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Functionalised ZnO-nanorod-based selective electrochemical sensor for intracellular glucose

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In this article, we report a functionalised ZnO-nanorod-based selective electrochemical sensor for intracellular glucose. To adjust the sensor for intracellular glucose measurements, we grew hexagonal ZnO nanorods on the tip of a silver-covered borosilicate glass capillary (0.7 µm diameter) and coated them with the enzyme glucose oxidase. The enzyme-coated ZnO nanorods exhibited a glucose-dependent electrochemical potential difference versus an Ag/AgCl reference micro-electrode. The potential difference was linear over the concentration range of interest (0.5 µM – 1000 µM). The measured glucose concentration in human adipocytes or frog oocytes using our ZnO nanorod sensor was consistent with values of glucose concentration reported in the literature; furthermore, the sensor was able to show that insulin increased the intracellular glucose concentration. This nanoelectrode device demonstrates a simple technique to measure intracellular glucose concentration.

KEYWORDS: ZnO nanorods; functionalisation; intracellular glucose; electrochemical sensor

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1. Introduction

Glucose, also known as grape sugar or corn sugar, is a fundamental carbohydrate in biology. Glucose is one of the main products of photosynthesis and serves as the human body's primary source of energy. Living cell uses it both as a source of energy and as a metabolic intermediate in the synthesis of more complex molecules such as fats. When glucose levels in the bloodstream are not properly regulated, diseases such as diabetes can develop. Because of the high demand for blood-glucose monitoring, significant research has been devoted to producing reliable methods for in vitro or in vivo glucose measurement, such as fluorescence spectroscopy (Ballerstadt and Schultz, 2000), diffraction spectroscopy (Asher et al., 2003), surface-enhanced Raman scattering (Shafer et al., 2003), a wireless magnetoelastic sensor (Cai et al., 2004), an electrochemical transistor sensor (Forzani et al., 2004; Raffa et al., 2003), an enzyme-based amperometric sensor (Zen et al., 2003; Hrapovic et al., 2004; Lin et al., 2004; Yang et al., 2004; Zhou et al., 2005), a nanoenzymetric amperometric sensor (Park et al., 2003), nuclear magnetic resonance spectroscopy (Cline et al., 1998) and a potentiometric sensor (Shoji et al., 2001). Since the development of the first glucose biosensor, improvement of the response performance of enzyme electrodes has been the main focus of biosensor research (Raitman et al., 2002). In particular, searches for new materials and methods for immobilising enzymes are still very important subjects toward more active and stable biosensors (Yang et al., 2002; Tsai et al., 2005).

In general, a biosensor consists of a bio-sensitive layer that either contains biological recognition elements or consists of biological recognition elements covalently attached to the transducer. Different biosensor experimental setups have been used for the real-time detection, diagnosis, and classification of different forms of biochemical reactions within single cells in

order to understand cellular behaviour. This work offers enormous potential to cellular biology research (Vo-Dinh et al., 2006; Koukin et al., 2005; Fasching et al., 2005; Firtel et al., 2004). In most of these biosensors, indirect methods or large experimental setups are required. A robust and simple technique that utilises direct intracellular measurement would be of great interest.

Since the discovery of ZnO nanorods, they have been the target of numerous investigations due to their unique properties. The diameters of these nanostructures are comparable to the size of the biological and chemical species being sensed, which intuitively makes them excellent primary transducers for producing electrical signals. ZnO nanorods, nanowires, and nanotubes have recently attracted considerable attention for the detection of biological molecules (Kang et al., 2005; Batista and Mulato., 2005; Bashir et al., 2002; Wei et al., 2006; Kim et al., 2006; Kumar et al., 2006). These nanostructures have unique advantages, including high surface-to-volume ratio, non-toxicity, chemical stability, electrochemical activity, and high electron-communication features, which make them one of the most promising materials for biosensor application (Sun and Kwok., 1999). In addition, ZnO can be grown as vertical nanowires, is biosafe, has high ionic bonding (60%), and is not very soluble at biological pH-values. These properties make ZnO suitable for sensitive intracellular ion measurements. These advantages should allow for stable and reversible signals with respect to glucose concentration changes. Among a variety of nanosensor systems, our nanostructured electrochemical probe can offer high sensitivity and real-time detection. The detection sensitivity of the glucose sensor can be increased to the single-molecule level of detection by monitoring the very small changes in electrochemical potential caused by the binding of biomolecular species on the surface of the probe.

In a previous investigation, we measured concentrations of extracellular and intracellular Ca^{2+} using ZnO nanorods (Asif et al., 2008; Asif et al., 2009). Intracellular determination of glucose is of great interest, and ZnO nanorod technology has potential for such measurements. The focus of the current study is the demonstration of a ZnO-nanorod-based sensor suitable for intracellular selective glucose detection. Our main effort has been directed towards the construction of tips that are selective for glucose and capable of penetrating the cell membrane, as well as the optimisation of electrochemical potential properties. Tips of borosilicate glass capillaries (0.7 μm in diameter) with grown ZnO nanorods have proven to be a convenient and practical choice, as we have demonstrated with our previously developed intracellular Ca^{2+} and pH nanosensors (Asif et al., 2009; Al-Hilli et al., 2007).

