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Original Publication:

Syed Usman Ali, Naveed Ul Hassan Alvi, Zafar Hussain Ibupoto, Omer Nur, Magnus Willander and Bengt Danielsson, Selective potentiometric determination of uric acid with uricase immobilized on ZnO nanowires, 2011, SENSORS AND ACTUATORS B-CHEMICAL, (152), 2, 241-247.
http://dx.doi.org/10.1016/j.snb.2010.12.015

Selective Potentiometric Determination of Uric Acid with Uricase Immobilized on ZnO Nanowires

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Abstract: In this study, a potentiometric uric acid biosensor was fabricated by immobilization of uricase onto zinc oxide (ZnO) nanowires. Zinc oxide nanowires with 80-150 nm in diameter and 900 nm to 1.5 μm in lengths were grown on the surface of a gold coated flexible plastic substrate. Uricase was electrostatically immobilized on the surface of well aligned ZnO nanowires resulting in a sensitive, selective, stable and reproducible uric acid biosensor. The potentiometric response of the ZnO sensor *vs* Ag/AgCl reference electrode was found to be linear over a relatively wide logarithmic concentration range (1 to 650 μM) suitable for human blood serum. By applying a Nafion membrane on the sensor the linear range could be extended to 1 to 1000 μM at the expense of an increased response time from 6.25 s to less than 9 s. On the other hand the membrane increased the sensor durability considerably. The sensor response was unaffected by normal concentrations of common interferents such as ascorbic acid, glucose, and urea.

Index Terms: ZnO nanowires, potentiometric nanosensor, uricase, uric acid, Nafion® membrane and electrochemical nanodevices,

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1. INTRODUCTION

Uric Acid (UA), a major nitrogenous compound in the urine, is the product of purine metabolism in the human body and is related to many clinical disorders [1]. It is important to determine the concentration of uric acid dissolved in human urine and blood to be able to diagnose diseases caused by disorders of purine biosynthesis and/or purine catabolism, such as gout, hyperuricemia, and Lesch-Nyhan syndrome [2-6]. Several epidemiological studies have suggested that the production of excess uric acid in human serum is also a risk factor for cardiovascular disease [7]. Thus, the screenings of UA in human physiological fluids are indispensable for the diagnosis of patients suffering from a range of disorders associated with altered purine metabolism. Various uric acid biosensors have emerged from laboratories, because of the advantages of simple measurement, a short response time, high sensitivity, and high selectivity. Most uric acid biosensors are on amperometric principles [8–11], the detection of oxygen consumption [12-14], chemiluminescence [15-16], and the detection of fluoride ions [17]. The main problem in the practical application of many amperometric biosensors is that the electrode must be held at approximately 0.7 V [18]. The relatively high electrode potential enables other biological electroactive molecules to react on the surface of the electrode [19]. However, interferences can be reduced by opting potentiometric configuration as described in literature [20-23]. Moreover, compared to amperometric biosensors, since no potential is required that may result in unwanted electrochemical reactions; potentiometric biosensors can have an advantage in selectivity. However, a limitation of ion sensitive electrodes (ISEs) is that only charged molecules can be directly detected. This obstacle can be overcome by letting the analyte undergo a reaction, such as an enzyme reaction, that produces a detectable ion in an amount proportional to the concentration of the analyte in the sample. In the enzyme field effect transistor (EnFET), this

is taken a step further by combining the enzyme reaction with an ion sensitive field effect transistor (ISFET) was first introduced by Caras and Janata [24].

Recent advances in electrochemical biosensing based on a wide variety of nanostructures such as ZnO nanowires, nanotubes and nonporous materials have attracted great interest due to their remarkable properties like non-toxicity, bio-safety, excellent biological compatibility, high-electron transfer rates, enhanced analytical performance, increased sensitivity, ease of fabrication and low cost [25-29]. ZnO nanomaterials can be used in a variety of electrochemical biosensing schemes due to their unique advantages in combination with immobilized enzymes. They can maintain the activity of the enzyme due to the desirable microenvironment, and enhance the direct electron transfer between the enzyme's active sites and the electrode. The high isoelectric point (IEP) of ZnO (9.5) makes it a good matrix to immobilize low isoelectric point acidic proteins or DNA by electrostatic interactions with high binding stability [30-31]. In addition, ZnO has high ionic bonding (60%), and it dissolves very slowly at normal biological pH values. The enzymes uricase has a good electrostatic interaction with ZnO nanowires due to its low IEP at a pH of 4.3 and shows a good thermal stability and high selectivity to uric acid [32]. A ZnO thin film may also be used for the determination of uric acid but we chose ZnO nanowires due to the higher surface-to-volume ratio thus capturing more ions on the ZnO structure. Also ZnO nanowires exhibited better performance in perspective of signal to noise ratio as compared to ZnO thin film [33] because the nanosurfaces have other chemical properties as well. These are the reasons why nanowires are more sensitive, have an extended detection limit and possess a fast response time as compared to a thin films [24-35].

In this work, a simple fabricated and sensitive potentiometric uric acid sensor based on ZnO nanowires grown on a gold coated flexible plastic electrode has been successfully demonstrated using electrostatic process for the immobilization of uricase enzyme. The good

performance of this proposed uricase/ZnO sensor in buffer solutions to determine uric acid level were investigated. It showed good features of selectivity, fast response, reproducibility, linearity, thermal stability and negligible foreign interferences.

