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MODULATION OF CALCIUM METABOLISM ON OSTEOSARCOMA CELLS USING ULTRASOUND-ACTUATED PIEZOELECTRIC NANOGENERATORS

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ABSTRACT

Piezoelectric materials respond to mechanical stimuli producing a voltage. When using piezoelectric nanostructures, they can convert tiny amounts of mechanical energy into electricity. These devices are commonly named as nanogenerators [1]. Recently, they are being used for biological application to stimulate cells [2]. The main objective of this work is to prove that microdevices with piezoelectric ZnO nanosheets (NSs) respond to the application of biomedical-range ultrasounds (US) and are capable of stimulate cells.

In this work, we report that these microdevices produced a signal in the order of millivolts when powered with 3 MHz – 1.0 Wcm^{-2} US. Besides, in contact with Saos-2 osteosarcoma cells, a part of the population was stimulated in response of the US actuation, observed using calcium (Ca^{2+}) imaging.

KEYWORDS

Piezoelectric, microdevices, stimulation, excitable cells, ZnO, nanostructures, bioelectronics.

INTRODUCTION

Microfabrication techniques allow creating highly reproducible structures in the micro- and nanoscale [3]. Millions of devices can be obtained from a single wafer. Microfabrication processes, combined with the use of smart materials that can be activated in response to stimuli at a given time, allow us to create smart devices in the micro- and nanoscale.

Similar microdevices to the ones assessed in this work were previously successfully fabricated and characterized, and their cytocompatibility was tested [4]. Here, we explore the application of these piezoelectric microdevices to electrically stimulate cells.

The microdevices are composed by a 3 x 3 μm^2 microfabricated platform of silicon dioxide (SiO_2) with a nanostructured layer of zinc oxide (ZnO). These nanostructures were grown into a shape of nanosheets through a hydrothermal process [5]. The NSs have an average thickness of 32 ± 4 nm. The piezoelectric layer can react to mechanical stimuli, such as ultrasonic waves. This means that the piezoelectric layer can be powered with US to produce an electric potential. The voltage produced by a whole surface covered by microdevices was characterized in response to US at 3 MHz frequency and 1.0 Wcm^{-2} intensity, which are in the biomedical range.

We hypothesized that the voltage generated by the piezoelectric NSs in contact with the cell was able to open the voltage-gated calcium channels (VGCCs) in the membrane of electrically-excitable cells [2]. To demonstrate this hypothesis, we studied the calcium flux in cells in response to the wireless stimulation of the piezoelectric microdevices with ultrasounds. Triggering the calcium pathways of the cell could activate subsequently other metabolic routes that lead to cell proliferation, cell differentiation, cell migration, or cell death, yet to be determined.

EXPERIMENTAL METHODS AND RESULTS

Electrical characterization of the piezoelectric layer

In order to do the electrical characterization of the piezoelectric NSs, we fabricated a silicon chip with patterns of 3 x 3 μm^2 where ZnO NSs were grown. Then, a crystal coverslip etched to obtain a rough pattern that contacted the ZnO NSs and metalized with 50 nm of Al was placed on top. The sandwiched structure consisting of first electrode, piezoelectric nanostructures and second electrode was covered with epoxy resist to make it robust and waterproof. The electric potential of the whole structure under the application of US, through an acoustic-coupling gel, was measured using an oscilloscope (HMO3004, Rohde&Schwartz).The voltage obtained can be observed in Figure 1.

Applying the ultrasonic wave at 3 MHz and 1.0 Wcm^{-2}, a stable signal of 7.6 ± 0.9 mV was obtained. However, without the application of ultrasounds, a noise voltage of 2.1 ± 0.2 mV was also measured. The effective generated signal in response to the ultrasound stimulus is around 5.5 mV.

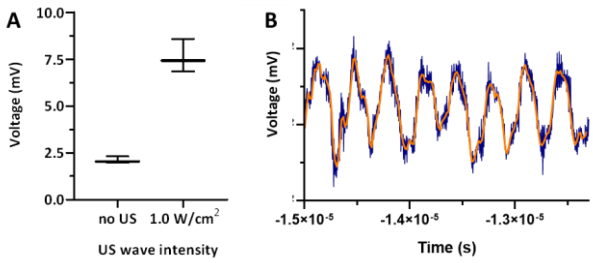


Figure 1. *Voltage measurement of a layer of ZnO NSs actuated with US at 3 MHz and 1.0 Wcm^{-2}. A) represents the voltage value obtained versus the control samples. B) shows the stable signal measured in response to US. In blue, the original signal and, in orange, after applying a MATLAB low pass filter with a cut-off frequency of 50 Hz.*

Calcium imaging of cells in response to US

The response of the electrical stimuli was experimentally tested with Saos-2 osteosarcoma cells. In a setup stablished at the confocal laser scanning microscope (CLSM Leica SP-5, Leica) at 37 ºC. The cells, loaded with Fluo-4 AM (Thermo Fisher Scientific) were evaluated in response to US.

