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Functionalized ZnO nanorod-based selective magnesium ion sensor for intracellular measurements

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ZnO nanorods were grown on a silver-coated tip of a borosilicate glass capillary (0.7 µm in tip diameter) and used as selective potentiometric sensor of intracellular free Mg^{2+}. To functionalize the ZnO nanorods for selectivity of Mg^{2+}, a polymeric membrane with Mg^{2+} -selective ionophores were coated on the surface of the ZnO nanorods. These functionalized ZnO nanorods exhibited a Mg^{2+}-dependent electrochemical potential difference versus an Ag/AgCl reference microelectrode within the concentration range from 500 nM to 100 mM. Two types of cells, human adipocytes and frog oocytes, were used for the intracellular Mg^{2+} measurements. The intracellular concentration of free Mg^{2+} in human adipocytes and frog oocytes were 0.4 – 0.5 and 0.8 – 0.9 mM, respectively. Such type of nanoelectrode device paves the way to enable analytical measurements in single living cells and to sense other biochemical species at the intracellular level.

Key words; ZnO nanorods, Potentiometric, Mg^{2+}-selective membrane, Human adipocyte, Frog oocyte

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1. Introduction

The magnesium ion (Mg^{2+}) is the most abundant intracellular divalent cation in living cells, where it plays a major biological role in its involvement with phosphate compounds and phosphate metabolism, binding in chlorophyll pigments, and acting as a critical cofactor in numerous enzymatic reactions (Frausto da Silva and Williams, 2001). Mg^{2+} also influences the nervous impulse, tension development in muscle, and modulates amongst others the ionic transport in nerve and mitochondria (Gunther, 1977; Shine, 1979; Acherman et al., 1983; Frausto da Silva and Williams, 2001; Elinder and Århem, 2003). There is an increased demand for selective, sensitive, and fast chemical sensors to explore the physiological environment. In particular, concentrations of compounds in the intracellular compartments are difficult to measure.. Here we describe a new Mg^{2+}-selective probe to measure intracellular Mg^{2+} concentration. The development of miniaturized ion-selective electrodes has received extensive attention during the recent years and these microelectrodes are possible to use for intracellular measurement as we have demonstrated in previous investigations (Asif et al., 2009; 2010).

Potentiometric solid-state ion sensors covered with, ionophore-doped polymeric membranes have been used because of their excellent selectivity toward the ion of interest and there is a wide range of ions for which ionophores are available. Potentiometric sensors are also easy to miniaturize and usually provide a large dynamic range. In conventional ion-selective electrodes, polyvinyl chloride is the most commonly used matrix as the selective membrane (Mascini and Pallozzi, 1974; Seo et al., 1997). The ion-selective membrane exhibits the selectivity with which the sensing material responds to the analyte and an electrochemical equilibrium is reached. The resulting

potential difference, formed between the phases, will then be governed by the activity of this specific ion in the two solution phases, (Ganjali et al., 2006; Faridbod et al., 2007).

Zinc oxide (ZnO) nanostructures is a semiconducting and piezoelectric material that has potential applications in optoelectronics, piezoelectric sensors, resonators, surface acoustic wave devices and transducers (Wang, 2005; Zhao et al., 2004). It has relatively high piezoelectric coefficients, a wide band gap (3.4 eV), near UV emission, and transparency to visible light. ZnO has attracted interest for applications in chemical sensing. ZnO nano-rods can be employed as bio-chemical physiological sensors with improved sensitivity and selectivity. They can be chemically customized to suit a wide variety of applications. With their ability to react rapidly and with extreme sensitivity such new materials may dramatically improve sensing technology and in combination with other functional multi-scale materials, they can open many new opportunities for sensors in biomedical applications. Before proceeding, we define the sensors of interest here to be those called electrochemical sensors. They are divided into conductometric, impedimetric, potentiometric and amperometric. An electrochemical sensor is a sensor that deals with the electron transfer, consumption, or generation during a chemical or biochemical process. It is also important to note that, a potentiometric sensor measuring a voltage, such as the ion-sensitive field-effect transistor or ion-selective electrode, is scale invariant, while amperometric and conductometric sensors on the other hand measures currents and are sensitive to miniaturization. The reduction in sensor size is necessary to sense compounds intracellularly. To illustrate this, we consider the sensitivity of a sensor as we miniaturize our electrodes. Researchers are integrating functionalized ZnO nanorods for a variety of sensor applications to meet urgent needs in fields ranging from

biomedicine to biochemistry (Zhao et al., 2003; Batista and Mulato, 2005; Kang and Ren, 2005; Dulub et al., 2005; Arab et al., 2003). For this investigation a miniaturized sensor is used for the smallest possible sample size to detect the smallest concentration of molecules of interest.

Changes in the intracellular concentration of Mg^{2+} affect many different processes in cells and have for instance been coupled to metabolism and control of glucose homeostasis (Paolisso et al., 1990). In adipocytes, a major cell type for the control of energy homeostasis, the intracellular concentration of free Mg^{2+} has been shown to be affected by pioglitazone, which belongs to a class of antidiabetic agents that affect insulin sensitivity (Nadler and Scott, 1994). Likewise, dietary supplementation with Mg^{2+} improved insulin sensitivity (Paolisso et al., 1994). However, not much is known about the control of intracellular concentrations of Mg^{2+} in adipocytes. A major reason for this is the lack of suitable detection techniques. In previous investigations we have measured the concentrations of intracellular calcium and glucose using ZnO nanorods (Asif et al., 2009; Asif et al., 2010). The focus of the current study is the demonstration of a ZnO nanorods-based sensor suitable for selective detection of intracellular Mg^{2+}. We have demonstrated their utility by determining the concentration of Mg^{2+} in isolated human fat cells and frog oocytes.

