Tailoring of analytical performances of urea biosensors using nanomaterials

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Abstract. This paper is a contribution to the study of enzymatic sensors based on nanoparticles of iron oxide (FeNPs). Urease enzyme was immobilized on FeNPs using layer-by-layer (LbL) deposition method. FeNPs were first coated with polyelectrolytes (PE): Poly (allylamine hydrochloride), PAH and Poly (sodium 4-styrenesulfonate), PSS for enzyme immobilization and then with enzyme. It has been confirmed through zeta potential measurements of FeNPs that the enzyme is immobilized on the surface. We evaluated the sensitivity of biosensors for urea by potentiometric and capacitive measurements on silicon / silica / FeNP-LBL-urease structures. The recorded capacity-potential curves (C-V) show a significant shift of flat band potential towards negative potentials in the presence of urea, the observed values of sensitivity vary between 30 and 40 mV/pM[urea]. It has been shown that the proposed method for the immobilization of urease can increase the dynamic range of urea detection (10⁻⁴M to 10⁻¹M) compared to the immobilization of urease without FeNP (10⁻³.5 M to 10⁻².5 M). When the number of PAH-PSS layers was increased the sensitivity of detection was modified. This effect is due to partial inhibition of the enzyme in presence of FeNPs, which was shown by measurements in homogeneous phase.

Keywords: Biosensors; polyelectrolyte; nanoparticles; silica; urease.

1. Introduction
Magnetic particles were traditionally used for concentration, separation, purification and identification of molecules and specific cells. Inorganic nanoparticles (NPs) with specific size and morphology have attracted increasing interest due to the influence of their shape and size on their electronic, optical, magnetic and catalytic properties [1]. Inorganic NPs play most important roles in the development of the nanoelectrochemistry, because inorganic NPs exhibit higher surface area to volume ratios than their bulk counterparts, so inorganic NP modified electrochemical interfaces will provide larger electrochemically active areas and therefore probably lead to higher detection sensitivity for target
molecules; then, some novel NPs, particularly metal NPs, can easily act as enhancing agents for effective acceleration of electron transfer between electrode and detected molecules, so leading to more rapid current response for target molecules; further inorganic NPs can act as a supramolecular assembling unit with advanced functional properties for constructing a variety of architectures on the surface of electrodes and further tailoring of an electrochemical-sensing interface [2]. A review from N. Jaffrezic-Renault and al presents the use of magnetic nanoparticles for biosensor applications. They covered three biosensing systems: affinity biosensors, enzymatic biosensors and bio-bar codes [3]. In recent years, the magnetic properties of some NPs have also been used as labels in biosensing [4, 5]. So, Cheng proposed a sensitive electrochemical biosensor for DNA sequence mutation detection based on the separation and enrichment of magnetic nanobeads. [6]. It can be seen that the application of nano-materials greatly improves the detecting sensitivity. Electrostatic layer-by-layer (LbL) self-assembly provides a convenient and versatile method to build micro/nanostructures on two- or three-dimensional substrates at the molecular level. [7, 8], this technique was developed first by Decher et al. [9, 10]. The construction of LbL shells involves consecutive polyelectrolyte adsorption on a colloid-template followed by decomposition of the template core. Different materials, including linear polyelectrolytes (synthetic and natural), lipids, proteins, and inorganic nanoparticles can be used in nanoassembly processes [11]. Through the LbL method, Elaissari et al. used polyelectrolytes to encapsulate oil in water magnetic emulsion and obtained magnetic particles [12]. Further, Souiri et al used the LbL technique involving the immobilization of a bioactive protein (or peptide) by trapping it onto a previously “conditioned surface” obtained by alternatively adsorbing oppositely charged polyelectrolyte layers [13]. In this study we propose to work with the enzymatic biosensors based on iron NPs using the LbL technique.

The aim of this work is to develop a potentiometric and capacitive biosensors for urea detection. The enzymatic reaction catalyzed by urease is the following one:

$$ CO\left(NH_2\right)_2 + H^+ + 2H_2O \xrightarrow{Urease} 2NH^+_4 + HCO^-$$. 

The reaction can be monitored either by measuring the local production of ammonium ions or by measuring the local variation of pH. The enzymatic reaction was monitored in homogeneous medium (solution) using a potentiometric ammonium electrode. For an enzymatic biosensor, the local variation of pH, when urease enzyme was immobilized, was transduced by an insulator-semiconductor structure, SiO2/Si3N4/SiO2/Si. The electrochemical response of the developed urea biosensor was evaluated using the capacitance potential measurements [14]. Variation of the flat-band potential of the Electrolyte-Insulator-Semiconductor structure varies when concentration of urea in solution varies. The effect of the conditions of immobilization of urease on the insulator surface (without and with FeNPs) where studied and the analytical features of the obtained biosensor were determined.

