Nitrate Biosensor Based on the Ultrathin-Film Composite Membrane Concept

ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 1998

Impact Factor: 5.64 · DOI: 10.1021/ac970798d

5 AUTHORS, INCLUDING:

Ligia Maria Moretto
Università Ca' Foscari Venezia
77 PUBLICATIONS **1,285** CITATIONS
SEE PROFILE

Paolo Ugo
Università Ca' Foscari Venezia
140 PUBLICATIONS **2,166** CITATIONS
SEE PROFILE

Anal. Chem. **1998,** *70,* 2163−2166

Nitrate Biosensor Based on the Ultrathin-Film Composite Membrane Concept

Ligia M. Moretto,[†] Paolo Ugo,*[,†] Micaela Zanata,[†] Paolo Guerriero,[‡] and Charles R. Martin[⊥]

Department of Physical Chemistry, University of Venice, S. Marta 2137, I-30123 Venezia, Italy, ICTIMA/CNR, Area della Ricerca, Padova, Italy, and Department of Chemistry, Colorado State University, Fort Collins, Colorado 80524

This paper describes the construction and characterization of an electrochemical nitrate biosensor based on the ultrathin-film composite membrane concept. The composite membrane is prepared by electropolymerization of a thin anion-permselective coating of 1-methyl-3-(pyrrol-1-ylmethyl)pyridinium across the surface of a microporous support membrane. This film separates the analyte solution from an internal sensing solution which contains the enzyme nitrate reductase and an electrocatalyst (methyl viologen). The ultrathin anion-permselective film prevents loss of these components from the internal sensing solution, yet allows the analyte, NO_3^-, to enter the internal solution. A glassy carbon working electrode is immersed into the internal solution. The nitrate is reduced enzymatically, yielding the oxidized form of the enzyme, which is reduced again by electrogenerated methyl viologen radical cation. The nitrate concentration in the analyte solution is proportional to the catalytic methyl viologen reduction current. This sensor shows good sensitivity to nitrate, with a detection limit of 5.4 μM and a dynamic range which extends up to 100 μM NO_3^-.

Methods for nitrate analysis[1] include spectrophotometric,[2,3] ion-chromatographic,[4] and electrochemical techniques.[5,6]

Voltammetric and polarographic determinations of nitrate are based on the electrocatalytic reduction of NO_3^-, which takes place on some electrode materials[6−9] or in the presence of electroactive catalysts either dissolved in solution[5,10−13] or incorporated in chemically modified electrodes.[14−16] These methods, however, suffered from poor selectivity, being sensitive to several interferences such as nitrite and phosphate.

Two recent papers[17,18] reported on electrochemical biosensors which used the enzyme nitrate reductase (NR) immobilized within polymeric coatings. Among these sensors, only the one proposed by Cosnier et al.,[18] who entrapped highly purified NR (not commercially available) within a film of an amphiphilic polypyrrole derivative, showed analytically useful responses in the amperometric time scale.

When developing a nitrate biosensor which employs commercially available NR and not specifically purified enzyme as in ref 18, one has to face the limiting factor caused by the low purity of the commercial products, whose activities typically do not exceed 900 units/g. This problem could be overcome by immobilizing a very large amount, of the order of tens of units per square centimeter, of such a low-activity preparation of the enzyme at the electrode surface. However, it seems unlikely that the enzyme concentration achievable via immobilization within a polymer matrix (as per the above examples[17,18]) would ever be high enough to allow such a large quantity of enzyme to be immobilized in this way.

Concentrations in the order of tens of units per milliliter of NR can be achieved by simply dissolving the enzyme in aqueous solution. A new concept in sensor design, the ultrathin-film composite membrane sensor,[19] allows for the use of an aqueous solution of the enzyme while keeping a fast response time. An ultrathin film composite membrane (UTFCM) consists of an ultrathin-film of the desired chemically selective material (typically a polymer) that has been coated across the surface of a microporous support membrane. The UTFCM separates the analyte solution from an aqueous internal sensing solution that contains the enzyme and electrocatalyst; the ultrathin film prevents these materials from leaching from the internal solution but allows the

* Corresponding author (phone, +39-41-5298503; fax, +39-41-5298594; e-mail, ugo@unive.it).
[†] University of Venice.
[‡] ICTIMA/CNR.
[⊥] Colorado State University.

