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An intracellular glucose biosensor based on nanoflake ZnO

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Abstract:

In this study, a potentiometric intracellular glucose biosensor was fabricated by immobilization of glucose oxidase on nanoflake ZnO. Nanoflake ZnO with a wall thickness around 200 nm was grown on the tip of a borosilicate glass capillary and used as a selective intracellular glucose biosensor for the measurement of glucose concentrations in human adipocytes and frog oocytes. The results showed a fast response within 4 s and a logarithmic linear glucose-dependent electrochemical potential difference over a wide range of glucose concentration (500 nM-10 mM). Our measurements of intracellular glucose were consistent with the values of intracellular glucose concentrations reported in the literature. The monitoring capability of the sensor was demonstrated by following the increase in the intracellular glucose concentration induced by insulin in frog oocytes. In addition, the nanoflake ZnO material provided 1.8 times higher sensitivity than previously used ZnO nanorods under the same conditions. Moreover, the fabrication method in our experiment is simple and the resulting nanosensor showed good performance in sensitivity, stability, selectivity, reproducibility, and anti-interference. All these results demonstrate that the nanoflake ZnO can provide a promising material for reliable measurements of intracellular glucose concentrations within single living cells.

Key Words:

Glucose oxidase (GOD), Intracellular, Potentiometric biosensor, Nanoflake ZnO, Nafion membrane

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1. Introduction

Diabetes mellitus is a worldwide public health problem. This metabolic disorder originates from insulin deficiency and hyperglycemia. It is reflected by blood glucose concentrations higher or lower than the normal range of 4.4-6.6 mM [1]. Diabetes can lead to higher risks of heart disease, kidney failure, and blindness. Diagnosis and treatment of diabetes require a tight daily monitoring of the blood glucose level. Fast and accurate determination of glucose concentration are therefore of great importance.

In recent years, tremendous amount of attention and efforts have been paid to develop reliable glucose biosensors including electrochemical [2], surface-enhanced Raman scattering [3], chemiluminescence [4], electrochemical transistor sensor [5, 6], potentiometric sensor [7], and other methods [8]. Intracellular sensors for glucose, metabolic precursors, and signaling ligands such as amino acids have also been used for real-time detection, diagnosis, and classification of different forms of biochemical reactions within single cells in order to understand cellular behavior and the complex roles of these small molecules in biology. Such biosensors offer enormous potential to the development of cell-biology research [9-12]. The use of nanomaterials has allowed the introduction of many new signal-transduction technologies in biosensors resulting in improved sensitivity and performance. Nano-sensors, nano-probes, and other nano-systems have also allowed simple and rapid analysis of *in vivo* measurements because their sub-micron dimensions are comparable to the size of target biological and chemical species being sensed, which render them excellent material choice for producing electrical signals [13]. However, most of the intracellular biosensors involve indirect methods, or large experimental setups. A robust simple technique for intracellular measurement is thus highly desired.

Among all glucose biosensors, enzyme-based electrochemical glucose biosensors have been in the main focus of biosensor research because of their simplicity, relatively low cost, and high sensitivity [14-16]. The intrinsic advantages of electrochemical biosensors are their robustness, easy miniaturization, excellent detection limits, also with small sample volumes, and ability to be used in

turbid biofluids. In enzyme-based electrochemical biosensors, enzyme immobilization is regarded to be one of the most important issues. Since proper immobilization of enzymes on a suitable matrix and their stability are important factors in the fabrication of biosensors, the search of support materials that provide large surface area for higher enzyme loading and a compatible microenvironment that helps enzyme bioactivity is thus of great importance.

Recently, zinc oxide (ZnO) nanostructures have attracted considerable interest in the applications of biosensors due to many advantages, including non-toxicity, bio-safety, excellent biological compatibility, high electron-transfer rates, enhanced analytical performance, increased sensitivity, and easy preparation [17-22]. In addition, it is important to note that ZnO is relatively stable around biological pH-values, which makes ZnO compatible with biological fluids and species [23]. Furthermore, the high isoelectric point (IEP) of ZnO (IEP 9.5) makes it a good matrix for immobilizing low IEP acidic proteins or DNA by electrostatic interactions with high binding stability [24-26]. This will suit glucose oxidase (GOD) which has an IEP of 4.5 and is widely employed in most of the glucose biosensors due to its stability and high selectivity to glucose. All these advantageous properties render ZnO suitable for sensitive intracellular ion measurements and can allow for stable and reversible signals with respect to glucose concentration changes.

In previous investigations, we have successfully demonstrated that ZnO nanorods can be used to measure the intracellular Ca^{2+} and glucose concentrations in human adipocytes and frog oocytes [27, 28]. This has proved that ZnO nanostructures have the potential to measure intracellular biological species in living cells. However, there has been no report of using other alternative materials to improve the performance of this intracellular glucose biosensor. Many methods, such as covalent bonding [29], embedding [30], and cross-linking [31-33], have been used to immobilize GOD on different supporting materials. As compared to ZnO nanorods and nanowires, ZnO nanotubes and nanoporous material possess several interesting unique properties such as highly dispersed structure and large surface area. There have been reports on the use of tubular ZnO structures as amperometric extracellular glucose

biosensors with improved performance and higher sensitivity compared to ZnO nanorods and nanowires [34, 35]. It has also been reported that the low IEP enzyme GOD binds well on the ZnO nanoporous material [36], resulting in enhanced sensitivity of the glucose biosensors.

The focus of the current study is to demonstrate an intracellular glucose biosensor based on a nano-honeycomb ZnO as an alternative to ZnO-nanorod-based sensors. Our main effort has been directed to the construction of tips coated with functionalized nanoflake ZnO that are selective to glucose and capable of gently penetrating the cell membrane. The new design differs from the previous ones by the introduction of a nanoflake ZnO that provides a larger surface area for the immobilization of GOD, which will lead to higher sensitivity and stability due to higher capture efficiency provided by higher surface-to-volume ratio. This offers a new simple and reliable way to measure intracellular glucose concentrations within single living cells.

2. Experimental Methods

2.1 Materials

Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger* 360 U/mg was purchased from BBI Enzymes (UK) Ltd. Bovine serum albumin (BSA≥98%), glutaraldehyde (50% solution), Nafion (5 wt%), D-(+)-glucose (99.5%), [$Zn(NO_3)_2.6H_2O$], and hexamethylenetetramine ($C_6H_{12}N_4$) 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 (PBS) 10 mM solution was prepared from Na_2HPO_4 and KH_2PO_4 (Sigma Aldrich) with 0.135 M NaCl and pH was adjusted to 7.4. Glucose stock solution was kept at least 24 h after preparation for mutarotation. All chemicals used (Sigma Aldrich) were of analytical reagent grade.

2.2 Preparation of human adipocytes and frog oocytes

Human adipocytes were isolated by collagenase digestion of pieces of subcutaneous adipose tissue [37], 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 Honor [37], and used in a Krebs-Ringer solution buffered with 20 mM HEPES, pH 7.4 [38].

Oocytes from female *Xenopus laevis* were obtained as previously described [39]. In brief, a *Xenopus laevis* was anesthetized in a bath with tricaine (1.4 g/L, Sigma Aldrich) and ovarian lobes were cut off through a small abdominal incision (procedure approved by the local ethics committee). Oocytes were manually dissected into smaller groups and defolliculated by enzymatic treatment with liberase (Roche Diagnostics, Sweden) for 2.5 h. 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 (in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 15 HEPES, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, pH adjusted to 7.6 by NaOH) supplemented with 2.5 mM sodium pyruvate, 25 U/mL penicillin, and 25 µg/mL streptomycin (all from Sigma Aldrich) at 11°C for 1-5 days before measurements.

