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Research Paper

# Ultrasound assisted electrochemical distinction of normal and cancerous cells

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ABSTRACT

Cyclic voltammetry (CV) is applied to monitor the sonoporative behavior of normal and cancer breast cells. Novel electrochemically produced microbubbles in the solution induce acoustic cavitation in the cells by ultrasonic stimulation (US). This makes sonoporation in the cells which exchanges ionic species between inner and outer parts of the membrane. Such ionic variations in culture media are detected by CV measurements. The differences in the CV patterns of the normal (MCF-10A) and cancerous (MCF-7) cells before and after US is related to the changed ion gradient between inner and outer parts of the cells. As a supporting result, cell membrane voltage in control cells, as an indicative of the membrane ion gradient, was measured by patch-clamp method which reveals the detected changes in CV diagrams. This phenomena would assist to achieve a new electrochemical method for cancer detection.



## 1. Introduction

Low intensity ultrasound waves have been an interesting issue in medical applications such as sonodynamic therapy, non-thermal therapy and diagnosis for decades [1–3]. Upsurge in membrane penetrability, uptake of various drugs and therapeutic compounds and rush of ions into or out of the cells are all the consequences of the so-called sonoporation as the main bioeffects of ultrasonic stimulation of the cells [4–6].

Microbubble generation is the crucial pre-step of sonoporation. Even though the microbubbles are produced inherently in the process of ultrasonic stimulation [7,8], commercial types of microbubble contrast agents (polymeric microspheres) [9–11] are applied in most US-based studies to lessen the threshold of cavitation and augment the subsequent sonoporation efficiency [12].

Monitoring the biological response of the cells to ultrasonic stimulation is an important step in evaluating sonoporation process. Moreover, cells exhibited different ionic functions during their phenotypic changes (such as normal and cancerous) [13,14]. So, their ion exchanging activities due to US would be induced from their phenotypes. Hence, analyzing the response of the normal and cancer cells to sonoporation could lead to a diagnostic indication correlated with ion exchange parameters of the cell. Investigation of cellular disorders based on material exchanges was reviewed by many researchers [15,16].

Here we introduced an electrochemical approach to diagnose breast cancer cells from that of normal phenotypes by monitoring the sonoporation induced ionic exchanges confirmed by membrane voltage dissimilarities in the cells. The electrochemical biosensor was fabricated on nanoroughened PMMA substrate by Au/Ti bilayer in integrated working, counter and reference electrode architectures. Extended interface between exchanged ions and electrodes surface provided by nanoroughened substrate, improves the level of ionic signal extraction. To enhance the effect of sonoporation, microbubbles were formed by a novel developed method based

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on applying electrolytic current through the working and counter electrodes. Then, the bubbles were stimulated by US and induced sonoporation in the cells. The anodic/cathodic peaks of the breast both normal (MCF10A) and cancer (MCF-7) cells in before and after US states were recorded by the assistance of cyclic voltammetry approach followed by measuring the voltage membrane by patch-clamp technique. The correlation between the cells' electrochemical responses and membrane voltages before and after applying US stimulation (US duration: 2 s, frequency: 20 kHz, Intensity: 0.9 and 1.8 w/cm$^2$) with similar parameters were investigated as a cancer diagnostic profile.

FE-SEM, confocal microscopy and florescent imaging were taken to investigate the reliability of electrochemical response due to the formation of sonopores, ionic transfer through cell membrane and sonoporation efficiency of the produced microbubbles. This study could shed new lights in combined ultrasonic-electrochemical diagnostic approaches.

## 2. Materials and methods

### 2.1. Experimental setup and ultrasound parameters

As presented in Fig. 1-A, the three electrode sensor is placed at the bottom of a chamber and the cells are cultured on the working electrode. Then, the chamber is filled with cell culture solution (RPMI containing 10% fetal bovine serum (FBS) and 1% antibiotics) which serves as an electrolyte for CV measurement as well as a medium for wave propagation of the ultrasonic stimulation. The ultrasonic horn (Top Sonics UP400-A, The Ultrasonic Tech. Development Co. (UTDC), Iran) is 4 cm above the working electrode. 20 kHz and 2 s were selected for the frequency and time duration of stimulation, respectively, and the US intensity was set to 0.9 w/cm$^2$ and 1.8 w/cm$^2$.