(1) Carpenter, E. J.; Capone, D. G., Eds. *Nitrogen in the Marine Environment*; Academic Press: New York, 1983.
(2) Taras, H. J. *Colorimetric Determination of Nonmetals*; Interscience Publishers: New York, 1958; pp 135−137.
(3) Deutsche Einheitsverfahren zur wasser-, Abwasser- und Schlammuntersuchung DIN 38 405-D 9-2; ISO 7890-1-2-1986.
(4) Fritz, J. S.; Giede, D. T.; Pohlardt, C. *Ion Chromatography*; Huthig: Heidelberg, 1982; p 84.
(5) Kolthoff, I. M.; Harris, W. E.; Matsuyama, G. *J. Am. Chem. Soc.* **1944**, *66*, 1782−1786.
(6) Davenport, R. J.; Johnson, D. C. *Anal. Chem.* **1973**, *45*, 1979−1980.
(7) Carpenter, N. G.; Pletcher, D. *Anal. Chim. Acta* **1995**, *317*, 287−293.
(8) Fogg, A.; Scullion, S. P.; Edmonds, T. E.; Birch, B. J. *Analyst* **1991**, *116*, 573−579.
(9) Horanyi, G.; Rizmayer, E. M. *J. Electroanal. Chem.* **1985**, *188*, 265−272.
(10) Collat, J. W.; Lingane, J. J. *J. Am. Chem. Soc.* **1954**, *76*, 4214−4218.
(11) Markušová, K. *Anal. Chim. Acta* **1989**, *221*, 131−138.
(12) Boese, S. W.; Archer, V. S.; O'Laughlin, J. W. *Anal. Chem.* **1977**, *49*, 479−484.
(13) Taniguchi, J.; Nakashima, N.; Matsushida, K.; Yasukouchi, K. *J. Electroanal. Chem.* **1987**, *224*, 199−209.
(14) Neuhold, C.; Kalcher, K.; Diewald, W.; Cai, X.; Raber, G. *Electroanalysis* **1994**, *6*, 227−236.
(15) Ma, L.; Zhang, B.-Y.; Li, H.-L.; Chambers, J. Q. *J. Electroanal. Chem.* **1993**, *362*, 201−205.
(16) Ugo, P.; Moretto, L. M.; Ballarin, B. *Electroanalysis* **1995**, *7*, 129−131.
(17) Willner, J.; Katz, E.; Lapidot, N. *Bioelectrochem. Bioenerg.* **1992**, *29*, 29−45.
(18) Cosnier, S.; Innocent, C.; Jouanneau, Y. *Anal. Chem.* **1994**, *66*, 3198−3201.
(19) Ballarin, B.; Brumlik, C. J.; Lawson, D. R.; Liang, W.; Van Dyke, L. S.; Martin, C. R. *Anal. Chem.* **1992**, *64*, 2647−2651.

S0003-2700(97)00798-1 CCC: $15.00
Published on Web 04/08/1998

analyte species to permeate from the analyte solution into the sensing solution.

The advantages of ultrathin-film composite membranes over modified dialysis membranes[20] lie in the much thinner thickness of the polymeric layer used in UTFCM, which allows shorter response time.[19] Moreover, the residual negative charge which characterizes cellulose acetate dialysis membranes[21] would slow the permeation of anionic substrates, such as nitrate. An ultrathin-film composite membrane-based biosensor for nitrate is described here. At variance with the glucose sensor described in ref 19, which used an UTFCM impermeable to anions, the nitrate sensor requires the development of an UTFCM which allows the free permeation of anions and avoids leaching of cations.

MATERIALS AND METHODS

Materials. All chemicals were of reagent-grade quality. Milli-Q water was used to prepare aqueous stock solutions of the reagents and the aqueous supporting electrolyte solutions. The enzyme used was the nitrate reductase (NAD[P]H, EC 1.6.6.2), available from Sigma as lyophilized powder from *Aspergillus* species. Two different batches of products, with certified specific activities of 900 and 440 units/g, were used (1 unit reduces 1 μmol of nitrate per minute in the presence of β-NADPH at pH 7.5 at 25 °C). Nonaqueous supporting electrolyte solutions were prepared by dissolving tetrabutylammonium tetrafluoroborate [(TBA)BF_4, Aldrich, dried overnight under vacuum at 50 °C] with commercially available acetonitrile (Aldrich, 99.9+%).