2. Materials and Methods

2.1. Fabrication of the microelectrodes

To selectively measure the intracellular Mg^{2+} concentration, we used two microelectrodes: 1) An Ag/AgCl electrode (Fig 1A) as the intracellular reference

microelectrode, and 2) a ZnO nanorod-decorated electrode (Fig 1B-D) coated with ionophore-containing membrane (Fig 1F) as the intracellular Mg^{2+}-sensing microelectrode. The electrochemical potential difference recorded in this way measures the difference in electrochemical surface potential generated near the electrodes. The details and optimization steps regarding preparations of silver-coated microelectrodes are given in the references (Asif et al., 2009; Asif et al., 2010). The silver film was connected with a copper wire (0.5 mm in diameter) and fixed with of high-purity silver conductive paint. The Ag/AgCl reference microelectrode was made by dipping the silver-coated end of the microelectrode in 0.2 M HCl solution and then by electrolyzing the silver film to form AgCl by polarizing to 1.0 V for one minute. A 3 cm long Ag/AgCl layer was coated on the tip of the microelectrode and covered with insulating material, leaving 3 mm of Ag/AgCl exposed at the tip.

The other silver-coated microelectrode, the Mg^{2+}-sensing microelectrode, was made by growing ZnO nanorods on the outer tip using a low-temperature method (Asif et al., 2009). This microelectrode is shown by Field emission Scanning electron microscope at different magnifications in Fig. 1(B-D). The ZnO nanorods are uniform in size with a hexagonal cross section of around 70 nm in diameter and a length of about 1μm and primarily aligned along the perpendicular direction to the electrode.

A ZnO nanorod grown on the glass tip has been studied by high resolution analytical transmission electron microscopy (TEM) using Tecnai G2 UT instrument operated at 200 kV with 0.19 nm point resolution. The TEM specimen was made by scraping the nanorods onto a copper grid with carbon film. TEM images exhibit that the rod axis is [0001] direction Fig.1G.

2.2. Covering of ZnO nanorod with a Mg^{2+} -selective membrane

The ZnO nanorod layer on the silver-coated microelectrode was coated with ionophore-containing liquid polymeric membrane by a manual procedure (Fig 1F). 120 mg polyvinyl chloride was dissolved in 5 mL tetrahydrofuran together with 10 mg of the plasticizer dibutyl phthalate and 10 mg of the Mg^{2+}-specific ionophore 4,5-Bis(benzoylthio)-1,3- dithiole-2-thione (Bz2dmit). This ionophore exhibits the best result for potentiometric sensing of Mg^{2+} (Zamani et al., 2008). All chemicals were from Sigma-Aldrich. The ZnO-coated microelectrode was dipped twice into the prepared solution. After each dip the electrodes were allowed to dry at room temperature. Finally to condition the microelectrode for selectivity of Mg^{2+}, it was dipped into a 10 mM $MgCl_2$ solution (Asif et al., 2008).

2.3. Electrochemical measurements

In a complete potentiometric cell, the Mg^{2+}-selective microelectrode is used in conjunction with a reference microelectrode. The electrochemical potential between the Mg^{2+}-selective and the Ag/AgCl reference microelectrodes was measured with Metrohm pH meter model 827. The Ag/AgCl reference microelectrode was calibrated externally versus an Ag/AgCl bulk reference electrode. Calibration shows approximately constant potential difference using $MgCl_2$ solutions through the Mg^{2+} range from 500 nM to 100 mM.

2.4. The cellular preparations

After making the calibration curve with different $MgCl_2$ solutions, the probe was used to selectively measure the Mg^{2+} in two types of cells: human adipocytes (fat cells

Fig 2A) and frog oocytes (egg cells Fig 2A). The experimental setup for the intracellular measurements is shown in Fig.2A Fig. 2B shows the role of intracellular Mg^{2+}. Only 1% to 3% of the total intracellular magnesium exist as free ionized form (James et al., 1999).

Primary 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. Cells were incubated overnight before use as described in (Danielsson et al., 2005). For the experiments cells were transferred to a modified Krebs-Ringer solution buffered with 20 mM Hepes, pH 7.4, as detailed in (Danielsson et al., 2005). A glass slide (5 cm length, 4 cm width, and 0.17 mm thickness) with sparsely distributed fat cells was placed on the prewarmed microscope stage set at 37°C. The Mg^{2+}-selective and the reference microelectrodes, mounted on a micromanipulator, were gently micromanipulated a short way into the cell by using hydraulic fine adjustments, through the cell membrane.

Oocytes were isolated from ovarian lobes cut off through a small abdominal incision from female *Xenopus laevis* frogs anesthetized in a bath with tricaine (procedure approved by the local Animal Care and Use Committee at Linköping University). Stage III and VI oocytes (approximately 1 mm in diameter) without spots and with clear delimitation between the animal and vegetal pole were selected. All details regarding preparation of oocytes and the solutions used are described in (Börjesson et al., 2010).

