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Urea potentiometric enzymatic biosensor based on charged biopolymers and electrodeposited polyaniline

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ABSTRACT

A potentiometric biosensor based on urease was developed for the quantitative determination of urea concentration in aqueous solutions for biomedical applications. The urease was either physisorbed onto an electrodeposited polyaniline film (PANI), or immobilized on a layer-by-layer film (LbL) assembled over the PANI film, that was obtained by the alternate deposition of charged polysaccharides (carboxymethylpullulan (CMP) and chitosan (CHI)). In the latter case, the urease (Urs) enzyme was either physically adsorbed or covalently grafted to the LbL film using carbodiimide coupling reaction. Potentiometric responses of the enzymatic biosensors were measured as a function of the urea concentration in aqueous solutions (from 10^{-6} to 10^{-1} mol L^{-1} urea). Very high sensitivity and short response time were observed for the present biosensor. Moreover, a stability study showed a higher stability over time for the potentiometric response of the sensor with the enzyme-grafted LbL film, testifying for the protective nature of the polysaccharide coating and the interest of covalent grafting.

1. Introduction

Enzymatic biosensors based on electrochemically polymerized films constitute an important area of biomedical research. Enzymes interact specifically with some substrates, and can thus be used for the detection of these substrates as, for instance, glucose by glucose oxidase, creatinine by creatinine amidohydrolase, or urea by urease. Among these analytes, urea has received considerable attention and many different techniques have been developed and proposed for its detection in clinical, food and environmental samples.

In human metabolism, urea is the main end-product of proteins; it has thus a great significance in clinical chemistry where urea nitrogen is an important indicator of kidney dysfunction (Gutierrez et al., 2007; Spencer, 1986; Abdel and Guibault, 1990). The physiological level of urea in serum ranges from 15 to 40 mg dL^{-1} (3.0 to 7.5 mmol L^{-1}) depending on the diet (Singh et al., 2008). Very high urea levels can be caused by severe renal dysfunction, often referred to as uremic syndrome and are eventually fatal if not treated by dialysis or kidney transplantation (Koncki, 2007). High level of urea causes chronic or acute renal failure, urinary tract obstruction, dehydration, shock, burns and gastrointestinal bleeding, whereas a low level of urea causes hepatic failure, nephrotic syndrome, cachexia (Rajesh et al., 2005).

In dairy milk, urea levels can be used to track and restrain adulteration used for commercial benefits, or assess the nutritional program of cows and particularly lactating dairy cows (Luong et al., 1997; Kaura, 2005; DePeters and Ferguson, 1992). Finally, urea can cause environmental stress because it acts as a nitrogenous fertilizer. It decomposes to ammonia, which is very toxic, thereby polluting the streams and rivers into which it ends up (Emsley, 1999).

Although direct spectroscopic methods can be used for the determination of urea, these methods usually require a sample pretreatment or conditioning and cannot be used for on site monitoring. This explains the need for biosensors using urease as a sensitizer since this enzyme catalyzes urea hydrolysis to ammonium and bicarbonate ions according to the following reaction:

$$H_2N\text{–}CO\text{–}NH_2 + 3\,H_2O \rightarrow 2\,NH_4^+ + HCO_3^- + OH^-. \quad (1)$$

Urease has often been used as the sensitizing material for the fabrication of urea sensors, like thermal (Xie and Danielsson, 1996; Xie et al., 1995; Fulton et al., 1980; Hauser et al., 1995), conductometric (Sheppard et al., 1996; Jdanova et al., 1996; Castillo-Ortega et al., 2005), piezoelectric (Xu et al., 1996; Yang et al., 2007; Yang et al., 2008), optical (Picioreanu et al., 2000; Koncki et al., 2001; Tsai and Doong, 2005), laser (György et al., 2010) and amperometric (Rajesh et al., 2005; Vostiar et al., 2002; Pizzariello et al., 2001; Stredansky et al., 2000) sensors. However, potentiometric