The monomer used to prepare the ultrathin polymer film, 1-methyl-3-(pyrrol-1-ylmethyl)pyridinium tetrafluoborate (MPP), was prepared using literature methods.[22,23] The starting material, 3-(pyrrol-1-ylmethyl)pyridine (Aldrich), was purified on an alumina/acetonitrile column before use. Anopore (SPI) alumina filters (200-nm pore diameter, 65% porosity, 55-μm thickness and 47-mm diameter) were used as the support membrane for the ultrathin-film composites.[19]

Apparatus and Procedures. All electrochemical measurements were carried out in a thermostated cell maintained at 25.0 ± 0.2 °C under a nitrogen atmosphere. The reference was a KCl-saturated Ag/AgCl electrode. The working electrode was a glassy carbon disk (diameter, 3 mm) sealed into epoxy. Common commercially available electroanalytical instrumentation was employed. Deposition of gold films, for the preparation of ultrathin-film composite membranes, was carried out using a Baltzer MED 010 coating unit.

Nitrate and nitrite analysis of natural samples were performed using Dr. Lange Küvetten-Test LCK 339 and LCK 341 field analysis kits (Dr. B. Lange, Milan, Italy) and the LASA 10/20 portable photometer. Measurements are based on the reaction of nitrate ion with 2,6-dimethylphenol[3] and of nitrite with aromatic primary amines,[24] respectively.

Well and ditch waters were sampled in an agricultural area in the inland near Venice, Italy; they were diluted 20 times with 0.05 M Tris-HCl (tris(hydroxymethyl)aminomethane) buffer, pH 7.5, before the analysis.

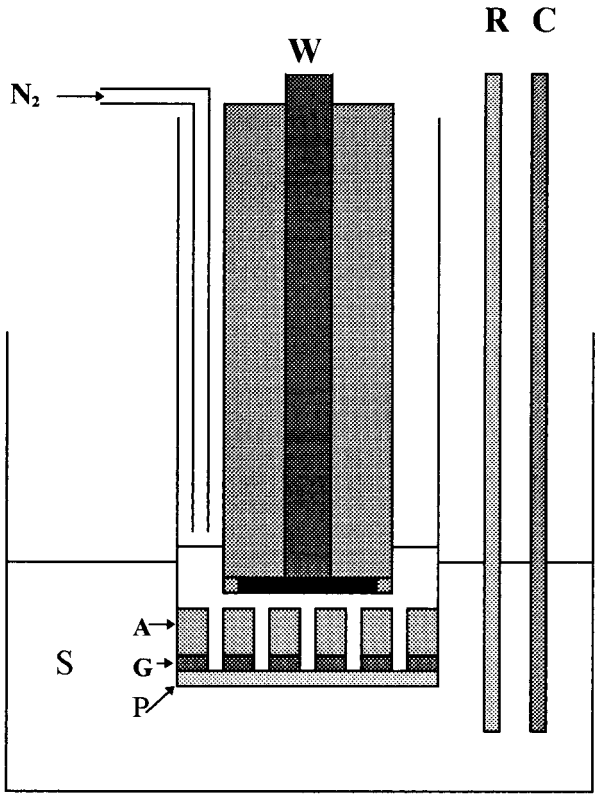
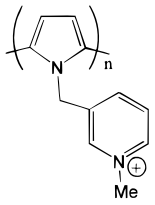


Figure 1. Schematic diagram of the ultrathin-film composite membrane-based nitrate sensor: (A) anopore membrane, (G) gold film, (P) poly-MPP coating, (W) working electrode, (R) reference electrode, (C) counter electrode, (S) sample solution.

Membrane Preparation and Sensor Assembly. The composite membranes were prepared by electropolymerization of an ultrathin film of MPP across the surface of the alumina support membrane.[25]



Poly-MPP

The "nonfiltration" side of the support membrane (according to the manufacturer's nomenclature) was first coated with a 50-nm layer of gold. The oxidative polymerization to deposit the poly-MPP film on the gold layer was carried out in 25 mM MPP and 0.1 M (TBA)BF_4 acetonitrile solutions by imposing a constant current of 0.8 mA cm^{-2}.[22,23] Unless otherwise noted, the deposition time was 180 s, which gives a film thickness of 1 μm (as determined via electron microscopy).

