CELL MEMBRANE ANALYSIS USING MODULATED DIELECTROPHORESIS

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Abstract. Dielectrophoresis – the manipulation of suspended particles by non-uniform AC electric fields – has been demonstrated to be an effective mechanism for determining the electrical properties of suspended cells. However, the use of the technique has been limited at low frequencies (typically below the kHz range) by the presence of other physical effects, such as AC electroosmosis and electrode screening that disrupt cell movement. This is a significant limitation, because these low frequencies potentially allow examination of the variation in membrane conductance due to conduction through ion channels and surface conductance of the membrane. In this paper we describe a method of examining this low-frequency region using a low frequency signal to modulate a 1 MHz carrier wave, allowing this region to be probed for the first time. Furthermore, by examining DEP spectra before and after the application of ion channel blockers, characteristic conductance peaks similar to those observed by patch-clamp methods, are shown.

Key words: Dielectrophoresis, K562 cells, membrane conductivity, ion channel blockers, quinine, verapamil, 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB).

INTRODUCTION

DIELECTROPHORESIS FORCE

Dielectrophoresis (DEP) is a phenomenon of induced force on suspended particles, which has been shown to be highly effective in elucidating the electrophysiological properties of mammalian cells, bacteria and viruses [4, 11, 14, 23, 29]. DEP can be used as a non-invasive and label free tool to characterise bioparticles from over 60 μm to less than 1 μm [3, 4, 6, 14, 19, 22, 25]. It has been

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used to differentiate between viable and dead yeast [10, 15] study the progress of apoptosis [5], characterise stem cells [16] and understand cancer biology [24].

The dielectrophoretic force, F_{DEP}, acting on a spherical body, is given by:

$$\mathbf{F}_{\mathrm{DEP}} = 2\pi r^3 \varepsilon_{\mathrm{m}} \mathrm{Re}\left[K(\omega)\right] \nabla \mathbf{E}^2 \tag{1}$$

where r is the particle radius, ε_m is the permittivity of the suspending medium, ∇ is the gradient operator, E is the amplitude of electric field and Re[$K(\omega)$] the real part of the Clausius-Mossotti factor, given by:

$$K(\omega) = \frac{\varepsilon_{\mathrm{p}}^* - \varepsilon_{\mathrm{m}}^*}{\varepsilon_{\mathrm{p}}^* + 2\varepsilon_{\mathrm{m}}^*} \tag{2}$$

where ε^*_m and ε^*_p are the complex permittivities of the medium and particle respectively, and $\varepsilon^* = \varepsilon - j*\sigma/\omega$ with σ the conductivity, ε the permittivity and ω the angular frequency of the electric field applied. The frequency-dependence of Re[$K(\omega)$] indicates that the force acting on the particle varies with the frequency. The magnitude of Re[$K(\omega)$] varies depending on whether the particle is more or less polarisable than the medium. If Re[$K(\omega)$] is positive, then particles move to regions of highest field strength (positive dielectrophoresis); the converse is negative dielectrophoresis where particles are repelled from these regions. By "nesting" iterations of equation (2) [13], $K(\omega)$ becomes dependent on the properties of many concentric layers of a particle, such as the cell membrane and cytoplasm.

Researchers typically analyse cells using a frequency range between approximately 10 kHz – 20 MHz, which allows the determination of parameters such as membrane conductance and capacitance, and cytoplasmic conductivity. This lower frequency limit is in place because as the energizing frequency becomes lower, effects related to change accumulation in the electrical double layer across the cells start to dominate, which affects the solution in several ways. First, the capacitor effects across the double layer can cause a drop in the potential applied across the bulk medium, leading to much-reduced DEP forces on little signal. Secondly, the interaction between the accumulated charge and the non-uniform electric field causes AC electro-osmosis (ACEO), a phenomenon whereby the suspending medium is driven to form vortices at the electrode edges. These redistribute the cells more rapidly than they can be analyzed by DEP, again nullifying the signal and resulting in little or no observable measure of the DEP force. Thirdly, low frequencies can lead to electrolysis of the solution, resulting in bubble formation and electrode destruction. As a consequence, low-frequency (DC to 1 kHz) DEP has generally been used for simple trapping rather than characterisation, for example in insulated-based "electrodeless" DEP [27, 28]. This is significant because whilst DEP can determine the steady-state conductance across the lipid bilayer itself, it is unable to elucidate the changes in electrical conduction across the ion channels found within the membrane. At low frequencies, the DEP behaviour is dominated by the conductivities of membrane

