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ZINC OXIDE NANORODS AS AN INTRACELLULAR *p*H SENSOR

Safaa Al-Hilli[a)], *R. Al-Mofrarji*[a)], *P. Klason*[a)], *N. Gutman*[b)], *A. Sa'ar*[b)], *A. Öst*[c)], *P.Strålfors*[c)], *and M. Willander*[a)]

[a)]Department of Physics-Gothenburg University, SE-41296, Gothenburg, Sweden
[b)]Racah Institute of Physics and The Center for Nanoscience and Nanotechnology, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel
[c)]Department Of Cell Biology, Linköping University, SE-58185 Linköping, Sweden.

ABSTRACT

pH measurements using two kind of samples, namely zinc oxide (ZnO) nanorods of 300nm in diameter and 10µm in length grown on 2D macro-porous periodic structures (2DMPPS) and plane n-Si substrates and ZnO nanorods of 60nm in diameter and 500nm in length grown on the silver coated tip of glass capillary (D=0.7µm). We found that the sensitivity of ZnO nanorods increases with reductions in size from (35mV/pH for D=300nm and L=10µm) to (58mV/pH for D=50nm and L=1µm) using the site binding model. The potential difference for the ZnO nanorods electrode vs. Ag/AgCl electrode showed a high sensitivity range for ZnO nanorods grown on 2DMPPS n-Si, as compared to plane n-Si, and had a sensitivity equal to 51.88mV/pH at 22°C for the ZnO on the capillary tip for pH (4-12) in buffer solutions. Vertically nanoelectrodes of this type can be applied to penetrate a single living cell without causing cell apoptosis.

1. INTRODUCTION

ZnO nanorods, nanowires, and nanotubes have recently attracted considerable attention for the detection of biological molecules [1-6]. Among a variety of nanosensor systems, our nanostructure electrochemical probe is one which offers high sensitivity and real-time detection. *p*H sensor miniaturization is highly important since the large surface-to-volume ratio leads to short diffusion distance of the analyte towards the electrode surface, thereby providing an improved signal to noise ratio, faster response time, enhanced analytical performance and increased sensitivity [7]. These results enable the sensitive and rapid detection of biochemical and physiological processes, which are essential to basic biomedical research applications. However, our research is at a very primitive stage and many additional efforts are necessary to obtain reliable instrumentation for intracellular measurements. The sensor in this study was used to detect and monitor real changes in cell behavior using changes in the electrochemical potential at the single cell/ZnO nanorod surface interface in the intracellular microenvironment. An advantage of ZnO nanorod sensors is their small size, which allows intracellular sensing of physiological and biological parameters in nanoenvironments, and a strong, stable, and reversible signal with respect to *p*H changes. The detection sensitivity of the *p*H sensor is achieved by monitoring minimal changes in electrochemical potential caused by binding of biomolecular species on the surfaces of the probe, owing to the high isoelectric point of the material comprising the sensor (in the case of ZnO it is between 9-10) [8]. When a solid emerges in a polar solvent or an electrolyte, a surface charge will develop through one or more of the following mechanisms: preferential adsorption of ions; dissociation of surface charged species; isomorphic substitution of ions; accumulation or depletion of electrons at the surface; physical adsorption of charged species onto the surface. The polar and nonpolar surface structures of *ZnO* nanorods are of interest in understanding the mechanism of interaction of these surfaces with the medium surrounding them. The sensing mechanism is the polarization-induced bound surface charge by interaction with the polar molecules in the liquids. Significant progress in understanding the surface properties of *ZnO* was achieved recently [9,10] stimulated by the importance of this material for a number of applications ranging from cosmetics and medicine to heterogeneous catalysis. The focus of the current study is divided into two stages, first we examine the electrochemical potential response of *ZnO* nanorod surfaces grown on (2DMPPS) and plane *n-type Si* substrates to variations in *pH* (4-11) for buffer and *NaCl* electrolyte solutions using both theoretical (site binding method) and experimental (electrochemical potential method) methods [11]. Second we got nanostructure ZnO nanorods suitable for intracellular pH sensing [12]. Our main effort has been

directed towards the construction of tips capable of penetrating the cell membrane as well as optimization of the electrochemical potential properties. To demonstrate the electrode performance, we apply it in biological media. Our results indicate that the electrode act as an extremely sensitive intracellular pH sensor.

