference electrode while Pt wire placed in a separate compartment containing the supporting electrolyte, was used as counter electrode. All the potentials are referred vs this electrode. All measurements were conducted in a Metrohm electrochemical cell thermostated at 30 °C containing aqueous solutions purged and maintained under argon atmosphere to avoid any oxygen interference. An autolab 100 potentiostat was used to carry out the electrochemical experiments. Koutecky–Levich plots were carried out using rotating disk electrodes (diameter: 5 mm) with a CTV 101 Speed Control Unit from Radiometer analytical. The surface of the glassy carbon electrode were polished with a 2 μm diamond paste purchased from Presi (France), sonicated and rinsed successively with acetone, ethanol and water.

2.2. Chemicals

Laponite is a synthetic hectorite (monovalent cation exchange capacity = 0.74 mmol g^{-1}) obtained from Rockwood Specialities Inc (Princeton, NJ). Nitrate reductase (NR) was a gift from Prof. J. Pommier and G. Giordano of the Laboratoire de Chimie Bactérienne (Marseille, France). Glutaraldehyde was purchased from Aldrich. Monomers 1 (*N*-methyl-*N*′-[*N*(13-pyrrol-1-y(-4′,7′,10′-trioxtridecanyl)-propionamido)]-4,4′-bipyridinium ditetrafluoroborate) and **2** (N-methyl-N′-(12-pyrrol-1-yldodecyl)-4,4′-bipyridinium ditetrafluoroborate) were synthesized as described in [8,19], respectively (Scheme 1). All other chemical reagents were of analytical grade. Water was doubly distilled in quartz.

2.3. Modification of the electrodes

An aqueous colloidal suspension was prepared by dispersing laponite (2 mg mL^{-1}) in deionized water overnight. An enzyme solution (NR, 1 mg mL^{-1}) was prepared with the laponite dispersion. The NR-clay electrode was prepared by spreading an aqueous suspension (22 μl) containing 1 mg mL^{-1} of laponite, NR and 6 μg mL^{-1} L of glutaraldehyde on the glassy carbon surface. In order to remove water, the resulting mixture was dried under vacuum, leading to adhering clay film with entrapped enzymes. The modified electrode was immersed into stirred 0.1 M Tris buffer (pH 7.5) for 20 min before use to remove the NR molecules not firmly entrapped in the clay matrix.

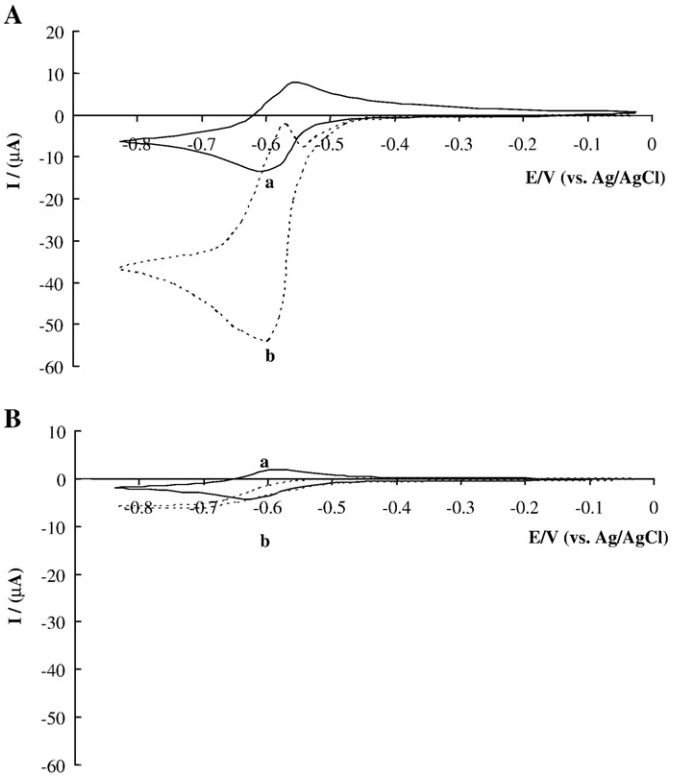

Fig. 1. (A) Cylic voltammograms of NR-clay glassy carbon electrode in deoxygenated 0.1 M Tris–HCl (pH 7.5) buffer containing methyl viologen (2.5 mM) in the absence (a) and presence of nitrate (2 mM) (dotted line). (B) the enzyme electrode was profusely rinsed with 0.1 M Tris–HCl and same experiments were performed without methyl viologen in the absence (a) and presence of nitrate (2 mM) (dotted line). Scan rate: 5 mV s^{-1}.