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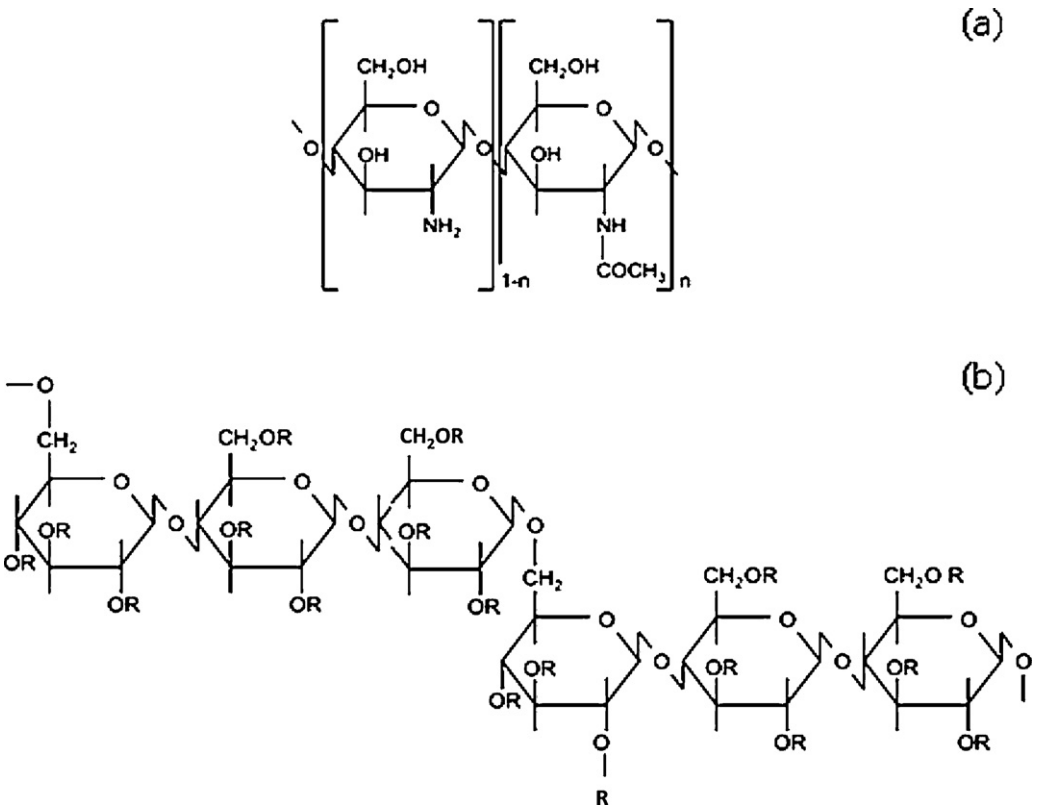

Fig. 1. Molecular structures of (a) chitosan repeating unit (with n = DA or degree of acetylation) and (b) carboxymethylpullulan (with R=H or CH_2COO^-).

biosensors are perhaps the most attractive sensors since they can be easily mass-fabricated in tiny formats using silicon or thin-film technologies, and allow for a simple mode of detection. Thus, several potentiometric enzymatic biosensors have been developed, e.g., for ammonia gas (Lui et al., 1997; Narinesingh et al., 1991), ammonium ions (Zamponi et al., 1996; Gracia et al., 1996; Trivedi et al., 2009) or pH sensing (Kuralay et al., 2005; Soldatkin et al., 2003; Lakard et al., 2004; Ahuja et al., 2008).

One possible approach for urea biosensor design involves urease immobilization on a conducting polymer film. Indeed, conducting polymers are attractive materials for biosensing applications since they enhance the speed and sensitivity of biosensors, in addition to the low cost of the monomer, high chemical stability and easy oxidation. In this context, this study aims at elaborating a potentiometric urea sensor based on polyaniline as conducting polymer transducer, polyaniline being sensitive to pH changes generated by the hydrolysis of urea (Eq. (1)).

Urease immobilization plays a fundamental role in the performance of the biosensor since the product of the enzymatic reaction should be generated as close as possible to the surface of the electrodes. The enzyme can be immobilized by various techniques like physical adsorption or covalent binding (Ahuja et al., 2007). Adsorption (Khan and Wernet, 1997; Minett et al., 2002) can be used to immobilize the enzyme on the outer layer of the conducting polymer and overcome the problem of entrapment. However, this technique suffers from inevitable enzyme desorption problems which can be solved by adding a covalent zero-length link between the enzyme and the polymer film. Covalent grafting has the additional advantage of improving the stability of the enzyme in the polymer film (Lakard et al., 2004; Ahuja et al., 2008). However, the accessibility of the enzyme active site and its activity have to be preserved after the surface reaction for a maximal sensitivity.

In this work, enzyme immobilization is ensured by covalent binding of the enzyme on a LbL film (Magnin et al., 2008) deposited beforehand on the conducting polyaniline film. The LbL film is aimed at improving both the enzyme immobilization and stability, in order to increase the lifetime of the urea biosensor for biomedical applications.

