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Intracellular K^+ determination with a potentiometric microelectrode based on ZnO nanowires

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Abstract: The fabrication and application of an intracellular K^+-selective microelectrode is demonstrated. ZnO nanowires with a diameter of 100–180 nm and a length of approximately 1.5 µm are grown on a borosilicate glass microcapillary. The ZnO nanowires were coated by a K^+-ionophore-containing membrane. The K^+-selective microelectrode exhibited a K^+-dependent potentiometric response versus an Ag/AgCl reference microelectrode that was linear over a large concentration range (25 µM - 125 mM) with a minimum detection limit of 1 µM. The measured K^+ concentrations in human adipocytes and in frog oocytes were consistent with values of K^+ concentrations reported in the literature. The sensor has several advantages including ease of fabrication, ease of insertion into the cells, low cost, and high selectivity features that make this type of sensor suitable to characterize physiologically relevant ions within single living cells.

***Index Terms* — ZnO nanowires, intracellular potassium ions, potentiometric nanosensor, ionophore membrane, human adipocytes, frog oocytes, nanotechnology.**

1. INTRODUCTION

Potassium ions (K^+) and other alkali metal ions play important roles in biological systems [1]. For example, K^+ participates in the regulation of the resting membrane potential of a cell and consequently affects excitability and other physiological functions of nerve and muscle [2-3].

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Only about 2% of the total body K^+ resides in the extracellular fluid and its levels are crucial to normal homeostasis [4]. Hypo- and hyperkalemia are the most commonly encountered electrolyte abnormalities in hospitalized patients [5-6], and both alter the resting membrane potential, which can lead to ventricular fibrillation, cardiac arrest and sudden death without any warning clinical signs [7]. Since the abnormal K^+ concentration in blood correlates with several diseases, the monitoring of K^+ in blood has become an important challenge. Various studies have described K^+ sensors based on ion-selective electrodes [8], homogeneous fluorescent K^+ sensing [9-10] and K^+ sensing based on quadruplex-forming oligonucleotides [11-12]. However, K^+ not only plays a critical role in the extracellular solution. K^+ is the main intracellular ion in the body where it participates in several vital functions, such as a cell growth, maintenance of cell volume, DNA and protein synthesis, enzymatic activity, acid base balance, and cellular apoptosis [13]. The purpose of the present investigation is to develop a simple method to determine the intracellular K^+ concentration.

Recently, chemical sensing based on a wide variety of nanostructures such as ZnO nanowires have attracted great interests in the applications of biosensors due to their remarkable properties like non-toxicity, bio-safety, excellent biological compatibility, high-electron transfer rates, enhanced analytical performance, increased sensitivity, easy fabrication and low cost [14-19]. The high isoelectric point of ZnO (IEP 9.5) makes it a good matrix to immobilize low isoelectric point acidic proteins

or DNA by electrostatic interactions with high binding stability [20-22]. In addition, ZnO has high ionic bonding (60%), and its dissolution is very slow at biological pH-values. Due to these various advantages, stable and reproducible signals with respect to analyte-concentration changes are expected to be obtained. Among a variety of nanosensor systems, our proposed nanowire electrochemical sensor is one that can offer high sensitivity, high selectivity and real-time detection, suitable for diagnostic applications.