The ultrasonic transducer was directly applied to the Dulbecco's Modified Eagle Medium (DMEM) cell culture medium of the culture plate at 3 MHz frequency and 1.0 Wcm^{-2} intensity. Time-lapse videos of the cell culture were recorded for 3 min before the US stimulation and 5 min after the stimulation. At least three replicate samples, for both control and cells with microdevices, were obtained. The US stimulus was applied in a continuous wave for 10 s.

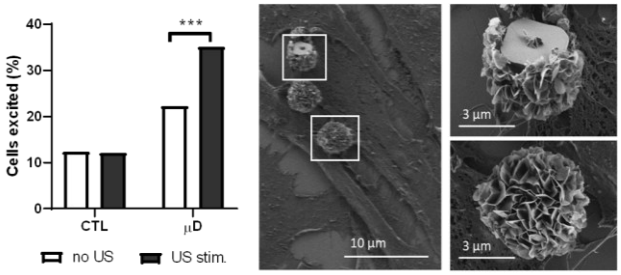


Figure 2. *Percentage of excited cells before and after applying a US wave of 3 MHz, 1.0 Wcm^{-2}. Scanning electron microscope (SEM) images depict how the cell and the microdevice are interacting.*

The results from Figure 2 show the percentage of cells excited in the overall population of cells. The significant differences were obtained by chi squared test. In the control samples, before the application of US, 12.3% of cells produced calcium peaks, while after the application of US, this percentage remained at 12.1%. This part of the cell population has an active basal metabolism not related to the piezoelectric material. For the cells in contact with microdevices, 22.2% of the cells were excited before the application of ultrasounds. We hypothesize that this increase in the number of cells excited with respect to the control is due to the force exerted by the cell on the material, which would also have a positive feedback triggering the VGCCs of the cell [2]. However, after the actuation with US, the population of cells excited increased up to a 35.1%. The cells were probably excited because of the voltage generated by the ZnO NSs reacting to the US wave.

Finite Element Modelling of the piezo-cell interaction

To understand the cell-microdevice interaction, we simulated a model of the cell membrane to interact with a single ZnO NS actuated under the same US conditions in aqueous medium using COMSOL Multiphysics 6.0 (COMSOL, Sweeden).

ZnO NS dimensions in the model were defined based on the measurements performed by SEM. A ZnO NS was anchored to the microparticle, defining the microparticle position as a fixed constrain. The upper edge of the ZnO NS is free to move, close to the cell membrane at a 5 nm distance. The membrane was modelled with a 10 nm thickness. The whole system was embedded in a sphere of saline water. Parameters such as the relative permittivity (ε_r) of the cell membrane were obtained from bibliography [6].

The study performed was time-dependent. An acoustic pressure of 1.2 Pa, equivalent to 1.0 Wcm^{-2} intensity, was applied in the model. The US wave of 3 MHz was transmitted from the bottom of the system (-z) at 45º. We analyzed the behavior of the system from the start of the acoustic stimulus to the stationary phase.

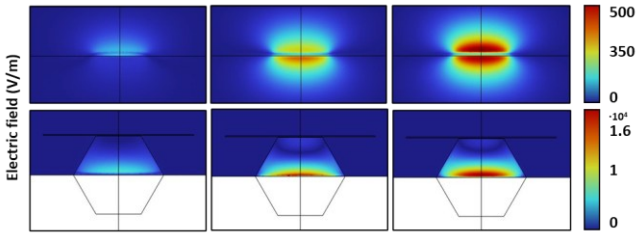


Figure 3. *COMSOL results from the evaluation of the cell-ZnO NS interaction. The first row shows the EF present at the cell membrane. The second row, the EF produced by a single ZnO in response to the US wave applied. The maximum EF present in the cell membrane has a magnitude of 500 V/m (5 V/cm). The maximum EF produced by a single ZnO NS has a magnitude of $1.6 \cdot 10^4$ V/m (160 V/cm). The first column depicts the values for an acoustic pressure close to 0 Pa. In the second and the third column, an acoustic pressure of 1 Pa and -1 Pa and their corresponding values of EF are shown respectively.*