2. Materials and Methods

2.1. Materials

Magnetic ferric oxide nanoparticles (FeNPs) were obtained from Sigma - Aldrich. The nanoparticle size was less than 50 nm. These nanoparticles were suspended in ultrapure water. Cationic polyelectrolyte used in this study, Poly(allylamine Hydrochloride) (PAH) and anionic polyelectrolyte is Poly(sodium 4-styrenesulfonate) (PSS) were purchased from Sigma - Aldrich (Figure 1). Bovine serum albumin (BSA), glutaraldehyde (GA) (grade II, 25% aqueous solution), NaH2PO4 (> 99%) and Na2HPO4.12H2O (> 99%) were obtained from Sigma - Aldrich. Acetone, trichloroethylene and isopropanol were purchase from Fluka. Urease (Type IX from Jack Beans, 0.28G solid 70400 U/g), Stock concentrated solutions were prepared in 10 mM phosphate buffer solution (pH 7.6) and stored at 4°C. All aqueous solutions were prepared using 18 MΩ cm⁻¹ ultrapure water produced by a Millipore Mili-Q system.
2.2. Potentiometric measurements of ammonium ions produced by the enzymatic reaction

The ammonium electrodes with polyvinyl polymeric chloride membrane (PVC) and the reference Ag/AgCl used in this study were provided by the Nico2000 Company. The potential difference between both electrodes was measured by a potentiometer (ELITS 8051) provided Nico2000 company.

2.3. Determination of electrophoretic mobility of coated FeNPs

To control the final surface composition of LbL NPs, we used polycation (PAH) or polyanions (PSS) as the outermost layer for the shells NPs. The zeta potential of polyelectrolyte NPs with different outermost layers were measured using dynamic light scattering (DLS) by a Zetasizer Nano-ZS (Malvern Instruments). Measurements on very dilute samples were performed at ionic strength (10⁻³ M NaCl). The average value of at least five measurements was taken at a given condition.

2.4. Preparation of the urea biosensor

2.4.1. EIS substrate and capacitance-voltage (C-V) measurements. The Electrolyte-Insulator-Semiconductor (EIS) were purchased from the Institute of Microtechnology of the University of Neuchatel (Switzerland). The EIS capacitors were fabricated by thermally growing 80 nm SiO2 on <100> p-type silicon wafer with 3-5 Ω.cm resistivity and 400μm thickness. Silicon oxynitride of 100 nm thickness was then deposited by LPCVD. The nitride surface was then oxidized for obtaining a 50 nm thick oxide layer. The ohmic contact was obtained by deposition of a gallium–indium mixture on the back side of the Si/SiO2/Si3N4 structures (Figure 2). Before the deposition of the biomembrane, the IS structures were treated using an optimized cleaning procedure: surface transducers were cleaned in successive baths of trichloroethylene, acetone, isopropanol and sulphochromic mixture (sulphuric acid and chromic acid), washed with ultrapure water, dried under nitrogen atmosphere at room temperature and placed at 70 °C for 10 min. The EIS structures were characterized by capacitance-voltage (C-V) method. This latter were performed using an impedance analyzer (Voltalab 40, Hach Lange) at a frequency of 10 kHz and with a signal amplitude of 10mV, the potential being varied from -0.5 V to +2.5 V. The solution used for testing the urea sensitivity was 10 mM phosphate buffer at a fixed pH value of 7.6.
2.4.2. Coating of ferric oxide nanoparticles. Nanoparticles (FeNPs) were first homogenized under mechanical stirring for 15 min in ultrapure water (Fe3O4:1%w/w). The nanoparticles were then coated with an initial “preconditioning” layer of PAH (positive charge), then a layer of PSS of opposite charge was deposited to form the first PE bilayer. Each PE coating step was performed in aqueous solutions of filtered ultrapure water at 5mg/mL of PE, under similar stirring conditions during 15 min. The nanobeads were then recovered by applying a mild magnetic field (using a small magnetic stir bar). The PE rich supernatant phase was then eliminated and the beads were rinsed twice in filtered ultrapure water under mechanical stirring during 15 min, and separated again under a mild magnetic field (Figure 3).

2.4.3. Urease immobilization. The globally negatively charged enzyme (purchased at Sigma–Aldrich) was immobilized on the nanoparticles coated with three layers of polyelectrolytes (FeNP-(PAH-PSS-PAH)) by electrostatic interaction in a 1mg/mL urease/phosphate buffer at pH 7.6. This step was performed by separating the particles from the urease solution, rinsing in phosphate buffer, separating them via magnetic field application (Figure 3).

In this study, two procedures were followed for the biosensor preparation:

• without FeNPs: 10 µL of a 10 mM phosphate buffer pH 7.6 containing 6% (m/v) BSA, 10% (m/v) glycerol with 4% (w/w) of urease was deposited on the surface of the IS structure. The sensors were then placed in saturated Glutaraldehyde (GA) vapours for 30 min. After incubation, the membranes were dried in free air for 15–30 min.
• with FeNPs: 10 µL of a 10 mM phosphate buffer pH 7.6 containing 6%(m/v) BSA, 10% (m/v) glycerol with 1% (w/w) of urease/PE/NPs was deposited on the surface of the IS structure. The sensors were then placed in saturated Glutaraldehyde (GA) vapours for 30 min. After incubation, the membranes were dried in free air for 15–30 min.