Intracellular sensors for nutrients such as sugars, metabolic precursors and signaling ligands such as amino acids will help to elucidate the complex roles of these small molecules in biology. Different biosensors have been used for real-time detection, diagnosis, and classification of different forms of biochemical reactions within single cells in order to understand cellular behavior. Such biosensors offer an enormous potential to cell biology research development [23-26]. In most of the intracellular biosensors, an indirect method or a large experimental setup is usually required. A simple technique that utilizes direct intracellular measurement would be of great interest. In our earlier investigations, we have used ZnO nanorods to measure the concentrations of intracellular Ca^{2+} and intracellular glucose [27-28]. In this work, we describe the construction of a simple and highly K^+-selective sensor, capable of penetrating the cell membrane. The sensor is based on ZnO nanowires coated with a thin ionophore-containing polyvinyl chloride (PVC) membrane. A ZnO thin film may also be used but we chose ZnO nanowires due to the higher surface-to-volume ratio thus capturing more ions on the ZnO structure also because the nanosurfaces have other chemical properties. These are the reasons why nanowires are more sensitive, have an extended detection limit and a fast response time as compared to a thin film [29-30]. The PVC-based polymer membranes have been successfully applied in many fields such as the monitoring of food production, environmental pollution and pathological specimens [31-33]. The K^+-selective microelectrode exhibited a linear electrochemical response (EMF) versus an Ag/AgCl reference microelectrode over a wide range of K^+ concentrations, and it was used in measuring K^+ in human adipocytes and *Xenopus laevis* oocytes. The results showed good performance in sensitivity, stability, selectivity, reproducibility, and anti-interference.

2. *EXPERIMENTAL DETAILS*

2.1 Materials and preparation of the K^+- selective membrane

Valinomycin, PVC, potassium tetrakis (4-chlorophenol) borate, bis-(2-ethylhexyl) sebacate, dibutyl phthalate (a plasticizer), $Zn(NO_3)6H_2O$ and hexamethylenetetramine, 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) solution contained 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 135 mM NaCl, with pH adjusted to 7.4. All chemicals used were of analytical reagent grade. The PVC membrane is a thin monolayer and contains the ionophore valinomycin, which has an excellent selectivity for K^+. The molecular structure of valinomycin reminds about the selectivity filter in a K^+-selective ion channel [34-35]. K^+ will then, selectively, interact with the ZnO nanowires. By adjusting the number of the ionophores in the membrane, the life time and the response time of the sensor can be improved. The coating is conformal. The K^+-selective PVC cocktail had the following composition: 1% ionophore, 33% PVC membrane and 66% plasticizer [36]. Finally the membrane coating was prepared by dissolving the composition in tetrahydrofurane.

2.2 Fabrication of the K^+-selective microelectrode

To develop K^+-selective and Ag/AgCl reference microelectrodes for the electrochemical measurements, we appended the borosilicate glass capillaries carefully inside a flat support of the vacuum chamber of an evaporation system (Evaporator Satis CR725) to uniformly deposit chromium and silver films (with thick nesses of 10 and 125 nm, respectively) at the outer surface of the capillary tips. After the evaporation steps, the silver covered microelectrode was dipped into 0.2 M HCl solution and a 3-cm long Ag/AgCl layer was coated on the tip by electrolyzing the silver film by polarizing it at 1.0 V for 1min. Finally the electrode was covered with insulating material, leaving 3 mm of Ag/AgCl exposed at the end of the tip to serve as a reference electrode.

To fabricate the K^+-selective microelectrode, we grew ZnO nanowires on another chromium and silver-coated capillary glass tip using a low-temperature method [37-39]. To grow the nanowires on the capillary tip, the capillary tip was first carefully dipped into a seed solution for two minutes and then dried in air. This procedure was repeated twice. The seed solution contained 0.025 M $Zn(NO_3)_2$ and 0.025 M hexamethylenetetramine. The solution was kept at 90 °C during the growth of ZnO nanowires. Subsequently, the capillary tip was carefully washed by deionized water and dried at room temperature. The ZnO-nanowire layer covered a small part of the silver-coated film (0.01-3 mm). The grown ZnO nanowires aligned perpendicularly to the capillary surface, as shown in figure 1(a-b). The nanowires were uniform in size with a diameter of 100–180 nm and a length of approximately 1.5 µm. The K^+-selective membrane was coated onto the ZnO nanowires by dipping the electrode in the membrane solution for few minutes and leaving it to dry in the air at room temperature for 2.5 hours. A scanning-electron microscope image of membrane-coated ZnO nanowires is shown in figure 1(c). The electrical contact was made on the other end of the Ag film for obtaining an electrical

signal during measurements. 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. These characteristics are necessary for effective bending and gentle penetration of the flexible cell membrane.