Layer-by-layer (LbL) assembly of polycations and polyanions has emerged as a facile technique to fabricate functional multilayered films. LbL, which primarily exploits the electrostatic attraction between oppositely charged species alternately adsorbing on a surface from an aqueous solution, has been used to modify the surface of substrates of varying nature, shape, and size, using a large variety of compounds (Decher, 1997; Bertrand et al., 2000). The LbL film used in this work is composed of 2.5 bilayers of two biopolymers: negatively charged carboxymethylpullulan and positively charged chitosan. The enzyme is covalently attached to the outer layer of the LbL film (carboxymethylpullulan) through a carbodiimide coupling reaction as previously studied (Magnin et al., 2008). This reaction simultaneously crosslinks the polysaccharide film, by reacting the amine groups of chitosan with the carboxylic acid moieties of carboxymethylpullulan which contributes to the stabilization of the film. To demonstrate the improvement of the detection properties and lifetime of the devices by this enzyme immobilisation strategy, sensors obtained by covalent grafting after LbL modification of the PANI transducer are compared with sensors in which urease is directly adsorbed on polyaniline or on a LbL film without carbodiimide coupling reaction.

2. Material and methods

2.1. Materials

The structures of the polysaccharides used in this study are presented in Fig. 1. Chitosan, Protosan UP CL 213 (CHI, Fig. 1a), with a M_w between 150,000 and 400,000 g mol^{-1} and a degree of deacetylation, DD, between 75 and 90%, was provided by

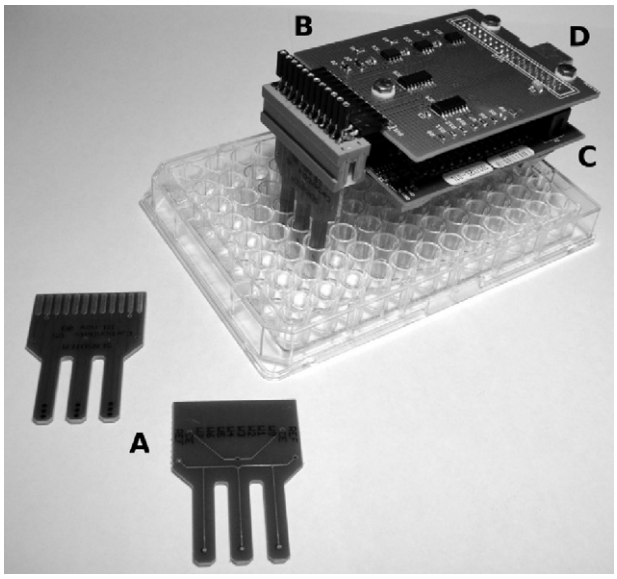

Fig. 2. Picture of the home-made USB potentiostat and electrodes.

Novamatrix (Drammen, Norway). Carboxymethylpullulan (CMP, Fig. 1b) with M_w = 426,000 g mol^{-1} and a substitution degree in carboxymethyl groups of 0.9 ± 0.05 per anhydroglucose unit was prepared from pullulan (Hayashibara Biochemical Laboratory, Japan) according to a procedure already described (Bataille et al., 1997). Aniline, HCl, urea, urease (Urs, EC 3.5.1.5, from Jack Bean, 50 U/mg), 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, 98+%), N-hydroxysulfosuccinimide (NHS, 98+%), sodium phosphate monobasic (NaH_2PO_4, 99+%), and sodium phosphate dibasic (Na_2HPO_4, 98%) were purchased from Sigma-Aldrich. Phosphate buffer was homemade (PBS; 0.2 M Na_2HPO_4 and NaH_2PO_4, pH 6.5). The water used in all experiments was purified by a Millipore system (Milli-Q water of 18.2 MΩ cm resistivity).

To test the efficiency of the developed biosensors, a synthetic urine solution was prepared that contained 14.1 g NaCl, 2.8 g KCl, 17.3 g urea, 1.9 mL of 25% v/v ammonia solution, 0.6 g $CaCl_2$ and 0.43 g $MgSO_4$, made up to 1 L with 0.02 M HCl (Tanaka and Hayashi, 1986). NH_3, HCl and all these salts were purchased from Sigma-Aldrich. This solution was diluted ten times to obtain a urea concentration of 2.88 × 10^{-2} M.

2.2. Analytical methods

Portable potentiostat. The portable potentiostat is home-made and can be seen in Fig. 2 (Yunus et al., 2010; Kwakye and Baeumner, 2007; Gopinath and Russell, 2006). This potentiostat is computer controlled via a National Instrument DAQ USB-6009 multipurpose control and acquisition card (C) that is partially visible and located below the potentiostat card (B). This instrument is connected to a computer via a USB bus (D). The programs to control the instrument are built and compiled with Microsoft VisualBasic 6.0. With this setup, the full scale range of the potentiostat is 5 V (−2.5–2.5 V). The potentiostat input is controlled via the card DAC (Digital to Analog Converter). As this DAC has a 12 bit resolution the voltage resolution is $5/2^{12}$ V (∼1.22 mV). The potentiostat output (current to voltage converter) is connected to the ADC (Analog to Digital Converter) that has a 14 bit resolution.