2. EXPERIMENTAL DETAILS

2.1 Materials

Uricase (E.C. 1.7.3.3), 25 units/1.5 mg from *Arthrobacter gloiformis*, uric acid (99.8% purity), β-D-glucose (99.5%), Nafion (1% in methanol), zinc nitrate hexahydrate and hexamethylenetetramine were purchased from Sigma Aldrich. Phosphate Buffer, 10 mM solution (PBS) was prepared from Na_2HPO_4 and KH_2PO_4 (Sigma Aldrich) with sodium chloride in 0.135 mM and the pH was adjusted to 7.4. A stock solution of 10 mM uric acid was prepared in PBS, and stored at 4 ^{0}C. The low concentration standard solutions of the uric acid were freshly prepared before the measurements. All chemicals used (Sigma, Aldrich) were of analytical reagent grade.

2.2 Fabrication of sensor electrode with ZnO nanowires

To prepare the sensor electrodes, we affixed the flexible plastic substrate on a flat support inside the vacuum chamber of an evaporation system (Evaporator Satis CR725). In the first step, titanium film was uniformly deposited with a thickness of 10 nm and in the second step, 50 nm of gold was deposited on the surface of the plastic substrate. The AFM image showing the surface roughness of deposited films is shown in figure 1 (a). A 4 cm long clean, piece of gold coated plastic electrode (1 mm in width) was first rinsed with acetone followed by rinsing in de-ionized water and drying at room temperature. To grow ZnO nanowires on the gold electrode, a low temperature chemical approach was adopted [36]. First the electrode was dipped into a seed solution containing zinc acetate for two minutes and then dried in air. This procedure was repeated twice. Then the electrodes were placed in an aqueous solution of

0.025 M zinc nitrate hexahydrate [(Zn $(NO)_3)_2$ $6H_2O$)] and 0.025 M hexamethylenetetramine [$C_6H_{12}N_4$] that was kept in an oven for 2-4 hours at 90°C. After the growth was completed, the grown nanowires were cleaned in de-ionized water and dried at room temperature. Typical ZnO nanowires grown on gold coated plastic electrode using this procedure are shown in figure 1(b). This SEM images clearly show that ZnO nanowires of 80-150 nm diameters with uniform density and spatial distribution had been grown. These nanowires were perpendicular relative to the surface of the strip. The morphological and structural characteristics of the grown nanowires can be controlled by adjusting the growth process parameters such as the concentration of the seed solution, the reagent stoichiometry, the temperature and the pH of the growth solution [37].

2.3 Construction of uric acid sensor and electrochemical measurements

Two types of uricase electrodes with and without membrane were prepared for the experiments. For the first type, a uricase solution was prepared in 10 mM PBS pH 7.4. Uricase was electrostatically immobilized by dipping the ZnO nanowire-coated electrode into the enzyme solution for 15 minutes at room temperature and then letting it to dry in air for 60 minutes as shown in figure 1 (c). To immobilize the uricase on the second type, the electrode was dipped into enzyme solution for 15 minutes and then left in air at room temperature for 60 minutes. After drying, 5 μL of Nafion solution (1 % in methanol) were applied onto the electrode surface to prevent possible enzyme leakage and eliminate foreign interferences. All enzyme electrodes were stored in dry condition at 4^oC when not in use. After completing these steps, both types of sensors were checked potentiometrically in uric acid solutions with an Ag/AgCl reference electrode purchased from Metrohm. A pH meter (Model 744, Metrohm) was used to measure the potentiometric output voltage of the ZnO nanowires based sensors presented here. For the time response measurements, a model 363A potentiostat/galvanostat (EG & G, USA) was used. Atomic force microscopy images were

acquired using a Dimension 3100 Scanning Probe Microscope (Digital Instruments) in tapping mode with Si cantilevers.

3. RESULTS AND DISCUSSION

3.1 Response time of potentiometric uric acid sensors

The construction of the standard two-electrode electrochemical potential cell can be described by the following representation

$$ZnO\ s\ |Zn^{+2}\ PBS\ sol||Ag/AgCl\ PBS\ sol\ |Ag$$

The cell voltage varies when the composition of the test electrolyte is changed. These changes can be related to the concentration of ions in the test electrolyte following the calibration procedure. The measurements started after conditioning the sensor electrode in PBS buffer at pH 7.4 for 30 minutes and when the electrodes were inside the PBS, a constant potential was observed. When freshly prepared 500 nM uric acid solution was tested then an unstable signal changed was observed and a stable and repeatable signal within 6 to 9 s was observed when 1 μM uric acid solution was tested. Hence we have chosen 1 μM as minimum stable detection limit of our proposed sensor. This response behavior indicates that the fabricated sensor is sensitive to a specific analyte in question. During all experiments the proposed sensor followed the Nernst's expression

$$E = E_0 - 0.05916\ V/\ n \log\ [Reduced]\ /\ [Oxidized]$$

A response of approximately 127 mV without membrane and 128 mV with membrane was observed for a 100 μM uric acid solution. The same experiment was repeated with different volumes ranging from 0.25 mL to 20 mL with the same length of 1 mm of the sensor electrode dipped into the 100 μM of uric acid solution. The response stayed within 127 to 128 mV for both types of sensors regardless of the sample volume. The sensing mechanism

of most electrochemical uric acid sensors is based on an enzymatic reaction catalyzed by uricase according to the following:

$$C_5H_4N_4O_3\,(\text{Uric acid}) + O_2 + 2H_2O \xrightarrow{\text{Uricase}} C_4H_6N_4O_3\,(\text{Allantoin}) + CO_2 + H_2O_2 \quad \text{---------- (1)}$$