2.3 Fabrication of the aluminum electrodes covered with nanoflake ZnO for the glucose-sensing microelectrode and of the Ag/AgCl reference microelectrode

A main effort has been directed to make the tip geometry of intracellular electrodes extremely sharp (submicrometer dimension) and long enough (> 10 µm) to be manipulated into small living cells.

The selective intracellular glucose measurements were accomplished by a potentiometric method utilizing two electrodes: 1) An Ag/AgCl electrode as the intracellular reference microelectrode, and 2) an intracellular aluminum electrode covered with nanoflake ZnO with attached enzyme molecules.

The reference microelectrode was prepared from a borosilicate glass capillary fixed on a flat support in the vacuum chamber of an evaporation system (Evaporator Satis CR725). First chromium and then silver (with a thickness of 10 nm and 125 nm, respectively) were uniformly deposited onto the outer surface of the capillary tip. The AgCl tip coating was prepared electrochemically by dipping the silver coated end of one capillary in 0.2 M HCl solution and then by electrolyzing the silver film to form AgCl

by polarizing it at 1.0 V for one minute.

Another borosilicate glass capillary was fixed on a flat support in the vacuum chamber of an evaporation system and titanium and aluminum (with a thickness of 10 nm and 200 nm, respectively) were uniformly deposited onto the outer surface of the capillary tip. After that the tip was first dipped into a seed solution for 2 min and then baked for 3min at a temperature of 110 °C as described in Ref. [40]. In the second step, nanoflake ZnO was grown by a hydrothermal process as described in Ref. [41]. In brief, the growth solution contained 0.025 M [$Zn(NO_3)_2.6H_2O$] and 0.025 M hexamethylenetetramine. The solution was kept at 90 °C for 4 h to form nanoflake ZnO as shown in figiure 1a. Before functionalization, the glucose-sensing microelectrode was carefully cleaned with de-ionized water and dried in the air. The electrical contact was attached to the surface of the prepared electrodes for obtaining electrical signal during measurements.

2.4 Functionalization of the glucose-sensing microelectrode and electrochemical measurements

Before immobilization of the enzyme, the ZnO covered microelectrode was rinsed with PBS to generate a hydrophilic surface. Enzyme solution was prepared by dissolving 10 mg GOD and 20 mg BSA in 200 µL PBS. The electrode was dipped into the enzyme solution for 15 minutes where after it was left in the air for 20 minutes to dry. The cross-linking procedure was carried out by adding 2 µL aqueous solution containing 2.5% glutaraldehyde and 0.5% Nafion onto the electrode surface. After drying at room temperature, 2 µL of 0.5% Nafion solution was further applied onto the electrode surface to prevent possible enzyme leakage and to eliminate foreign interferences. All enzyme-covered microelectrodes were stored in dry condition at 4 °C when not in use.

The morphology of ZnO was observed by field emission scanning electron microscopy, and the crystal structure was identified by X-ray diffraction. Elemental analysis was performed by energy-dispersive X-ray spectroscopy. All the electrochemical experiments were carried out using a Metrohm pH meter model 744 (Metrohm Ltd, Switzerland). For the time response measurements, a model 363A potentiostat/galvanostat (EG&G Ltd, USA) was used.

3. Results and Discussions

3.1 Characterization of the fabricated biosensor

Figure 1a shows scanning electron microscopy images of the nanoflake ZnO grown on the tip of the borosilicate glass capillary before enzyme immobilization. As clearly seen, nano-honeycomb structures with a wall thickness around 200 nm with uniform density and spatial distribution had been formed on the tip of the electrode.

It has been shown in previous studies that aluminum directs the growth of the nano-honeycomb ZnO and suppresses the growth along the [001] direction [41]. Aluminum substrates are known to favor the synthesis of ZnO nanomaterials with 2D morphology [42]. The dissolution of aluminum is a prerequisite and since aluminum is an amphoteric metal, it can be dissolved under alkaline conditions in the presence of an amine (such as hexamethylenetetramine) forming $Al(OH)_4^-$ In addition, the additive ions usually act as regulators to promote or inhibit the growth by capping the ZnO surface [43]. Presumably $Al(OH)_4^-$ binds to the Zn^{2+} terminated (001) surface suppressing the growth along [001] direction. This triggers the lateral growth that forms oriented nanoflake ZnO [41].

The X-ray diffraction pattern of the nanoflake ZnO is shown in Figure 1b. All three peaks that belong to ZnO indicate formation of single-phase ZnO with wurtzite structure. Figure 1c shows energy-dispersive X-ray spectroscopy analysis, indicating that the nano-honeycomb structure consists of Zn and O. Figure 1d shows a scanning electron microscopy image of the nanoflake ZnO after modification with GOD and Nafion.

3.2 Response behavior of the intracellular glucose-sensing microelectrode

The sensing mechanism of most electrochemical glucose sensors is based on the enzymatic reaction catalyzed by GOD according to the following reaction:

$$H_2O + O_2 + \beta - D - glu\cos e \overset{GOD}{\Rightarrow} \delta - D - gluconolactone + H_2O_2 \qquad (1)$$

As the result of this reaction, $\delta - D - gluconolactone$ and H_2O_2 are produced. The concentration of

these two products and the oxygen consumption can be used for the glucose determination. With H_2O available in the reaction, gluconolactone is spontaneously converted to gluconic acid, which at neutral pH, form the charged products of gluconate$^-$ and H^+, according to the equation below:

$$\delta - D - gluconolactone \xrightarrow{spontaneous} D - gluconate^- + H^+ \quad (2)$$

This proteolytic reaction results in a decrease of the pH and can be used for determination of the glucose concentration [44]. In our case, it is the resulting change in ionic distribution around the ZnO crystal structure that causes a change of the overall potential of the glucose-sensing microelectrode.

First, the extracellular function of the GOD immobilized on the ZnO electrode was tested. A two-electrode configuration with the glucose-sensing and Ag/AgCl microelectrodes was employed for micro-liter volumes in the electrochemical measurements. The extracellular potentiometric response of the two microelectrodes was studied for calibration purpose in a buffer (PBS pH 7.4) with glucose concentrations ranging from 500 nM to 10 mM. The response time when glucose was added to the solution was fast, reaching 95% of the steady-state potential within 4 s. A typical example of the response for 50 μM glucose is shown in Figure 2a. The steady-state potential showed linear dependence with the logarithm of the glucose concentration over the complete glucose concentration range studied (Figure 2b). This is an improvement over the previously proposed ZnO-nanorod-based intracellular glucose-sensing microelectrode [28], which failed to show linear response for glucose concentration over 1mM. This wider range could be due to the different immobilization technique used in the new sensor, in which immobilized GOD retained its enzymatic activity for a longer duration and the prevention of possible enzyme leakage from the electrode by the Nafion membrane coating which also reduces foreign interferences. The sensitivity of the nanoflake ZnO sensor was -65.2 mV/decade (Figure 2b), which is 1.6-1.8 times higher sensitivity than for earlier reported glucose sensors [28, 45]. The higher sensitivity may be ascribed to the fact that nano-honeycomb structures provide a larger and more accessible surface area, which might lead to different enzymes coverage and a different distribution of reactants on the ZnO surface at equilibrium.