The ultrathin-film composite membrane-based sensor prepared is shown schematically in Figure 1. The composite membrane was glued to the end of a glass tube (0.8-cm i.d.), which formed

(20) Blaedel, W. J.; Engstron, R. C. *Anal. Chem.* **1980**, *52*, 1691−1697.
(21) (a) Colton, C. K.; Smith, K. A.; Merrill, E. W.; Farrel, P. C. *J. Biomed. Mater. Res.* **1971**, *5*, 459−479. (b) Wang, J.; Hutchins, L. D. *Anal. Chem.* **1985**, *57*, 1536−1541.
(22) Mao, H.; Pickup, P. G. *J. Electroanal. Chem.* **1989**, *265*, 127−142.
(23) Ugo, P.; Sperni, L.; Moretto, L. M. *Electroanalysis* **1997**, *9*, 1153−1158.
(24) Deutsche Einheitsverfahren zur wasser-, Abwasser- und Schlammuntersuchung DIN 38 405-D 10; ISO 6777-1984.
(25) Liu, C.; Espenscheid, M. W.; Chen, W.-J.; Martin, C. R. *J. Am. Chem. Soc.* **1990**, *112*, 2458−2459.

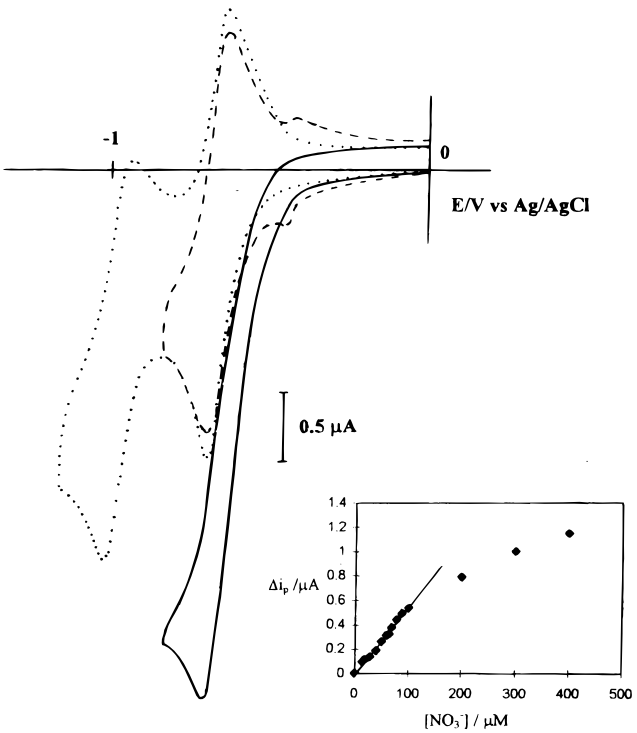


Figure 2. Cyclic voltammograms for the mediator methyl viologen (0.2 mM) in the sensor inner compartment before (dotted-line) and after the addition of nitrate reductase (9 units/mL) in the inner compartment in the absence (dashed line) and in the presence (full line) of 1 mM NO_3^- in the sample solution. Supporting electrolyte, 0.1 M phosphate buffer, pH 7.5; scan rate, 20 mV s^{-1}. Inset: Calibration plot for the UTFCM sensor.

the body of the sensor. Unless otherwise noted, the solution used in the inner compartment was 200 μL of carefully degassed 0.1 M phosphate buffer, pH 7.5, in which 2 units of nitrate reductase (i.e., 10 units/mL) and 0.2 mM methyl viologen (MV) were dissolved. A freshly prepared sensor was equilibrated for 1 h in the buffer prior to use. When not in use, the sensor was stored in buffer solution.

Preliminary experiments showed that the reference and auxiliary electrodes could be placed either inside[19] or outside of the sensor body; however, to keep the volume of the inner solution as low as possible, the reference and counter electrodes were placed outside of the sensor, in the analyte solution. It is also important to point out that the gold layer deposited on the membrane was used as working electrode only for the poly-MPP deposition. While the gold layer could be used as the working electrode for the sensor,[19] it proved easier to make electrical contact with a glassy carbon working electrode that was simply inserted into the body of the sensor (Figure 1), located as close as possible to the inner face of the membrane.

RESULTS AND DISCUSSION

Preliminary permeation experiments showed that 1-μm-thick films (0.144 C/cm^2) of poly-MPP were optimal in preventing the leaching of MV^{2+} while allowing the permeation of anions. Thinner films did not completely block MV^{2+} transport, indicating that these thinner films contain defects, a common problem in ultrathin-film composite membranes.[25] Thicker films also blocked MV^{2+} transport but, as would be expected, provided lower rates for anions.