3. Results and discussion

3.1. Characterization and calibration of ZnO nanorods

Field emission scanning electron microscope and high resolution transmission electron microscopy showing the structure of the ZnO nanorods grown by aqueous

chemical method on capillary glass tip is shown in Fig. 1. The potentiometric measurements were carried out for different Mg^{2+} concentrations varying from 500 nM to 100 mM. The experimental setup for the intracellular measurements is shown in Fig. 2A. The electrochemical cell voltage (electromotive force) is changed when the composition of the test electrolyte is modified. These changes can be related to the concentration of ions in the test electrolyte via a calibration procedure (Fig. 3A). The actual electrochemical potential cell can be described by the diagram below:

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

After functionalizing the ZnO nanorods with the Mg^{2+}-selective membrane, the measurements were started for calibration. The electrochemical potential difference between the Mg^{2+}-selective and the reference microelectrodes was measured for different Mg^{2+} concentration in the range from 500 nM to 100 mM and shows that the Mg^{2+} dependence is linear and has sensitivity equal to 26.1 mV/decade at around 23°C (Fig. 3A). This linear dependence implies that such sensor configuration can provide a large dynamical range.

3.2. Selectivity and stability of the setup

Selectivity is the most important characteristic which describe the specificity towards the target ion in the presence of other ions (interfering ions). There are a number of different methods to check the potentiometric selectivity (Ganjali et al., 2006): 1) The separate solution method, 2) the mixed solution method, 3) the matched potential method, and 4) the unbiased selective coefficients. Instead of using the above mentioned methods, we checked the selectivity and stability of the sensor by output response curve with (black and green) and without (red) interfering ions as shown in Fig. 3B. The red curve

shows the output response without any interfering ions in 1 µM $MgCl_2$ solution which show the good stability after reaching at stable point. The black curve shows that as soon as we introduced the 10 µM interfering ions such as Ca^{2+}, Na^{+}, K^{+} in a 1 µM $MgCl_2$ solution, we disturbed the stability for a short time, but after these initial disturbances the signal became stable and reached the same value of as before the introduction of the interfering ions. The green curve shows the output response with same amount of interfering ions in 100 µM $MgCl_2$. Here only small peaks were observed during the measurements. The pH dependence of the electrode was tested over the pH range from 2.0 to 9.0 for 500 nM to 100 Mm Mg^{2+}. The corresponding potential values obtained were independent of pH in the range 4.5 – 8. Above and below these pH values deviations in potential were observed. In order to check the reproducibility of the fabricated sensor, we have used three electrodes to measure Mg^{2+} concentration in frog oocyte and human adipocyte. Fig 3C shows the good reproducibility of the developed sensors for intracellular measurements.

Mg is a strong acid. Consequently there should be reasonably good interaction between oxygen atoms and Mg^{2+}. It is expected that the covalent functionalization is a chemical process in which a strong bond is formed between the nanostructured material and the biological or chemical species. In most cases, some previous chemical modification of the surface is necessary to create active groups that are necessary for the binding of biological or chemical species (Takahashi et al., 1987).

3.3. Measurements of intracellular free Mg^{2+} concentrations

We measured free concentration of intracellular Mg^{2+} in single human adipocytes and frog oocytes by micromanipulating the Mg^{2+}-selective and the reference

microelectrodes gently into the cells (Fig. 2A). Once the microelectrodes were inside the cell, that is isolated from the extracellular buffer solution, an electrochemical potential difference signal was detected identifying the activity of Mg^{2+}. The measured intracellular Mg^{2+} concentration for human adipocytes was 0.4 to 0.5 mM and for frog oocyte it was 0.8 to 0.9 mM, closely consistent with the intracellular concentration reported in the literature (Nadler and Scott, 1994; Gabriel and Gunzel, 2007; Vemana et al., 2008; Lyashchenko and Tibbs, 2008).

When the functionalized working electrode was removed outside the cells after measurements, it was monitored by scanning electron microscope (Fig. 1E), which shows that the ZnO nanorods were not fully dissolved. The viability of the cells depends strongly on the size of the ZnO nanorods. We can improve the life time of cells by reducing the size of the used nanorods.

4. Conclusion

Hexagonal ZnO nanorods were grown on a sub-micron silver-covered capillary glass tips. Then the ZnO nanorods were functionalized by covering them with the Mg^{2+}-selective membrane. The potential difference between the Mg^{2+}-selective and the reference microelectrodes was found to be linear over a wide concentration range of Mg^{2+} (500 nM to 100 mM). The measured intracellular Mg^{2+} concentrations in single human adipocytes and frog oocytes were consistent with values found in the literature. ZnO nanostructures have unique advantages including high surface to volume ratio, nontoxicity, chemical stability, electrochemical activity, and high electron communication features, with high ionic bonding (60%) which make them one of the

most promising materials for biosensor application. Moreover, the fabrication method in our experiment is simple and excellent performance of the developed nanosensor regarding sensitivity, stability, selectivity, reproducibility and anti-interference was achieved. These results pave the way to perform biologically relevant measurements of Mg^{2+} inside living cells.