Various methods for immobilisation of glucose oxidase on different supporting materials have been proposed, including covalent binding (Piro et al., 2000), embedding methods (Cosnier et al., 1999), cross-linking methods (Muguruma et al., 2000; Yang et al., 1998; Wu et al., 2004) and physical adsorption (Sun et al., 2008; Topoglidis et al., 2001). In this study, electrostatic enzyme immobilisation has been used, drawing on the fact that there is a large difference in the isoelectric points of ZnO and glucose oxidase. The isoelectric point of ZnO is about 9.5, making it suitable to immobilise low-IEP proteins or enzymes such as glucose oxidase (IEP ~ 4.2) by electrostatic adsorption in proper buffer solutions around neutral pH (Usman Ali et al., 2009; Wink et al., 1997).

In the human body, the hormone insulin only stimulates glucose transport into muscle and fat cells. However, insulin has been found to affect glucose uptake in oocytes from frog *Xenopus laevis* (Simon and Cushman, 1986: Janicot and Lane, 1989). The large size of these cells makes it possible to microinject specific reagents that interrupt or activate signal

transmission to glucose. In this study, we used an intracellular electrochemical glucose sensor based on ZnO nanorods to measure intracellular glucose concentration in human adipocytes and *Xenopus laevis* oocytes and to demonstrate a glucose transport system that is markedly activated by insulin in both cells.

2. Experimental Details

2.1 Materials

Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger*, type GO3A360 U/mg was purchased from BBI Enzymes (UK) Ltd)., D-(+)-glucose (99.5%), zinc nitrate hexahydrate $Zn(NO_3)_2$ $6H_2O$ and hexamethylenetetramine (HMT) were purchased from Sigma-Aldrich. Borosilicate glass capillaries (sterile Femtotip® II with tip inner diameter of 0.5 µm, tip outer diameter of 0.7 µm, and length of 49 mm) were purchased from Eppendorf AG, Hamburg-Germany. Phosphate-buffered saline 10 mM solution (PBS) was prepared from Na_2HPO_4 and KH_2PO_4 with 0.138 M NaCl, and the pH was adjusted to 7.40. Glucose stock solution was kept at least 24 hours after preparation for mutarotation. All chemicals used (Sigma-Aldrich) were of analytical reagent grade.

2.2 Fabrication of sensor and reference electrodes

To prepare the sensor and reference electrodes, we affixed the aforementioned borosilicate glass capillaries inside a flat support of the vacuum chamber of an evaporation system (Evaporator Satis CR725) to uniformly deposit chromium and silver films (with thicknesses of 10 nm and 125 nm, respectively) the outer surface of the capillary tips. After some optimisation, the reference electrode Ag/AgCl tip was electrochemically prepared by dipping the coated end of a capillary in 0.2 M HCl solution and then by electrolysing the silver film to form

AgCl by polarising it at 1.0 V for one minute. A 3-cm-long Ag/AgCl layer was coated on the tip of the capillary and covered with insulating material, leaving 3 mm of Ag/AgCl exposed at the tip to serve as a reference electrode. The outer end of the Ag/AgCl layer was connected to a copper wire (0.5 mm in diameter and 15 cm in length) and fixed by means of high-purity-silver conductive paint. To prepare the working electrode, we grew hexagonal single crystals of ZnO nanorods on another silver-coated capillary glass tip using a low-temperature method (Greene et al., 2003; Vayssieres et al., 2001; Kumar et al., 2005). The ZnO nanorod layer covered a small part of the silver-coated film. The part of the capillaries covered with ZnO nanorods varied from 3 mm down to 10 µm. The nanostructure had a rod-like shape with a hexagonal cross-section and primarily aligned along the perpendicular direction, as shown in Figure 1. The nanorods are uniform in size with a diameter of 100-120 nm and a length of 900-1000 nm. The electrical contact was made on the other end of the Ag film for obtaining an electrical signal during measurements.

Careful efforts were taken to ensure sufficiently small tip geometry. Intracellular electrodes must have extremely sharp tips (sub-micrometer dimensions) and must be >10 µm in length. These characteristics are necessary for effective bending and gentle penetration of the flexible cell membrane.

2.3 Immobilisation of the enzyme

Glucose oxidase solution, 5 mg/ml, was prepared in 10 mM PBS containing 1.5 mM Na_2HPO_4, 8 mM KH_2PO_4, 0.138 M NaCl, and 2.7 mM KCl pH 7.40. Glucose oxidase was electrostatically immobilised by dipping the tip of a borosilicate glass capillary with well-aligned ZnO nanorods into 2 mL of the enzyme solution for 15 minutes at room temperature and then

drying it in air for more than 20 minutes. Figure 1c shows ZnO nanorod with immobilized GOD. All enzyme electrodes were stored in dry condition at 4^{o}C when not in use.

2.4 Electrochemical measurements

The selective intracellular glucose measurements were performed by a potentiometric method utilising two electrodes. A ZnO-nanorod-decorated electrode coated with enzyme served as the intracellular working electrode, and an Ag/AgCl electrode was used as the intracellular reference microelectrode. The electrochemical response of the glucose probe was measured with a Metrohm pH meter model 827 versus the Ag/AgCl reference microelectrode, which had been calibrated externally versus an Ag/AgCl bulk reference electrode. This calibration showed approximately constant potential difference using glucose solution with concentrations ranging from 0.5 µM to 1000 µM. Subsequently, the potentiometric response of the glucose probe was studied in glucose solutions within the same concentrations range. A very fast response time was noted over the entire concentration range, reaching 95% of the steady-state voltage within one second, as shown in Figure 4(c). After the extracellular measurements, the probe was used to selectively measure the intracellular concentration of glucose in two types of cells: human adipocytes (fat cells) and frog oocytes (egg cells). The experimental setup for the intracellular measurements is shown in Figure 2.