2. ZnO NANORODS ON 2DMPPS AND PLANE n-Si ELECTRODES

2.1. Site Binding Method Calculation

Based on the site binding model, *ZnO* is an inorganic metal oxide with amphoteric surface sites. These sites can protonate or deprotonate, leading to a surface charge and a surface potential that is dependent on the electrolyte solution *pH*. This means that the surface hydroxyl groups may act as proton donors or acceptors, depending on the electrolyte *pH*. The corresponding reactions are:

$$ZnO_{(S)} + 2H_2O = Zn(OH)^{-}_{3(S)} + H^{+}$$
$$ZnO_{(S)} + H^{+} = ZnOH^{+}_{(S)}$$

where K_a and K_b are the dissociation constants. To calculate the surface potential between the sensitive layer and electrolyte interface based on the site binding model is:

$$\psi = 2.303 \cdot \frac{kT}{q} \cdot \frac{\beta}{\beta + 1} \cdot \left(pH_{pzc} - pH\right)$$

where k is the Boltzmann's constant, q is the electronic charge, and β is a parameter, which can be expressed in terms of the acidic and basic equilibrium constants of the related surface reactions, and it is given by:

$$\beta = \frac{2q^2 N_S (K_a K_b)^{1/2}}{kTC_{DL}}$$

Here N_s is the total number of sites per unit area (density of surface OH groups), C_{DL} is the double layer capacitance at the interface which is given by the Gouy-Chapman-Stern model [13], of which the value is mainly determined by the ion concentration of the bulk solution via the corresponding Debye length. Fig. 1 shows the surface potential response to the variation in *pH* for the *ZnO* nanorods length 1μm and diameter 100*nm* grown on plane *Si* substrate and for *ZnO* nanorod length 10μm and diameter 300*nm* grown on 2DMPPS *Si* substrates. From the figure, we observed that the *pH* sensitivity increases with reduction in nanorod size. We observed the Nernstian sensitivity is 58.86*mV/pH* for *ZnO* nanorod size (D=50*nm* and L=1μm) and 35.82*mV/pH* for size (D=300*nm* and L=10μm) at 25°C with pH_{pzc} equal to 9.55. By increasing the surface-to-volume ratio, it is clear from these results that the *ZnO* nanorod sensitivity increases.

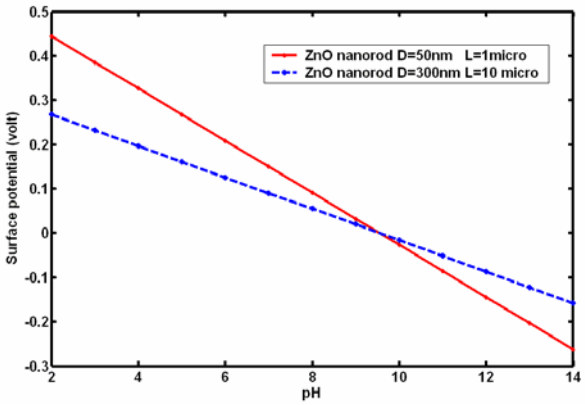

Fig.1. Effect of changing *ZnO* nanorod size on the surface potential using the site binding method simulation.

2.2. Experimental

We assume the electrochemical potentiometric device used here consists of using Ag/AgCl as a reference electrode supplied at a constant potential $E_{Ag/AgCl/Cl-}$, against which we measure the potential of the *ZnO* nanorods redox electrode. The diagram of the electrochemical potential cell in this study can present as:

$$Ag \mid AgCl_{(s)} \mid KCl_{(aq,1M)} \vdots H_2O \mid ZnOH^{+}_{(S)} \mid ZnO_{(S)}$$

for which the device e.m.f. (E) is the potential difference between the supplied potential of the *ZnO* redox working electrode $E_{ZnO/ZnOH+}$ and the supplied potential of the silver/silver chloride reference electrode $E_{Ag/AgCl/Cl-}$:

$$E = E_{ZnO|ZnOH^{+}} - E_{Ag|AgCl|Cl^{-}}$$
$$E = E^{o} + (0.05915)\lg\left(\frac{[ZnOH^{+}]}{[ZnO]}\right) + (0.05915)\cdot pH - E_{Ag|AgCl|Cl^{-}}$$

where E^o is the standard electrode potential of the *ZnO* redox electrode. For the dilute solutions used here, ion activities have been substituted by molar concentrations.