Table 1. Effect of Different Amounts of Nitrate Reductase on the Peak Current of the $MV^{2+}/MV^{+\bullet}$ Voltammogram When the Sensor Is Dipped in 1 mM NO_3^- Solution, 0.1 M Phosphate Buffer, pH 7.5, Scan Rate 20 MV s^{-1}

[NR]/units mL^{-1}	$i_{p\ red}/\mu A$
0	1.95
1.5	2.54
3	2.75
9	3.95

The dotted line in Figure 2 shows the cyclic voltammogram obtained for methyl viologen in the internal solution, in the absence of nitrate and nitrate reductase (NR). Two reversible voltammetric waves are observed, the first associated with the $MV^{2+}/MV^{+\bullet}$ couple (i.e., on the forward scan reduction of the dication to the cation radical) and the second associated with the $MV^{+\bullet}/MV^0$ couple. Only the first wave was used for these investigations. The main characteristics of the voltammogram are kept even after 1 or 2 days, indicating that there is no leakage of the mediator from the sensor.

The dashed line in Figure 2 shows the effect on the $MV^{2+}/MV^{+\bullet}$ voltammogram of the presence of NR (again, no NO_3^-); the forward peak is slightly diminished, and a very small prewave is observed. The magnitude of these minor changes scales with the quantity of enzyme added; this suggests that they can be attributed to adsorption of NR on the surface of the working electrode. These very minor changes in the voltammogram do not affect the functioning of the sensor.

The full-line voltammogram in Figure 2 was obtained after adding 1 mM NO_3^- to the external (analyte) solution. The enhancement in the cathodic current is due to electrocatalytic reduction of the oxidized form of NR. This process can be represented as follows:

$$MV^{2+} + e \rightleftarrows MV^{+\bullet} \quad (1)$$

$$NR_{red} + NO_3^- \rightarrow NR_{ox} + NO_2^- \quad (2)$$

$$NR_{ox} + MV^{+\bullet} \rightarrow NR_{red} + MV^{2+} \quad (3)$$

where NR_{red} and NR_{ox} are the reduced and oxidized forms of the enzyme. The cathodic wave still has a peak (even at scan rates of 5 mV s^{-1}), indicating that the rate of the electrocatalytic process is controlled partially by eq 3 and partially by diffusion.[26] The electrocatalytic current is not observed if NR is not present in the internal solution.

Data listed in Table 1 show the effect of increasing the concentration of NR in the internal solution on the magnitude of the electrocatalytic current. The concentration of NO_3^- in the external solution was maintained at 1 mM. At this high concentration of substrate, the rate of the enzymatic reaction is undoubtedly at a maximum for each concentration of NR listed in Table 1. These data illustrate the importance of having high activities of NR in the internal solution.

(26) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods*; Wiley: New York, 1980; Chapter 11.

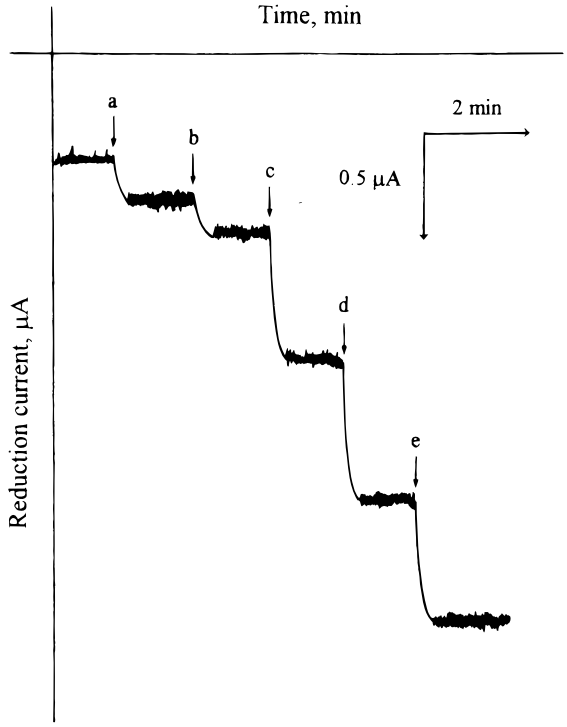


Figure 3. Amperometric response of the UTFCM sensor to successive additions of 10 (a, b), 20 (c, d), and 140 μM KNO_3 (e). The electrode potential was set at −0.700 V, and the solution was stirred at 800 rpm. Other experimental conditions are as in Figure 2.