3.3 Intracellular glucose measurements in two different cell types

In order to measure the intracellular glucose concentration in a single human adipocyte, a glass slide (5 cm length, 4 cm width, and 0.17 mm thickness) with sparsely distributed human adipocytes was placed on the microscope stage. The microelectrodes, mounted on a micropipette holder of a micromanipulation system, were then gently micro-manipulated into the cell using the hydraulic fine adjustments for penetrating the cell membrane and extending a short way into the cell as shown schematically in Figure 3. Once the microelectrodes were inside the cell, the electrochemical potential difference signal could be measured and the glucose concentration determined. The intracellular glucose concentration in a single human adipocyte was 60±15 μM (n = 5), corresponding well 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 [46]. As soon as we got a stable potential for intracellular measurement, a 3μL of 10 nM insulin was injected into the total volume of 0. 25 mL of extracellular solution, and the glucose concentration in the cell increased from 60±15μM to 130±10 μM (n = 5). It took a few minutes to reach the final potential for intracellular glucose uptake as shown in Figure 4a. The increase in intracellular glucose after insulin treatment was expected because insulin stimulates cellular glucose uptake as shown schematically in Figure 4b. Insulin binds to its receptor at the cell surface. This initiates an intracellular signal transduction causing translocation of the insulin-sensitive glucose transporter type 4 (GLUT4; encoded by the GLUT4 gene) from intracellular stores to the plasma membrane. After integration into the plasma membrane, GLUT4 allows glucose to enter the cell down a concentration gradient.

In the human body, the hormone insulin only stimulates glucose transport into muscle and fat cells. However, insulin has also been found to affect glucose uptake in oocytes from *Xenopus laevis* [47, 48]. The large size of these cells makes it possible to micro-inject specific reagents that interrupt or activate signal transmission to glucose. Thus, in another set of experiment, we used the microelectrodes to measure the intracellular glucose concentration in single frog oocytes. The intracellular concentration

was 110±20 μM (n = 5), which was slightly higher than what has been reported (< 50 μM) in a previous investigation using another method [49]. We do not know the reason for this difference, but one possibility is that the electrodes behave slightly different inside the oocyte than outside, where they were calibrated. To test if the electrode can monitor changes in the glucose concentration inside the frog oocytes, 10 nM insulin again was added to the cell medium and the glucose concentration in the frog oocytes increased from 110±20 to 225±10 μM. The increase in glucose response and stabilized on the new value could be noted within few minutes.

The introduction of the ZnO nanosensor into the cytoplasm of the single living cell did not visibly seem to affect cellular viability. The viability of the penetrated cells depends strongly on the size of the sensor. This study demonstrated that size of the proposed sensor made it a minimally invasive tool appropriate for monitoring glucose changes inside living cells of the actual size.

3.4 Reproducibility, stability, and anti-interference of glucose-sensing microelectrodes

To obtain an accurate and reusable glucose-sensing microelectrode, parameters such as reproducibility, stability, and anti-interference were examined. Figure 5a shows a reproducibility test of 5 independently developed ZnO nanosensor in human adipocytes. The relative standard deviation determined from this measurement was less than 5%.

Figure 5b displays the results of three experiments for the same glucose-sensing microelectrode showing good repeatability and linearity in various glucose concentrations PBS solution (pH 7.4). The glucose-sensing microelectrode was carefully washed with de-ionized water after each measurement to clean and to remove the residual ions from the surface of the electrode.

The selectivity of a glucose-sensing microelectrode depends on two major factors: The enzyme-analyte reaction and the selective measurements. The enzyme analyte reaction with $\beta-$ D glucose is highly specific without any major interfering reaction with other types of sugars. It could however, be useful to control for possible interferences from reducing agents such as ascorbic acid and uric acid, which are well known interferents with amperometric GOD biosensors. The results are shown in Figure

5c and clearly demonstrate that the addition of these potential interferents did not substantially change the signal. Addition of 100 μM of ascorbic acid or uric acid to 1000 μM glucose only generated some small transient signals. Also long-term measurements could be a problem because of the solubility of ZnO in aqueous solution although the solubility is lowest at neutral pH. Furthermore, the experiments described here were short and could be performed without influence of this drawback. However, if the long term stability really is an issue it can be improved to the same level as for most other sensors in use by a thin membrane coating [45].

4. Conclusions

Nanoflake ZnO was grown on the tip of an aluminum-coated glass capillary to be used as basis for an intracellular glucose biosensor as an alternative to previously used ZnO nanowires. By growing the ZnO on aluminum the crystal growth was directed towards a nano-honeycomb structure well suited for electrostatic immobilization of the glucose oxidase enzyme. For increased stability and selectivity the sensing layer was protected by Nafion. The sensor was tested in measurements of glucose in human adipocytes and frog oocytes giving results consistent with the values reported in the literature. It was also possible to monitor the time course of insulin-stimulated glucose uptake by the cells. The developed sensor showed reliable stability, selectivity, and reproducibility. It was insensitive to compounds known to interfere with amperometric measurements. The proposed biosensor showed a fast response within 4 s and an improved linear electrochemical response (EMF) over a wide range of glucose concentrations (500 nM-10 mM) as well as higher sensitivity than previous micro/nano sensor concepts. The fabrication method used for this intracellular sensor is simple and can be used to immobilize other enzymes and bio-molecular species with low isoelectric points. An advantage to potentiometric devices in intracellular measurements is that they can be tuned for very low analyte consumption, which is of particular importance in intracellular applications.

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References

[1] J. Wang, Electrochemical glucose biosensor, Chem. Rev. 108 (2008) 814.

[2] G. D. Liu, Y. H. Lin, Amperometric glucose biosensor based on self-assembling glucose oxidase on carbon nanotubes, Electrochem. Commun. 8 (2006) 251.

[3] D. A. Stuart, J. M. Yuen, N. Shah, O. Lyandres, C. R. Yonzon, M. R. Glucksberg, J. T. Walsh, R. P. V. Duyne, In vivo glucose measurement by surface-enhanced Raman spectroscopy, Anal. Chem. 78 (2006) 7211.

[4] Q. W. Li, G. A. Luo, Y. M. Wang, X. G. Zhang, Immobilization of glucose oxidase in sol-gel matrix and its application to fabricate chemiluminescent glucose sensor, Mater. Sci. Eng. C. 11 (2000) 67.

[5] J. Liu, M. Agarwal, K. Varahramyan, Glucose sensor based on organic thin film transistor using glucose oxidase and conducting polymer, Sens. Actuators. B. 135 (2008) 195.

[6] B. S. Kang, H. T. Wang, F. Ren, S. J. Pearton, T. E. Morey, D. M. Dennis, J. W. Johnson, P. Rajagopal, J. C. Roberts, E. L. Piner, K. J. Linthicum, Enzymatic glucose detection using ZnO nanorods on the gate region of AlGaN/GaN high electron mobility transistors, Appl. Phys. Lett. 91 (2007) 252103.

[7] C. W. Liao, J. C. Chou, T. P. Sun, S. K. Hsiung, J. H. Hsieh, J.H, Preliminary investigations on a glucose biosensor based on the potentiometric principle, Sens. Actuators, B.123 (2007) 720.

[8] G. W. Cline, B. M. Jucker, Z. Trajanoski, A. J. M. Rennings, G. I. Shulman, A in vivo novel^{13} C NMR method to assess intracellular glucose concentration in muscle, Am. J. Physiol. Endocrinol. Metab. 274 (1998) E381-E389.