When uric acid is oxidized in the presence of uricase it is turned into allantoin along with carbon dioxide and hydrogen peroxide. Due to the presence of water (H-OH), it is a high probability that allantoin will accept a proton from (H-OH) converting it to allantoinium ion, which in turn will interact with ZnO nanowires and produce a potential change at the electrode. As the concentration of ions changes in surrounding the ZnO nanowires the electrode potential will change [38]. The potentiometric responses of the sensor electrodes were studied in uric acid solutions made in buffer (PBS pH 7.4) with concentration ranging from 1 μM to 1000 μM. During the measurements it was observed that the produced carbon dioxide does not affect the stability of ZnO nanowires as shown in SEM image of Figure 1 (d) and we did not observe any substantial change in pH of the buffer solution (PBS). A very fast response time was noted over the whole concentration range with 95 % of the steady state voltage achieved within 6. 25 s as shown in figure 2 (a) with a sensitivity of 29 mV/decade for a sensor electrode without membrane and within 9 s with a sensitivity of around 32 mV/decade for a sensor electrode with membrane figure 2 (b). The tested sensor configuration showed large dynamic ranges with an output response (emf) that was linear *vs*. the logarithmic concentrations of the uric acid as shown in figure 3(a, b).

3.2 Reproducibility, linearity and stability of the sensor

The reproducibility and long term stability were evaluated by using 5 different uric acid sensor electrodes constructed independently under the same conditions; the relative standard deviation of the fabricated sensor electrodes in standard uric acid solutions was less than 7%. The sensor to sensor reproducibility in 100 μM uric acid solution is shown in figure

4. Figure 5 displays the results of three experiments showing good stability and linearity in pH 7.4 PBS solution. Here, the same ZnO sensor electrode was used for the three experiments to check the reproducibility. The sensor electrode was carefully soaked in PBS after each measurement. This sensor electrode had been periodically used and stored at 4 ^{0}C for more than three weeks; it retained up to 80% of its original activity and still showed a good response to uric acid. The morphology of the functionalized ZnO sensor electrode was checked by Scanning Electron microscopy (SEM) after measurements as shown in Figure 1(d). The minor dissolving phenomena of ZnO nanowires after several measurements was observed in figure 1 (d), it shows shorter and thinner nanowires tip as compared to figure 1(b).The dissolving phenomena of zinc oxide nanostructures in aqueous solutions of different pH values gradually results to impair the sensor performance.

4. Studies of pH effect, temperature and interferences

The pH of the working medium affects the activity of uricase and signal response of the sensor electrode. The pH dependence of sensor response was investigated in 250 μM uric acid solution over the pH range from 6 to 9 as shown in figure 6(a). The experimental results indicate that the optimum pH range is between 6.5 to 7.5 pH. However, the linearity is optimal at pH 7.4. Although the reported optimal pH of uricase was in the range pH 8.5 to pH 9.2 [9], the pH of most physiological fluids is below this range. The effect of varying temperature on the sensor response was also examined between 20 and 70 ^{o}C. As shown in Fig. 6(b), the EMF response gradually increases with increasing of temperature and immobilized enzyme showed an optimum reaction at temperature 35 to 40 ^{o}C. Therefore, it is concluded that the electrostatic adsorption caused an increase in the thermal stability of enzymes. After 40 ^{o}C, the response decreases which is caused by the natural thermal degradation of the enzymes. Although the biosensor shows a maximum response at 40 ^{o}C but the Room temperature (23±2) ^{o}C is still chosen for this work in order to prevent possible

solution evaporation at higher temperature and ease of operation. The selectivity of a uric acid sensor depends on two major factors that are the enzyme-analyte reaction and the selective mechanism. The enzyme analyte reaction is very specific due to the nature of the uricase functionality. The possible interferences present in blood that normally interfere with an amperometric uric acid biosensor include ascorbic acid (AA) urea (UR) and glucose (GL) [39]. Hence, ascorbic acid, urea and glucose were selected to affirm the selectivity of the potentiometric uric acid sensor in the present work. Upon adding glucose to 5mM, ascorbic acid to 100 μM, and urea to 1mM in a 100 μM uric acid solution the signal changed only slightly as is shown by the output response in figure 6. This was repeated several times on new, independently prepared sensors and continued to show little signal response to interferences. In practical measurements, however these changes in sensor response can be neglected.

5. Conclusion

In this article, we introduced a simple fabrication procedure for a highly sensitive uric acid biosensor based on ZnO nanowires which provided a suitable microenvironment for enzyme loading and an easy immobilization procedure. The uricase sensor retained its enzymatic activity due to strong electrostatic interaction between zinc oxide and uricase. The potentiometric response increase with increasing the concentrations of uric acid from 1 μM to 1000 μM with a sensitivity of 32mV/decade, Since the sensor is low cost with appreciable reproducibility, it may offers an easy extension to on-spot clinical diagnosis. It is also convenient to assemble into portable chip based sensing devices suitable to unskilled users.

Acknowledgement

The authors gratefully acknowledge Dr. Ari Laiho for acquiring AFM images.

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Figures Captions

Fig. 1:(a) AFM image shows a flat surface with a surface roughness of Ra=10nm and a typical SEM images of ZnO nanowires grown on gold coated plastic substrate using low temperature chemical growth. The figure showing (b) the nanowires without uricase (c) with immobilized uricase and (d) the same sensor after measurements.
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Fig. 2: Time response of the uric acid sensor in 100 μM uric acid solution (a) without membrane (b) with membrane.

Fig. 3: Calibration curves for the uric acid sensor (a) with membrane (b) without membrane.

Fig. 4: The sensor to sensor reproducibility of five (n = 5) ZnO nanowires/uricase/Nafion electrodes in 100 μM uric acid solution.

Fig. 5: Calibration curves from three different experiments using the same sensor electrode and Ag/AgCl reference electrode.