2.3 Measurements and calibration

The K^+-selective intracellular measurements were carried out by a potentiometric method using the two microelectrodes described above. The electrochemical response between the electrodes was measured with a Metrohm pH meter model 827. For the time response measurements, a model 363A potentiostat/galvanostat (EG & G Ltd, USA) was used. Initially we performed extracellular measurements for calibration using a standard Ag/AgCl reference electrode. The Ag/AgCl reference microelectrode was calibrated against the standard Ag/AgCl reference electrode. The calibration results showed approximately the same potential difference in all K^+ solutions for concentrations ranging from 1 µM to 125 mM. This potential difference was added to the data obtained with the reference microelectrode.

2.4 Preparation of adipocytes and oocytes

Human adipocytes were isolated by collagenase digestion of pieces of subcutaneous adipose tissue [40] 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 reference [40] and used in a Krebs-Ringer solution buffered with 20 mM HEPES, pH 7.4 and with additives, as in [41]. A glass slide (5cm × 4 cm and 0.17 mm thick) with sparsely distributed adipocytes was placed on a pre-warmed microscope stage set at 37°C.

Female *Xenopus laevis* were anesthetized 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 h. Stage 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 a modified Barth's solution (MBS in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 15 HEPES, 0.33 Ca $(NO_3)_2$, 0.41 $CaCl_2$ and 0.82 $MgSO_4$; pH adjusted to 7.6 by NaOH) supplemented with pyruvate (2.5 mM), penicillin (25 U/mL) and streptomycin (25 µg/mL) at 11°C for 1–5 days before measurements. The measurements were carried out at room temperature (20-23 ^{o}C). The experimental procedures are described in more detail by [42].

3. Results and discussion

The construction of the standard two-electrode electrochemical potential cell can be described by the diagram below:

$$ZnO_{\ s}\,|Zn^{+2}{}_{\ PBS\ sol}||Ag/AgCl_{\ PBS\ sol}\,|Ag''$$

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 three hours after ionophore coating of the ZnO nanowire microelectrode. After this we tested the fabricated microelectrodes inside a 100 µl drop of distilled water and did not obtain any signal from it. When the microelectrodes were inside a droplet containing 1 mM KCl in deionized water a change of the signal was observed within about 30 s. This response behavior indicates that the fabricated sensor is sensitive to a specific metallic ion, i.e. K^+. The latency for the detection of K^+ depends on the number of ionophores located in PVC membrane. With a low density of ionophores it will take a long time to reach equilibrium, while a high density increase the access to the ZnO nanowires hidden under the PVC cover, thus making the response time faster.

3.1 Measurements, calibration and specificity of the K^+-selective microelectrodes

After calibration (see Experimental Details), the electrochemical response (EMF) was measured in the K^+ solutions for the concentrations ranging from 1 µM and 125 mM. The tested sensor configuration showed a large dynamic range with an EMF linearly related to the logarithm of K^+ concentration between 25 µM to 125 mM. The slope was 41.5 mV/ decade as shown in figure 2. The selectivity is very important parameter which describes the specificity towards the target ion in the presence of other interfering ions. To investigate common interferents normally present inside cells we prepared two types of K^+ solutions. One solution was prepared with 85 mM KCl in deionized water. The other one was prepared with 85 mM K^+ in PBS in which we have introduced the possible interfering ions such as 235 mM Na^+, 1 µM Ca^{2+} and 500 µM Mg^{2+}. Good selectivity was observed from the output response curves with and without interfering ions. This can be clearly seen in figure 3(a) that shows that the response of the sensor is quite stable for K^+ in deionized water without interfering ions, while figure 3(b) shows negligible noise in the response due to the mixing of interfering ions in the solutions. The pH effect on the sensor performance was investigated over the pH range from 2 to 10 for 1 µM to 125 mM of K^+. The corresponding potential values obtained were independent of pH in the range 4 to 8. Deviations in sensor potential were observed at pH values above and below this range due to dissolving phenomena of ZnO nanowires.