[9] T. Vo-Dinh, P. Kasili, M. Wabuyele, Nanoprobes and nanobiosensors for monitoring and imaging individual living cells, Nanomedicine 2006, 2, 22-30.

[10] N. A. Kouklin, W. E. Kim, A. D. Lazareck, J. M. Xu, Carbon nanotube probes for single-cell experimentation and assays, Appl. Phys. Lett. 87 (2005) 173901.

[11] R. Fasching, E. Tao, S. J. Bai, K. Hammerick, L. Smith, R. Greco, F. Prinz, Nanoscale technology in biology systems, edited by Greco, R.; Prinz, F; Smith, R. L. (CRC, Boca Raton, FL, 2005), 55-72.

[12] M. Firtela, G. Hendersonb, I. Sokolov, Nanosurgery: observation of peptidoglycan strands in *Lactobacillus helveticus* cell walls, Ultramicroscopy. 101 (2004) 105.

[13] J. R. Chen, Y. Q. Miao, N. Y. He, X. H. Wu, S. J. Li, J. Nanotechnology and biosensors, Biotech. Advan. 22 (2004) 505.

[14] M. Florescu, C. M. A. Brett, Development and evaluation of electrochemical glucose enzyme biosensors based on carbon film electrodes, Talanta. 65 (2005) 306.

[15] X. Luo, A. J. Killard, M. R. Smyth, Reagentless Glucose biosensor based on the direct electrochemistry of glucose oxidase on carbon nanotube-modified electrodes, Electroanalysis. 18

(2006)1131.

[16] A. I. Gopalan, K. P. Lee, D. Ragupathy, S. H. Lee, J. W. Lee, An electrochemical glucose biosensor exploiting a polyaniline grafted multiwalled carbon nanotube/perfluorosulfonate ionomer–silica nanocomposite, Biomaterials. 30 (2009) 5999.

[17] F. F. Zhang, X. L. Wang, S. Y. Ai, Z. D. Sun, Q. Wan, Z. Q. Zhu, Y. Z. Xian , L. T. Jin, K. Yamamoto, Immobilization of uricase on ZnO nanorods for a reagentless uric acid biosensor, Analytica. Chimica. Acta. 519 (2004) 155.

[18] S. P. Singh, S. K. Arya, P. Pandey, B. D. Malhotra, S. Saha, K. Sreenivas, V. Gupta, Cholesterol biosensor based on rf sputtered zinc oxide nanoporous thin film, Appl. Phys. Lett. 91 (2007) 063901.

[19] P. H. Yeh, Z. Li, Z. L. Wang, Schottky-gated probe-free ZnO nanowire biosensor, Adv. Mater. 21 (2009) 4975.

[20] T. Y. Wei, P. H. Yeh, S. Y. Lu, Z. L. Wang, Gigantic enhancement in sensitivity using schottky contacted nanowire nanosensor, J. Am. Chem. Soc. 131 (2009) 17690.

[21] N. Kumar, A. Dorfman, J. I. Hahm, Ultrasensitive DNA sequence detection using nanoscale ZnO sensor arrays, Nanotechnology. 17 (2006) 2875.

[22] Q. Wan, Q. H. Li, Y. J. Chen, T. H. Wang, X. L. He, J. P. Li, C. L. Lin, Fabrication and ethanol sensing characteristics of ZnO nanowire gas sensors, Appl. Phys. Lett. 84 (2004) 3654.

[23] J. Zhou, N. S. Xu, Z. L. Wang, Dissolving behavior and stability of ZnO wires in biofluids: A study on biodegradability and biocompatibility of ZnO nanostructures, Adv. Mater. 18 (2006) 2432.

[24] E. Topoglidis, E. Palomares, Y. Astuti, A. Green, C. J. Campbell, J. R. Durrant, Immobilization and Electrochemistry of Negatively Charged Proteins on Modified Nanocrystalline Metal Oxide Electrodes, Elec. Anal. 17 (2005) 1035.

[25] S. M. Usman Ali, O. Nur, M. Willander, B. Danielsson, Glucose Detection With a Commercial MOSFET Using a ZnO Nanowires Extended Gate, IEEE Tran. Nanotech. 8 (2009) 678.

[26] J. X. Wang, X. W. Sun, A. Wei, Y. Lei, X. P. Cai, C. M. Li, Z. L. Dong, Zinc oxide nanocomb biosensor for glucose detection, Appl. Phys. Lett. 88 (2006) 233106.

[27] M. H. Asif, A. Fulati, O. Nur, M. Willander, C. Brännmark, P. Strålfors, S. I. Börjesson, F. Elinder, Functionalized zinc oxide nanorod with ionophore-membrane coating as an intracellular Ca^{2+} selective sensor, Appl. Phys. Lett. 95 (2009) 023703.

[28] M. H. Asif, S. M. Usman Ali, O. Nur, M. Willander, C. Brännmark, P. Strålfors, U. H. Englund, F. Elinder, B. Danielsson, Functionalised ZnO-nanorod-based selective electrochemical sensor for intracellular glucose, Biosens. Bioelectron. 25 (2010) 2205.

[29] B. Piro, V. A. Do, L. A. Le, M. Hedayatullah, M. C. Pham, Electrosynthesis of a new enzyme-modified electrode for the amperometric detection of glucose, J. Electroanal. Chem. 486 (2000) 133.

[30] S. Cosnier, A. Senillou, M. Grätzel, P. Comte, N. Vlachopoulos, N. J. Renault, C. Martelet, A glucose biosensor based on enzyme entrapment within polypyrrole films electrodeposited on mesoporous titanium dioxide, J. Electroanal. Chem. 469 (1999) 176.

[31] H. Muguruma, A. Hiratsuka, I. Karube, Thin-Film Glucose Biosensor Based on Plasma-Polymerized Film: Simple Design for Mass Production, Anal. Chem. 72 (2000) 2671.

[32] Q. L. Yang, P. Atanasov, E. Wilkins, Development of needle-type glucose sensor with high selectivity, Sens. Actuators, B. 46 (1998) 249.

[33] B. L. Wu, G. M. Zhang, S. M. Shuang, M. M. F. Choi, Biosensors for determination of glucose with glucose oxidase immobilized on an eggshell membrane Talanta. 64 (2004) 546.

[34] T. Kong, Y. Chen, Y. P. Ye, K. Zhang, Z. X. Wang, X. P. Wang, An amperometric glucose biosensor based on the immobilization of glucose oxidase on the ZnO nanotubes, Sens. Actuators, B. 138 (2009) 344.

[35] K. Yang, G. W. She, H. Wang, X. M. Ou, X. H. Zhang, C. S. Lee, S. T. Lee, ZnO nanotube arrays as biosensors for glucose, J. Phys. Chem. C 113 (2009) 20169.

[36] E. Topoglidis, A. E. G. Cass, B. O'Regan, J. R. Durrant, Immobilisation and bioelectrochemistry of proteins on nanoporous TiO_2 and ZnO films, J. Anal. Chem. 517 (2001) 20.

[37] P. Strålfors, R. C. E. Honnor, Insulin-induced dephosphorylation of hormone-sensitive lipase, FEBS Journal.182 (1989) 379.

[38] A. Danielsson, A. Öst, E. Lystedt, P. Kjolhede, J. Gustavsson, F. H. Nystrom, P. Strålfors, Insulin resistance in human adipocytes occurs downstream of IRS1 after surgical cell isolation but at the level of phosphorylation of IRS1 in type 2 diabetes, FEBS Journal.272 (2005) 141.