Fig. 6 (a,b): Calibration curves showing the study of emf response with the influence of varying pH and temperature

Fig. 7: Effect of potentially interfering substances on sensor response (emf) upon adding 5 mM glucose (GL),100 μM ascorbic acid (AA) and urea(UR) into 100 μM uric acid solution.

Figure 1(a)

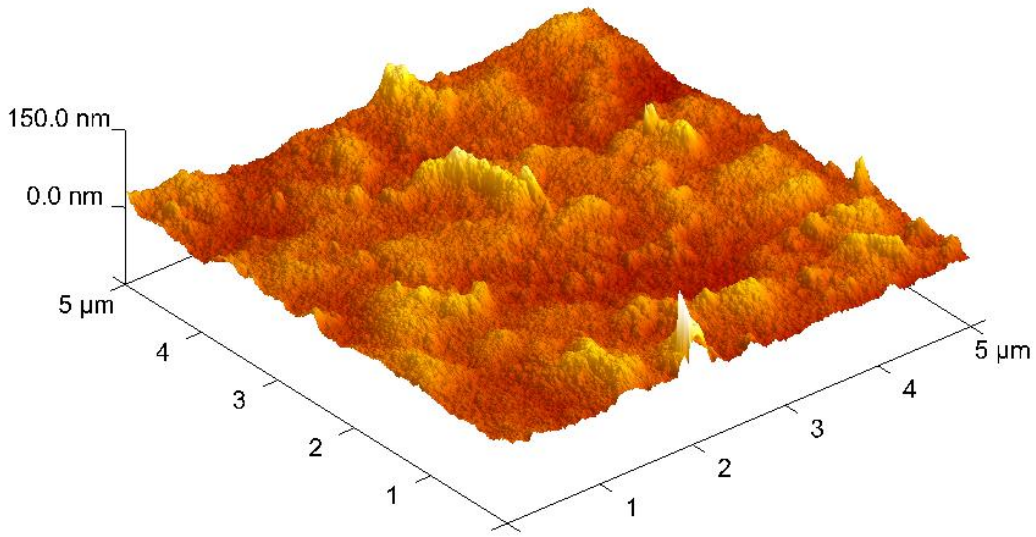
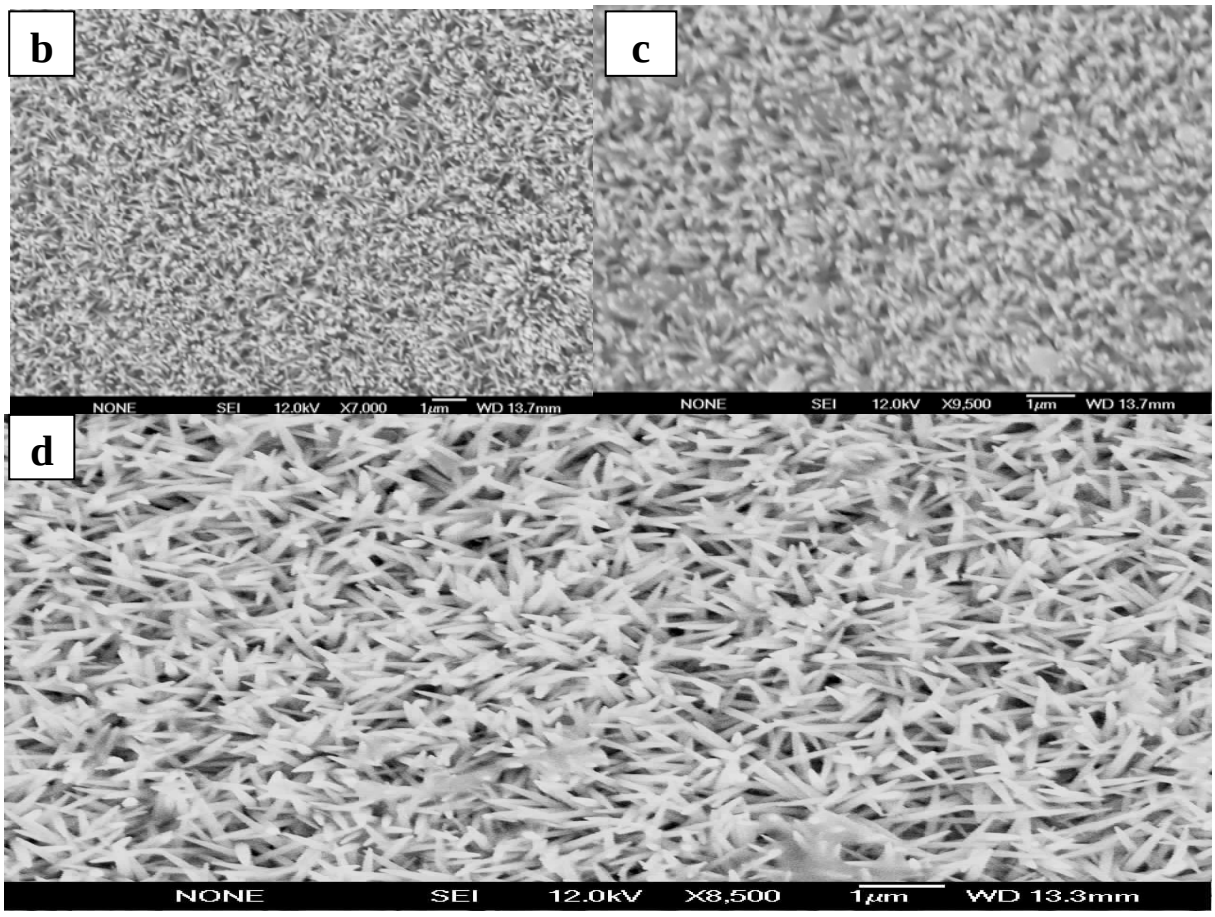