3.2 Measurements in two different cell types

To measure the electrochemical potential in human adipocytes, we used micromanipulators to move the two electrodes gently into the cell. When the K^+-selective and the reference microelectrodes were inside the cell, the electrochemical potential difference could be recorded and analyzed. The intracellular K^+ concentration in single human adipocyte was 83 ± 10 mM (n = 5), The only other published data we have found for adipocytes is 177 mM in primary rat adipocytes determined from the steady-state accumulation of $^{86}Rb^+$ [43].

In a second set of experiment, we measured the intracellular K^+ concentration in frog oocytes in a similar way. The intracellular concentration was 110 ± 20 mM (n = 5), which was close to what has been reported before: 92 (large oocyte), 117 (mature oocyte), 120 (oocyte with the follicle cell layer), and 150 mM (large oocyte) [44]. In order to study the stability and reproducibility of the fabricated sensor, eight sensor electrodes were prepared independently; four electrodes were used to measure the K^+ concentration in human adipocytes and four other electrodes were used to measure the K^+ concentration in frog oocytes. Figure 4(a-b) shows the good reproducibility for the measurements.

The insertion of thin K^+-selective ZnO-nanowire-based microelectrodes into the cytoplasm of single living cells did not visibly seem to affect cellular viability. However, larger microelectrodes affected the viability of the penetrated cells. Thus, the viability depends strongly on the dimensions of the electrode tip. This study demonstrated that the small size of the sensing microelectrode made it a minimally invasive tool appropriate for monitoring K^+ inside living cells of the size used in the present investigation. The morphology of the membrane-coated K^+-selective microelectrode was checked by scanning electron microscopy directly after measurements. Some debris from the cell and the cell membrane adhere to the electrode tip and possibly this contamination occurs mainly when the electrode tip is pulled out from the cell after the measurements (Fig. 1(d)). In any case the electrochemical 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. We have attempted to clean the stuck cell components from the electrode after intracellular measurements but due to the fragile nature of electrode tip, it is often broken down during cleaning process. Hence we recommend this proposed sensor as disposable sensor.

4. Conclusion

In conclusion, we have described the construction of a K^+-selective microelectrode based on ZnO nanowires grown on a thin glass tip and coated by a valinomycin-containing K^+-selective membrane. This sensor showed a response time of less than 30 s and a quite wide linear range from 25 µM to 125 mM K^+ with minimum detection level of 1 µM. The sensor showed a good performance in sensitivity, stability, selectivity, reproducibility and small interference from other ions. Furthermore, the sensor is easy to fabricate and easy to insert in large cells. The measured intracellular K^+ concentrations in single human adipocytes and frog oocytes were consistent with values found in the literature. These results pave the way to perform biologically relevant measurements of K^+ inside living cells.