A calibration curve of Δi_p vs concentration of NO_3^- in the analyte solution is shown in the inset in Figure 2. The dynamic range extends up to 0.1 mM nitrate. The sensitivity of the linear portion of the plot is 5.5×10^{-3} μA/μM. The detection limit, calculated as 3 times the background noise level (±0.01 μA) divided by the sensitivity,[27] is 5.4 μM.

As shown in Figure 3, the UTFCM can also be used as an amperometric sensor. The steady-state current recorded at −700 mV, in fact, increases linearly with the amount of nitrate in the sample solution, up to about 0.1 mM nitrate. The response time is on the order of 10 s. The sensitivity of the sensor in the amperometric mode increases slightly as the detection limit does. The net result is the lowering of the detection limit to 2 μM.

The addition to the analyte of NO_2^- (2 times the nitrate concentration) does not cause any measurable change in the sensor response; hence, this device does not suffer from nitrite interference. The sensor showed a satisfactory lifetime and can be used for at least 3 weeks when stored wet at +4 °C. When stored wet at room temperature, the sensor continued to respond to nitrate for over 1 week; however a decrease in sensitivity of 6% after 3 days and of 15% after 1 week was observed. Reliable quantification of nitrate concentrations within the 5−100 μM range can be performed even with aged sensors (1 week at room temperature and 3−4 weeks in the refrigerator) if the standard addition method is used.

Nitrate determination can also be performed using 0.05 M Tris-HCl buffer, pH 7.5, instead of phosphate. Such an alternative can be useful in the analysis of natural water samples, which contain cations which can form insoluble phosphate salts. The results of the determination of NO_3^- with the UTFCM in ditch and well samples are shown in Table 2, where they are compared with those obtained by the photometric method.[3]

Table 2. Determination of Nitrate in Natural Water Samples

	$[NO_3^-]$/mM	
sample	UTFCM sensor[a]	photometric method[3]
well water	1.94 (±0.09[b])	2.11 (±0.10[b])
ditch water	0.96 (±0.[b])	0.81 (±0.02[b])

[a] Experimental conditions: UTFCM sensor loaded with 10 units/mL NR and 0.2 mM methyl viologen in 0.05 M Tris-HCl buffer, scan rate 20 mV s^{-1}. [b] Standard deviation from three replicates.

The accuracy and precision of data obtained with the UTFCM appear satisfactory. It is worth noting that the ditch water contained 24 ± 1 μM nitrite (determined photometrically[24]), while in the well water the nitrite concentration was below the detection limit of the photometric method. The good accuracy of the nitrate result obtained with the UTFCM sensor in the presence of the high nitrite concentration in the ditch water sample confirms that the sensor does not suffer interference from this anion.

CONCLUSIONS

The UTFCM nitrate sensor proposed here shows good analytical performance and can be reliably used for the sensitive and selective determination of nitrate. To the best of our knowledge, this is the first report on a nitrate sensor which works properly (also in real samples) using commercially available NR. Previous sensors which employed commercial NR[17] suffered due to the low activity of the enzyme−polymer assembly, and the biocatalyzed process was not efficient enough to allow the observation of catalytic currents of analytical usefulness. The UTFCM sensor can find wider use than the sensor described in ref 18, whose application is limited by the availability of enzyme preparation of very high purity. The poly-MPP-based UTFCM allows the entrapment both of high enzyme loading and of low-molecular-weight mediators while keeping the response time short, even with respect to anionic substrates (as nitrate); such achievements are hard to reach with classical dialysis membranes, in which the presence of residual carboxyl groups on the cellulose chains can slacken the permeation of anions.[21]

ACKNOWLEDGMENT

This work was supported by CNR and Murst (Rome) and Project “Sistema Lagunare Veneziano”, by NATO (CRG 951182), and by the U.S. National Science Foundation (grant to C.R.M.). Thanks to Prof. M. Mascini (Florence) for his useful suggestions.

Received for review July 23, 1997. Accepted February 25, 1998.

AC970798D

(27) Long, G. L.; Winefordner, J. D. *Anal. Chem.* **1983**, *55*, 712A−724A.