DEVELOPMENT OF A UREA BIOSENSOR BASED ON A POLYMERIC MEMBRANE INCLUDING ZEOLITE

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Résumé

Un biodétecteur d'urée a été préparée par l'immobilisation de l'uréase sur la surface d'un transistor à effet de champ ammonium-sensible [FET] . La membrane de NH_4^+-sensible est basée sur un biodétecteur polymére zéolite incorporé dans la membrane (clinoptilolite). La sensibilité de la détection de l'ammonium est sous-nernstian $(32mV / pNH4^+)$. Le greffage de l'uréase sur la membrane de NH_4^+-sensible a été éffectuée à travers l'utilisation du glutaraldéhyde. La sensibilité de l'urée ENFET est de 15 mV/ p Uréa et ceci reste stable pendant 15 jours avec une limite de détection de 3.10^{-5} M.
En conclusion , afin d'examiner la performance du biodétecteur d'urée pour des applications environnementales, l'activité après exposition à l'enzyme empêchant des ions des métaux lourds tel que Hg (II) . En utilisant ces biodétecteurs d'urée la limite de détection est de 5.10^{-8} M a été obtenue pour Hg (II).
Mots clés: ISFET, Biodetecteur d'urée , Zéolite , Membrane polymere .

Abstract

A urea biosensor has been prepared by covalent biding of urease directly to the surface of an ammonium-sensitive field effect transistor (FET).The NH_4^+ -sensitive membrane is based on a zeolite-incorporated polymeric membrane biosensor (clinoptilolite). The sensitivity of ammonium detection is sub-nernstian ($32mV/pNH_4^+$) but the ISFET presents a high selectivity, which is interesting for measurements in biological media. The grafting of urease to the NH_4^+-sensitive membrane was permorfed by cross-linking with glutaraldehyde .The sensitivity of the urea ENFET is 15V/purea and this remains stable over 15 days with a detection limit of $3x10^{-5}$ M. Finally, in order to test feasibility of the urea biosensor for environmental applications, the remaining activity of the urease was determined after exposure to enzyme inhibiting heavy metals ions such as Hg(II).Using these urea biosensors, a detection limit of less than 5×10^{-8} M was obtained for Hg(II).
Keywords: ISFET ; Urea biosensor ; Zeolite ; Polymeric membrane

M. L. HAMLAOUI* [a]
N. BOUYAHIA[a]
N.JAFFREZIC-RENAULT [b]

[a] LPCI , Département de Chimie, Université de Annaba , BP 12 , El-Hadjar , Annaba , Algérie .
[b] *IFoS , UMR-CNRS 5621 , ECL-Lyon , 69134 Ecully Cedex , France*
*E-mail: l_hamlaoui@yahoo.fr

ملخص

تم تحضير كاشف بيولوجي لليوريا و هذا انطلاقا من تثبيث اليوريا على سطح اترانزيستور دو فعل حقلي **امونيوم+حساس** [FET] . -غشاء (سطح) الأمنيوم حساس(Sensible NH_4^+) مكونة أساسا من مكشاف بيولوجي بوليميري زيوليتي مدمج في الغشاء (السطح) -حساسية الكشف للأمونيوم هي عن نوع تحت ناريسته $(32mV / pNH_4^+)$ sous nersntienne عملية زرع اليورياء على الغشاء (السطح) ل NH_4^+- حساس (NH_{4-}^+ sensible) تمت عن طريق استعمال الغليتار الدهيد . رتبة 3.10^{-5} M. و كخلاصة عن أجل تقييم كفاءة الكاشف البيولوجي لليوريا من أجل تطبيقات خاصة بالبيئة الفعالية المتبقية لليوريه قد تم تحديدها و هذا بعد تعرضها للأنزيم لمنع أيرنات المعادن الثقيلة مثل $Hg(II)$. باستعمال هذا النوع بين الكواشف البيولوجية لليوريا، فان هذا الكشف هي عن رتبة $5.10^{-8} M$ من أجل $Hg(II)$.