and medium [2], and so variation in the spectrum is due to changes in the membrane conductivity; at low frequencies (from DC to approx. 10 kHz in typical mammalian cells), equation (2) can be expressed as:

$$K(\omega = 0) = \frac{\sigma_p - \sigma_m}{\sigma_p + 2\sigma_m} \tag{3}$$

where σ_m is assumed to hold a constant value. However, ion channels allow the passage of charges between cell interior and exterior, and should manifest as conductive components in the cell membrane at frequencies where the ions have sufficient time to move across the membrane, typically found in the sub-kHz frequency band, resulting in narrow peaks in transmembrane conduction [21]. If it was possible to examine this frequency range, it would offer potential for examination of the function of ion channels in a manner normally addressed using much more complex, expensive and low-throughput methods such as patch-clamp [20]. In this paper, we explore for the first time a mechanism for examining the DEP behaviour of cells at low frequencies using a modulated DEP wave.

DIELECTROPHORESIS UNDER MODULATED ELECTRIC FIELD

Amplitude modulation (AM) is a common technique to encode signals for radio transmission. A signal across a range of low frequencies is encoded as the change in amplitude of a single higher frequency. Once the signal is received, it is demodulated to obtain the original low frequency signal. The simplest demodulation circuit for amplitude modulation signals consists of a diode and a capacitor. In a suspended cell the membrane acts as a capacitor, while there are two methods in which ion channels can act as diodes to demodulate the signal. Ion channels will normally conduct along a concentration gradient of the particular ion they transport; however, DEP is normally performed in media with low ionic strength. This limits the ability of the ion channel to conduct in both directions since there is a much higher concentration of ions inside the cell than outside the cell, making the flow of a particular ion essentially one direction similar to the flow of electrons through a diode. Additionally, some ion channels move ions directionally [20], have an intrinsic directionality only allowing excreting or taking up a particular ion make behave as a diode even without a concentration gradient. Since an ion needs a characteristic time to be transferred though a particular ion channel, the electric field will only have an effect on ion channels that transfer change in less than half the period of the signal. This has been modeled as an inductive component in then ion channels response for some time [20] and gives rise to resonance conditions at well defined frequencies. These resonance peaks should be detectable in the DEP response of the cell. If we modulate the amplitude

of a high-frequency carrier wave (for example, a 1 MHz wave) using a lower-frequency signal, then we would anticipate that where there is a change in conductance to due to ion channel activity, the net force on the cell would be due to the superposition of the low-frequency signal acting across the membrane due to the demodulation effect, plus the effect of the high-frequency signal. This high-frequency component would depend solely on the interaction between the medium and cytoplasm [2], the membrane having been bypassed at these frequencies. It is therefore possible to deduct the high-frequency force component by measuring the force acting on cells when exposed to an unmodulated signal at the carrier frequency. Hence, by using a low-frequency signal modulated onto a MHz-frequency carrier signal, we can observe the DEP spectrum of cells at low frequency and observe changes to the spectrum when channel blockers and other chemical agents are used; to further clarify the effect, it is possible to examine the difference between DEP spectra of cells before and after drug treatment in order to observe the action of the drugs. Nodulation also confers a secondary benefit; since the electrical double layer formation is governed by the high-frequency alternating component of the applied field, there is insufficient time for the double layer to accumulate significant amounts of charge, eliminating problems with electrode screening and AC electro-osmosis (e.g. [26]) which act to interrupt DEP measurements at low frequencies by reducing the voltage gradient in the medium and moving the fluid sample, respectively. A similar approach has been recently adopted [1] for the study of homogeneous nanoparticle movement, but this work represents the first use of the approach to study biological particles.