Acknowledgement:

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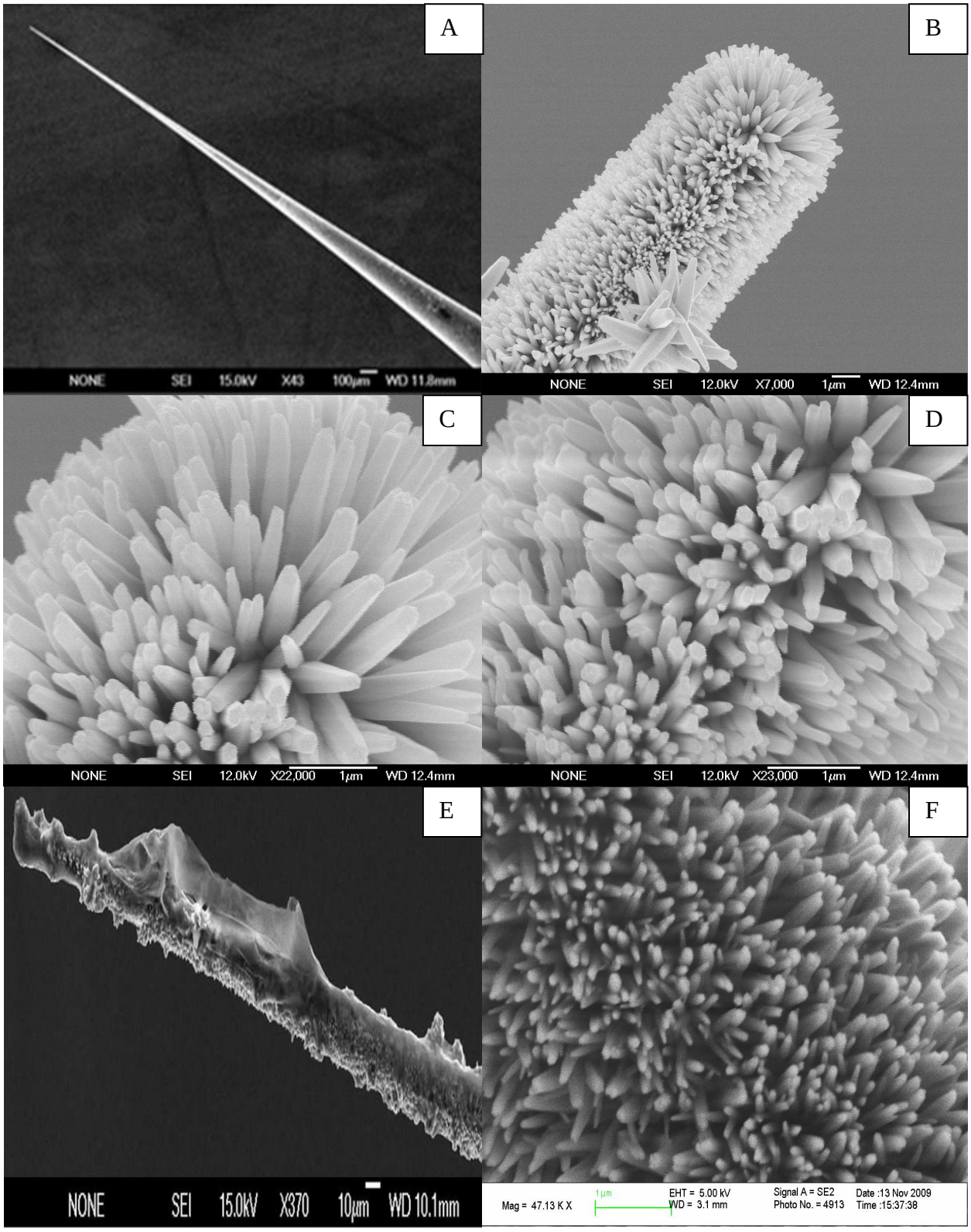
Figure captions

Fig. 1. (A-D) Field emission scanning electron microscope images at different magnifications of the Ag-coated glass capillary without (A) and with (B-D) grown ZnO nanorods using low temperature growth. The ZnO nanorod layer covered with transparent Mg^{2+}-selective membrane (F) and **(**E) shows the Mg^{2+}-selective electrode after intracellular measurements. High resolution analytical transmission electron microscopy images exhibit that the axis is 0001 direction is shown in (G).

Fig. 2: (A) Schematic diagram illustrating the setup for the measurement of the intracellular Mg^{2+} concentration with microscopic images of human adipocyte and frog oocyte. (B) The intracellular role of ionized Mg^{2+}.

Fig. 3. (A) A calibration curve showing the electrochemical potential difference between the Mg^{2+}-selective ZnO nanorod-covered and the Ag/AgCl reference microelectrodes versus the Mg^{2+} concentration. Insets show images of human adipocytes and frog oocytes with arrows pointing at measured intracellular levels of Mg^{2+} for the respective cells. (B) The output response with (black and green) and without (red) interfering ions. A mix of 10 μM Ca^{2+}, 10 μM Na^{+}, and 10 μM K^{+} is added to a 1 μM $MgCl_2$ control solution (after 35 s, black line) or to a 100 μM $MgCl_2$ control solution (after 40 s, green line). The red line is a 1 μM $MgCl_2$ control line with no addition of interferents. (C) The sensor-to-sensor reproducibility of three Mg^{2+} selective microelectrodes for frog oocytes (red) and human adipocytes (black).