The NR-composite electrodes were prepared first by spreading 20 µL of an aqueous dispersion of monomer 2 (4 mg mL^{-1}) and evaporating water under vacuum on glassy carbon disks. The resulting "dry" modified electrodes were transferred into a cell containing an aqueous 0.1 M $LiClO_4$ solution. Electrochemical polymerization of the adsorbed monomer 2 was carried out by controlled potential electrolysis for 15 min at 0.8 V vs. Ag/AgCl. Further, the poly 2 electrodes were coated with 37 µL of NR (1 mg mL^{-1}) and laponite (mg mL^{-1}) solution and dried under vacuum atmosphere for 20 min. Water removal leads to the formation of an adherent NR-laponite coating on the underlying poly 2 film. Finally, the modified enzyme electrode was soaked in an aqueous 0.1 M $LiClO_4$ solution containing monomer 1 (5 mM). The latter was electropolymerized within the enzyme-clay coating by controlled-potential electrolysis for 15 min at 0.8 V vs Ag/AgCl.

3. Results and discussion

Thanks to their unusual intercalation property, porosity and high hydrophilic character inorganic clays, especially laponite, preserve the biological activity of enzymes and hence have been widely exploited for the design of biosensors. As previously reported, the high cation-exchange property of laponite was exploited for the immobilization of cationic methylene blue into a diaphorase-laponite gel coating [19]. The resulting "redox clay" constituted the first example of biosensor based on clay containing an electrically wired enzyme. This concept was investigated with methyl viologen (MV^{2+}) to establish an electrical connection between the electrode surface and NR entrapped in a laponite film. After a cross-linking step of the entrapped NR by glutaraldehyde, the NR-clay electrode was transferred into of 0.1 M Tris–HCl solution (pH 7.5) containing methyl viologen (2.5 mM). The electrochemical behavior of MV^{2+} at the NR-clay electrode was investigated by cyclic voltammetry in the absence of oxygen. The cyclic voltammogram recorded at 5 mV s^{-1}, displays a reversible peak system at $E_{1/2}$=−0.57 V vs Ag/AgCl, the $E_{1/2}$ value being estimated from the midpoint of the anodic and cathodic peaks (Fig. 1A).

The latter corresponds to the one-electron reduction process, characteristic of the redox couple of MV^{2+} that diffuses within the microchannels and interlamellar spaces of the laponite coating. After three cycles, this electrochemical signal was stable and reproducible reflecting the rapid incorporation of MV^{2+} within the bioinorganic matrix by electrostatic interactions. The addition of the enzyme substrate (potassium nitrate) induced a strong increase in cathodic and a quasi-disappearance in anodic currents of the MV^{2+}/MV^{+} system (Fig. 1A). This electrocatalytic phenomenom indicates that NR can catalyze the reduction of nitrate into nitrite using the cation radical form of the freely diffusing viologen. MV^{2+} was reduced into MV^{+} at the electrode surface (Eq. (1)) and re-oxidized by NR that catalyzed the reduction of nitrates (Eq. (2)).

$$MV^{2+} + e^{-} \overset{\text{electrode}}{\rightarrow} MV^{\cdot +} \quad (1)$$

$$2\ MV^{\cdot +} + NO_3^- + H_2O \overset{\text{NR}}{\rightarrow} 2MV^{\cdot +} + NO_2^- + 2OH^- \quad (2)$$