الكلمات المفتاحيـــة : ISFET -كاشف بيولوجي لليوريا- زيوليتي- غشاء بوليميري

1. INTRODUCTION

The detection of urea is of great interest in biomedical and clinical analysis application.
Indeed, an increase of urea concentration in blood and a reduced level of urine is a strong indication of renal dysfunction. The determination of urea in body fluids is one of the most frequent analyses in clinical laboratories.The determination of urea is generally performed with enzyme –based biosensors .Enzymatic reactions of non-ionic bioproducts often produce ionic species. Therefore a variety of biosensors have been developed for the selective determination of many substances using ion-selective membranes in combination with suitable enzymes. For the determination of urea, enzymatic biosensors are based on urease.
The detection principle is based on the fact that urease catalyses the hydrolysis of urea according to the reaction.

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- \quad (1)$$

In the case of conventional urea sensors, pH [1- 6] and NH_4^+ [7- 9] selective electrodes have been used to detect hydrogen ions and ammonium ions , respectively , that are produced by enzymatic reaction. The major problem for pH-sensitive electrodes is that the sensor response is strongly dependent on the buffer capacity of the sample. Indeed , the change of pH, which occurs during the enzyme-catalysed reaction, is compensated by the buffer used , which leads to a narrow dynamic range and a loss in sensor sensitivity [10].
The biosensor presented in this study is based on a polymeric membrane incorporating a NH_4^+ - sensitive zeolite.
As urease can be inhibited by heavy metal ions such as mercury , these enzymatic biosensors can be used for the detection of these toxic ions. A very high bioaccumulation of heavy metals can take place in living organisms, especially in marine organisms .In particular, the influence of mercury on environment is a serious problem due to its strong toxicity and to an increasing level of its extensive use in industrial processes. The detectors using the enzyme inhibitor determination method can by very sensitive. Indeed; the reduction of enzyme activity by a single inhibitor molecule can be large thanks to amplification effects. Moreover enzymes are often specific to inhibitor families and in many cases the inhibition effect of the investigated pollutant is related to its biological toxicity. So the inhibition of enzymatic activity by mercury may be a good choice as a simple and sensitive screening test. Numerous enzyme have been used for inhibitive determination of mercury; e.g. peroxidase [11], xanthine oxidase [12], glucose oxidase [13] or butyrylcholinesterase [14] but urease is the most frequently used as it is relatively cheap and available. In this study, the analytical performances of the ISFET based on the zeolite-incorporated membrane are presented and discussed. After the functionalization of this NH_4^+-sensitive membrane by urease molecules, the enzymatic activity and the analytical characteristics of the resulting ENFET have been investigated concerning the detection of urea. In addition; the detection limit of heavy metals cations via an inhibition process of the enzyme activity has been evaluated .

2 . EXPERIMENTAL

2.1. ISFET sensors

pH-sensitive field effect transistors (FETs) with Si_3N_4 gate insulator were fabricated at Centro Nacional de microeletronica (CNM), Spain. The detailed manufacturing process has been described elsewhere [15]. The devices are based on a N-channel metal oxide semiconductor (MOS) process compatible with the standard CNM complementary MOS technology. In our case, the silicon nitride layer has been deposited on the ISFET gate by low temperature (380°C) plasma-enhanced chemical vapour deposition (PECVD).

2.2 Characterization of clinoptilolite

Zeolite are crystalline aluminosilicates displaying in a single materiel both ion exchange capacity and size selectivity properties [16]. This attractive feature was exploited in electrochemistry by designing a variety of zeolite-modified electrodes with applications in the field of molecular recognition, mediated electrocatalysis and electroanalysis [17-19] . Most of their use in electroanalytical chemistry were directed to : (1) voltammetric analysis after accumulation at open circuit; (2) direct electrocatalytic detection; (3) indirect amperometric detection of non-electroactive cations and (4) some biosensors applications [20-21] .
A number of investigations on zeolite-based composite membranes were also performed, in order to determine alkali metal species and some other ions in aqueous medium by potentiometric measurements [22-28] . Membranes were made of a dispersion of zeolite particles within a polymeric binder as low viscosity epoxy resins, polysulfone or polydimethylsiloxane. Both the zeolite nature and the membrane preparation procedure were found to strongly affect the potentiometric responses , as a function of the zeolite / polymer adhesion [25-26] .
A recent report describes the use of a pure zeolite membrane for potentiometric detection in non-aqueous medium [28] . Detection was always made by simple measurement of the emf across the membrane. Except in the case of obvious size exclusion [23], the selectivity observed for potentiometric sensing of non-size-excluded cations using zeolite-based membranes remains poor .
A possible explanation for such lack of performance is linked to the low ion exchange selectivity of the zeolites used, at least with respect to the alkali metal ion series , which is otherwise observed for many common zeolites [16] . A way to circumvent this limitation is to choose a zeolite structure liable to lead to high selectivity for the target analyte over other common interferences. For