Figure 1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c
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d
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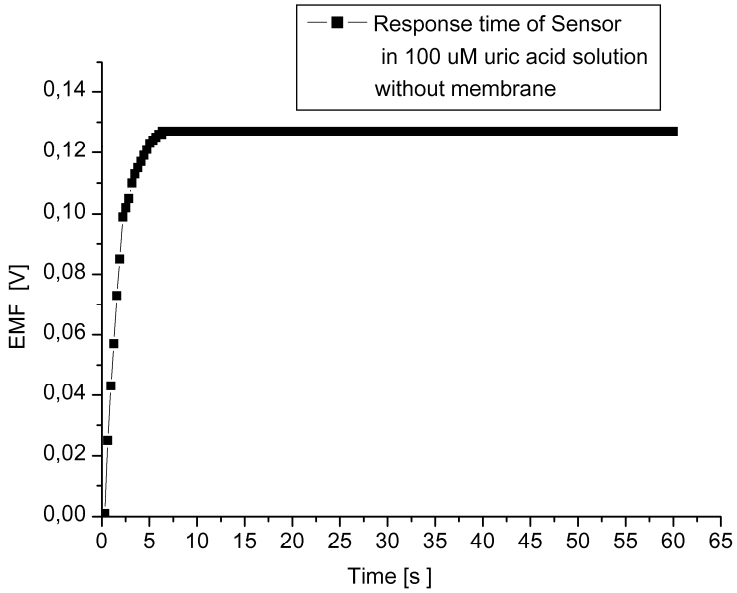
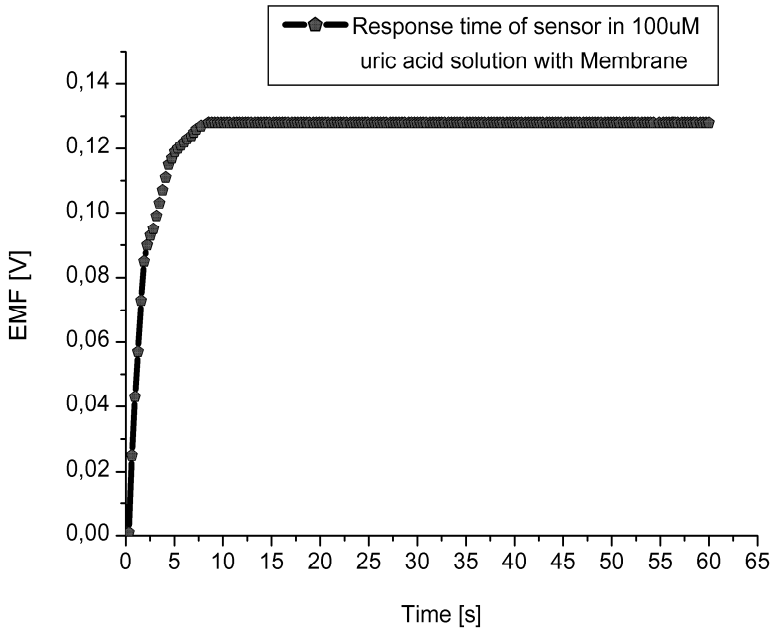
Figure 2
(a)


Response time of Sensor
in 100 uM uric acid solution
without membrane
EMF [V]
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Time [s]

(b)


Response time of sensor in 100uM
uric acid solution with Membrane
EMF [V]
0,14
0,12
0,10
0,08
0,06
0,04
0,02
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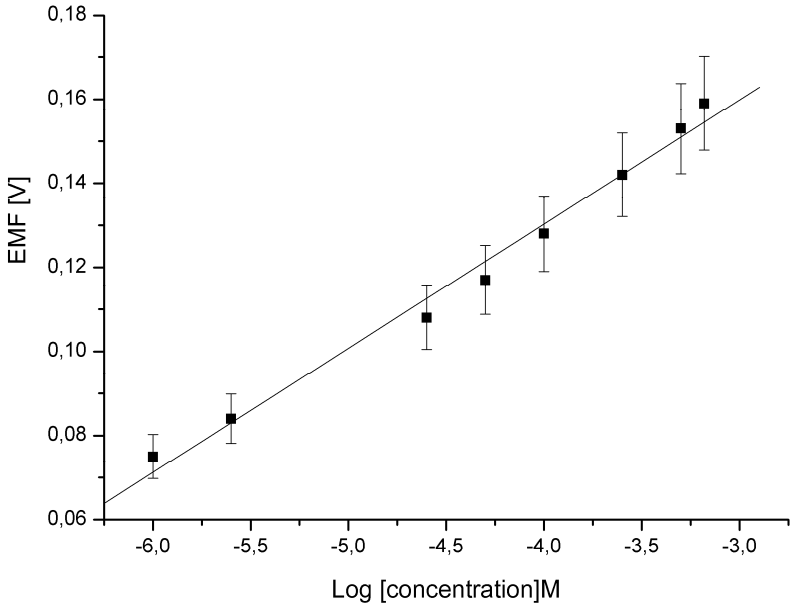
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Time [s]