The electrodes in Fig. 2(A) are interchangeable. The mini-potentiostat card and the electrodes consist in single sided layer printed circuit boards (PCB) and are fabricated by Elprinta (Belgium) using standard printed circuit boards realization techniques. Only the electrodes area are covered with a screen printed carbon layer having a resistance of approximately 14–20 Ohm/square at 25 μm dry film thickness (Peeters SD 2841 HAL-IR) since polyaniline cannot be electro-synthesized on copper, The rest of the copper circuitry is protected by a soldermask varnish.

As can be seen in Fig. 2(A), the electrode heads are composed of 3 × 4 = 12 carbon electrode spots. Each branch of these electrodes comprises 4 spots, 3 on the front side that are 2 working electrodes surrounding 1 counter electrode and 1 on the bottom side that is the reference electrode. These spots have a diameter of ∼1.3 millimeter (working area: 1.32 mm^2). The reference electrode is obtained by applying a small spot of solid Ag/AgCl amalgam (Dupont 5874 Silver/Silver Chloride Composition). This solid Ag/AgCl reference electrode has been checked for its stability, repeatability and reliability in different measurement setups from pH 2 to 12. This reference displays an electrode potential 100 mV higher (∼300 mV vs. SHE) than a commercial reference electrode (∼197 mV vs. SHE). Each of the 6 working electrodes is assignable by multiplexers present on the potentiostat card. These electrodes allow multianalyses. Indeed, the 6 electrodes are immersed simultaneously in a solution (here during 2 min for each urea concentration). During this immersion, the multiplexers of the potentiostat measure the potential of all electrodes one electrode after the other but at a high acquisition rate. Consequently, it can be considered that the measurements are done simultaneously on the 6 electrodes of the biosensor. This allows to obtain statistical data and to prove the reproducibility of the measurements.

SEM. Scanning electron microscopy (SEM) samples were dried with argon gas. The trident electrodes, coated with PANI, were fixed on an aluminum sample holder with a carbon-based adhesive tape. The samples were then coated with Cr for charge dissipation. Samples were observed with a high resolution FEG Digital Scanning Electron Microscope Leo982 Gemini from Zeiss.

2.3. Preparation of the Polyaniline/LbL assembly

2.3.1. Polyaniline electrodeposition

Polyaniline (PANI) was electropolymerized simultaneously on the six carbon working electrodes of the biosensor from 0.2 mol L^{-1} aniline in 2 mol L^{-1} HCl solutions. A galvanostatic method was used to perform the electropolymerization of polyaniline. A current of 15 μA was imposed to each carbon electrode leading to a current density of 11.8 μA mm^{-2} (electrode area = 1.32 mm^2). The current was imposed until reaching a charge per area q = 15 mC cm^{-2}, leading to a reproducible coating on from one carbon electrode to the other. The PANI films obtained present a porous structure formed of entangled nanowires (see supplementary information, Figures S1 and S2 for $U = f(t)$ obtained during synthesis and SEM image of the resulting film, respectively) typical of the electrodeposition process (Hao et al., 2010; Lakard et al., 2004).

2.3.2. LbL assembly procedure

Polyelectrolyte multilayers were built by the layer-by-layer technique (Decher, 1997) on the electrodeposited polyaniline films. LbL assembly was performed by immersing alternately the substrates for 5 min into aqueous solutions of CMP and CHI (1 mg mL^{-1} and pH 5.5 for each polyelectrolyte), each immersion in a polyelectrolyte solution being followed by two rinsing steps with milli-Q water for 2 min. The process was cycled to obtain an assembly composed of PANI-(CMP-CHI)$_2$-CMP (PANI-LbL in the following sections) (Magnin et al., 2008).

2.4. Enzyme immobilization procedures

Enzyme immobilization plays a fundamental role in the characteristics of biosensors since a large amount of the active

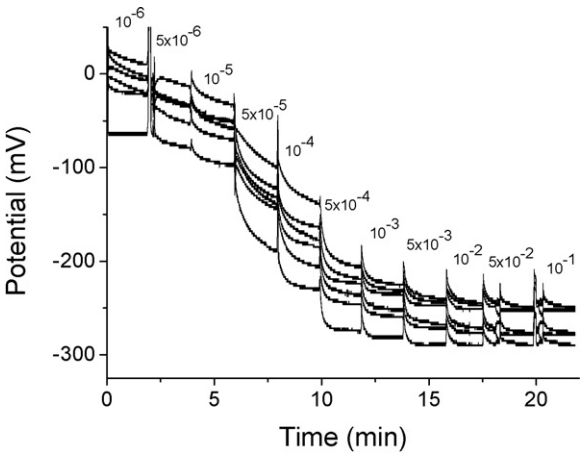

Fig. 3. Potentiometric responses of the 6 PANI-Urs urea biosensors to increasing concentrations of urea from 10^{-6} to 10^{-1} mol L^{-1}.