In the presence of NO_3^- (2 mM) and MV^{2+} (2.5 mM), the stability of the electrochemical catalytic signal indicated that the glutaraldehyde/laponite matrix did not act as a barrier towards the diffusion of methyl viologen and did not affect their electrochemical behavior. The catalytic signal appearance after nitrate addition in the electrolytic solution showed that the enzyme was not denaturated during the immobilization procedure. Such a strong catalytic current for immobilized NR was not often reported due to the low NR activity and the use of non biocompatible immobilization procedures. This corroborates thus the non-denaturating character of the NR entrapment by laponite gel. The enzyme electrode was then thoroughly rinsed with Tris–HCl buffered solution and transferred into 0.1 M Tris–HCl buffer (pH 7.5). In the absence of MV^{2+}, repeatedly scanning the potential over the range −0.20 to −0.83 V resulted in the continuous decrease in the current intensity of the reversible viologen peak system. The latter became negligible after 15 scans (Fig. 1B). In the same vein, the electrocatalytic signal observed in the presence of NO_3^- drastically decreased and disappeared highlighting the key role of MV^{2+} (Fig. 1B). These electrochemical evolutions unambiguously indicated the fast release of the redox mediator into the bulk solution.

In order to circumvent this problem of viologen release, the immobilization of the redox mediator was carried out by electrochemical polymerization in water of a hydrophilic viologen derivative functionalized by a pyrrole group 1 within the inorganic template (Scheme 1). With the aim to improve the electrical communication between the electrode surface and the subsequent redox polymer, the NR-composite electrodes were elaborated on an electropolymerized film of hydrophobic pyrrole viologen 2. Thanks to the cationic-exchange property of laponite nanoparticles, laponite-NR mixture was strongly adsorbed by electrostatic interactions onto this positively charged polypyrrolic film. Then, as previously observed for MV^{2+}, the water-soluble pyrrole viologen 1 was incorporated within the interlamellar space of the clay-NR coating and electropolymerized. Fig. 2 shows the electrochemical behavior of the resulting NR-composite electrode in 0.1 M Tris–HCl buffer. In the absence of nitrate and oxygen, the cyclic voltammogram exhibits a stable reversible peak system relative to the one-electron reduction of the polymerized viologen groups. The addition of nitrate (2 mM) induces the appearance of a strong irreversible cathodic peak at −0.60 V combined with the disappearance of the anodic wave related to the re-oxidation of the reduced polymerized viologen groups (Fig. 2). This catalytic signal reflects an efficient electrochemical wiring of the entrapped NR by the surrounding poly1 film electrogenerated in the laponite matrix.

In order to investigate more accurately the kinetic and mechanistic behavior of the mediated enzymatic reduction of nitrate, rotating disk experiments were carried out. The electrocatalytic reduction of nitrate was performed at different nitrate concentrations and variable rotation rates at −0.60 V. This potential value was more negative than the first reduction potential of the polymerized viologen groups to ensure that the electrocatalytic reaction between the immobilized NR and nitrate will be the rate limiting step instead of the electrical wiring of NR by the reduced form of viologen. The reduction current of NO_3^- at the enzyme electrode can be limited by mass transport of NO_3^- or by the kinetic of the enzymatic reaction.

$$NR_{red} + NO_3^- \overset{k_{obs}}{\rightarrow} NR_{ox} + NO_2^- \quad (3)$$

Actually, in rotating disk electrode conditions, the catalytic cathodic current generated by the electro-enzymatic reduction of nitrate (I_{cat}) may be composed of the current limited by the diffusion

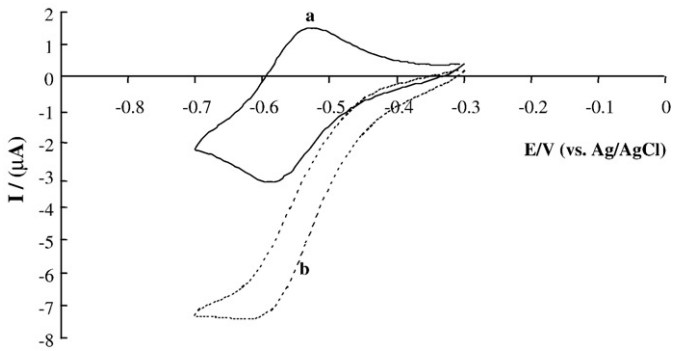

Fig. 2. Cyclic voltammograms of a NR-composite electrode in deoxygenated 0.1 M Tris–HCl (pH 7.5) buffer a) without nitrate, b) in the presence of 2 mM nitrate. Scan rate: 5 mV s^{-1}.