Figure 3
(a)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0,12
0,10
0,08
0,06
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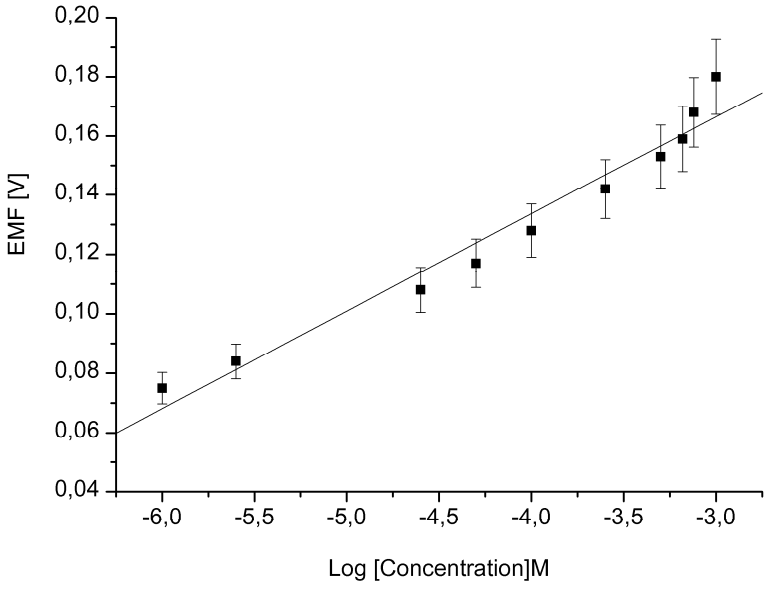
-5,0
-4,5
-4,0
-3,5
-3,0
Log [concentration]M

(b)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EMF [V]
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-5,0
-4,5
-4,0
-3,5
-3,0
Log [Concentration]M

Figure 4

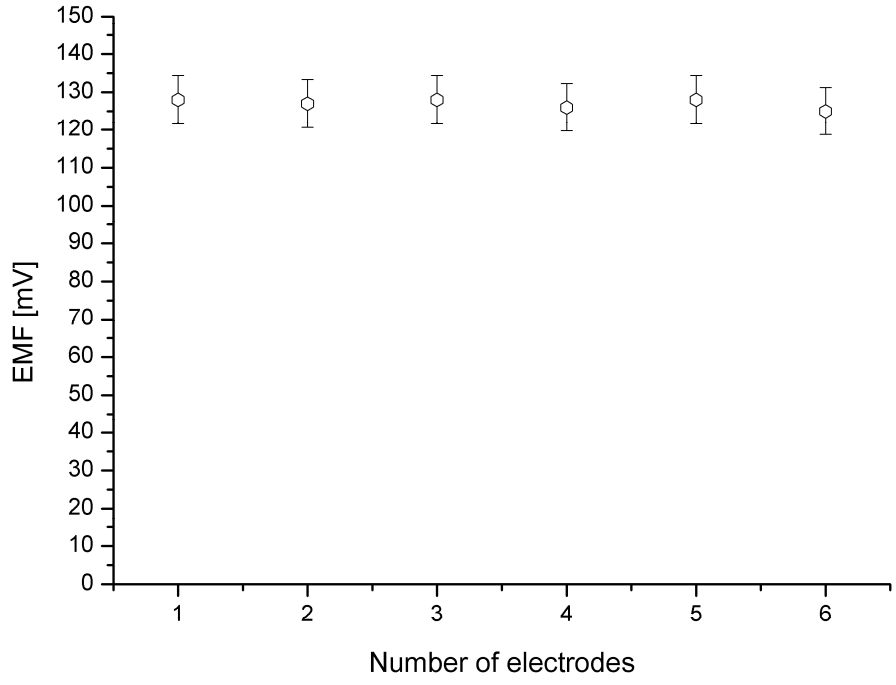

EMF [mV]
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Number of electrodes