Human adipocytes (fat cells) were isolated by collagenase digestion of pieces of subcutaneous adipose tissue (Strålfors and Honnor., 1989) obtained during elective surgery at the university hospital in Linköping, Sweden (all patients gave their informed consent, and procedures were approved by the local ethics committee). The adipocytes were incubated overnight before use as described by Strålfors and Honnor (1989) and used in a Krebs-ringer solution buffered with 20 mM HEPES, pH 7.40 and with additives, as in Danielsson et al.

(2005). A glass slide substrate (5 cm in length, 4 cm in width, and 0.17 mm in thickness) with sparsely distributed fat cells was placed on a prewarmed microscope stage set at 37°C. The indicator electrode and reference electrode were mounted and micromanipulated into the adipocytes according to the procedure described by Asif et al. (2009).

Female *Xenopus laevis* were anesthetised in a bath with tricaine (1.4 g/L, Sigma-Aldrich, Sweden), and ovarian lobes cut off through a small abdominal incision (procedure approved by the local ethical committee). Oocytes were manually dissected into smaller groups and defolliculated by enzymatic treatment with liberase (Roche Diagnostics, Sweden) for 2.5 hours. Stage-III and -VI oocytes (approximately 1 mm in diameter) without spots and with clear delimitation between the animal and vegetal pole were selected. Oocytes were kept in MBS solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.5 mM pyruvate, 25 mg/L penicillin-streptomycin; all from Sigma-Aldrich, Sweden) at 11ºC for 1-5 days before measurements. The experimental procedures are described in more detail by Börjesson et al. (2010). During measurements, oocytes were placed on a glass slide substrate and bathed in a PBS solution supplemented with 1 mM glucose. Measurements were carried out at room temperature (20-23ºC). The indicator electrode and reference electrode were mounted and micromanipulated into the oocytes according to the procedure described for adipocytes.

3. Results and discussion

The construction of a two-electrode electrochemical potential cell was as follows:

reference electrode | reference electrolyte || test electrolyte | indicator electrode

The electrochemical cell voltage (electromotive force) changed when the composition of the test electrolyte was modified. These changes can be related to the concentration of ions in the test electrolyte via a calibration procedure. The actual electrochemical potential cell can be described by the diagram below:

$$Ag\ |ZnO\ |\ buffer\ ||\ Cl^-\ |AgCl\ |\ Ag$$

The measurements were started 30 minutes after functionalising the enzyme-covered ZnO nanorods and the Ag/AgCl reference microelectrode in an electrolyte drop. Next, both microelectrodes were immersed inside a 30-µl drop of distilled water as a test sample. To test the response of the probe, a 30-µl drop of a 1 mM glucose solution was added to the drop of distilled water. The signal change from one level to another was recorded, giving the response behaviour of the ZnO-nanorod glucose sensor without stirring (controlled by diffusion to the sensor). The experimental setup for the intracellular measurements is shown in Figure 2. The response of the electrochemical potential difference of the ZnO nanorods to the changes in buffer electrolyte glucose was measured for the range of 500 nM to 1 mM and shows that this glucose dependence is linear and has sensitivity equal to 42.5 mV/decade at around 23°C (Figure 3). This linear dependence implies that such sensor configuration can provide a large dynamic range.

The sensing mechanism of the electrochemical glucose sensors is based on an enzymatic reaction catalysed by glucose oxidase (GOD) with β D -glucose, according to the following:

$$H_2O + O_2 + \text{D-Glucose} \xrightarrow{\text{GOD}} \text{D-gluconolactone} + H_2O_2 \quad \text{---------- (1)}$$

As a result of this reaction, D-gluconolactone and hydrogen peroxide are produced. These two products and the oxygen consumption can be used for glucose determination. With H_2O availability in the reaction, gluconolactone is spontaneously converted to gluconic acid, which at neutral pH forms the charged products of gluconate$^-$ and a proton (H^+) according to the following equation:

$$\text{D-Gluconolactone} \xrightarrow{\text{Spontaneous}} \text{Gluconate}^- + H^+ \qquad \text{---------- (2)}$$

This proteolytic reaction of D-gluconolactone to gluconic acid shown in equation (2), results in a decrease of the medium pH that can be used for determination of the glucose concentration (Shaw et al., 2003). In our case, it is the resulting change in ionic distribution around the ZnO nanorods that causes a change of the overall potential of the ZnO-nanorod electrode. Depending on the sample properties different selective mechanisms may be required to avoid influence by other ions present or other reactions taking place during the measurements. At glucose determination in serum samples by amperometric glucose oxidase methods, ascorbic acid and uric acid are well known interferents. In earlier studies [Usman Ali et al., 2010] it was shown that the proposed methods was not affected by these compounds. On the other hand the same study showed that the performance of the sensor could be improved by membrane coatings with respect to stability and measuring range. In the measurements described in the actual work the sensors were not used for repeated measurements in the cells and the measurement conditions were more constant. Sensor performance and stability were quite acceptable without any protective membranes or other selective measures.