[39] S. I. Börjesson, T. Parkkari, S. Hammarström, F. Elinder, Electrostatic Tuning of Cellular Excitability, Biophys. J. 98 (2010) 396.

[40] L. E. Greene, B. D. Yuhas, M. Law, D. Zitoun, P. Yang, Solution-grown zinc oxide nanowires Inorg. Chem. 45 (2006) 7535.

[41] J. P. Cheng, X. B. Zhang, Z. Q. Luo, Oriented growth of ZnO nanostructures on Si and Al substrates, Surf. Coat. Technol. 202 (2008) 4681.

[42] A. Rahm, G. W. Yang, M. Lorenz, T. Nobis, J. Lenzner, G. Wagner, M. Grundmann, Two-dimensional ZnO:Al nanosheets and nanowalls obtained by Al_2O_3-assisted carbothermal evaporation, Thin Solid Films. 486 (2005) 191.

[43] F. Xu, Z. Y. Yuan, G. H. Du, M. Halasa, B. L. Su, High-yield synthesis of single-crystalline ZnO hexagonal nanoplates and accounts of their optical and photocatalytic properties, Appl. Phys. A. 86 (2007) 181.

[44] T. V. Anh Dam, D. Pijanowska, W. Olthuis, P. Bergveld, Highly sensitive glucose sensor based on

work function changes measured by an ^{E}MOSFET, Analyst. 128 (2003) 1062.

[45] S. M. Usman Ali, O. Nur, M. Willander, B. Danielsson, A fast and sensitive potentiometric glucose microsensor based on glucose oxidase coated ZnO nanowires grown on a thin silver wire, Sens. Actuators, B. 145 (2010) 869.

[46] B. M. Jucker, A. J. Rennings, G. W. Cline, K. F. Petersen, G. I. Shulman, In vivo NMR investigation of intramuscular glucose metabolism in conscious rats, Am. J Physiol Endocrinol. Metab. 273 (1997) E139.

[47] I. A. Simpson, S. W. Cushman, Hormonal Regulation of Mammalian Glucose Transport, Annu. Rev. Biochem. 55 (1986) 1059.

[48] M. Janicot, M. D. Lane, Activation of glucose uptake by insulin and insulin-like growth factor I in Xenopus oocytes, Proc. Natl. Acad. Sci. 86 (1989) 2642.

[49] J. A. Umbach, M. J. Coady, E. M. Wright, Intestinal Na+/glucose cotransporter expressed in Xenopus oocytes is electrogenic, Biophys. J. 57 (1990) 1217.

Figure Caption

Figure 1: Properties of the glucose-sensing microelectrode. (a) Typical scanning electron microscopy images of the nanoflake ZnO grown on an aluminum-coated glass capillary before enzyme coating. The two panels show different magnifications. (b) X-ray diffraction pattern of the grown ZnO. All three peaks (002), (101), and (102) come from ZnO. (c) Energy-dispersive X-ray spectrum of the nanoflake ZnO. (d) Scanning electron microscopy image of the nanoflake ZnO coated with GOD.

Figure 2: Calibration of the glucose-sensing microelectrodes. (a) Time response of the microelectrode in a solution with 50 μM glucose. (b) Calibration curve showing the electrochemical potential difference between the glucose-sensing microelectrode and the Ag/AgCl reference microelectrode.

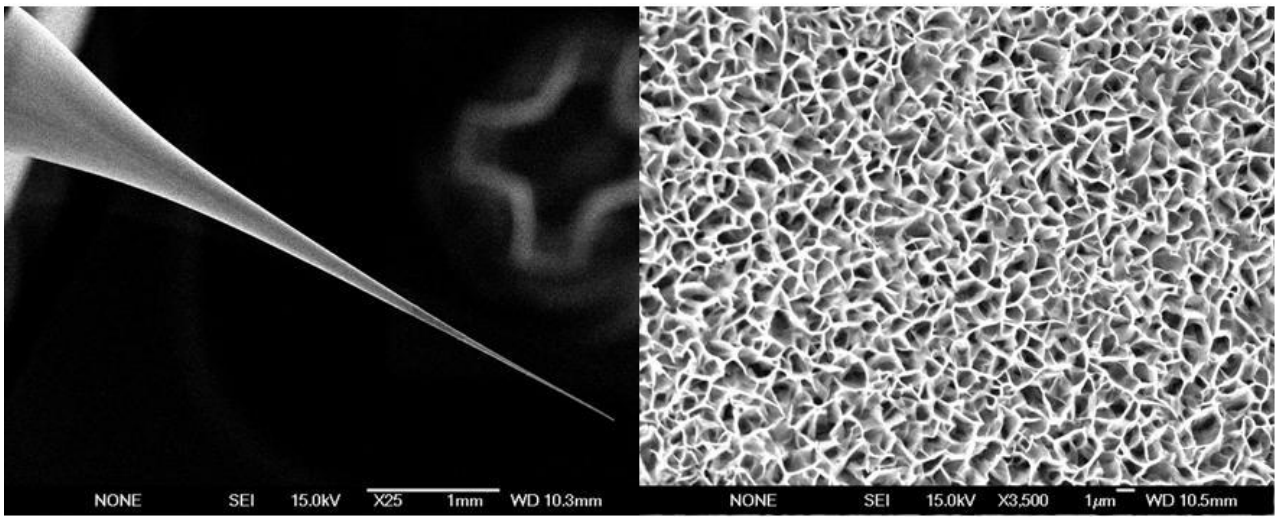
Figure 3: A schematic diagram illustrating the setup for the selective measurements of the intracellular glucose concentration. Typical microscope images of a single human adipocyte and a single frog oocyte during measurements are shown.

Figure 4: Insulin increases the intracellular glucose concentration. (a) The output response with respect to time when insulin is applied to the extracellular solution. (b) A schematic illustration showing the effect of insulin on glucose uptake. Insulin binds to the insulin receptor, which leads to the recruitment of glutamate transporters to the plasma membrane.

Figure 5: Reproducibility and stability of the glucose-sensing microelectrodes. (a) A sensor to sensor reproducibility test of five intracellular electrodes in human adipocytes. (b) Calibration curve from three different experiments using the same sensor electrode showing the electrochemical potential difference at different glucose concentration. (c) Measurements showing the lack of effect of other molecules. At indicated time points 0.1 mM ascorbic acid (AA) or 0.1 mM uric acid (UA) is added to the 1mM glucose solution.