Figure 5

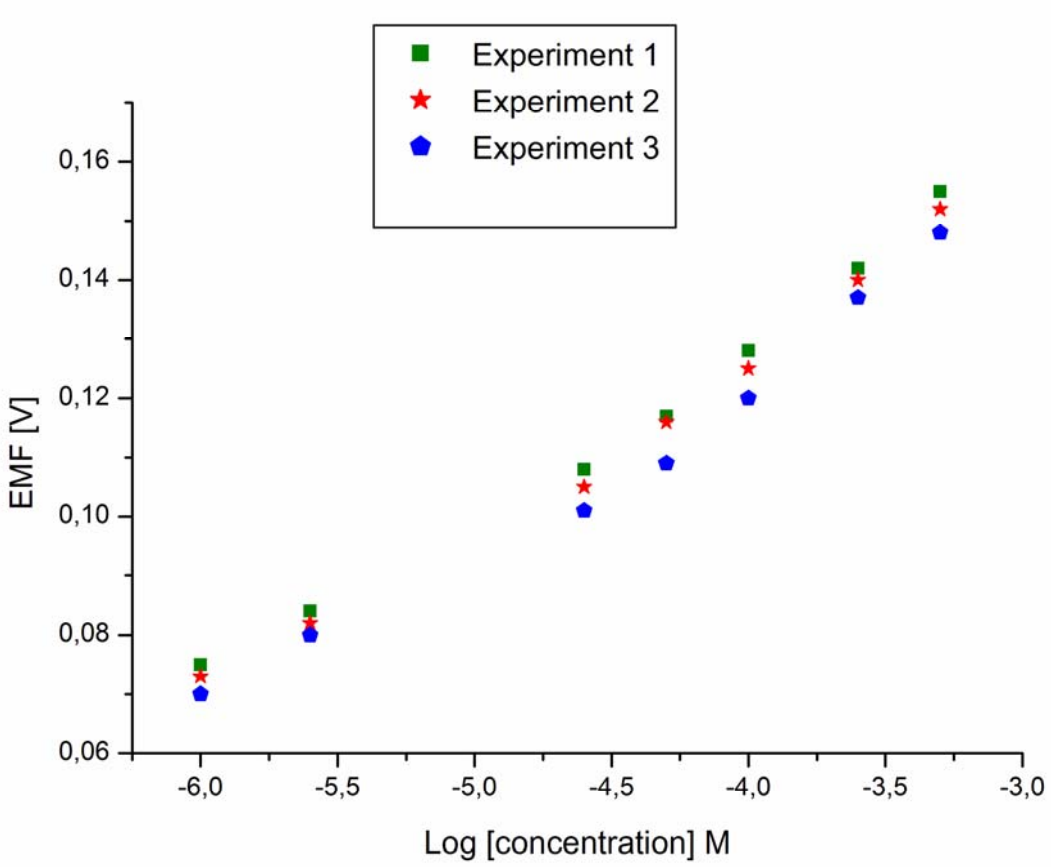
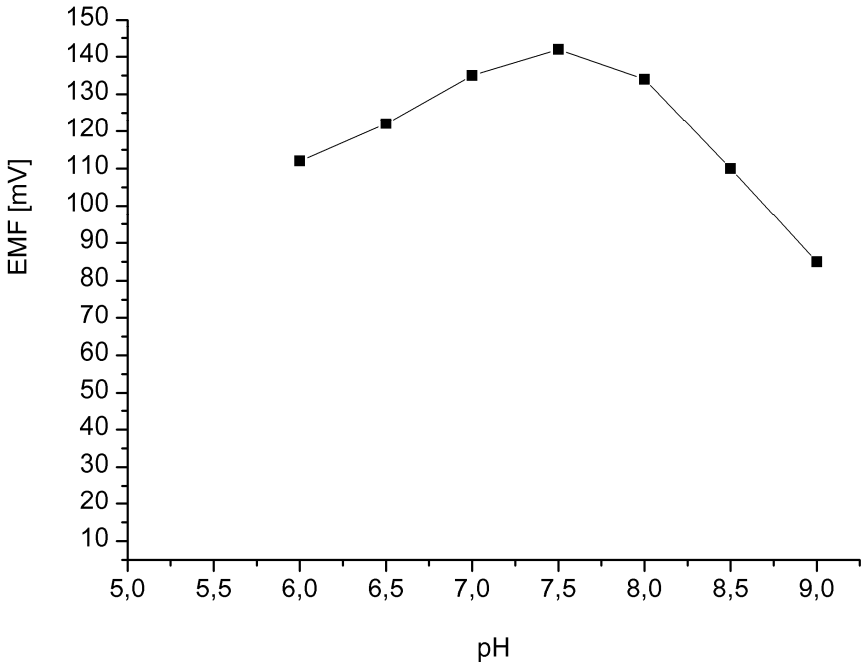

Experiment 1
Experiment 2
Experiment 3
EMF [V]
0,16
0,14
0,12
0,10
0,08
0,06
-6,0
-5,5
-5,0
-4,5
-4,0
-3,5
-3,0
Log [concentration] M

Figure 6
(a)


EMF [mV]
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
5,0
5,5
6,0
6,5
7,0
7,5
8,0
8,5
9,0
pH

(b)

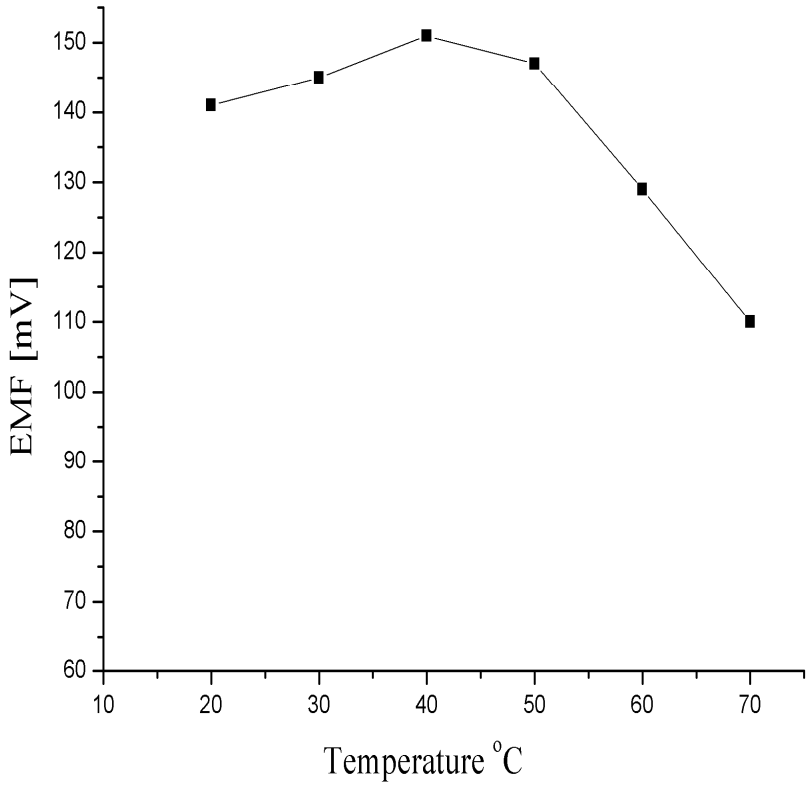
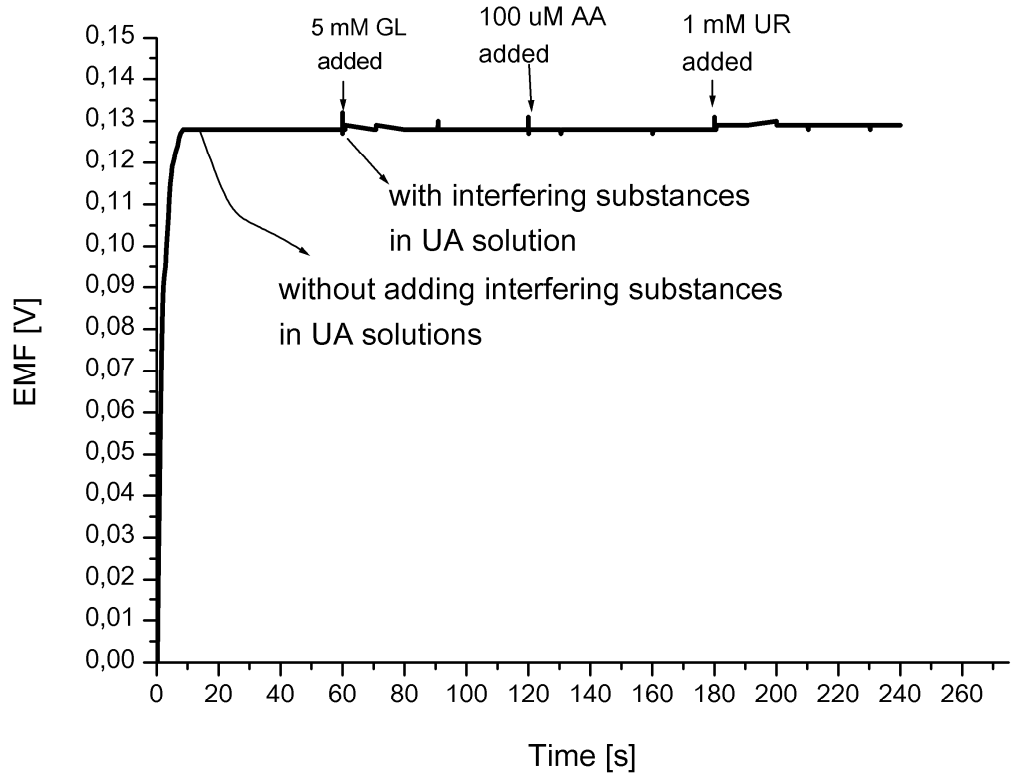