In our case, we assume the shape of the *ZnO* nanorods grown on 2DMPPS *Si* substrate with a varying radius between 300-500*nm* and lengths between 10-15μm (see Fig. 2a,b) and for that grown on plane *n-Si* substrate is hexagonal, with a varying radius between 50-100*nm* and lengths between 1-2μm (see Fig. 2c,d). At 25°C, the Ag/AgCl reference electrode is stated to have a potential of 0.22234V [13]. In Fig. 3a & b, where the solid line represents the calculated electrochemical potential difference, the dash line represents the buffer solution, and the dotted line represents the *NaCl* solution vs. *pH* ranging from 4 to 11 for *ZnO* nanorods grown on plane and 2DMPPS *Si* substrates, respectively. Simplified behavior is a function of *pH*, where *ZnO* nanowires serve as the working electrode vs. the Ag/AgCl reference

electrode, and the electrochemical potential difference decrease with increasing *pH*. We know from site binding simulation experiments that the *pH* sensitivity increases with reductions in nanorod size, but in our experiment we found *ZnO* nanorods with big dimensions that grew on 2DMPPS at higher sensitivity (70.017 for buffer and 63.7 for *NaCl* solutions) relative to the *ZnO* nanorods with small dimensions that grew on plane *Si* (44.271 for buffer and 53.703 for *NaCl*). We believe that the high sensitivity resulted from both *ZnO* nanorods and the use of 2DMPPS as a substrate, which increases the number of site binding and increases *ZnO* nanorod size. By reducing *ZnO* nanorod size, the sensitivity will increase because of the increased surface-to-volume ratio, and the hexagonal shape of the nanorod that includes many shape edges with defects in the crystal structure. We found that the calculated pH_{pzc} is 9.3 for the *ZnO* nanorod grown on plane *Si* substrate (D=50*nm* and L=1μm) and 10.8 for the *ZnO* nanorod grown on the 2DMPPS (D=300*nm* and L=10μm), which means that there is a pH_{pzc} shift to low values with a reduction in *ZnO* nanorod size. We expect that if the *ZnO* nanorods grown on 2DMPPS *Si* substrate have similar dimensions to that on plane *Si* substrate, the sensitivity of the device will be higher.

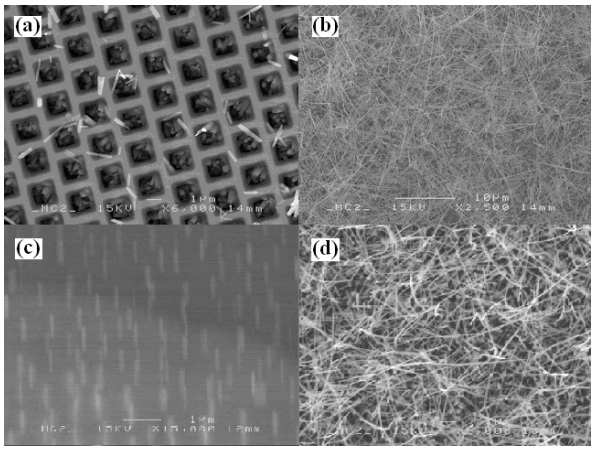

Fig. 2. SEM images of the *ZnO* nanostructures (a) nanorods (b) nanowires grown on 2DMPPS *Si* substrate; (c) nanorods (d) nanowires grown on plane *Si* substrate before the *pH* measurements.