Figure 3 shows the results obtained from the COMSOL modelling. The stress produced by the acoustic wave at 3 MHz frequency and 1.0 Wcm^{-2} intensity at its maximum acoustic pressure was capable of producing an area of influence on the cell membrane surrounding the nanostructure with an EF of 5 V/cm. However, at its base, the ZnO NS was capable of producing EF up to 160 V/cm, also at its maximum. The maximum module of the acoustic pressure exerted on the NS in the model was of 1.2 Pa.

It has to be noted that the acoustic pressure is related to the EF produced, a reduction of 1 V/cm of the EF present in the cell membrane is produced once the acoustic pressure is reduced every 0.4Wcm^{-2}. This was studied using a parametric sweep for the stationary model. Besides, the eigenfrequency value decreases directly proportional to the ZnO NS thickness and inversely proportional to the diameter of the nanostructure.

DISCUSSION

The main objective of this work was to demonstrate the piezoelectric potential produced by the fabricated microdevices and its effect on cells. Overall, that the results showed that the electric output produced by the piezoelectric layer of ZnO NSs in response to 3 MHz and 1.0 Wcm^{-2} US was capable of triggering a response in the cells.

The exact value of the electrical stimuli attaining the cell is still undefined, but it is in the range of the values previously found in literature around 14 V/cm [7]. Our approach with FEM simulations corroborated that a single ZnO NS was capable of producing an EF ten orders of magnitude above the 14 V/cm described in bibliography in response to the US applied and that this EF, with a reduced value, is also present at the cell membrane.

Previous works in the field used nanoparticles and other nanostructures with the same purpose of stimulating cells with interesting results [8, 9]. However, smart materials in the nanoscale have several parameters that cannot be precisely controlled. For instance, their size or

position with respect to the cell, most of them tend to be internalized and would not serve to our original purpose of stimulating the cell from the outside of the membrane to trigger VGCCs. On the other hand, there are several works that describe wireless devices in the millimeter scale to electrically stimulate excitable cells, such as neurons and muscle cells. However, the main disadvantage with this kind of devices is that they cannot attain a single-cell resolution [10, 11].

Another application type is the use of scaffolds made of piezoelectric materials [12] or integrating piezoelectric nanomaterials inside [13]. The results described for scaffolds with piezoelectric components indicated that the use of US greatly improved the results obtained with scaffolds without piezoelectric properties. However, due to their size, the application is restricted to implantation and wound healing [12, 13].

The size of the piezoelectric device is of great importance for cell stimulation and related applications. The microdevices have a size comparable to a cell. Indeed, the size can be modified to target different microdevice positions with respect to the cell [14, 15]. The NSs have a size in the range of nanometers. The nanostructures can directly interact with biomolecules at cellular level, which is interesting in terms of local effects.

FUTURE PERSPECTIVES

As future perspectives of this work, several parameters could be optimized. The set-up can be optimized by tuning the wireless microdevice actuation to maximize the number of cells stimulated. This can be done through several ways. First, by modulating the NS sizes modifying the hydrothermal growth parameters [5] to produce a higher electric field in response to the same ultrasonic wave. Second, by modifying the parameters of the ultrasonic wave (frequency, intensity, duty cycle, etc.) to optimize the electrical response of the microdevice. Third, increasing the number of microdevices per cell to enlarge the surface of ZnO NSs in contact with the cells. In parallel, these microdevices can be tested to stimulate other cell types.

Further interest in this topic is also to integrate other kind of piezoelectric materials, such a polymers or organic materials. Their main advantage is that they have a Young Modulus (E) more similar to most of the biological tissues [16].

CONCLUSIONS

US of 3 MHz frequency and 1.0 Wcm^{-2} intensity can actuate piezoelectric ZnO NSs to produce an electrical voltage in the order of millivolts. This electrical potential produced is capable of stimulating Saos-2 osteosarcoma cells in contact with the microdevices. The actuated microdevices triggered the increment of calcium concentration in the cytoplasm of Saos-2 cells via the opening of the VGCCs. The EF values reproduced through simulations are in the order of the minimum field intensity needed to trigger these ion gates.

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