Monitoring the dielectric response of single cells following mitochondrial adenosine triphosphate synthase inhibition by oligomycin using a dielectrophoretic cytometer

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One of the main uses of adenosine triphosphate (ATP) within mammalian cells is powering the Na^+/K^+ ATPase pumps used to maintain ion concentrations within the cell. Since ion concentrations determine the cytoplasm conductivity, ATP concentration is expected to play a key role in controlling the cytoplasm conductivity. The two major ATP production pathways within cells are via glycolysis within the cytoplasm and via the electron transport chain within the mitochondria. In this work, a differential detector combined with dielectrophoretic (DEP) translation in a microfluidic channel was employed to observe single cell changes in the cytoplasm conductivity. The DEP response was made sensitive to changes in cytoplasm conductivity by measuring DEP response versus media conductivity and using double shell models to choose appropriate frequencies and media conductivity. Dielectric response of Chinese hamster ovary (CHO) cells was monitored following inhibition of the mitochondria ATP production by treatment with oligomycin. We show that in CHO cells following exposure to oligomycin ($8\text{ }\mu\text{g/ml}$) the cytoplasm conductivity drops, with the majority of the change occurring within 50 min. This work demonstrates that dielectric effects due to changes in ATP production can be observed at the single cell level. © 2014 AIP Publishing LLC.

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I. INTRODUCTION

In recent decades dielectrophoresis (DEP) has emerged as a powerful technique for analysis, sorting and manipulation of cells.^{1,2} DEP has been employed in blood cells,^{3–5} stem cell differentiation,^{6,7} cancerous cells that are multidrug resistant (MDR),⁸ circulating tumour cells,⁹ viability,¹⁰ and apoptosis tracking.^{11,12} DEP of cells is influenced by many factors such as membrane capacitance, cytoplasm conductivity and cytoplasm permittivity. Unlike most other dielectric materials, cells adapt to environmental conditions using a host of active systems. Some of these active systems have significant impact on the dielectric properties of the cell. For example, the metabolic state is an important factor in controlling ion homeostasis and hence cytoplasm conductivity.^{13–15} Hence determining dielectric effects due to perturbations of cell metabolic state is important for many applications of DEP.

One of the main uses of ATP within mammalian cells is powering the Na^+/K^+ ATPase pumps used to maintain cell homeostasis. The two major ATP production pathways within the cell are glycolysis (2 ATP) within the cytosol and the electron transport chain (34 ATP) via the mitochondria.¹⁶ Both these pathways produce ATP, which is the most common form of energy used to drive cellular processes. Some compounds can be used to control specific aspects of

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this energy production. For example, oligomycin is known to inhibit the ATP synthase by blocking the proton channel on the mitochondria transmembrane within minutes of treatment.^{17–19} Consequently, the only channel to pump protons inside the mitochondria will be blocked and electron transport will be significantly reduced. Therefore, oligomycin inhibits the ATP production through the electron transport chain in the mitochondria. In addition, oligomycin is an inhibitor of the Na^+/K^+ ATPase pump in the cytoplasm membrane.^{20,21} These pumps transfer 2K^+ ions inside and 3Na^+ ions outside the cell at the expense of one ATP molecule to maintain normal homeostasis as shown in Fig. 1. Blocking Na^+/K^+ ATPase pumps impacts the ion concentration inside the cytoplasm and hence the cytoplasm conductivity. It has been expected that depleting ATP production levels within the mitochondria has direct impact on cytoplasmic ion concentrations.^{13–15}

Oxygen consumption rates and metabolism bi-products evaluation offers a method to estimate mitochondrial versus glycolysis ATP production. However, these methods are not applicable at the single cell level.²²

In this work, oligomycin is used to inhibit the mitochondrial ATP production in a cell population to observe their dielectric response using a DEP cytometer.¹² This study is focused on time course measurements within the first two hours of mitochondrial ATP synthase inhibition. Monitoring the cell response within the first hours of the treatment with oligomycin reveals important information on the ion regulations and the impact of mitochondrial ATP synthase inhibition while avoiding the impact of cell adaptability, which will affect longer time period studies.²³

II. MATERIALS AND METHODS

A. Background

In this work, the dielectric response of the cell is monitored before and after the inhibition of mitochondrial ATP production. A cell surrounded by a medium in a non-uniform electric field experiences a dielectrophoretic force. The dipolar force approximation is expressed by