Fig. 1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B
NONE SEI 12.0kV X7,000 1µm WD 12.4mm
C
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D
NONE SEI 12.0kV X23,000 1µm WD 12.4mm
E
NONE SEI 15.0kV X370 10µm WD 10.1mm
F
Mag = 47.13 K X
1 µm
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WD = 3.1 mm
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Time :15:37:38

Fig. 1G

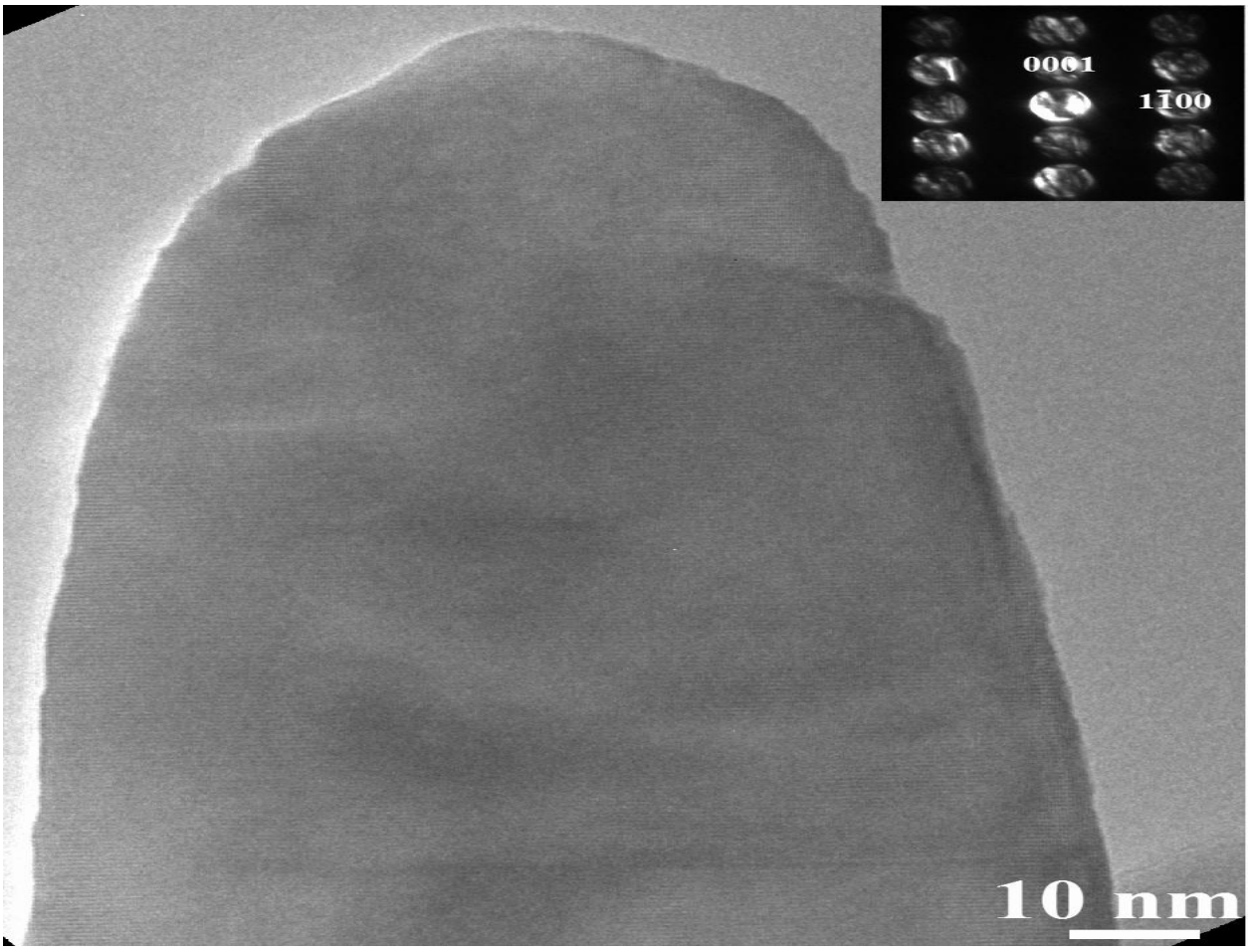
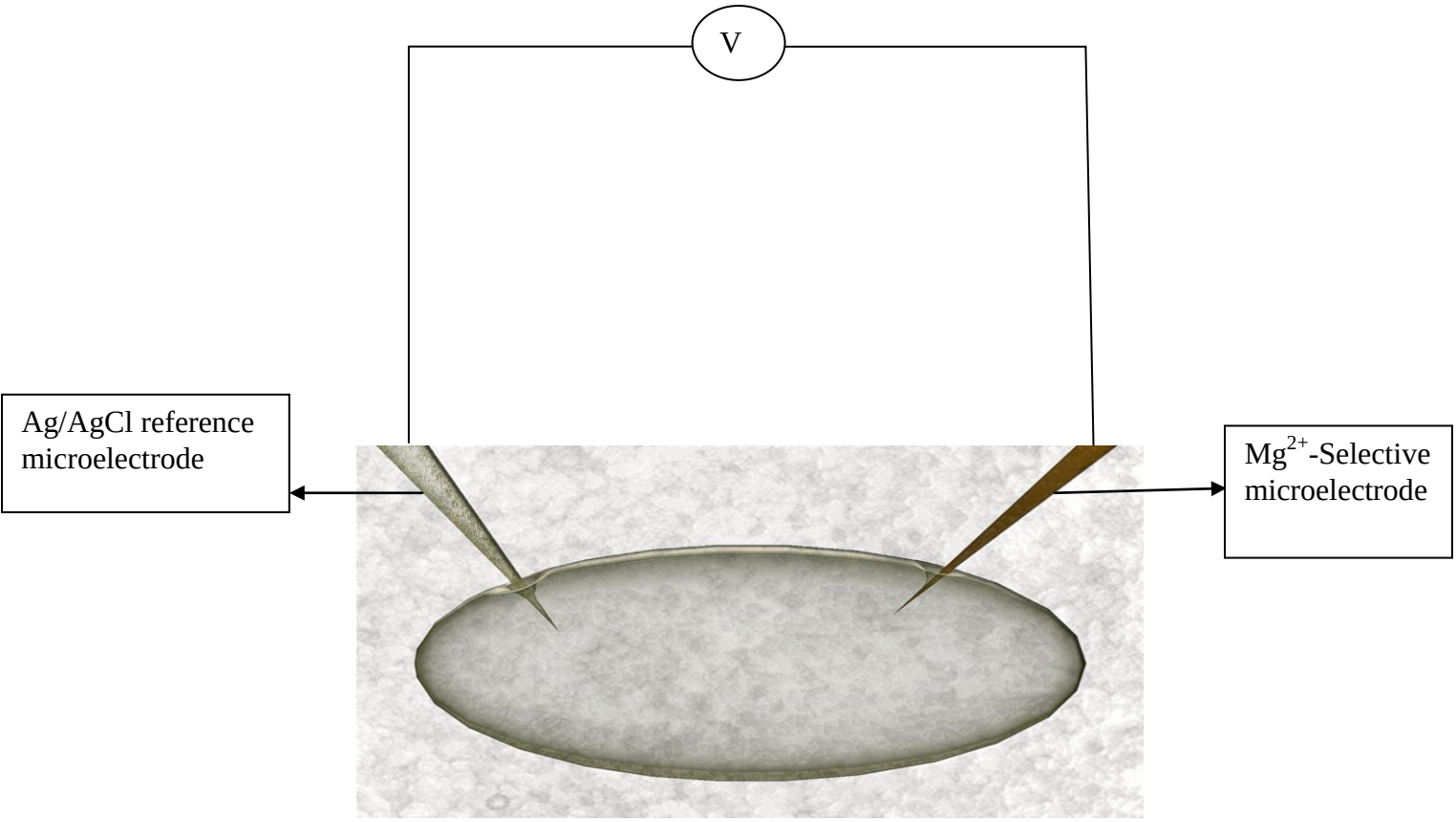