### 2.2. Sensor fabrication and characterization

The sensor is an integrated three electrode system (Fig. 1-B) with a circular working electrode (WE). The diameter of working electrode is 5 mm and is surrounded by a 1 mm-thicked ring shape counter electrode (C) as well as a reference electrode (R). The counter/reference electrodes are separated from working electrode with distance of 1 mm. In the case of sensor fabrication, PMMA (or tradename plexiglass®) was treated by Reactive Ion Etching (RIE) equipment in which the WE was roughened by $SF_6$, $H_2$ and $O_2$ gases (with typical flows of 100, 80 and 85 sccm) in the existence of RF Plasma (13.56 MHz). RF Plasma causes the ionization of $SF_6$ which plays the key role as etching radical. 190 w and 50 s were determined for plasma power and period of the bombarding, respectively. The sequential treatment of the PMMA by $SF_6$ followed by the combination of $H_2/O_2$ and $SF_6$ as passivating layer in the presence of RF plasma, roughens the surface. In comparison to the pristine PMMA surface (Fig. 1-C), FESEM analysis were done to track the formation of nanoroughened PMMA surface (Fig. 1-D). Moreover, surface roughness and topography of the both pristine and nanoroughened PMMA were collected by atomic force microscopy (AFM, NT-MDT Solver Next). 100 $\mu m^2$ of the surface was scanned for three times in different sections to evaluate the differences in roughness between prism and treated PMMA.

Using this method, nanoroughened pattern was obtained with roughness of ~70 nm as presented in AFM profile of the sensor (Fig. 1-E). After surface treatment of the PMMA, the substrate coated with 30 nm thickness gold using RF sputtering system. To promote the adherence of the gold to PMMA, prior to this step, a thin layer of Titanium (5 nm) sputtered on PMMA substrate. Finally, the desired pattern of sensor projected onto the Ti/Au nanoroughened PMMA substrate using standard photolithography technique.

### 2.3. Microbubble generation and electrochemical signal recording

Before beginning the experiment, microbubbles are generated to facilitate the sonoporation of the cells (Fig. 1-F). In this regards, an instantaneous potential of −1.7 V is applied to the three electrode system using RNF Potentio Galvanostat (Roshd Nano Fannavaran Co. Iran). Rapid potential stimulation gives a rise to the electrolysis of the solution and subsequently generation of microbubbles on the surface of the WE.

Before ultrasonic stimulation and to measure the electrochemical response of cell lines, cyclic voltammetry was performed in the voltage range of −600 mV to 600 mV with scan rate of about 100 mV/s. For all CV experiments these limits were kept constant. After recording the CV response of the nonstimulated cells, the US was applied and again the CV response was measured.

### 2.4. Cell culture

MCF-10A (human normal breast cell line) and MCF-7 (human noninvasive breast cancer cell line) were purchased from National Cell Bank of Pasteur Institute of Iran and cultured in Rose well Park Memorial Institute medium (RPMI, Sigma), supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics (Gibco). Cells were maintained in an incubator under standard cell culture conditions (37 °C, 5% $CO_2$) and the medium was changed every two days.

### 2.5. Fluorescent microscopy

Cytoskeletal actin microfilaments were assessed by inverted confocal microscopy system (Leica, TCS SP5, Germany) for cells stimulated and nonstimulated by ultrasound. At the first step, cells were fixed by 4% methanol-free formaldehyde solution and then permeabilized with 0.2% Triton-X100 in PBS for 20 min. In the next step, blocking was done by 1% BSA diluted with PBS in incubator for 1 h. At the end, actin dye Alexa Fluor® 488 Phalloidin (Invitrogen, USA) was added to the cells and maintained for 45 min in an incubator.

To detect cell membrane perforation, PI (Propidium Iodide) (100 μM; Sigma Aldrich) was added to the cell culture medium before the ultrasound application. PI is a charged dye which is expelled from entering the nonpermeable alive cells. Due to the disruption of cell membrane by US, PI enters the cell and after intercalating into DNA/RNA emits a red fluorescent color. ImageJ software was used to quantify the increase of red cells after US in the presence vs. absence of produced microbubbles.

### 2.6. Patch-clamp

Whole-cell configuration of patch-clamp method was used to determine the membrane potential of breast normal (MCF-10A) and cancer (MCF-7) cells. After culturing on plastic dishes cells were transferred on top of the objective lens of an inverted microscope. Signals were recorded by borosilicate glass electrodes (3–6 MΩ) which were pulled with a two-stage vertical puller (PC10, Narishige, Japan). After pipette preparation they were filled with an intracellular solution containing (mM) 140 K-Gluconate, 10 HEPES, 2 $MgCl_2$, 2 Na2-ATP, 1.1 EGTA, 0.1 $CaCl_2$ and 0.4 Na2-GTP. Data recording was performed using a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA) equipped with Digidata 1320 A/D converter (Axon Instruments, Foster City, CA). Upon achieving the whole cell configuration, the amplifier was switched to the current clamp mode and the membrane potential was measured.