FIG. 1. Oligomycin will inhibit ATP production in the mitochondria, but other pathways such as glycolysis will continue to produce ATP. Decreased ATP production reduces the rate of Na^+/K^+ ATPase pumps, resulting in a net out flow of ions and hence a drop in cytoplasm conductivity. (a) Medium: pumps and pores on the cytoplasm membrane are responsible for ion transport inside and outside of a cell. The Na^+ ion concentration inside the cell is lower than in the medium, however, the K^+ ion concentration is much higher inside the cell. Diffusion pathways for the ions are shown in purple. In addition, Na^+/K^+ ATPase pumps transfer 2K^+ ions inside and 3Na^+ ions outside the cell at the expense of one ATP molecule. (b) Cytoplasm: glucose is converted into pyruvate in the glycolysis pathway and can be further converted to ATP either through glycolysis or in the mitochondria. (c) Mitochondria: oligomycin blocks the ATP synthase in the mitochondria and inhibits ATP production within mitochondria.

$$\mathbf{F}_{DEP} = \frac{3}{2} \varepsilon_m V_{cell} \text{Re}\{K_{CM}\} \nabla E_{rms}^2, \quad (1)$$

where ε_m is the real part of the permittivity of the medium, V_{cell} is the volume of the suspended spherical cell, and $\text{Re}\{K_{CM}\}$ is the real part of the Claussius Mossotti factor defined by

$$K_{CM} = \frac{\tilde{\varepsilon}_c - \tilde{\varepsilon}_m}{\tilde{\varepsilon}_c + 2\tilde{\varepsilon}_m}, \quad (2)$$

where $\tilde{\varepsilon}_c$ and $\tilde{\varepsilon}_m$ are the complex permittivity of the cell and medium, respectively. The sign of the Claussius Mossotti factor determines if a polarized cell moves towards or away from the higher electric field region. If $\text{Re}\{K_{CM}\}$ is positive, electrostatic work is required to displace the particle from the higher electric field region. Therefore, a polarized particle moves to the higher density of the electric field and vice versa for negative $\text{Re}\{K_{CM}\}$ values. A double shell model of the cell includes one shell for the membrane surrounding the cell and a second for the nucleus.¹ The space between the outer membrane and the nucleus is the cytoplasm, and is electrically modelled by its conductivity and relative dielectric constant.¹ The remaining regions are modelled by their thickness, conductivity, and relative dielectric constant. The simulated double shell model of the Claussius Mossotti factor spectrum in Fig. 2 shows that in the 1–10 MHz frequency range, the value of $\text{Re}\{K_{CM}\}$ is dominated by the cytoplasm conductivity and hence the ionic composition of the cell. The parameters used in the double shell model are adopted from Asami *et al.*, Polevaya *et al.*, and Kotnik *et al.*^{24–26} The media conductivity was chosen to be 0.42 S/m so that the DEP force would be close to zero ($f=6$ MHz) at the expected pre-inhibition equilibrium cytoplasm conductivity of 0.42 S/m and to decrease the sensitivity to the cell radius. Since the loss of mitochondrial ATP is expected to result in the loss of ions in the cytoplasm, the frequency was chosen to be largely sensitive to changes in the cytoplasm

FIG. 2. Claussius Mossotti factor spectrum for a viable CHO cell, calculated by double shell model: (a) for 1–4 cytoplasm conductivity is varying from 0.22–0.52 S/m, $r=5.5 \mu\text{m}$ (b) for 1–4 cell radius is varying from 4.5–7.5 μm , cytoplasm conductivity = 0.42 S/m. All other parameters are fixed: medium conductivity = 0.42 S/m, nuclear radius = 3.25 μm , membrane thickness = 5 nm, nuclear envelope thickness = 40 nm, membrane permittivity = $6.8 \varepsilon_0$, cytoplasm permittivity = $60\varepsilon_0$, nuclear envelope permittivity = $28 \varepsilon_0$, nucleus permittivity = $52 \varepsilon_0$, membrane conductivity = 3×10^{-3} S/m, nucleus envelope conductivity = 6×10^{-3} S/m, nucleus conductivity = 1.35 S/m.

conductivity. To maximize sensitivity to these changes and hence ion concentration, a frequency of 6 MHz for DEP actuation of the cells was chosen.