Figure 1

(a)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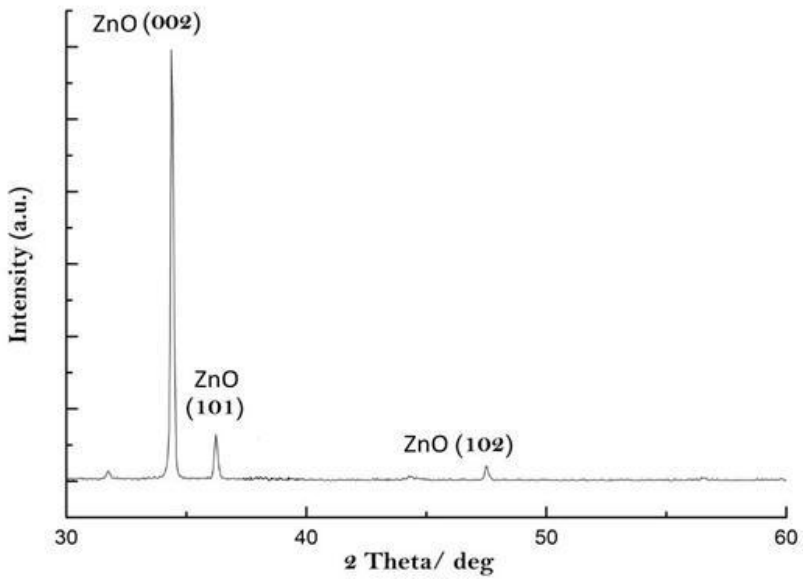
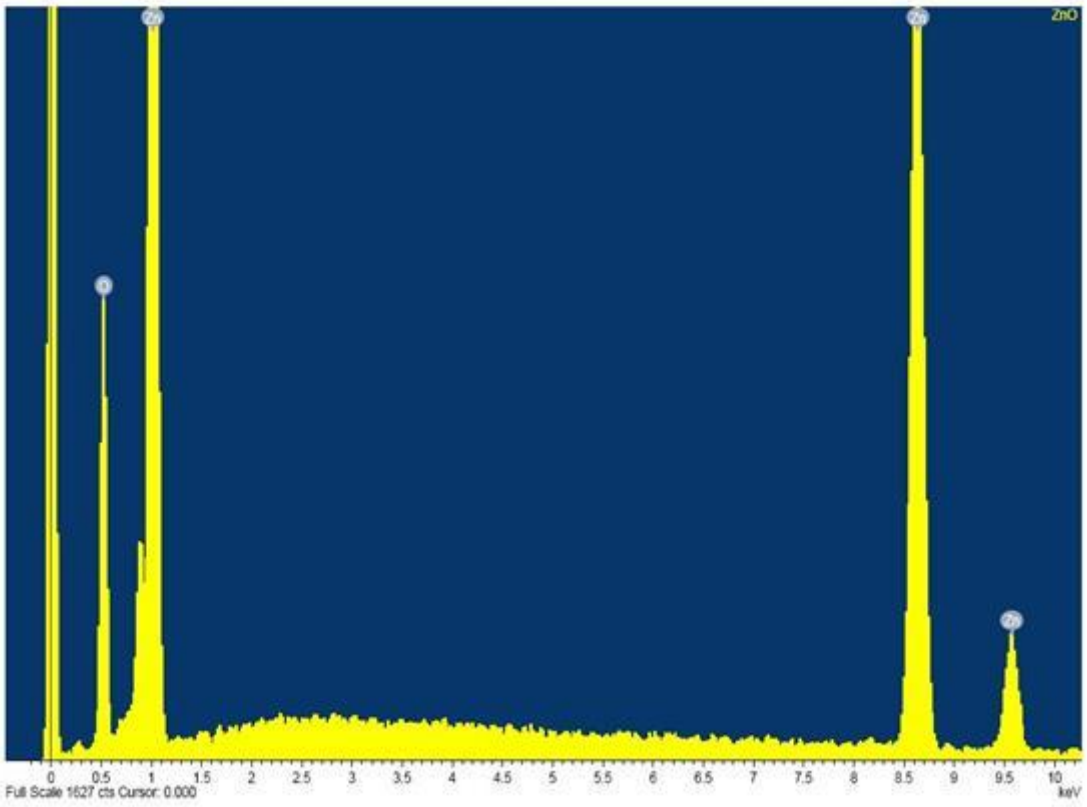
NONE SEI 15.0kV X3,500 1μm WD 10.5mm

(b)


ZnO (002)
ZnO (101)
ZnO (102)
Intensity (a.u.)
30
40
50
60
2 Theta/ deg

(c)


O
Zn
Zn
Zn
ZnO
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10
keV
Full Scale 1627 cts Cursor: 0.000

(d)

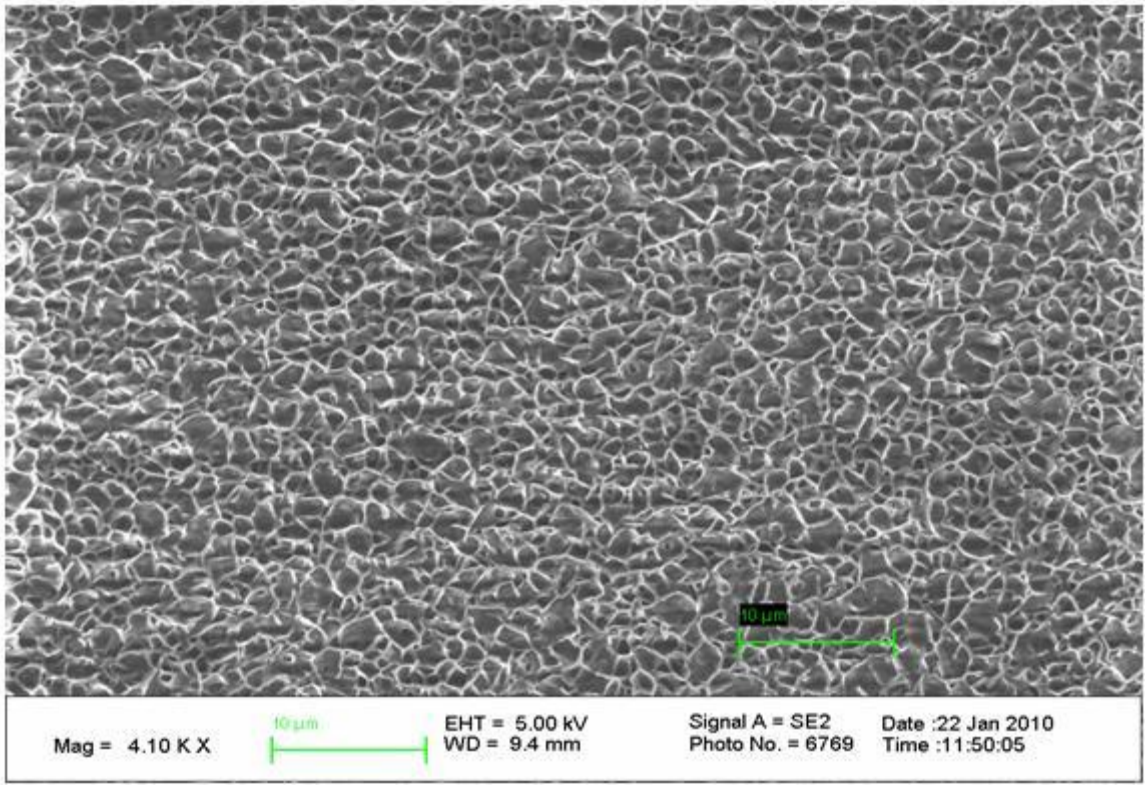
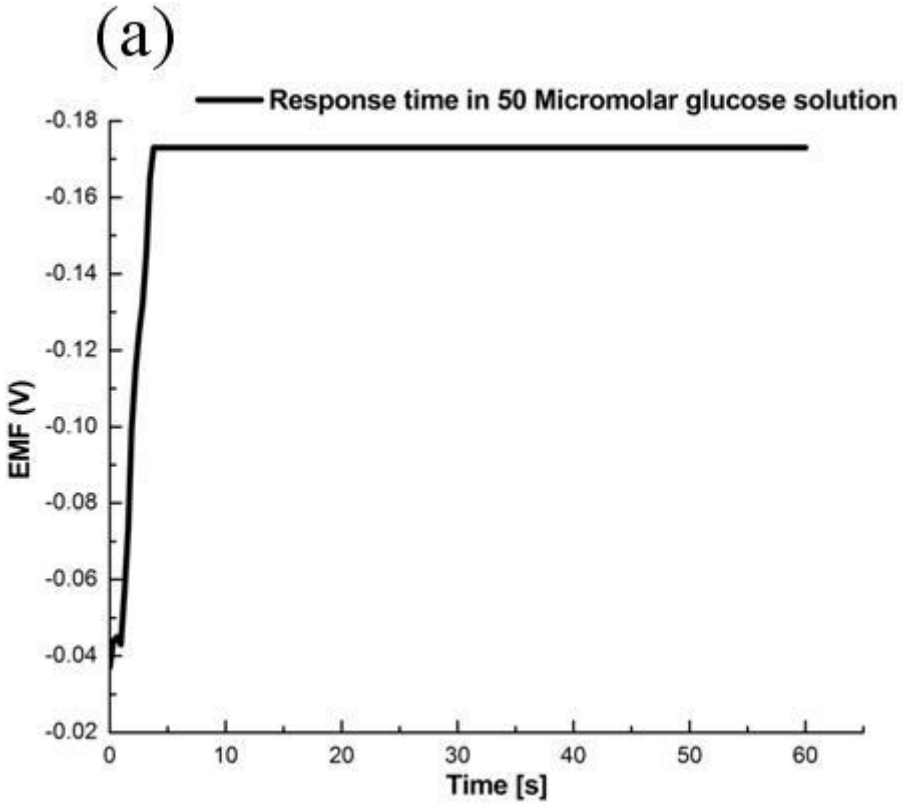