First, we used the nanosensor to measure the free concentration of intracellular glucose in a single human adipocyte. The glucose-selective nanoelectrode, mounted on a micromanipulator, was moved into position in the same plane as the cells. The ZnO nanoelectrode and the reference microelectrode were then gently micromanipulated a short distance into the cell (Figure 2). Once the ZnO nanorod working electrode and the Ag/AgCl reference microelectrode were inside the cell, that is, isolated from the buffer solution surroundings, an electrochemical potential difference signal was detected and identified as the presence of glucose. The intracellular glucose concentration was estimated to be 50 ± 15 μM (n = 5). This can be compared with the 70 μM intracellular concentration determined by nuclear magnetic resonance spectroscopy in rat muscle tissue in the presence of a high, 10 mM, extracellular glucose concentration (Cline et al., 1998). Insulin stimulates glucose uptake by binding to its receptor at the cell surface, which initiates intracellular signal transduction, causing translocation of insulin-sensitive glucose transporters (GLUT4, glucose transporter-4). After integration in the plasma membrane, GLUT4 allows glucose to enter the cell along a concentration gradient, as shown in Figure 4(a). Thus, when we achieved a stable potential for intracellular measurement, 10 nM insulin was added to the cell medium. After several minutes insulin, increased the glucose concentration in the cell from 50 ± 15 to 125 ± 15 μM (Fig. 4(b)). Insulin stimulates glucose uptake by binding to its receptor at the cell surface, which initiates intracellular signal transduction causing translocation of insulin-sensitive glucose transporters (GLUT4, glucose transporter-4). After integration in the plasma membrane GLUT4 allows glucose to enter the cell down a concentration gradient as shown in Figure 4(a).

In another set of experiments, we used the nanosensor to measure intracellular glucose concentration in single frog oocytes. The intracellular glucose concentration was 125 ± 23 μM (n

= 5). This is slightly higher than what has been reported earlier (<50 μM; Umbach et al., 1990). We do not know the reason for this difference, but one possibility is that the electrodes behave slightly differently inside the oocyte than outside, where they were calibrated. However, to test whether the electrode is measuring the glucose concentration inside the oocytes, we added 10 nM insulin to the cell medium to stimulate glucose uptake. Indeed, the glucose concentration in the frog oocytes increased from 125 ± 23 μM to 250 ± 19 μM. The viability of the penetrated cells strongly depends on the size of the ZnO nanorods. By reducing the size of ZnO nanorods, the total diameter of the tip will be reduced, which in turn increases the cell viability, and the sensitivity of the device is also expected to increase. The morphology of the functionalised ZnO intracellular sensor electrode was checked by scanning electron microscopy directly after measurements, shown in the images of Figure 5. Obviously some components from the cell and the cell membrane adhere to the probe and possibly this contamination occurs mainly when the probe is pulled out from the cell. In any case the glucose response of the electrode does not seem to be affected, which is in line with what could be expected from a potentiometric device as long as the blockage of the active surface is only partial. If proper cleaning in deionised water is performed, the immobilised glucose oxidase will retain its enzymatic activity due to the strong electrostatic interaction between ZnO and glucose oxidase. We have attempted to clean the stuck cell components from the electrode after intracellular measurements. Figure 5b shows the immobilized electrode after cleaning. As clearly seen in the figure, the immobilized ZnO nanorods are still in good condition and that some residues form the cell components are still stuck to the electrode.

4. Conclusion

In conclusion, we have demonstrated that functionalised hexagonal ZnO nanorods grown on sub-micron silver-covered capillary glass tips works as a selective sensor for intracellular glucose concentration in single human adipocytes and frog oocytes. The functionalised glucose oxidase retained its enzymatic activity due to excellent electrostatic interaction between ZnO and glucose oxidase. The proposed intracellular biosensor showed a fast response with a time constant of less than 1 s and has quite a wide linear range from 0.5 µM to 1000 µM. The performance regarding sensitivity, selectivity, and freedom from interference when the sensor was exposed to intra- and extracellular glucose measurements were quite acceptable. The stability of the sensing ZnO layer was, however, limited and should be improved although the experiments described here have a short duration and could be performed without influence of this drawback. The effect of the hormone insulin, which increased the concentration of intracellular glucose, was also demonstrated. These results demonstrate the capability to perform biologically relevant measurements of glucose within living cells. The ZnO-nanorod glucose electrode thus holds promise for minimally invasive dynamic analyses of single cells. All of these advantageous features can make the proposed biosensor applicable in medical, food or other areas. Moreover, the fabrication method is simple and can be extended to immobilise other enzymes and other bioactive molecules with small isoelectric points for a variety of biosensor designs. We also plan to take the same approach for other intracellular activity measurements.

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Figure Captions

Figure 1: Scanning electron microscopy images of the ZnO nanorods grown on Ag-coated glass capillaries using low-temperature growth, (a-b) before enzyme immobilisation and (c) after enzyme immobilization.

Figure 2: (a) Schematic diagram illustrating the selective intracellular glucose measurement setup. (b) Microscope images of a single frog (*Xenopus laevis*) oocyte and a single human fat cell (adipocyte) during measurements with a functionalised ZnO-nanorod coated probe as a working electrode and with an Ag/AgCl reference microelectrode.

Figure 3: A calibration curve showing the electrochemical potential difference versus the glucose concentration (0.5-1000 μM) using functionalised ZnO-nanorod-coated probe as a working electrode and an Ag/AgCl microelectrode reference microelectrode.

Figure 4: (a) Intracellular mechanism for insulin-induced activation of glucose uptake. (b) Output response with respect to time for intracellularly positioned electrodes when insulin is applied to the extracellular solution. (c) Output response with respect to time for glucose applied to the extracellularly positioned electrodes.

Figure 5: Scanning electron microscopy images showing the working electrode after intracellular measurements (a) before rinsing with de-ionized water and (b) after rinsing with deionized water.