3. ZnO NANORODS ON SILVER COATED OF GLASS CAPILLARY ELECTRDES

The fabrication of electrochemical potential ZnO nanorods was preformed by the growing of a hexagonal single crystal of ZnO nanorods on silver-coated capillary glass using a low temperature growth method described previously [14-16]. It can be seen that the nanostructure is a rod like shape with a hexagonal cross section and primarily aligned along the perpendicular direction of the capillary, a typical morphology of wurtzite ZnO structure. The nanorods are uniform in size with a diameter of 60-80nm and a length of 500-700nm [12].

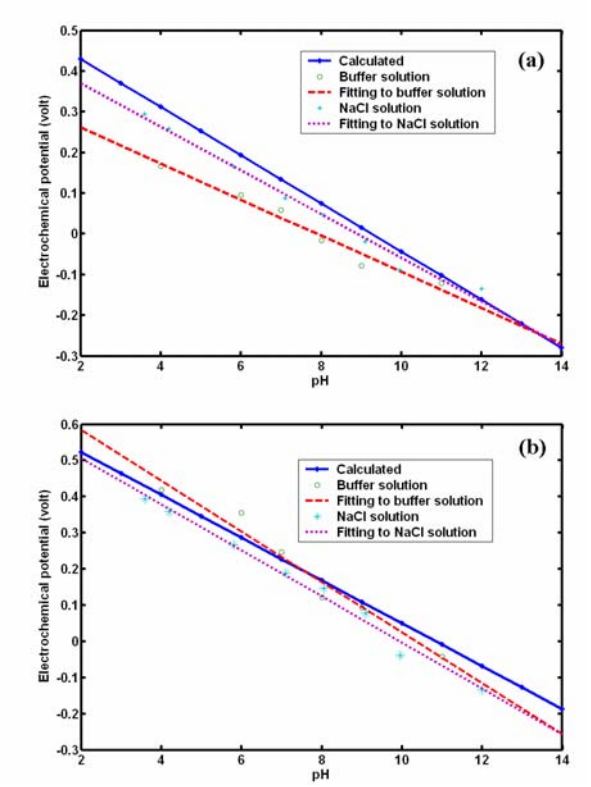

Fig. 3. Electrochemical potential vs. *pH* comparison curves between calculated and experimental measurements fitted to a linear equation for *ZnO* nanorods grown on (a) plane and (b) 2DMPPS *n-Si* substrates immersed in buffer and *NaCl* solutions.

3.1. Potentiometric Measurement

A two-electrode configuration was employed for microliter-volumes in electrochemical studies consisting of ZnO nanorods as the working electrode and Ag/AgCl as a reference micro-electrode. All electrochemical experiments were conducted using a Metrohm pH meter model 827 at room temperature (22 ± 2^{o}C). The response of the ZnO nanorod electrochemical potential difference (as a working electrode versus the Ag/AgCl reference micro-electrode) to the changes in standard buffers at room temperature (potassium phthalate pH 4.0, sodium-potassium phosphate pH 6.0, sodium phosphate-potassium phosphate pH 7.0, Hydrochloric acid-borate pH 8.0, Boric acid-sodium-potassium borate pH 9.0, and Boric acid-sodium-potassium borate pH 11) was measured and shows that this pH dependence is linear and has a sensitivity equal to 51.881 mV/pH at 22^{o}C (*see* **Fig.**

4). Electrodes reading less than 50 mV per pH were discarded. The measurements were started immediately after placing the ZnO nanorods and the Ag/AgCl reference microelectrode in the electrolyte drop. To make certain that variations in the tip potential of the reference side due to differences in the ionic strength of the standard buffers and the cytoplasm of the cell would not cause errors in pH measurement; we tested the electrodes in a potassium phosphate buffer simulating the internal environment of the cell. The measurement duration did not exceed five minutes to avoid significant changes in electrolyte concentration due to evaporation and to maintain the dissolving behavior and stability of the ZnO nanorods [17].