MATERIALS AND METHODS

CELL CULTURE AND ION CHANNEL BLOCKERS

Human chronic myelogenous leukaemia (K562) cells were grown in 20 mM HEPES modified RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), (Invitrogen, Paisley, UK), 2 mM L-glutamine and 100 units/mL penicillin-streptomycin. All cell culture reagents were obtained from Sigma Aldrich (Poole, UK), unless stated otherwise. The cells were grown under standard cell culture conditions with 5% CO_2 / 95% air at 37 °C.

The 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was dissolved in dimethylsulfoxide, and water was used for quinine and verapamil. All reagents were purchased from Sigma Aldrich, Poole, UK. A density of $2.5 \cdot 10^6$ cells per mL was used for ion channel blocker treatments, and the cells were incubated for two hours prior to DEP analysis. This procedure was followed for each agent: 20 μM verapamil, 15 μM NPPB and 20 μM quinine, 34 nM valinomycin.

DEP EXPERIMENTS

Data were collected using an adapted 3DEP (Deptech, Ringmer, UK) DEP-well instrument, as described in detail elsewhere [8]. In summary, well electrodes were energised and the motion of cells within the well electrodes was monitored using a Nikon eclipse E400 microscope fitted with a AVT Dolphin F145B camera. Light intensity measurements related to the motion of cells (and by inference, the force on those cells) were measured and processed using Matlab (the Mathworks, Natick USA). The system was adapted by energising the electrodes with a Thurlby-Thandar 2002 TG sweep function generator whose output was modulated (using the modulation input) using a Grundig/Digimess FG100 signal generator at both 1 MHz and 5 MHz. There was no observable difference between the two carrier frequencies. Data were collected at 5 points per decade. In order to reduce noise, spectra were filtered using a weighted rolling average with a 0.4 log window (i.e. two data points either side). To remove the effects of the carrier frequency, one data point was recorded at the carrier frequency. This was removed to compensate for the peak-to-peak voltage and the gradient of the squared field divided data.

The cells were centrifuged at room temperature at 190×g for 5 minutes. The pellets were washed and resuspended in isotonic medium consisting of 8.5% (w/v) sucrose plus 0.3% (w/v) dextrose buffer. The medium conductivity was adjusted to 10 mS/m using KCl and the final conductivity, before use, was verified with a conductivity meter (RS components Ltd, London, UK). The final cell population was counted using a haemocytometer and adjusted to approximately 2.5×106 cells/ mL (±15%) for DEP measurements. In order to reduce the effect of variation in cell number in each sample, the experiments were repeated many times (generally 4–6) with different populations, which were summed prior to modeling.

RESULTS

EFFECT OF MODULATION

Without modulation, the DEP spectrum of K562 cells is as shown in Figure 1. As can be seen, above 10 kHz the polarisability exhibits a rise and fall in accordance with the Clausius-Mossotti model of the behaviour of shelled spheres, which in turn allows the determination of electrical properties. However, as frequency drops towards 1 kHz the polarisability appears to move towards zero as the effects of electrode screening and ACEO reduce the potential and redistribute the cells.