Figure 1

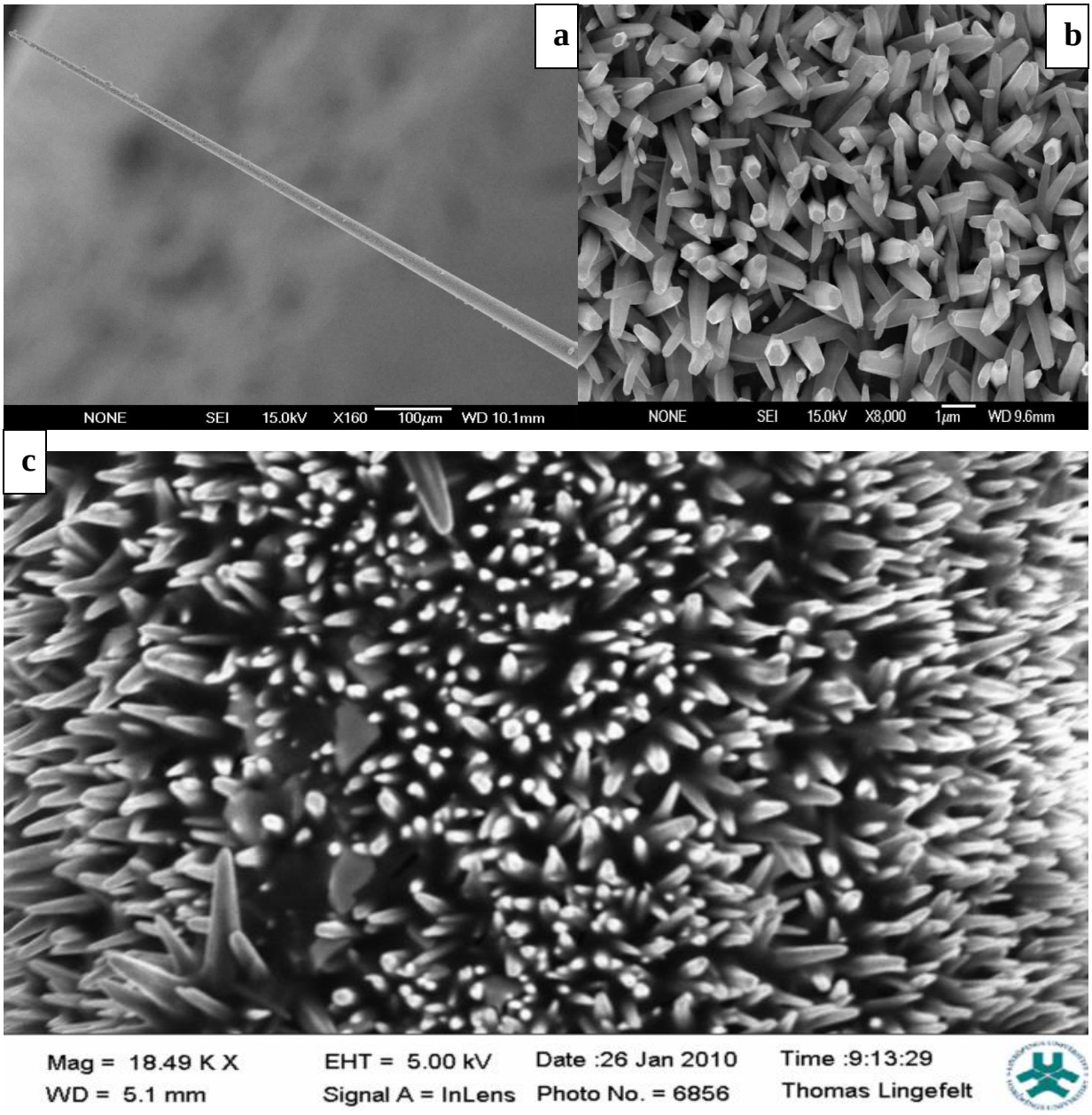
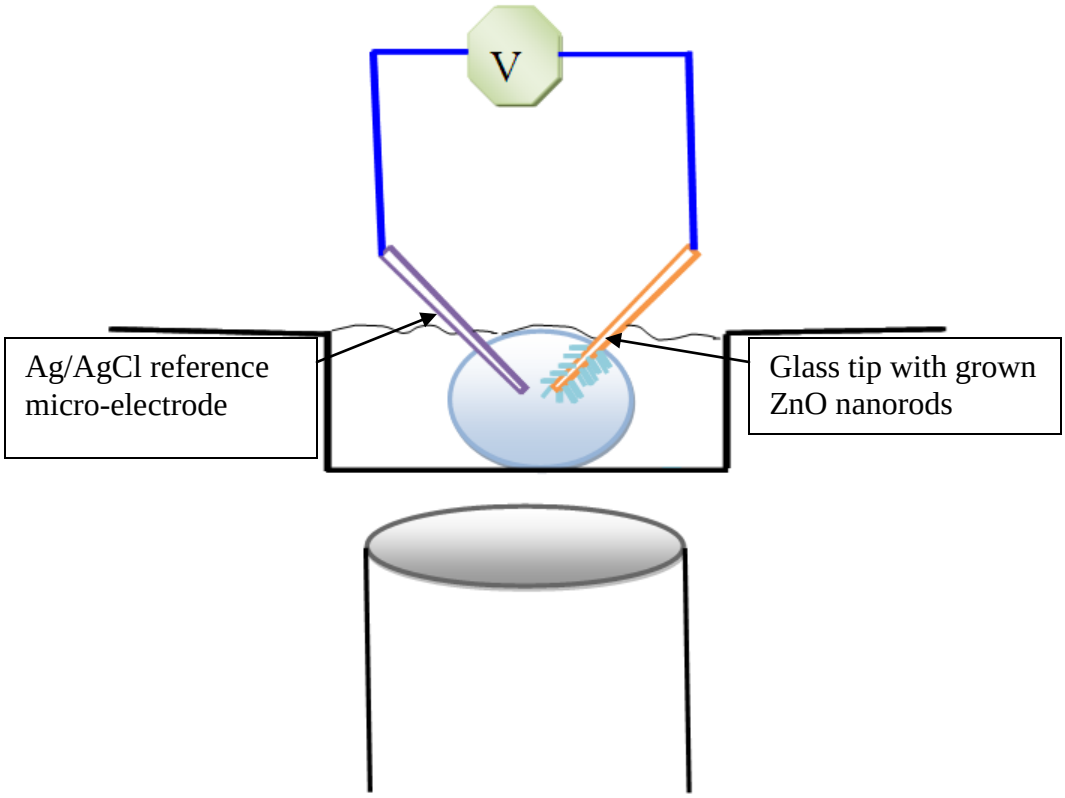