B. DEP cytometer

The DEP cytometer is based on differential detection of single cells before and after DEP actuation in a microfluidic channel and has been described in detail by Nikolic-Jaric *et al.*²⁷ Briefly, the cell first passes over a first pair of detection electrodes, Fig. 3(c), and produces a signal with a peak value of P_1 . A DEP field produced between the DEP electrode and ground then actuates the cell. The cell then passes over a second pair of detection electrodes, Fig. 3(c), and produces a signal with a peak value of P_2 . The actuation and detection voltages are applied in greatly different frequencies to prevent coupling between the actuation and detection signals. The non-uniform DEP field is provided by applying a sinusoidal $4V_{p-p}$ voltage at 6 MHz to the DEP electrode as shown in Fig. 3 and detection takes place at 1.5 GHz with a 300 mV_{p-p} signal. A microwave interferometer approach is employed for detection to sense the dielectric change due to cells flowing through the analysis volume.²⁸ As can be seen in Fig. 2, in the 1–2 GHz region the relative dielectric response is largely due to the difference in polarizability of the materials within the cell relative to the surrounding medium and is independent of cytoplasm conductivity or membrane properties. The magnitude of the signal measured for each cell is due to the position of the cell as it passes over the electrodes and the size of the cell. Larger cells and cells passing closer to the electrodes produce larger signals.²⁷

The two peaks form the detection signature for each cell. When a DEP voltage is applied, the cells will be attracted or repelled from the higher density electric field region; meaning depending on the magnitude and sign of the Clausius Mossotti factor the second peak will be larger or smaller than the first peak. The Clausius Mossotti factor is monotonically dependant on the cell conductivity (Fig. 2(a)), and hence the forces on the cell will also be dependent on the cell conductivity. The cytoplasm conductivity is determined by the concentration of ions in the cytoplasm, taking into account the mobility of the ions within the cytoplasm as compared with the surrounding media.^{29,30}

We quantify each cell signature using a normalized differential response called the “Force Index” defined by

$$\text{Force Index} = \frac{P_2 - P_1}{P_2 + P_1}, \quad (3)$$

where P_2 and P_1 are the leading and trailing peaks illustrated in Fig. 3, respectively. A threshold is used to identify events with P_2 and P_1 above noise and interference. The Force Index depends on $\text{Re}\{K_{CM}\}$ and the height at which the cell enters the analysis region, cell horizontal velocity and cell radius. For each cell a Force Index is measured and histograms of Force Index are used to monitor the evolution of the dielectric response of the cells.

FIG. 3. (a) Microfluidic chip. (b) Top view of the microfluidic channel, detection and DEP actuation electrodes. (c) Cell trajectory in the microfluidic channel. Here, the cell is experiencing an attractive DEP force. $P_2 > P_1$ and Force Index > 0 . (d) Different detection signals for a cell experiencing nDEP force, no DEP actuation and pDEP force.²⁷

C. Cell preparation

Chinese Hamster Ovary (CHO) cells expressing a human llama chimeric antibody (EG2) were used for this work. Yves Durocher of the NRC, Canada kindly provided the cell line (CHODG44-EG2-hFc/clone 1A7).³¹ The cells were cultured in 250 mL baffled shaker flasks (VWR International, Radnor, PA) at 120 rpm in an incubator at 37 °C with 10% CO₂ overlay. The cells were grown in BioGro-CHO serum-free medium (BioGro Technologies, Winnipeg, MB) supplemented with 0.5 g/L yeast extract (BD, Sparks, MD), 1 mM glutamine (Sigma, St. Louis, MO), and 4 mM GlutaMax I (Invitrogen, Grand Island, NY).

For the DEP cytometer measurement a sample was taken from the shaker and centrifuged at 377 g for 1 min. After the supernatant was removed the cell pellet was reconstituted in fresh growth medium (37 °C) and low conductivity (~ 0.067 S/m) medium (37 °C) [22.9 mM sucrose (Sigma), 16 mM glucose (Fisher), 1 mM CaCl₂ (Fisher), 16 mM Na₂HPO₄ (Fisher)]²⁶ using a ratio of 6:17 (fresh: low conductivity medium) diluting the cell sample to $\sim 2 \times 10^5$ cells/mL and reaching a conductivity of 0.42 S/m as measured by a conductivity meter (Orion 3-Star Plus, Thermo Scientific, Waltham, MA). The oligomycin treated sample was prepared by adding oligomycin (Sigma), reconstituted in dimethyl sulfoxide (DMSO), at the concentration of 8 μ g/mL to the diluted sample. Control samples were prepared by adding the same volume of DMSO without oligomycin to the sample. For each test run the cells were in passages 35–40 and came from a 3 day old culture. This consistency is important as the age of a cell is known to affect the ratio of mitochondrial to glycolysis ATP production.³²