example, clinoptilolite is know to display marked preference for ammonium ions (over other small cations) and this unique property was exploited for the selective ammonium removal from aqueous effluents [29, 30] .
Chemical composition was determined by elemental analysis (carried out by fluorescence), resulting in the unit cell formula " ($Na_{0.10}K_{0.57}$); ($Ca_{0.47}$ $Mg_{0.15}$); ($Al_{1.97}$ $Fe_{0.12}$); ($Si_{9.96}$ $Ti_{0.02}$) $O_{24}.7H_2O$" [31].

2.3. Composition and deposition of the membrane

For preparing the membrane of ammonium –ISFET , a typical procedure was applied using siloprene and cross-linker in ratio 10:1 (w/w) diluted in THF , with different concentrations of zeolite (w/w %) . Before deposition of the siloprene membrane , a treatment of the ISFET insulator surface with a sulphochromic mixture was performed .The siloprene membranes were formed by depositing 0.1 µl of the aforementioned siloprene /zeolite suspension on the ISFET surface . The solvent was then evaporated at ambient air . The sensor was then soaked for 6h in a 0.1M NH_4NO_3 solution in order to achieve appropriate conditioning of the siloprene/zeolite membrane.
Urease (type IV from Jack beans , 118 U/ mg) and bovine serum albumin (BSA) were purchased from Sigma . BSA enzymatic membrane was prepared by co-reticulation , by mixing 0.27 ml of the aqueous enzymatic solution of urease , 5 mg BSA , 10 µl glycerol in 90 µl of phosphate buffer (1mM , pH = 8.0) [32] .Glycerol was used as a plasticiser to avoid the formation of cracks in the enzymatic membrane during storage and also result in a better homogeneity of the membrane and better adhesion to the surface of the transducer .
A glutaraldehyde drop and then the previous mixture (0.5µl) were deposited on the clinoptilolite-based NH_4^+-sensitive membrane. Then, the electrode was placed in a saturated
glutaraldehyde vapour for 30 min. The sensors were stored in a dry and dark environment at 4°C in order to preserve enzymatic activity.

2.4 Measurements

The output voltage of modified ISFETs or ENFETs immersed in 5mM phosphate buffer were measured with the source and drain follower type ISFET amplifier. This system allowed the source voltage (V_s) to be measured, while the drain voltage (V_d) and drain current (I_d) remained constant. V_s was directly plotted on a recorder. The voltage was measured against a saturated calomel electrode .

3. RESULTS AND DISCUSSION

3.1.Analytical characteristics of the ammonium ISFET
3.1.1. Effect of zeolite content of membrane on ISFET sensitivity
The sensitivities of detection of ammonium ions obtained in 0.1M KNO_3 solutions , with ISFETs covered by membranes with different zeolite contents (w/w%), are presented in Table1.A very low sensitivity for ammonium ions (3 mV/ pNH_4^+) is obtained with the siloprene membrane alone. This sensitivity is low compared to the plasticised PVC membrane one (as high as 20mV/pNH_4^+) [33]. Such an interesting feature of siloprene membrane renders them convenient for the elaboration of reference field effect transistor (REFET) in differential measurements. The main drawback of the zeolite-incorporated membrane biosensor was a rather long response time (5min).

Table1: Sensitivities of detection of ammonium ions obtained in 0.1 M KNO_3 solutions , with ISFETs covered by membranes with different zeolite contents (w /w %) .