enzyme should be directly attached to the electrode's surface. Four enzyme immobilization procedures were used (summarized in Table S1 and Figure S3). Briefly, one electrode was simply coated by urease through adsorption (PANI-Urs), one electrode was treated by EDC-NHS before adsorption of the urease (PANI-EN-Urs), and two electrodes were prepared by LbL treatment before binding of the enzyme, in one case through adsorption on the LbL film (PANI-LbL-Urs), and in the second case by covalent grafting after activation of the LbL surface through EDC-NHS treatment (PANI-LbL-EN-Urs).

2.5. Covalent Enzyme immobilization

Where stated, covalent grafting was performed by immersing the electrodes for 15 min in PBS (pH 6.5) containing 0.5 mg mL^{-1} EDC and 0.7 mg mL^{-1} NHS. The electrodes were then rinsed with PBS and milli-Q water before being spotted with 5 μL drops of the urease solution (5 mg mL^{-1}) and left overnight at +4 °C. In the presence of LbL films, the amine residues of the enzyme therefore attached covalently to the exposed free carboxylate groups from CMP through a carbodiimide-activated amidation reaction. An identical reaction occurs in the LbL film which is therefore crosslinked after reaction. After fabrication and between each use, the electrodes were rinsed with water and stored in PBS at +5 °C.

3. Results and discussion

3.1. Response characteristics of the urea biosensors

The three pairs of enzyme-modified electrodes of the sensor were immersed simultaneously in aqueous, non buffered, urea solutions of increasing concentrations (from 10^{-6} to 10^{-1} mol L^{-1}). Consequently, the potentials between each of the 6 enzyme-modified electrodes and the reference electrodes were measured simultaneously. During measurements, the potential reached its equilibrium value in less than 2 min for all urea concentrations tested. This is, for example, the case of the sensor prepared by simple adsorption of urease on PANI films, PANI-Urs, as shown in Fig. 3. The electrodes were rinsed with water between each measurement. The narrow peaks observed are an artifact occurring during the change of solution and are not of interest. For the first potential measurements, corresponding to the lowest urea concentrations, the potential was nearly independent of urea concentration. Then, a dramatic decrease of the potential occurred with urea concentration, before stabilizing for the highest urea concentrations (10^{-3}–10^{-1} mol L^{-1}). This trend is similar for each electrode, indicating a good reproducibility of the responses of the sensors. Similar curves (data not shown) were obtained for the other enzyme immobilization procedures, the only noticeable difference lying in the difference observed between the lowest and highest potential values.

The potential variations measured are related to pH changes. Indeed, as the enzymatic reaction products are alkaline (Eq. (1)), an increase in urea concentration causes a pH increase inside the enzyme layer at the surface of the transducer, leading to a decrease in potential according to the Nernst equation. Consequently, the immobilization of urease on the PANI films did not affect the well-known proton sensitivity of polyaniline films (Lakard et al., 2005; Pandey and Singh, 2001; Karyakin et al., 1999).

3.2. Sensitivity and reproducibility of the potentiometric response

Calibration curves presenting the evolution of the measured potential (after 2 min in a solution of given urea concentration) as a function of the logarithm of the urea concentration (pUrea = −log [Urea]) were build for the four types of urea biosensors (Fig. 4). The calibration curves exhibit a sigmoidal response against urea concentration with a satisfactory regression coefficient r ($r > 0.999$) independently of the immobilization procedure used. This result is not surprising since the common calibration graphs of pH-based urea sensors are known to exhibit this type of trend (Mourzina et al., 2004; Koncki et al., 1999; Koncki, 2007; Karyakin et al., 1999). In fact, besides the electrochemical equilibrium, the curve shape also depends on the kinetic parameters of the enzyme reaction (Walcerz et al., 1995). The plateau at high urea concentration results from a change in the kinetic order of the catalytic reaction: in this range an increase in urea concentration does not raise anymore the concentration of reaction products, and consequently does not lead to a potential change. On the other hand, at lower urea concentrations, the conditions are generally favorable to a sigmoidal response of the biosensor.