D. Choosing the media conductivity to reduce unwanted sensitivity

In order to improve the detection sensitivity to the cytoplasm conductivity and decrease the size and velocity variability effects, medium conductivity has been chosen such that the mean Force Index is zero for the untreated cells. This was done by measuring the mean Force Index for a range of media conductivities and finding the conductivity where the mean Force Index was zero. The mean for each point was found by averaging the Force Index for ~ 500 cells. The uncertainty estimate was found by standard error of the mean (SEM) calculated by dividing the standard deviation to the square root of the sample size. The conductivity was set by using low conductivity (~ 0.067 S/m) medium (37 °C) [22.9 mM sucrose (Sigma), 16 mM glucose (Fisher), 1 mM CaCl₂ (Fisher), 16 mM Na₂HPO₄ (Fisher)]²⁶ and then adding the appropriate volume of growth media required (17:6) to reach the chosen cell density and conductivity, as measured by a conductivity meter (Orion 3-Star Plus, Thermo Scientific, Waltham, MA). Cells were suspended in the new medium conductivity for ~ 10 min before starting each experiment. A media conductivity (0.42 S/m) was then used for the remainder of the tests. This minimizes unwanted sensitivities due to size, as shown in the simulation Fig. 2(b).

III. RESULTS AND DISCUSSION

A. Dielectric response versus media conductivity

The dielectric response (Force Index) was measured for medium conductivities ranging from 0.17 S/m to 0.45 S/m. At each conductivity the mean Force Index was measured for ~ 500 cells. Cells with no DEP voltage applied are expected to have a near zero Force Index, but a measurable off set is evident. This may be due to asymmetries in the electrodes yielding slightly larger signals from one pair of detection electrodes.³³ The crossover from positive DEP (pDEP) to negative DEP (nDEP) occurs at a medium conductivity of 0.42 S/m and therefore this medium conductivity was used for the remainder of the tests, shown in Fig. 4. By using this media conductivity the sensitivity to cell size is minimized (see Fig. 2(b)). In addition since the mean Force Index for the untreated cells is near zero, deviations in Force Index are easier to observe.

The value of the medium conductivity where a zero Force Index occurs can be used to estimate the cytoplasm conductivity. Using a double shell model with parameters as in Fig. 2(a), the K_{CM} spectra were calculated for cytoplasm conductivities from 0.22 to 0.52 S/m. For a null in the dielectrophoresis force at 6 MHz the estimated cytoplasm conductivity is 0.42 S/m.

FIG. 4. Mean Force Index for 0.17–0.45 S/m medium conductivities. Solid circles indicate the case in which DEP is applied. Hollow circles indicate the case in which DEP is not applied. Therefore, cell elevation does not change in the channel and we expect Force Index to be zero. Slight discrepancy from zero is resulted from electrode asymmetries.³³

However, it should be noted that the media conductivity that will result in a null of the Force Index is frequency dependent.

B. Monitoring force index after exposure to 8 μ g/ml oligomycin

Control sample (without oligomycin) and oligomycin treated sample were monitored for 100 min after exposure to DMSO for control, and DMSO with oligomycin for the treated samples. The Force Index was averaged in time blocks of 5 min (approximately 100–150 cells).

As shown in Fig. 5, the control sample Force Index changes by less than 0.01 over 100 min. The fact that the control response does not decrease, even though the external media conductivity has been changed from 1.43 S/m to 0.42 S/m during sample preparation, is in agreement with modified Hodgkin Huxley models for non-excitable cells.¹⁴ The Modified Hodgkin Huxley models for non-excitable cells predict that the cytoplasm potassium concentration will be relatively insensitive to the media potassium concentration.¹⁴ Since potassium is the dominant species, with regards to cytoplasm conductivity, if the potassium is unchanged the conductivity would also expect to be unchanged. A decrease of about 0.05 in the Force Index is observed for the oligomycin treated sample. The majority of the change occurs within the first 50 min. Force Index histograms were gathered for cells exposed between 50 and 100 min. As shown in Fig. 6, the Force Index histograms for the oligomycin treated sample signatures are shifted from the control sample. The drop in Force Index implies a drop in the K_{CM} and hence a drop in cytoplasm conductivity. Therefore, the exposure to oligomycin has produced a decrease in cytoplasm conductivity.

These results are in agreement with computational models of ion homeostasis. Modified Hodgkin Huxley models for non-excitable cells predict that after a 30–60 min inhibition of the ATPase pumps the cell dynamically reaches equilibrium.^{13–15} This equilibration time depends on the numbers of pores and pumps on the membrane and the initial state of ions compared to the medium. During this time the cell experiences a decrease in the K^+ concentration (efflux) and an increase in the Na^+ ion concentration inside the cytoplasm (influx).