Fig. 2A



Human adipocyte

Frog oocyte

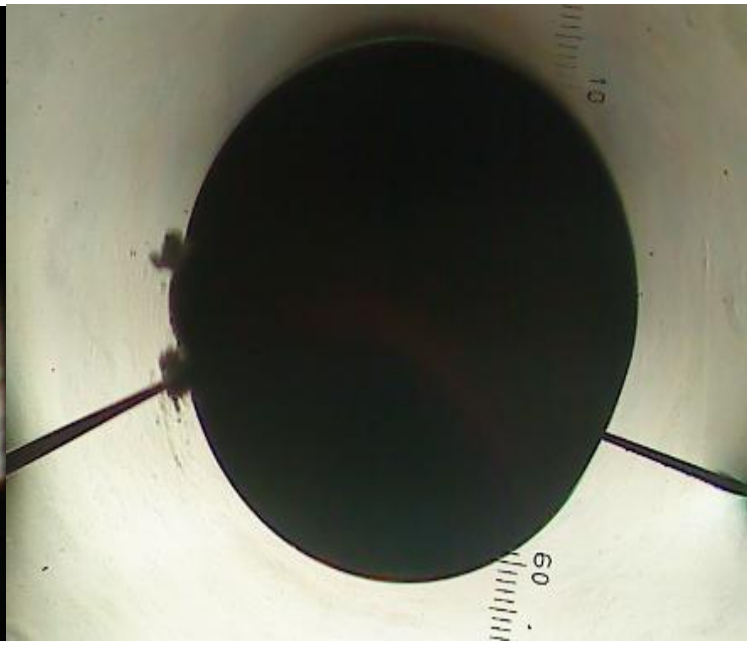
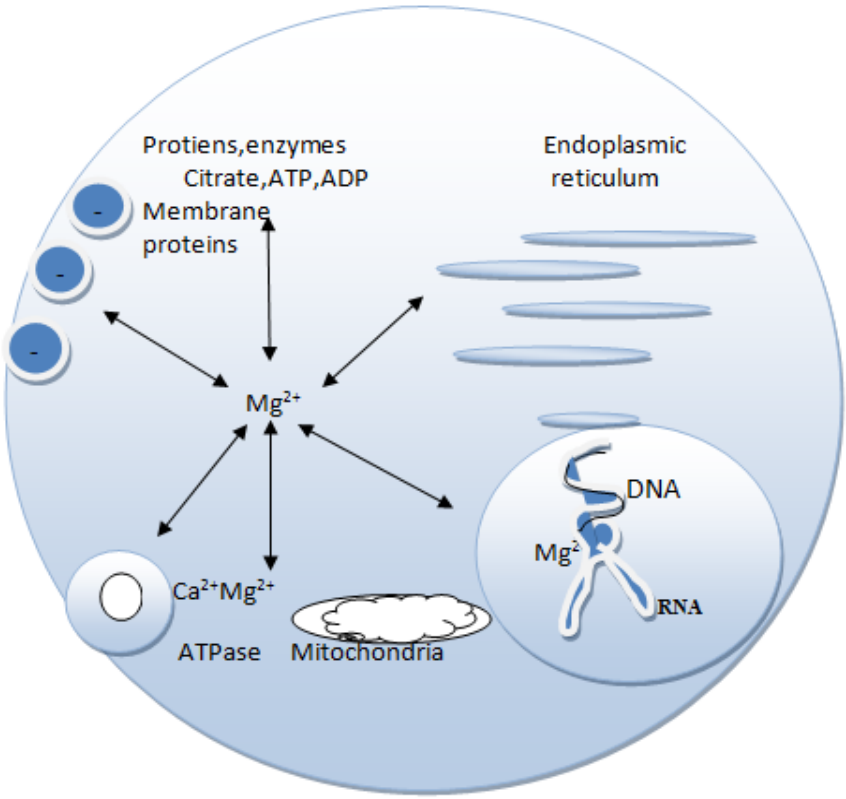