Zeolite content (w / w %)	0	17	35	43	50
Sensitivity (mV / pNH_4)	3.3	11.2	12.5	31.5	21.1

Sensitive of detection of ammonium ions obtained in 0.1 M KNO_3, solutions , with ISFETs covered by membranes with different zeolite contents (w/w%) .
When the zeolite content increases, sensitivity for ammonium detection increases up to 43 w/w % .Upon increasing the zeolite content above this value , the elastomer properties of the siloprene membrane become progressively destroyed by the high mineral content (i.e.50w/w %) which decreases membrane conductivity . It has been shown that conductivity is an important parameter in order to approach nernstian behaviour for a polymeric membrane [34]. The optimal zeolite content of 13 w/w % was used for all further tests.

3.1.2. Effect of the nature of alkaline ion as support electrolyte on ammonium detection

Output voltage of ammonium –ISFET versus log [NH_4^+] is presented in Fig.1. In the presence of potassium ions and sodium ions , sensitivity of ammonium detection is 32 mV / pNH_4^+. This sensitivity is rather low compared to that obtained in the case of NH_4^+-sensitive membrane based on zirconium titanium phosphate [35] (40 mV per decade) or on nonactin/polysiloxane [36] or nonactin/ PVC [33] (46-50 mV per decade) for example. The sensitivity of ammonium ions does not depend on the nature of alkaline ion present . The detection limit is 10^{-8} M which is very important compared to the limit of detection obtained in the cases mentioned ([NH_4^+] > 5 x 10^{-6} M. In 0.1 M NH_4NO_3 solution , sensitivities of detection of proton (between pH = 2.0 and 9.0) sodium and potassium ions (between 10^{-5} and 10-^{2} M) are quite low , less than 3 mV per decade. Such a value has to be compared to 32 mV per decade obtained for ammonium ions. This ammonium ISFET is highly selective for ammonium. Both the high sensitivity and high selectivity

constitute a considerable improvement with respect to the previously reported potentiometric sensing devices based on zeolite-incorporated membranes (for which typical linear response was observed in the rnge of 1.5 x 10^{-5} to 10^{-2} M, with detection limits of about 1.5 x 10^{-5} M) [24, 26, 27,37] The high selectivity for ammonium detection is clearly due to the particular zeolite (clinoptilolite) used for this purpose, displaying an intrinsically high preference for ammonium over other common ions [16, 29, 30]. The increase in sensitivity is less easily explained and could be assigned to a good adhesion between the polymer and zeolite particles, which has been reported as a key factor affecting the response of ISE-based zeolite-modified electrodes [25, 26, 37], together with an efficient combination of the zeolite / siloprene membrane with the FET device. No decrease of ammonium sensitivity is observed after using the ammonium ISFET for more than 1 month .

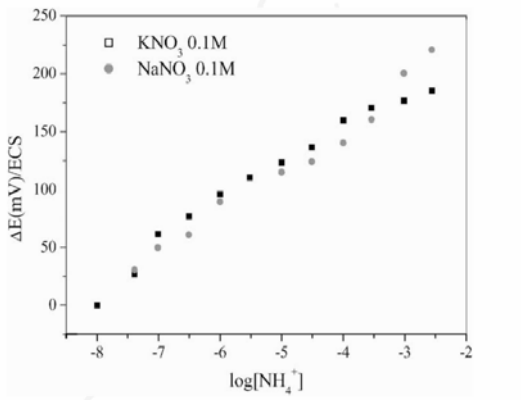

Fig.1. Output voltage of ammonium –ISFET vs. log $[NH_4^+]$ in KNO_3 0.1 M and $NaNO_3$ 0.1 M.

3.2. Urea biosensors

The curve corresponding to enzymatic sensor response, prepared by deposition of enzymatic membrane on NH_4^+-sensitive membrane , versus urea concentration is presented in Fig. 2 The biosensor operation was evaluated by measuring the variation of V_s versus concentration
of urea (E versus –log [urea]) in 5 mM phosphate buffer pH = 7.0. From the curve , it can be conclude that the biosensor has a linear response in the range of 3 x 10^{-5} to 5 x10^{-3} M urea in buffered solution.The response time of the biosensor with urease immobilized on clinoptilolite membrane was the same as that without enzyme (5 min) . The sensitivity of the biosensor is 15 mV / purea , its sensitivity was approximately constant over a period 15 days . After 15 days, adhesion of enzymatic membrane becomes poor. Deviations between individual response values are about 10 % . The sensitivity is low compared to urea biosensors based on double matrix membrane NH_4^+ -sensitive membrane [9] or nonactin / PVC membrane [36] but the ranges of detection are similar in each case .