of nitrate (I_{lim}) described by the Levich equation and the current (I_{kin}) limited by the kinetic of the enzyme reaction:

$$I_{lim} = 0.620nFAv^{-1/6}D^{2/3}\left[NO_3^-\right]\omega^{1/2} \quad (4)$$

$$I_{kin} = nFA\Gamma k_{obs}\left[NO_3^-\right] \quad (5)$$

The mass transfer limited current (I_{lim}) depends on the rotating velocity ($s\omega$) and the concentration of NO_3^- while the kinetically limited current depends on the NO_3^- concentration. F is the Faraday constant; n is the number of electron (2) involved in the reduction of NO_3^-, D (1.8×10^{-5} cm^2s^{-1}) is the diffusion coefficient of NO_3^-, Γ is the enzyme surface density (mol cm^{-2}) while ν (0.01 cm^2s^{-1}) represents the kinetic viscosity of water [20]. The general equation that describes the dependence of the electro-catalytic current on the experimental parameters is commonly reported following the Koutecky–Levich equation [21]:

$$1/I_{cat} = 1/I_{lim} + 1/I_{kin} = 1/\left(0.620nFAv^{-1/6}D^{2/3}\left[NO_3^-\right]\omega^{1/2}\right) + 1/\left(nFA\Gamma k_{obs}\left[NO_3^-\right]\right) \quad (6)$$

Only the first term of the Eq. (6) is dependent upon the rotation rate of the disk electrode. Therefore, a plot of $1/I_{cat}$ versus $1/\omega^{1/2}$ presents a linear behavior with a positive intercept whose value depends on k_{obs}.

As previously described by Willner and co-workers, the mechanism of the electro-enzymatic reduction of nitrate by an electrically wired NR involved the formation of an enzyme-substrate complex following a Michaelis Menten kinetic model [20] and a general equation describing the dependence of k_{obs} on these kinetic constants was thus reported:

$$NR + NO_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} NRNO_3^- \xrightarrow{k_2} NR + NO_2^- \quad (7)$$

$$\frac{1}{k_{obs}} = \frac{K_M + \left[NO_3^-\right]}{k_2} \quad (8)$$

where $K_M=(k_{-1}+k_2)/k_1$.

With the aim to estimate the heterogeneous second order reaction rate, k_{obs} and other kinetic constants, cyclic voltammograms of the NR-composite electrode were recorded at 5 mV s^{-1} scan rate for different electrode rotation speeds and nitrate concentrations.

The observed catalytic currents (I_{cat}) were determined in the steady state domain of the cyclic voltammogram (−0.60 V vs Ag/AgCl) and their inverse values ($1/I_{cat}$) were plotted versus $1/\omega^{1/2}$.

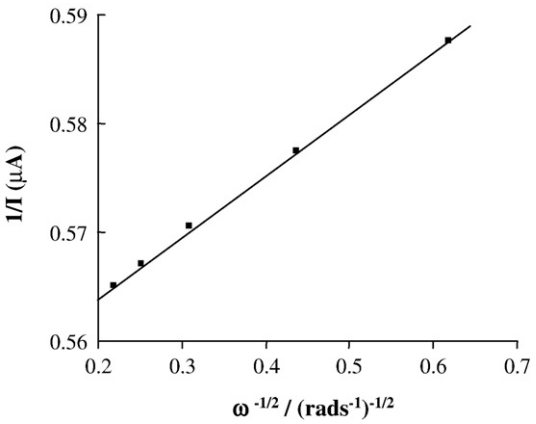

Fig. 3. Koutecky–Levich plot obtained from catalytic current recorded at NR-composite rotating disk electrode at −0.60 V (vs Ag/AgCl) in presence of 10 μM NO_3^- as a function of the rotational velocity. Correlation coefficient: 0.999. Experimental conditions as in Fig. 1.