Fig. 2B


Protiens,enzymes
Citrate,ATP,ADP
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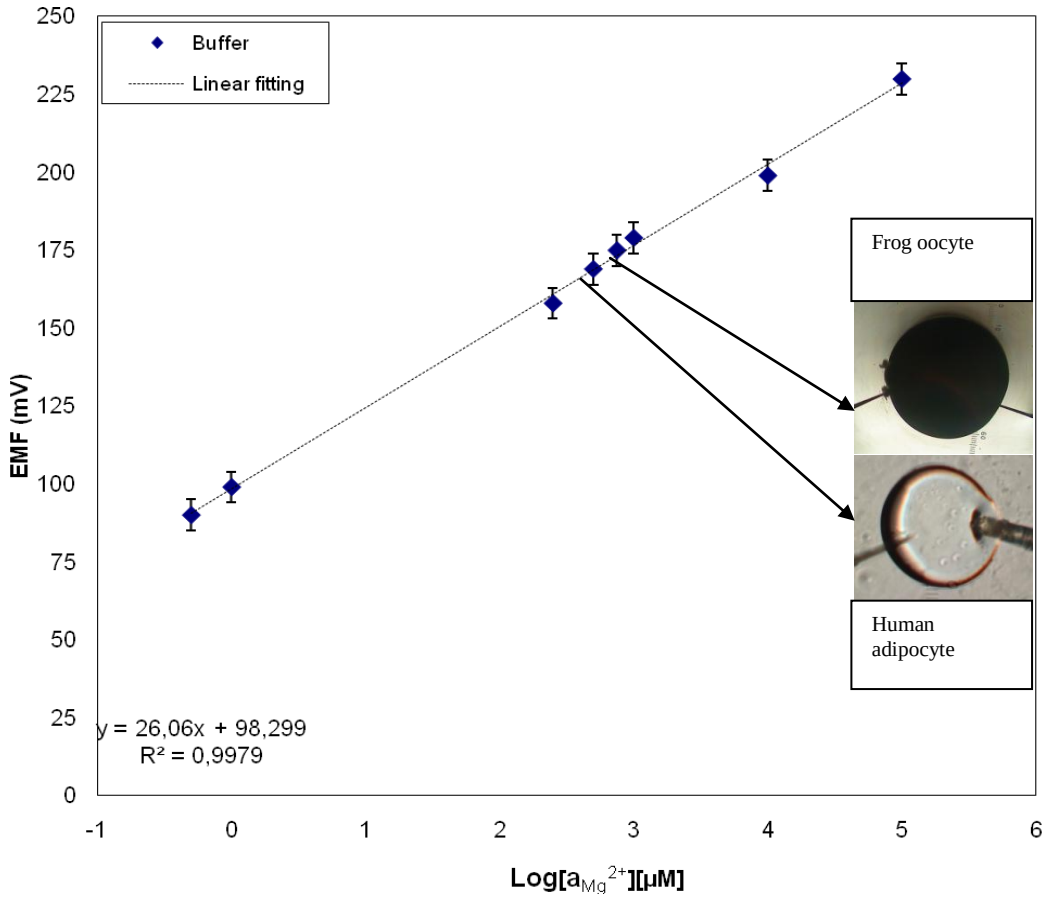
proteins
Endoplasmic
reticulum
Mg2+
DNA
Mg2
RNA
Ca2+Mg2+
ATPase
Mitochondria