The oligomycin concentration used in this study is high enough to assure the ATP synthase inhibition in the mitochondria following 10 min of exposure.¹⁷ If all the ATPs were produced through the mitochondrial pathway, the K_{CM} and hence the Force Index would be expected to drop more significantly. The magnitude of the decrease in the Force Index after exposure to oligomycin is evidence that other energy production pathways are active within the cell; i.e., glycolysis in cytoplasm.

Using the double shell model we have calculated the Clausius Mossotti factor for each point in Fig. 4 and plotted them versus Force Index in Fig. 6(a). A linear fit yields a Clausius

FIG. 5. Force Index of control (a) and oligomycin treated (b) samples monitored for 100 min. Each marker represents the average Force Index for 5 min intervals (100–150 cells). The dashed line represents the overall mean Force Index for the control sample. (c) Force Index histograms for the last 50 min of the experiment. The Force Index histogram for the oligomycin treated sample is shifted from the control sample.

Mossotti factor slope of 2.4 times the Force Index. Using double shell models with parameters derived from bulk dielectric and time domain reflectometry the Claussius Mossotti factor was calculated for cytoplasm conductivities from 0.5 to 0.1 S/m.^{24,25} The cytoplasm conductivity, after oligomycin exposure, was estimated using the factor 2.4 and the Force Index measured in Fig. 5 between 60 and 100 min and also for the results in the supplementary figures.³⁹ These points are then plotted as filled circles in Fig. 6(b). For example, in Fig. 5 the after 60 min of exposure to oligomycin the Force Index drops by 0.05. Using the slope from Fig. 6(a), the estimated change in the Claussius Mossotti factor is -0.12 . Assuming linearity for negative Force

FIG. 6. (a) The real part of the CMF calculated using a double shell model is plotted against the measured Force Index, using results from Fig. 4. Each point is due to a different media conductivity (0.45, 0.35, 0.245, and 0.17 S/m). A slope of 2.4 was estimated from the linear fit. (b) The cytoplasm conductivity was estimated using the factor 2.4 and the Force Index measured in Fig. 5 between 60 and 100 min and also from the data in the supplementary figures.³⁹ A total lack of ATP conductivity boundary was estimated using the factor of 3 to 5 drop in potassium seen in apoptosis.^{35–37} For example, with ouabain the ATPase pumps are strongly inhibited and the potassium concentration dropped from 140 mM to 20 mM.³⁵

Index, this corresponds to a change in cytoplasm conductivity from 0.42 to 0.27 S/m. If ATP production were to cease altogether the conductivity would drop even further. With the onset of apoptosis, ATP levels drop and cytoplasm conductivity also drops.^{11,34,40} In K562 cells the cytoplasm conductivity dropped from 0.23 S/m to 0.05 S/m with the onset of apoptosis.¹¹ In apoptosis factors of 3 to 5 drop in potassium are observed.^{35–37} With ouabain the ATPase pumps are strongly inhibited and the potassium concentration dropped by a factor of from 140 mM to 20 mM.³⁵ Potassium makes up the majority of ions contributing to the conductivity. Therefore, a drop in the conductivity by a factor of 3–5 times is expected with a total loss of ATP. The bar at the bottom left of Fig. 6(b) shows this range of conductivities. The points after exposure to oligomycin are all above the level expected for a complete lack of ATP, indicating that the ATPase pumps are still partially active. This would most likely be due to glycolysis producing ATP.²³ Strong evidence the pumps continue to function is the fact the cells continue to control ion concentrations many hours after the treatment with oligomycin.³⁸ For 20–30 h the Force Index histograms remain close to those of untreated cells.³⁸ The cells are controlling ion concentrations, which is only possible if ATP is present to run the ATPase pumps. Since the mitochondrial path has been shut down other processes must be contributing ATP.

IV. CONCLUSION

The metabolism plays a critical role in determining the overall dielectric response of cells in either analysis or manipulation of cells by DEP.

In this work, we demonstrated that perturbations to the dielectric response of cells due to loss of mitochondrial ATP can be detected. This detection was enhanced by the judicious choice of DEP signal frequency and medium conductivity. This work demonstrates that the inhibition of mitochondrial ATP synthase causes a decrease in cytoplasm conductivity and a reduction of the K_{CM} of the cell. One can imagine using this approach to quantify mitochondrial versus glycolysis ATP production by measuring dielectric responses with and without oligomycin treatment. Having a dielectric assay for quantifying the mitochondria versus glycolysis ATP production at the single cell level will be useful.

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