10 µm
Mag = 4.10 K X
10 µm
EHT = 5.00 kV
WD = 9.4 mm
Signal A = SE2
Photo No. = 6769
Date :22 Jan 2010
Time :11:50:05

Figure 2


(a)
Response time in 50 Micromolar glucose solution
EMF (V)
-0.18
-0.16
-0.14
-0.12
-0.10
-0.08
-0.06
-0.04
-0.02
0
10
20
30
40
50
60
Time [s]

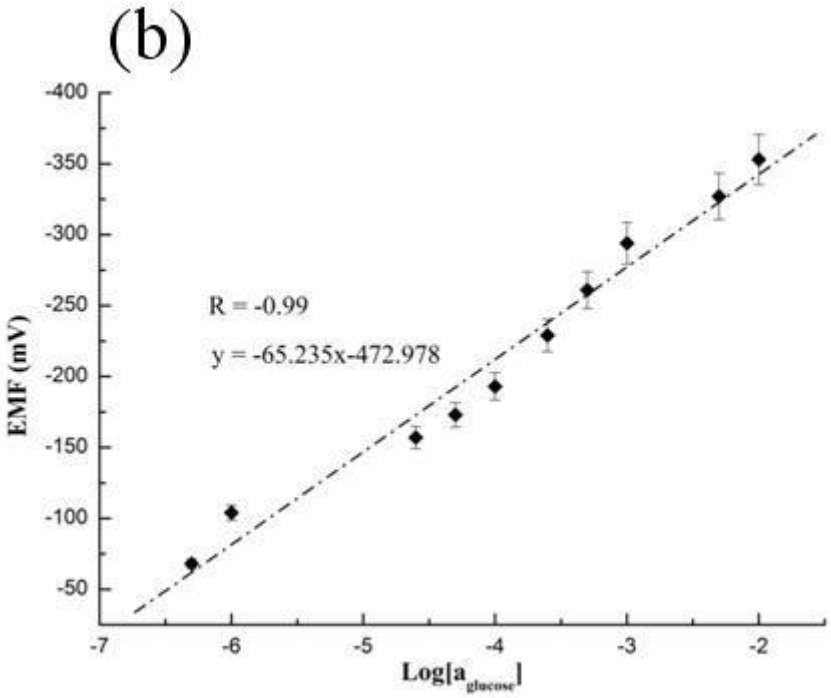

(b)
EMF (mV)
-400
-350
-300
-250
-200
-150
-100
-50
R = -0.99
y = -65.235x-472.978
-7
-6
-5
-4
-3
-2
Log[a glucose]

Figure 3

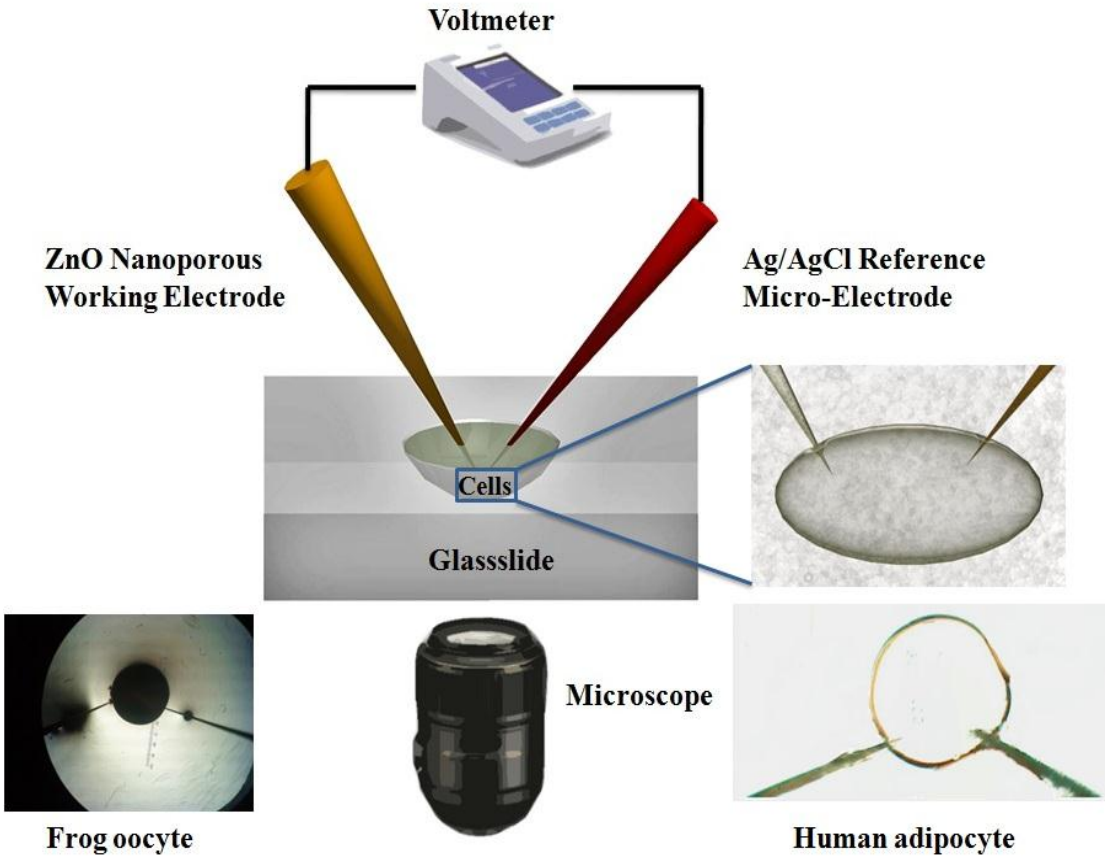

Voltmeter
ZnO Nanoporous
Working Electrode
Ag/AgCl Reference
Micro-Electrode
Cells
Glassslide
Microscope
Frog oocyte
Human adipocyte