a
NONE SEI 15.0kV X160 100µm WD 10.1mm
b
NONE SEI 15.0kV X8,000 1µm WD 9.6mm
c
Mag = 18.49 K X
WD = 5.1 mm
EHT = 5.00 kV
Signal A = InLens
Date :26 Jan 2010
Photo No. = 6856
Time :9:13:29
Thomas Lingefelt

Figure 2


V
Ag/AgCl reference micro-electrode
Glass tip with grown ZnO nanorods

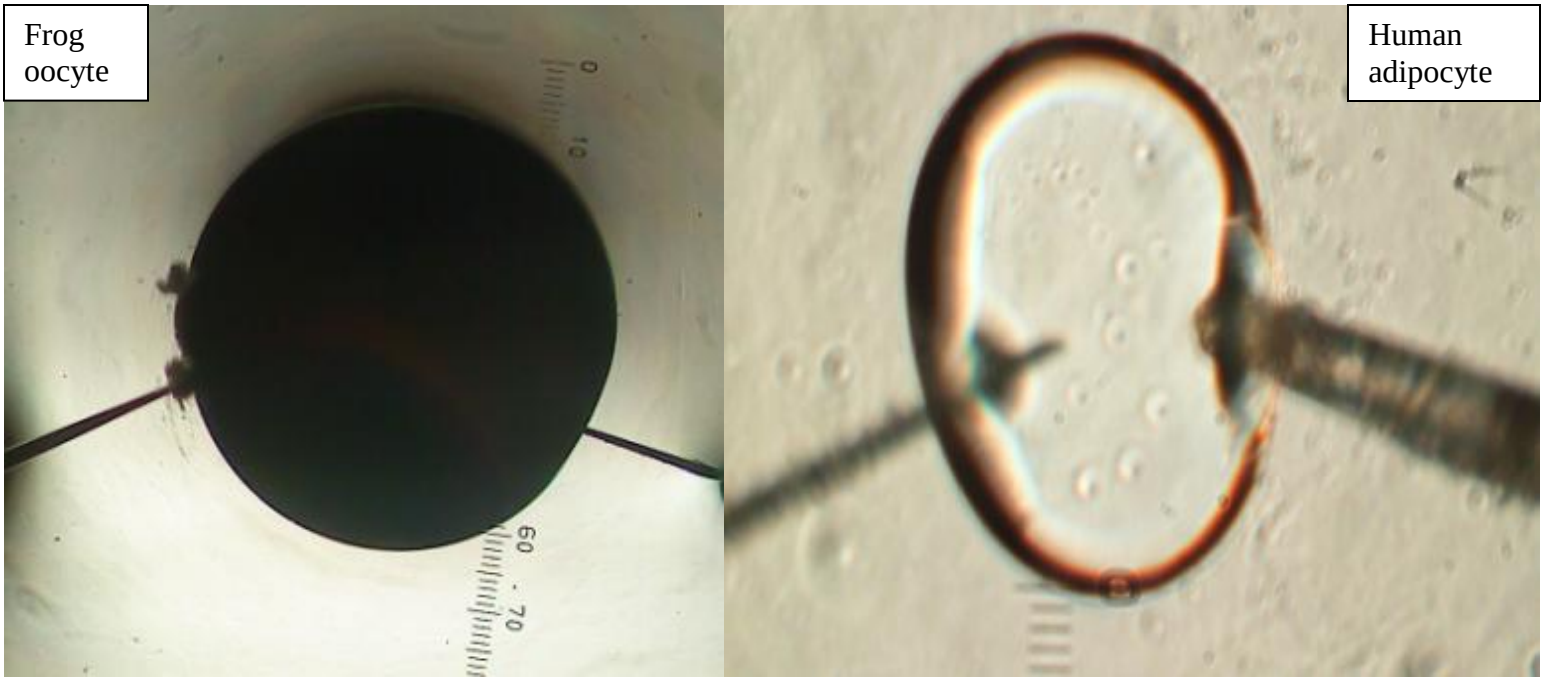

Frog oocyte
Human adipocyte

Figure 3

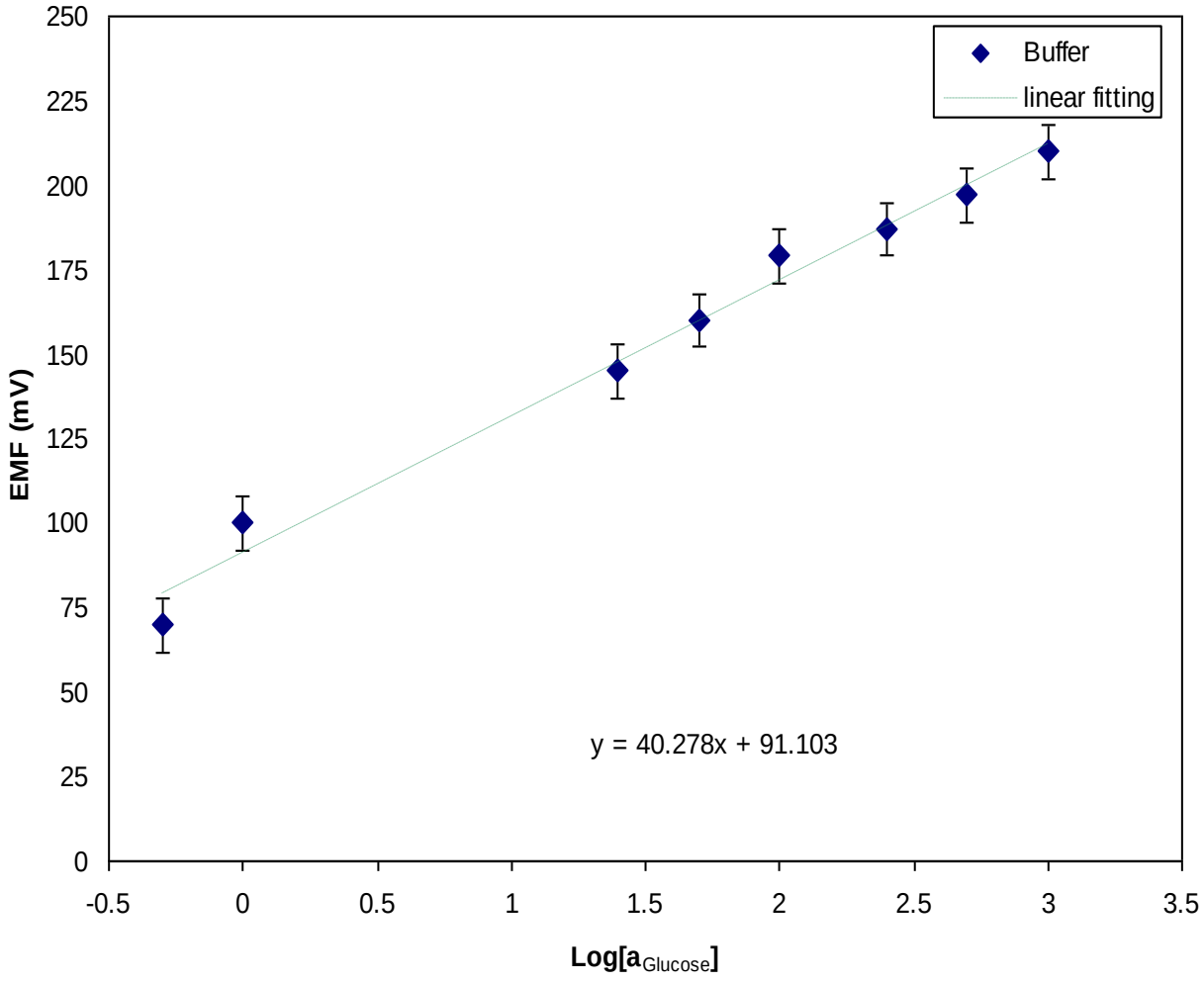
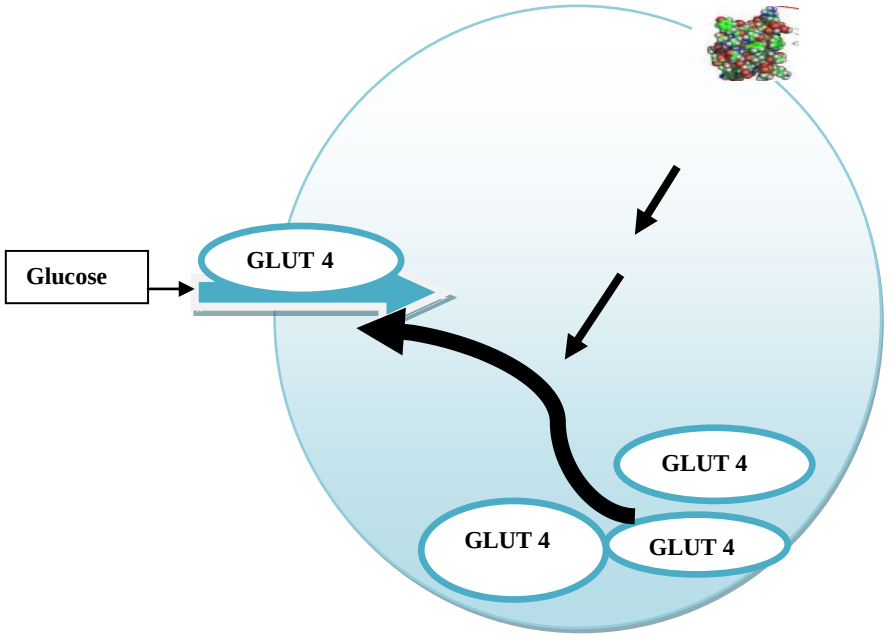