The sensitivity of the different urea biosensors can be quantified using the parameters obtained by fitting the potential against pUrea curves. The curves appear to be governed by the following Boltzmann equation since the correlation coefficients obtained with this model were superior to 0.998 for all sensors (Table S2):

$$y = A_2 + (A_1 - A_2)\,(1 + \exp\left(\frac{(x - x_0)}{(D)}\right)^{-1} \quad (2)$$

where A_1 is the limit of the potential for the most concentrated solutions, A_2 is the limit of the potential for the most dilute solutions, x_0 is the center of the sigmoid and D its width. All these parameter values were compiled in Table S2.

The potential sensitivity of the biosensor, denoted as S, was then calculated as:

(3)$S = A_1 - A_2$for each biosensor. In addition, the average calibration curve was calculated for each type of biosensor from the 6 calibration curves (Figure S4), the standard deviation was also evaluated for the 4 urea sensors.

The best sensitivity was obtained for PANI-Urs (S = 240.3 mV) and PANI-EN-Urs (S = 275.5 mV) urea biosensors. The sensitivities of PANI-LbL-Urs (S = 219.7 mV) and PANI-LbL-EN-Urs (S = 111.8 mV) were lower but comparable to the ones observed in the literature for potentiometric urea biosensors using polymers developed for biomedical applications (Table 1).

Adsorption leads to the most sensitive sensors since it does not damage strongly the native structure and function of enzymes. Additionally, with this immobilization technique, the enzyme is closer to the solution/polyaniline transducer interface, leading to higher sensitivity. However, there might be more than one monolayer of enzyme adsorbed, which also certainly contributes to increase the sensitivity. In contrast, the chemical methods of

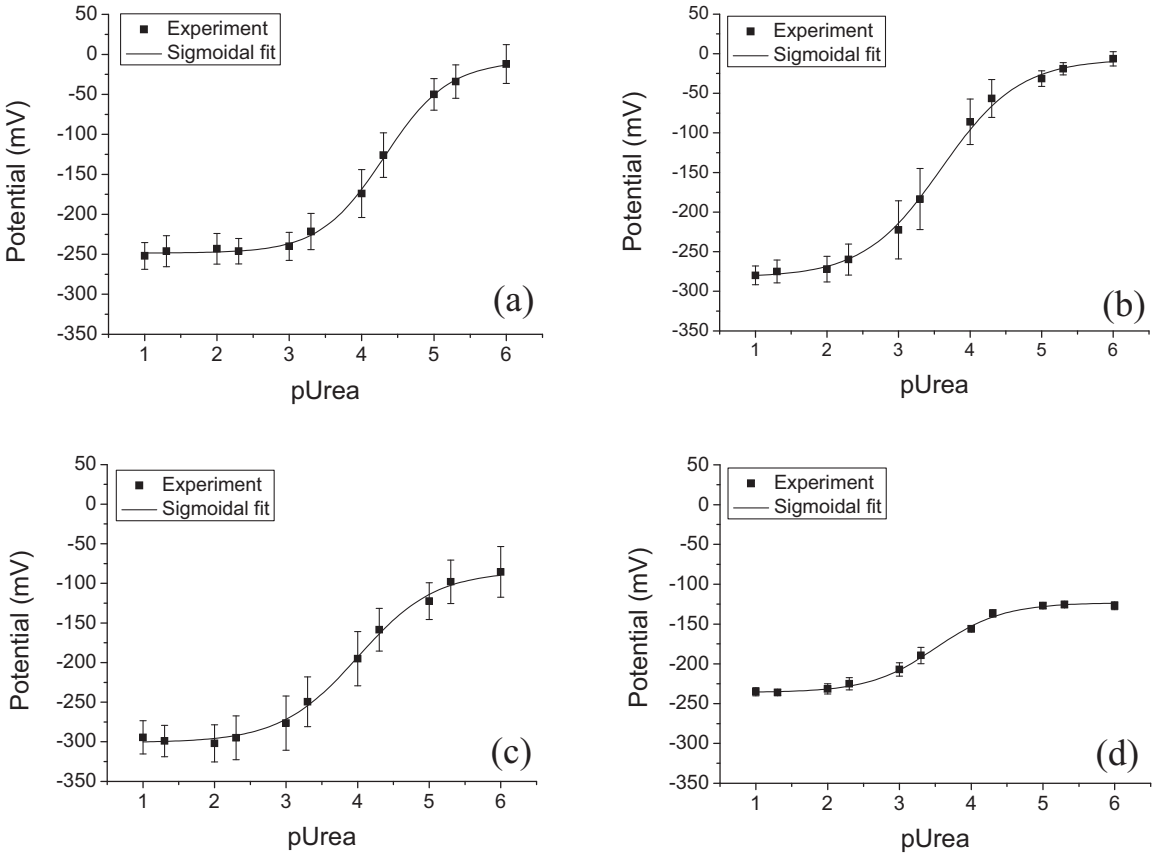

Fig. 4. Average calibration curve for each urea biosensors: (a) PANI-Urs, (b) PANI-EN-Urs, (c) PANI-LbL-Urs, (d) PANI-LbL-EN-Urs (each curve is the average of 6 calibration curves).