Fig. 3A


Buffer
Linear fitting
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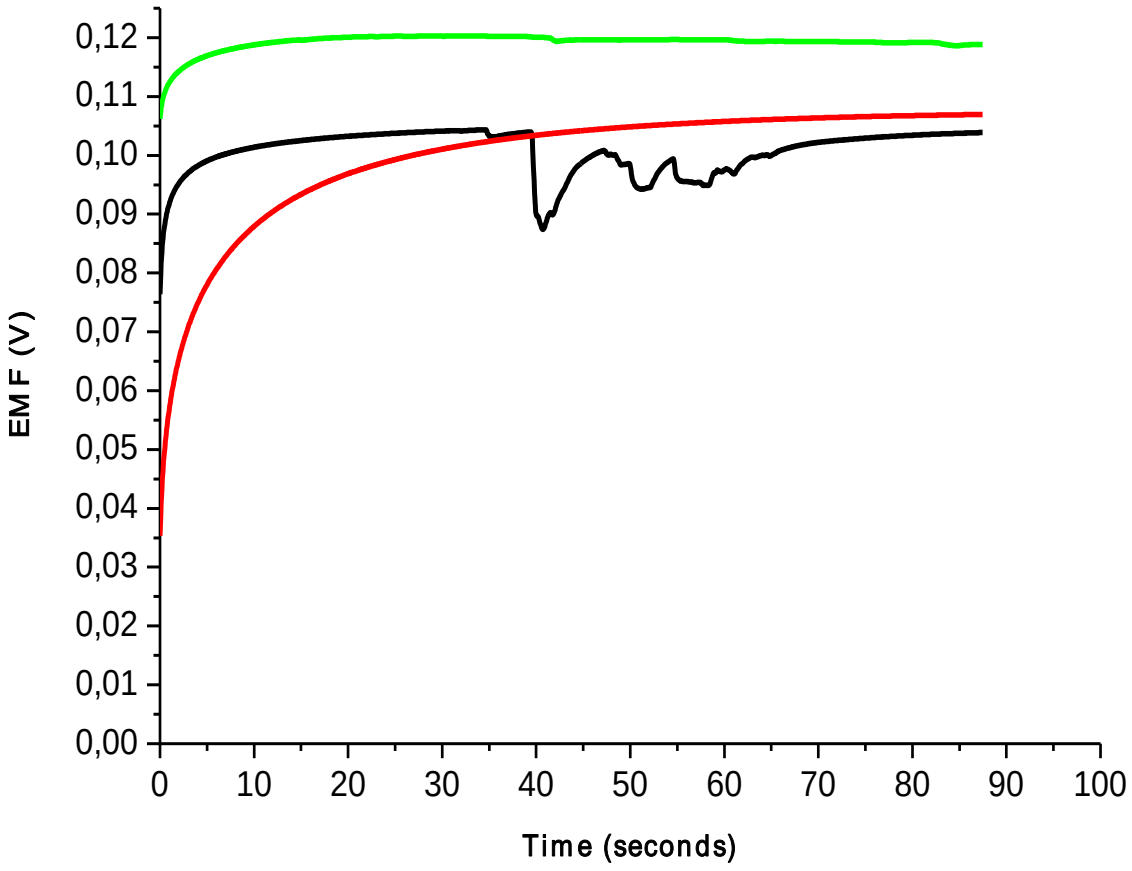
Log[a$_{Mg^{2+}}$][µM]
y = 26,06x + 98,299
R² = 0,9979
Frog oocyte
Human adipocyte

Fig. 3B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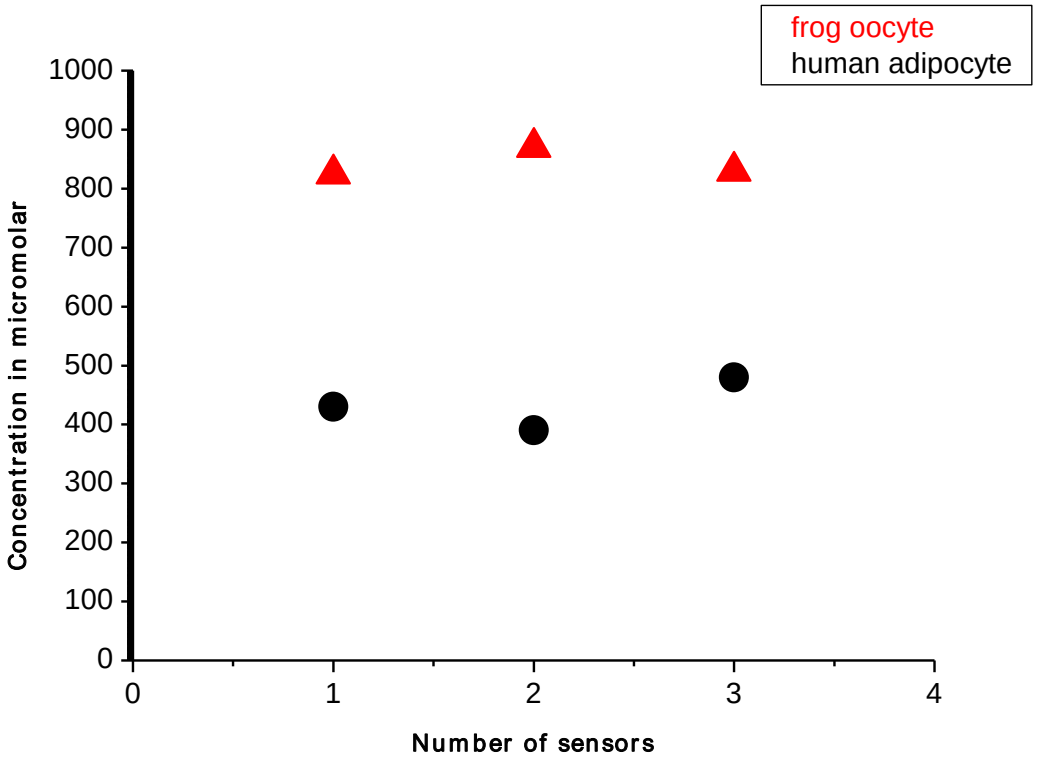
30
40
50
60
70
80
90
100
Time (seconds)

Fig. 3C


frog oocyte
human adipocyte
Concentration in micromolar
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
Number of sensors