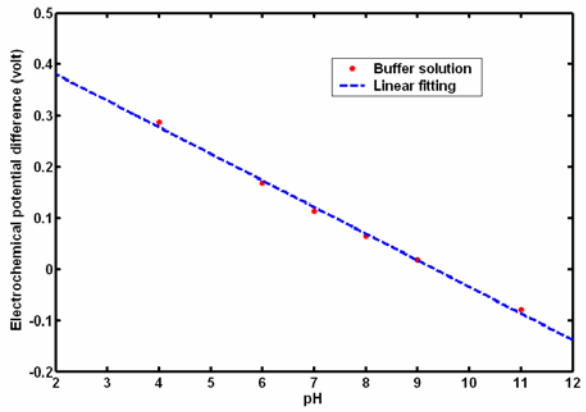

Fig. 4. Calibration curve showing the electrochemical potential difference for the ZnO nanorods as a working electrode with an Ag/AgCl reference micro-electrode, versus pH changes for buffer solution.

3.2. Intracellular pH in A Single Human Adipocyte or Fat Cell

The acid and base properties of electrolytes in living cells play an important role in any biological process, as the *p*H value is the most critical parameter in chemical and biochemical reactions. We used the ZnO nanosensor to measure intracellular pH in a single human adipocyte or fat cell. A glass slide substrate (5cm length, 4cm width, and 0.17mm thickness) with sparsely distributed fat cells was placed on the pre-warmed microscope stage set at 37^{o}C. The pH nano-electrode, mounted on a micropipette holder of a micromanipulation system, was moved into position in the same plane as the cells. The ZnO nano and reference electrodes were then gently micro-manipulated into the cell using the hydraulic fine adjustments. They were inserted past the cell membrane and extended a short way into the cell. Once the ZnO nanorod working electrode and the Ag/AgCl reference micro-electrode were inside the cell, the electrochemical potential difference signal detected and identified the proton activity (pH). A signal reading was taken with the nano-electrode inside the cell (pH=6.81) (*see* **Fig. 5**). A typical experimental measurement required approximately five minutes. In this work, we found the measured pH value (6.81) to be close to reported values for intracellular pH (6.95-7.57) in rat brown adipocytes [18] or (6.85-7.05) in rat hepatocytes [19], using an indirect determination of pH.

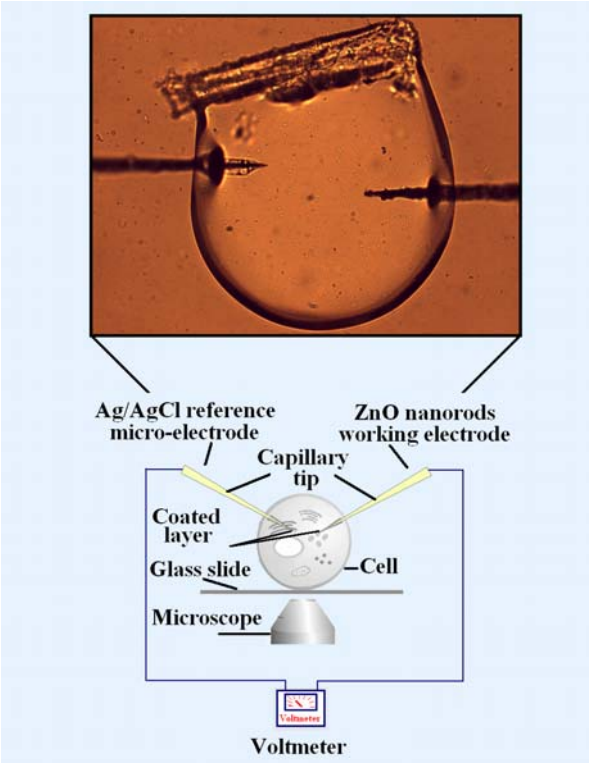

Fig. 5. Optical image and schematic diagram illustrating intracellular pH measurements performed in a single human fat cell using ZnO nanorods as a working electrode with an Ag/AgCl reference micro-electrode.

3.3. Cell Viability

Placing pH microelectrodes inside cells causes some damage, but the membrane potential is measured and the damage can be assessed. The damage usually consists of a 'leak' around the electrodes. This leak rarely causes a large change in intracellular pH because intracellular buffering power is high and the pH gradient across the cell membrane is low. The extent to which the membrane potential reflects the amount of damage is, of course, dependent upon the input resistance of the cell. The damage to large cells is less than that of small cells. However, in both small and large cells, the damage, if

sufficient, will lead to a large influx of other ions such as calcium and sodium. This physical damage to the integrity of the cell membrane has limited the used of pH microelectrodes to large cells and represents a constant source of anxiety for those using ion-sensitive microelectrodes.