enzyme immobilization have the benefit of firmly anchoring the enzyme to the polymer film, with the drawback of rendering some sites of the transducer unable to capture protons, therefore reducing sensitivity. Additionally, the presence of the LbL film introduces a larger distance between the enzyme and the polyaniline film leading to a slightly lower efficiency.

The reproducibility of the potentiometric responses is an interesting parameter that can easily be estimated since 6 biosensors were simultaneously tested for each immobilization procedure. Calculating the standard deviation, it appeared that PANI-LbL-EN-Urs sensors have very reproducible potentiometric responses since their average sensitivity is 111.8 ± 3.8. The reproducibility of the other sensors is lower since their sensitivities are: 219.7 ± 10.8 mV for PANI-LbL-Urs sensors, 240.3 ± 7.1 mV for PANI-Urs and 275.5 ± 7.4 mV for PANI-EN-Urs sensors.

3.3. Stability with time of the urea biosensors

Adsorption has been proven to generate the most sensitive urea biosensors. However, despite leaving the enzyme relatively free of structural and functional damage, adsorption generally leads to a poor long-term stability in terms of immobilization. This poor stability generally results from a slow enzyme desorption from the surface even when the surface has been coated by a polyelectrolyte film, and not from the degradation of the polyaniline films since additional experiments (not shown here) demonstrated that the sensitivity of polyaniline films to pH changes remains constant during more than one month. We have tested the stability of the sensors by measuring their sensitivity after different storage times ranging from 1 to 21 days after construction, while keeping the sensors in PBS at 4 °C between two measurements.

Table 1
Detection characteristics of previously reported urea sensors based on conductive polymer transducers, and of the device reported in this work.

Polymer transducer	Range[a] (in mmol L^{-1})	Sensitivity[b] (in mV/decade)	Uses/stability in time	References
Electrodeposited poly(vinylferrocenium)	0.05–100	50	3 uses	Kuralay et al. (2005)
Electroinactive polypyrrole	0.1–30	32	n.a.	Komaba et al. (1997)
Electroinactive polypyrrole/polyion complex	0.3–30	110	More than 10 uses	Osaka et al. (1996)
Poly(N-3-aminopropylpyrrole-co-pyrrole)	0.4–6300	27	2 months	Rajesh et al. (2005)
Polypyrrole on carbon paper	3.8–1220	52	1 month	Syu and Chang (2009)
Polyaniline coated by LbL	0.01–1	From 56 (PANI-LbL-EN-Urs) to 133 (PANI-EN-Urs)	More than 21 days and 6 uses	Present work

[a] The range was defined as the range of concentrations corresponding to the beginning and to the end of the potential variations.
[b] The sensitivity was defined as the difference of potential in the range of concentrations (previously described).

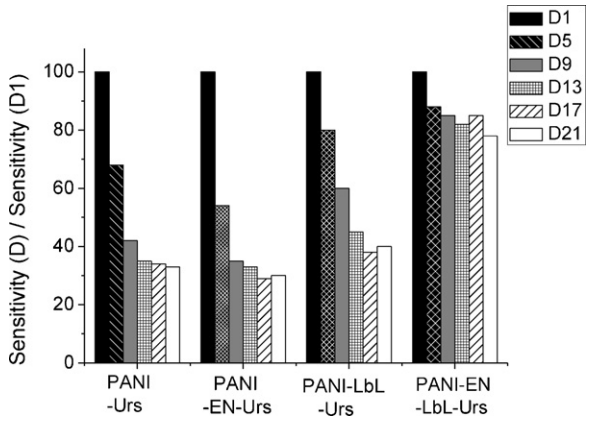

Fig. 5. Stability with time of the urea biosensors. Experiments performed *n* days after sensor construction are denoted as 'D*n*'. Between two experiments, the sensors were kept in PBS at 4 °C.

The poor long-term stability of sensors comprising adsorbed enzymes is here evidenced by a dramatic loss of sensitivity of PANI-Urs and PANI-EN-Urs sensors after a few days only (Fig. 5 and Table S3). Indeed, the response of PANI-Urs reached 68% of the initial sensitivity after 5 days and 42% after 9 days, and the response of PANI-EN-Urs reached 54% of the initial sensitivity after 5 days and 35% after 9 days. Consequently, despite their high sensitivity, these urea biosensors present less interest for applications because of their low reproducibility and their low stability in time. The main reason for this lower performance is the continuous desorption of urease from the transducer.