Figure 2. Scheme of the EIS structure with urease functionalized FeNPs.
3. Results and discussion

3.1. Characterization of coated FeNPs through electrophoretic mobility of coated FeNPs

The measured values of zeta potential of the uncoated and coated FeNPs are presented in Table 1. When the outermost layer is a polycation (PAH), a positive surface potential is observed whereas when the outermost layer is a polyanion (PSS) a negative surface potential is observed. When urease is the outermost layer, a negative potential is observed showing the successful immobilization of urease.

| Name                          | Temperature (°C) | Zeta Potential (mV) |
|-------------------------------|------------------|---------------------|
| Uncoated FeNP                 | 25               | -12.1 ± 0.3         |
| FeNP + PAH                    | 25               | 25.5 ± 0.7          |
| FeNP + PAH + PSS              | 25               | -38.9 ± 0.2         |
| FeNP + PAH + PSS + PAH        | 25               | 12.4 ± 0.7          |
| FeNP + PAH + PSS + PAH + Enzyme | 25           | -24.7 ± 0.7         |

3.2. Variation of the enzyme activity in presence of iron oxide nanoparticles

Heavy metals inhibit urease activity. The mechanism of the inhibition of urease is based on interactions between the heavy metal ions and the groups of thiol/methylthiol of cysteine/methionine presented at the active centre of the enzyme. Consequently, several urease biosensors are reported in the literature for the detection of heavy metal ions through enzyme inhibition [15-21]. Activity of urease is monitored in homogeneous phase by measuring produced ammonium ions (cf. Figure 4). When 1% of FeNPs is added, the concentration of produced ammonium ions decreases for more than 50%. The green curve shows that the presence of copper ions (Cu^{2+}) leads to a complete inhibition of enzyme activity by reducing the signal to zero. These results confirm the inhibition of urease in presence of FeNPs.
3.3. Analytical features of the EIS biosensor

3.3.1. Urease immobilized without nanoparticles. Figure 5 shows the potentiometric response of EIS-urease structure for different urea concentration. The modified electrode shows a linear response to urea addition from $10^{-3.5}$M to $10^{-2.5}$M with a sensitivity of 26 mV/pUrea.

3.3.2. Urease immobilized with nanoparticles. When urease is immobilized on FeNP-PAH-PSS-PAH, and deposited on the surface of the EIS, the effect of urea concentration is presented in Figure 6. It is shown that the proposed method for the immobilization of urease can increase the dynamic range of urea detection ($10^{-4}$M to $10^{-1}$M) compared to the immobilization of urease without FeNP ($10^{-3.5}$M to $10^{-2.5}$M).
10^{-2.5}\text{M}). This effect is due to partial inhibition of the enzyme by these particles, it was shown by measurements in homogeneous phase (cf. Figure 4).

![Figure 6. Capacitance response with urease immobilized on FeNP/PAH/PSS/PSS](image)

### 3.3.3. Effect of the number polyelectrolyte layer on FeNP on the response of biofunctionalized EIS.

The effect of the number of PAH-PSS layers deposited on FeNP on the biosensors response was investigated. The obtained results are gathered in Table 2. An increase of the urea biosensor sensitivity is observed when the number of PAH-PSS layers increases. This effect can be explained by a higher protection of urease enzyme against inhibition by iron ions.

| Composition of the biofilm on EIS structure | Sensitivity (mV/p[Urea]) | Domain of linearity | Limit of detection |
|-------------------------------------------|--------------------------|---------------------|-------------------|
| Urease without nanoparticles              | 26                       | $10^{-3.5}\text{M} - 10^{-2.5}\text{M}$ | $10^{-3.7}\text{M}$ |
| FeNP/PAH/PSS/PAH/Urease                   | 32                       | $10^{-4}\text{M} - 10^{-1}\text{M}$     | $10^{-4}\text{M}$  |
| FeNP/PAH/PSS/PAH/PSS/PAH/Urease           | 33.4                     | $10^{-3.5}\text{M} - 10^{-1.7}\text{M}$ | $10^{-3.4}\text{M}$ |
| FeNP/PAH/PSS/PAH/PSS/PAH/PSS/PAH/Urease  | 34                       | $10^{-3.7}\text{M} - 10^{-2.5}\text{M}$ | $10^{-3.8}\text{M}$ |

### 4. Conclusion

In this paper, layer-by-layer technique has been used for the immobilisation of urease on FeNPs for the fabrication of an EIS urea sensor. This procedure allows increase of the sensitivity of detection (26 mV/p[Urea] 33 mV/p[Urea]) and tailoring of dynamic range of the biosensor. It is expected that such a promising concept of inhibitor-based biosensors will be of interest for different applications of this urea sensor.

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6. References

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