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Figure 1

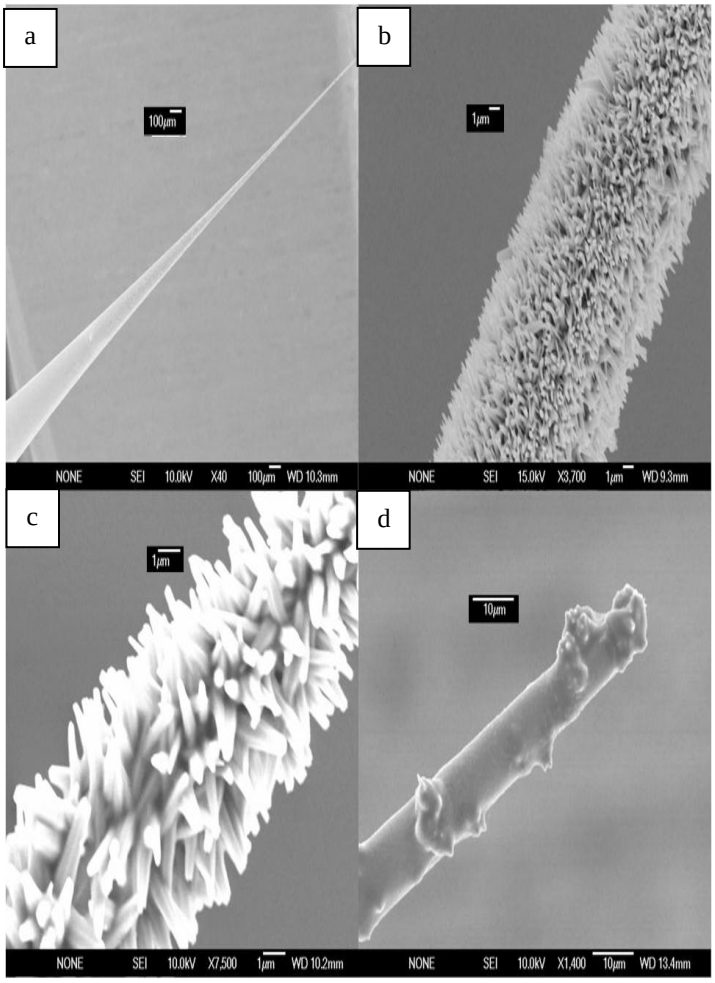


Fig.1 (**a-b)** Scanning electron microscope images at different magnifications of the ZnO nanowires grown on Ag-coated glass capillary using low temperature growth.

Fig.1 (c) The ZnO nanowires covered with transparent layer of K^+ selective membrane.

Fig.1 (d) The K^+ selective electrode after intracellular Measurements.

Legend: Fig.1 (a-b) the size of the scale bars is 100 µm and 1µm respectively where as in parts (c-d) the size of the scale bars are 1 µm and 10µm respectively.

Figure 2

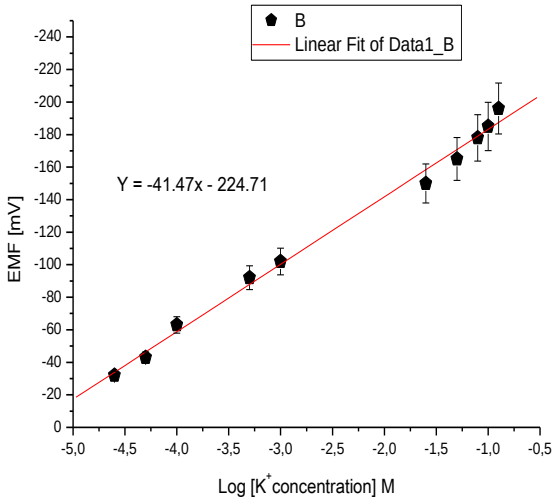
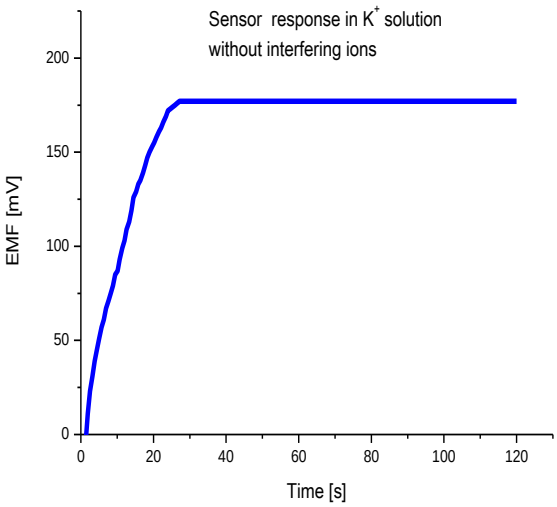


Fig. 2

Calibration curve showing the electrochemical potential difference between the K^+-selective ZnO nanowire-coated microelectrode and the Ag/AgCl reference microelectrode in solutions only containing KCl.