On the contrary, the firm anchoring of the enzyme to the matrix resulting from the combination of polyaniline, LbL and EDC-NHS treatment, leads to biosensors having a far better stability in time. Indeed, the response of PANI-LbL-EN-Urs reached 88% of the initial sensitivity after 5 days and is still 78% after 21 days.

The biosensors where PANI was coated with a LbL film but did not undergo the EDC-NHS treatment, exhibited an intermediary behavior since their response reached still 80% of the initial sensitivity after 5 days but only 40% for the final test performed after 21 days. The comparison between PANI-LbL-Urs and PANI-Urs sensors demonstrates a positive impact of the LbL treatment on the stability of the potentiometric response, indicating a better preservation of the enzyme activity. Moreover, the comparison between PANI-LbL-EN-Urs and PANI-LbL-Urs proves that EDC-NHS greatly improves the stability of the sensors and the activity of the enzyme.

From these results, it is clear that polyaniline is an efficient transducer, that LbL allows a good preservation of the enzyme structure and function and that the EDC-NHS treatment allows for a strong covalent immobilization of the urease. Consequently, urea biosensors based on this careful construction present a short response time, a good sensitivity (even if adsorption leads to better sensitivities), a good reproducibility and a good stability in time.

3.4. Urea detection in synthetic urine solutions

Synthetic urine solutions were used to estimate the efficiency of the developed biosensors. These solutions were composed of NaCl, KCl, $CaCl_2$, $MgSO_4$, NH_3 and urea in 0.02 M HCl (Tanaka and Hayashi, 1986). A calibration was then performed using 0.02 M HCl solutions containing different urea concentrations in the range 10^{-3}–10^{-1} M since the concentration of the synthetic urine solution was 2.88×10^{-2} M (after a tenfold dilution).

Thus, for example in the case of PANI-Urs biosensor, a potential of −135 mV was measured in the urine solution with a calibration curve fitting the equation: E (mV) = −183.4 + 31.9 pUrea. From this equation, the expected potential for the urine solution [Urea] = 2.88×10^{-2} M) was −134 mV, that is very similar to the experimental potential measured in the urine solution (less than 1% of difference from the experimental value). Consequently, PANI-Urs urea biosensors were found to be applicable to urine solutions and their response was not affected by the numerous ions composing synthetic urine. Other biosensors were also tested and led to a variation of approximately 10% between the urea concentration measured in urine solution and in urea solution (containing only urea and 0.02 M HCl). More precisely, the underestimation of the urea concentration due to the interference of the ions composing urine solution was found to be 11.3% for PANI-EN-Urs, 10.3% for PANI-LbL-Urs and 9.6% for PANI-LbL-EN-Urs.

4. Conclusions

In this work, potentiometric biosensors were elaborated by electrodeposition of polyaniline, followed by the assembly of a LbL multilayer of polysaccharides and by the covalent grafting of urease through amide bonds, or by direct adsorption of urease on the electrodeposited polyaniline film or on the LbL film. The potentiometric responses of the enzymatic biosensors were obtained as a function of urea concentration in aqueous solutions from 10^{-6} to 10^{-1} M urea. The obtained response curves all assumed a sigmoidal shape and were fitted in order to quantify sensing parameters such as sensitivity, detection range and reproducibility. Although the highest sensitivity was obtained using the simple adsorption method, the best overall compromise in terms of sensitivity, stability over time, and reproducibility was obtained for biosensors obtained by chemical grafting of the enzyme over the polysaccharide LbL multilayer. The present study thus confirms that polyaniline is an efficient transducer, and shows that a polysaccharide LbL preserves the enzyme structure and function and that covalent binding provides a long-term stability to the biosensor. The urea biosensor that is described in our paper exhibited a high sensitivity (from 55 to 125 mV/decade) in the 10^{-5}–10^{-3} M urea concentration range (corresponding to the concentration of urea in human body). This concentration range is wide compared to previously developed sensors, and the sensitivity obtained is among the most important of published potentiometric urea biosensors. Moreover, the potential signal of the sensor remains equal to 80% of the initial potential after 3 weeks, proving that its stability is very good. Finally, preliminary tests were successfully done using synthetic urine solutions to estimate the potentialities of the urea biosensors for use in biomedical applications. Consequently, the sensor developed in this paper is highly competitive with the previously published urea biosensors; furthermore, the principles demonstrated here to graft the enzyme should be of interest for other types of devices as well.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.04.009.

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