The viability of the penetrated cells depends strongly on the size of ZnO nanorods. We used ZnO nanorods (80nm in diameter and 700nm in length) grown on one side of the capillary glass with a 0.7μm tip diameter, so the total diameter of the tip is 1.5μm. By reducing the size of ZnO nanorods, the total diameter of the tip was reduced, which, in turn, increased cell viability and the sensitivity of the device increases. (*see* **Fig. 6**).

The introduction of ZnO nanorod pH sensors into a single cell's cytoplasm to measure the intracellular pH does not visibly seem to affect cellular viability. This has been empirically established in several experiments in which, after ZnO nanorod penetration and equilibration for five minutes, the electrode was withdrawn and the cells were monitored by microscope. This study demonstrated that ZnO nanorods are minimally invasive tools appropriate for monitoring pH changes inside living cells.

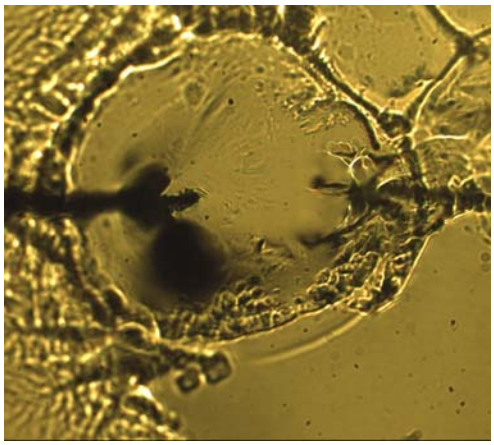

Fig. 6. Dead adipocyte during the intracellular pH measurement using ZnO electrode.

3.4. Probe Usability

The ZnO nanorod electrode was used to obtain only one measurement at a time and was not reused. We made calibration measurements of the solution surrounding the cell after the measurement inside the cell to obtain a quantitative estimation of the detection signal. For these calibration measurements, the ZnO nanorod electrode was placed in the solution surrounding cells directly after the intracellular measurement and a pH reading of 6.77 was obtained where the actual pH value of the surrounding solution was 7.4. We believe that the difference between actual and measured pH values resulted from the strong association of cell materials with the electrode (*see* **Fig. 7**).

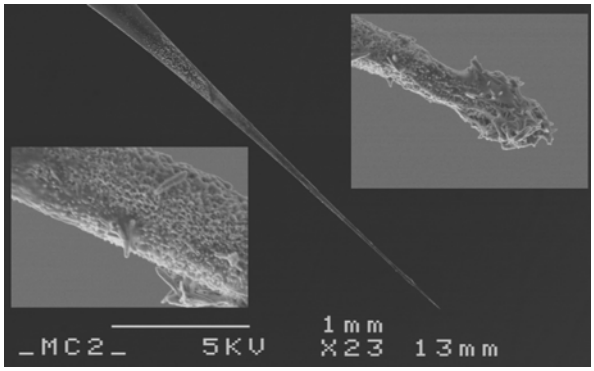

Fig. 7. The strong association of binding to cell materials with the ZnO nanorod electrode after the experiment.

4. SUMMARY

It is clear that these molecules are bonded by van der Waals type interactions and they generate surface charge changes induced by *ZnO* polarization. Different chemicals are likely to exhibit different degrees of interaction with the *ZnO* surface. The linear response to changes in the *pH* range 4-12 for *ZnO* nanorod structures suggested that this results from the high value of number of sites on the nanorod surface. We have used ZnO nanorods as electrochemical nanobiosensors and used them to detect the intracellular pH of a human fat cell. These results demonstrated the capability of performing biologically relevant measurements inside single living cells. The ZnO nanorod pH electrode thus holds promise for minimally invasive dynamic analyses of proton and hydroxyl groups in biochemical pathways within single living cells. Future applications of the ZnO nanorod pH electrode could include reducing the size of the tip sensing part and getting more multi-analyte detection measurements.

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