Buffer
linear fitting
y = 40.278x + 91.103
EMF (mV)
Log[a Glucose]
250
225
200
175
150
125
100
75
50
25
0
-0.5
0
0.5
1
1.5
2
2.5
3
3.5

Figure 4

a


Glucose
GLUT 4
GLUT 4
GLUT 4
GLUT 4

b

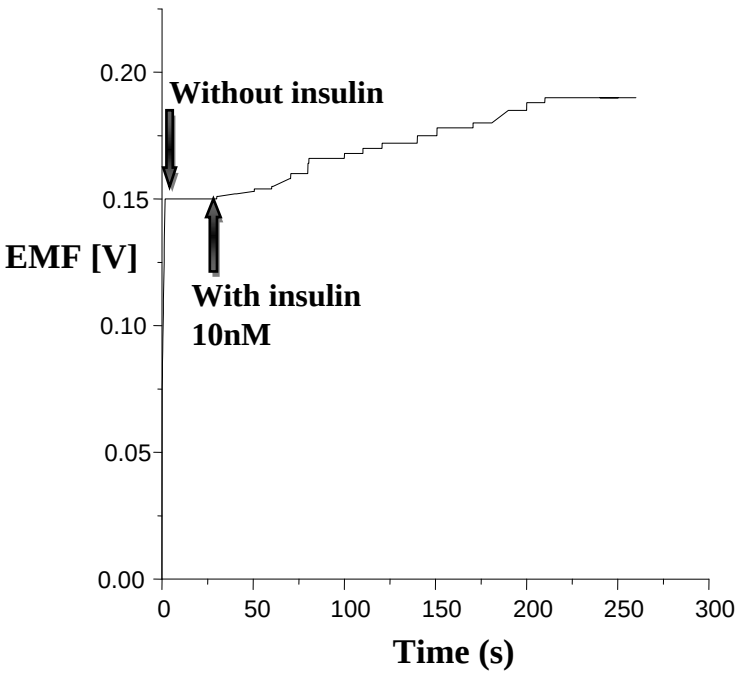

Without insulin
With insulin
10nM
EMF [V]
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
300
Time (s)

Figure 4

c

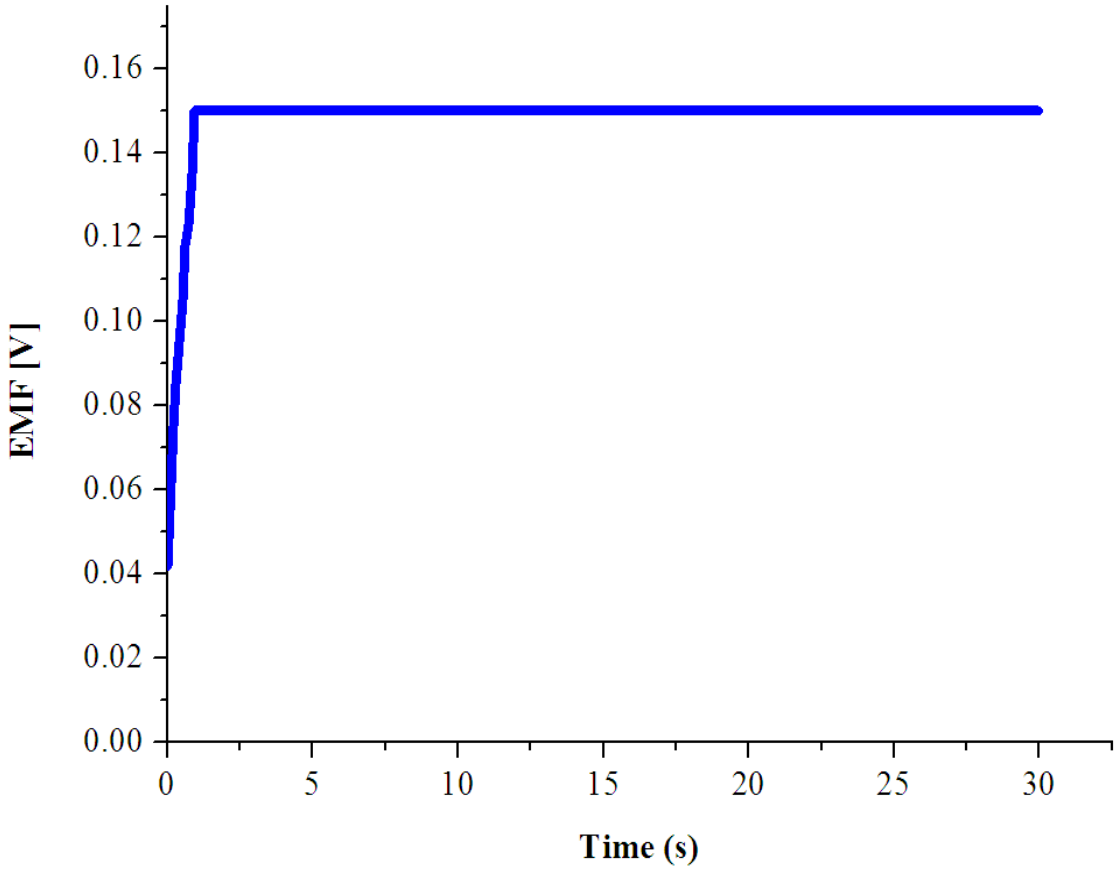


Figure 5