Figure 3
(a)



(b)

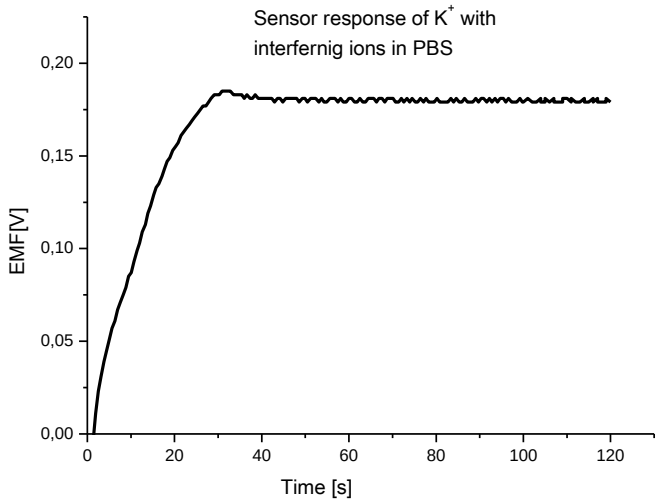
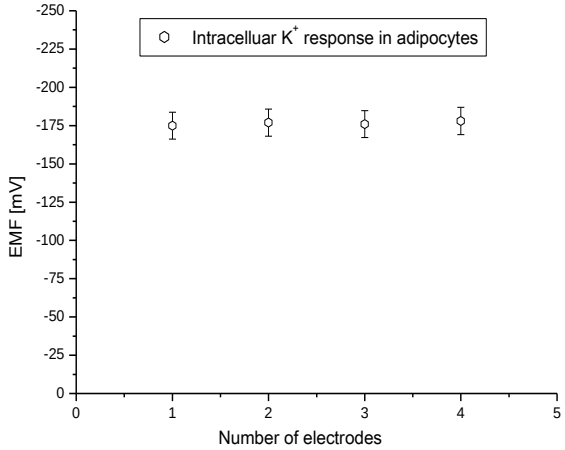


Fig.3 (a-b) The time response of the measurement of the signal in two K^+ solutions of 85 mM KCl without and with interfering ions (Na^+,Ca^{2+} and Mg^{2+})

Figure 4
(a)



(b)

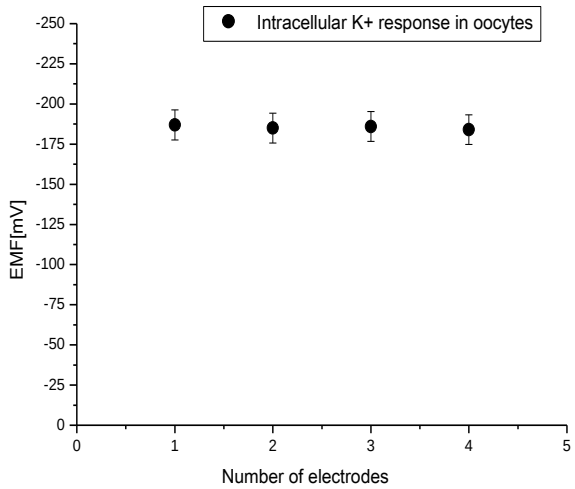


Fig.4 (a-b) The sensor-to-sensor reproducibility of four K^+-selective microelectrodes for human adipocytes (a) and frog oocytes(b)

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Syed Muhammad Usman Ali received the B.E degree in Electronic Engineering from (DCET) NED University of Engineering & Technology Karachi, Pakistan in 1993 and the M Sc. (Electrical Engineering) in Power electronics and computer systems in 2000 from NED university of Engineering & Technology Karachi, Pakistan.

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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.

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