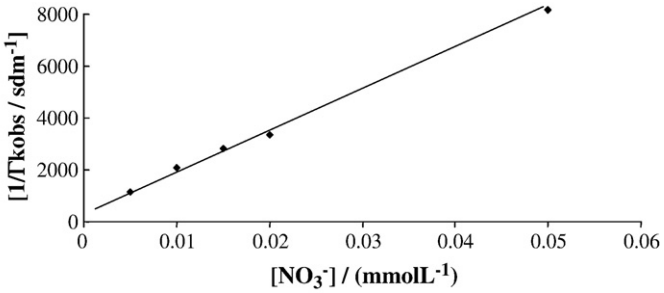

Fig. 4. Variation of $1/\Gamma k_{obs}$ for the nitrate reduction at the NR-composite electrode as a function of nitrate concentration (correlation coefficient: 0.99). Experimental conditions as in Fig. 1.

For instance, Fig. 3 shows the linear Koutecky–Levich plot obtained at 10 μM NO_3^- leading to $k_{obs}=6.34\times10^3$ $M^{-1}s^{-1}$. Koutecky–Levich plots were performed for different nitrate concentrations, namely 5, 10, 15, 20 and 50 μM providing a linear evolution with correlation coefficient comprised between 0.987 and 0.999.

From the intercept of the Koutecky–Levich plots, $1/\Gamma k_{obs}$ values were extracted from Eq. (5) and plotted as a function of nitrate concentrations. Fig. 4 shows the resulting linear relationship between $1/\Gamma k_{obs}$ and $[NO_3^-]$ (correlation coefficient = 0.99). According to Eq. (8), $1/\Gamma k_2$ and $K_M/\Gamma k_2$ were then calculated from the slope and the intercept at zero nitrate concentration respectively.

Taking into account that the deposited amount of NR was 37 μg, the enzyme surface density was estimated at 7.4×10^{-10} mol cm^{-2}. According to Eq. (8), the kinetic parameters were evaluated, namely $k_2=10.7$ s^{-1} and $K_M=7$ μM. It should be noted that the k_2 value is markedly lower than that ($k_2=4.7\times10^4$ s^{-1}) reported by Willner's group for an electrically wired NR monolayer [20]. Although the electrical connection was not assured by a viologen group, this difference in k_2 values may reflect the steric constraints due to the NR entrapment in the laponite gel. In addition, the NR molecules were dispersed within the whole structure of the composite coating compared to the close proximity at the molecular level offered by the direct formation of a NR monolayer on a redox microperoxidase-11 monolayer. Nevertheless, this k_2 value that reflects the efficiency of the electro-enzymatic reduction of nitrate, is of the same order of magnitude than those previously reported for biosensors based on electrically wired enzymes [22,23]. Moreover, the estimation of the kinetic parameter K_M from the electrochemical Lineweaver–Burk plot ($1/I$ versus $1/[NO_3^-]$) leads to an apparent K_M value (16 μM) which is in good agreement with that calculated according to the Koutecky–Levich treatment. This low K_M value illustrates the high substrate–enzyme affinity and hence the biocompatible microenvironment due to the clay template.

4. Conclusion

The successful immobilization of a nitrate reductase in laponite clay matrix containing a polypyrrole film functionalized by viologen groups has been described. In the presence of nitrate, analytical expressions describing the voltammetric response of the enzyme electrode operated under rotating disk conditions have demonstrated the efficient electrical wiring of the entrapped NR. The beneficial influence of the clay template on the immobilized enzyme and the efficiency of the electro-enzymatic reduction of nitrate were illustrated by K_M and k_2 values respectively. It is expected that the attractive properties offered by inorganic lamellar materials such as laponite, combined with electrogenerated redox polymers will be exploited for the development of biosensors based on wired enzymes.

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