EMF [mV]
150
140
130
120
110
100
90
80
70
60
10
20
30
40
50
60
70
Temperature °C

Figure 7


5 mM GL
added
100 uM AA
added
1 mM UR
added
with interfering substances
in UA solution
without adding interfering substances
in UA solutions
EMF [V]
Time [s]
0,15
0,14
0,13
0,12
0,11
0,10
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
0
20
40
60
80
100
120
140
160
180
200
220
240
260

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 Zafar Hussain Ibupoto did M.Sc in Physical Chemistry in 2001 from S.A.L University, Khairpur Mirs, Sindh, Pakistan. Zafar Hussain Ibupoto is working as a lecturer in Dr. M.A Kazi Institute of Chemistry, University of Sindh, Jamshoro, Pakistan. He is currently joined as a PhD student the Department science and Technology, (Physical Electronics and Nano-technology Division) campus Norrköping, Linköping University, SE-601 74 Norrköping, Sweden. His ongoing research interests are based on ZnO nanostructures characterization and medical applications based on ZnO nonmaterial's.

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 Magnus Willander has M.Sc degrees from Lund University, (physics), Uppsala University (engineering physics) and Stockholm University (economy) and PhD degree in physics from Royal Institute of Technology in Stockholm. Dr Willander worked five years with electronic design in different industries in the 70s and 80s. In the 80s he did pioneering work on SiGe, SiC and polymer transistors as associate professor in Linköping University. In 1995 he was appointed to full professor in nanoscience in Gothenburg University, where he

continued to work on more fundamental problems related to tunneling, collective phenomena like BEC, stochastic phenomena etc. In the beginning of 2000 Prof. Willander extended his work to more soft materials and liquids. Around 2002 he started his work on ZnO nanostructures. In 2005 Willander become professor in Linköping University where he has continued to work on ZnO nanostructures and its interaction with soft materials etc. During 2006 and 2009 he was also guest professor in Gothenburg University. He has also several times been guest scientist in nanoscience in Tokyo Institute of Technology, Tokyo. In the above mention research areas Prof. Willander has published numerous numbers of experimental and theoretical refereed articles and eight books.

Omer Nur completed the B.Sc. Honors in Physics during 1986 from the University of Khartoum, Sudan and the Ph.D. degree during 1996 in Device Physics from the University of Linköping, Sweden. His research interest is in device physics and technology. At present Dr. Omer Nur is an associate professor and holds a senior lecturer position at the Department of Science and Technology, Campus Norrköping, Linköping University, Sweden. His current research interest is synthesis, characterization and device development based on ZnO nano-structures for technical and medical applications. He has published over 120 articles in international journal and in reviewed conference proceedings.

Bengt Danielsson joined Pure and Applied Biochemistry, Lund University 1975 realizing various biosensor developments, such as the ‘enzyme thermistor’ and "enzyme transistors". He became PhD in biochemistry 1979 and associate professor (docent) in biochemistry 1982. He joined the medtech company Acromed Invest in Lund 2007. His current research interests are focused on bioanalysis and biosensor development and practical biomedical and environmental applications including miniaturized sensor-chips for home and *in* and *ex vivo* monitoring. Studies on thermometric and optical sensors as well as electrochemical and optothermal techniques has resulted in about 300 publications. Recent work involves nanotechnology (e.g. ZnO nanowires), bioaffinity arrays and micropattern formation studied by surface plasmon resonance, ellipsometry, scanning probe microscopy and chemiluminescent and fluorescent immuno- and molecular imprinting assays.