Sensitivities recorded with pH-sensitive membrane are more important but the range of detection is smaller [38, 39] .The use of such sensors in biological media , where interferences due to alkaline ions can take place , makes the clinoptilolite membrane interesting due to its selectivity.

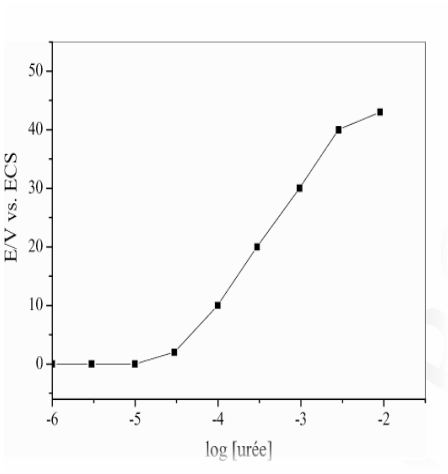

Fig .2 . Potentiometric response of urea biosensors using 5mM phosphate buffer to urea concentration increase .

3.3 Inhibition by heavy metal ions

The postulated mechanism of enzyme inhibition by heavy metal ions is based on the interaction of metal ions with exposed thiol groups of protein amino acids , which often form the active site of the enzyme. The strongest interaction takes place in the case of mercury , copper and silver , therefore those metals exhibit the largest inhibition effect . As a consequence , the enzymatic activity of the biosensor is decreased or totally suppressed.

The urea ENFET was applied to the determination of mercury ions as mentioned in literature [37] . The sensor was immersed into the phosphate buffer into which was added, firstly an amount of urea and secondly an amount of Hg (II) .

The equilibration time was rather long after injection of Hg (II) (15 min) . The remaining activity of enzyme after inhibition with mercury ions versus log [Hg^{2+}] is presented Fig.3.

The remaining activity decreases up to 10^{-4} M Hg^{2+} concentration for which the enzyme is totally inhibited . The detection limit for Hg (II) , obtained with these urea biosensors is less than 5x 10^{-8} M which is comparable with results recorded in such biosensors based on urea [33]. The sensor based on zeolite is not selective to mercury ions but the mercury concentration could be obtained by using a multienzymatic sensor network associated with a statistical treatment of data.

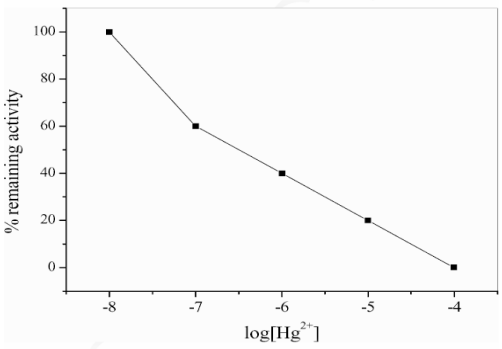

Fig. 3 . Dependence of remaining activity of the biosensors on the concentration of the inhibitor metal ion Hg (II).

CONCLUSION

In this report , we have described the analytical characteristics of an ammonium ISFET based on a zeolite-incorporated polymeric membrane . Despite its low sensitivity (32 mV / p $NH4^+$) , the limit of detection (10^{-8} M) and the high selectivity of this ISFET towards ammonium made it very attractive .

After functionalisation of the ISFET by urease molecules , the biosensor was tested for urea and heavy metal ion detection. The results are interesting since the detection limits reached were respectively 3 x 10^{-5} M for urea and 5 x10^{-8} M for heavy metal ions .

The high selectivity of this ammonium –based biosensor will be exploited for in vitro and in vivo urea measurements in biological media rich in alkaline ions .

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