Figure 4

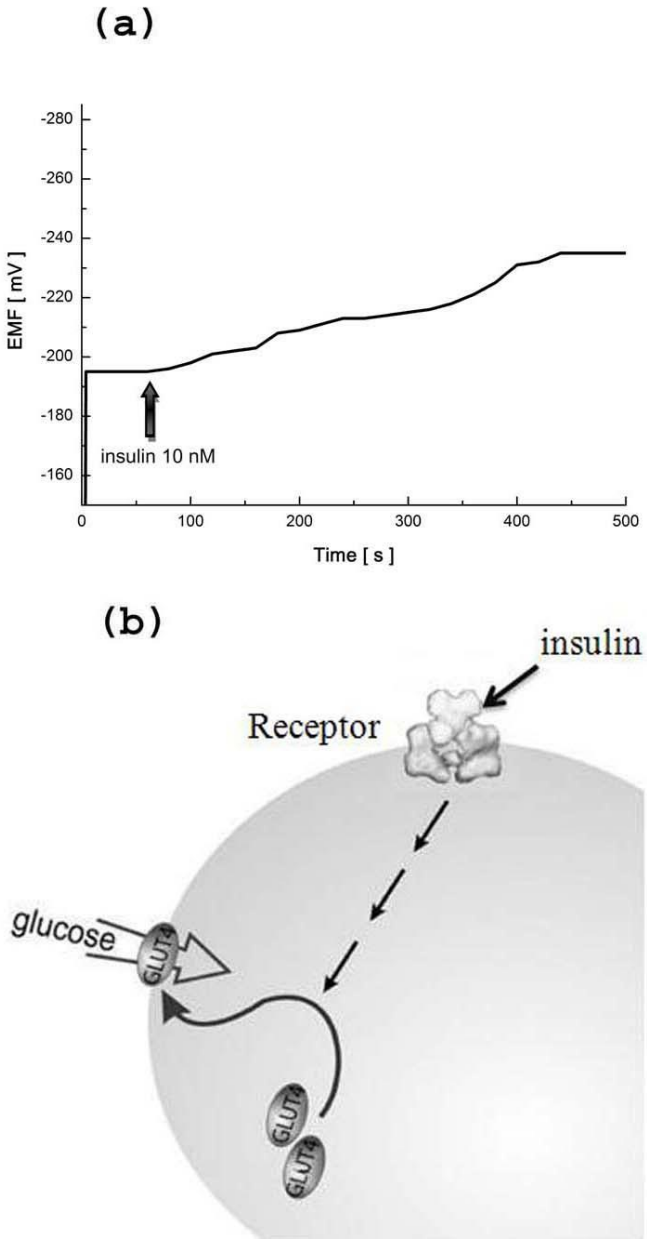
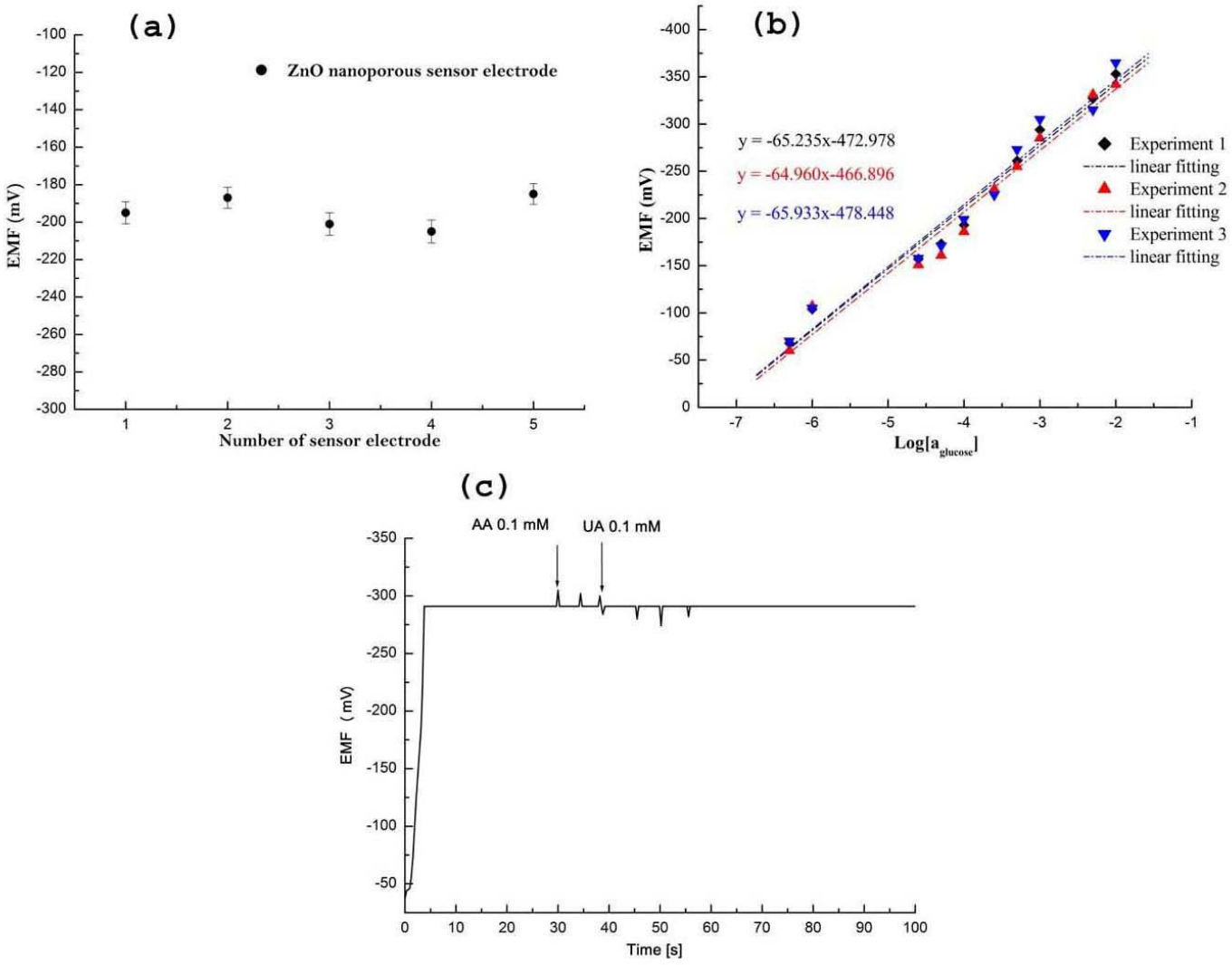

(a)
EMF [mV]
-280
-260
-240
-220
-200
-180
-160
insulin 10 nM
0
100
200
300
400
500
Time [s]
(b)
insulin
Receptor
glucose
GLUT4
GLUT4
GLUT4

Figure 5


(a)
ZnO nanoporous sensor electrode
EMF (mV)
Number of sensor electrode
(b)
y = -65.235x-472.978
y = -64.960x-466.896
y = -65.933x-478.448
Experiment 1
linear fitting
Experiment 2
linear fitting
Experiment 3
linear fitting
EMF (mV)
Log[a glucose]
(c)
AA 0.1 mM
UA 0.1 mM
EMF (mV)
Time [s]

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.


Magnus Willander has M.Sc degrees from Lund (physics), University, 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 seven books.


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. 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 over 200 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.


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