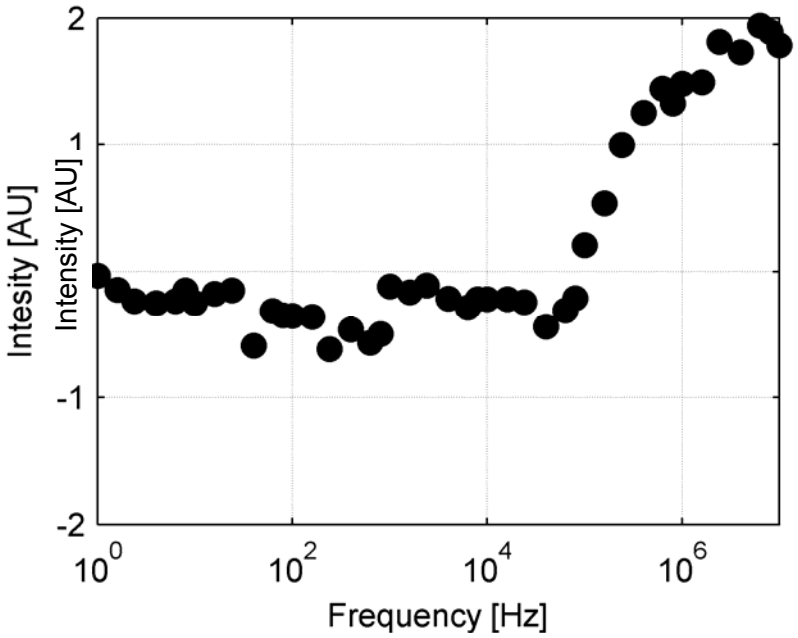

Fig. 1. DEP spectrum for K562 cells in the absence of modulation, showing the reduction in observed effects at low frequency. AU = arbitrary units relating to light intensity change (see [8] for details).

Figure 2 shows a close-up of the 1–10,000 Hz frequency range after the application of modulation. As can be seen, a change in behaviour is apparent; where the non-modulated signal produces a band of mild negative dielectrophoresis, the cells receiving a modulated signal exhibit slight positive dielectrophoresis. Of particular note is the behaviour below 100 Hz, where the polarisability – and by implication at low frequencies, the trans-membrane conductivity – exhibits a sharp rise. This is particularly notable as similar behaviour has been observed in cells measured when experiencing low-frequency electrorotation (ROT). ROT is a technique similar to dielectrophoresis in which cell rotation is observed in a rotating electric field [13], and cell properties can be elucidated using similar means to that used in DEP. However, it is constrained by requiring longer periods to perform the analysis whilst being applicable to a very small numbers of cells (often one cell being measured at a time). Previous studies [12, 30] have used electric fields rotating in the field regions we are examining here and have indeed observed changes in the ROT spectrum that parallels the results we observe here. For example, Hölzel [9] observed rotational shift at frequencies below 1 kHz in yeast, but did not attribute the behaviour to any particular origin, focusing instead on higher-frequency effects.

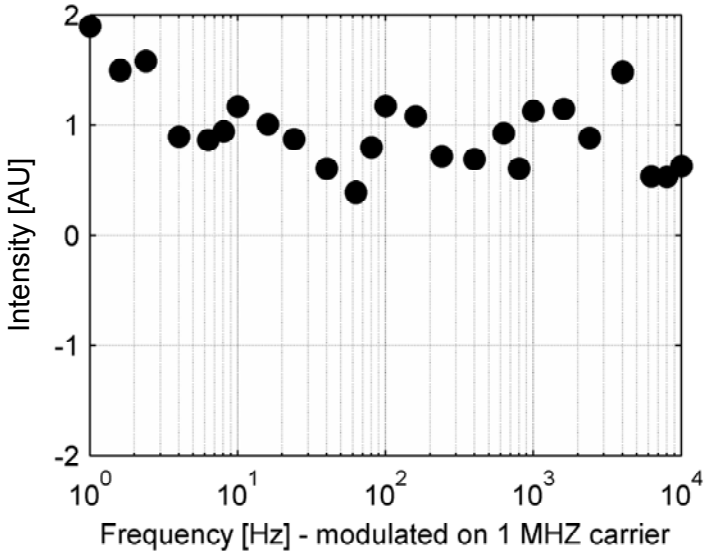

Fig. 2. Low-frequency (1 Hz – 10 kHz) DEP spectrum of K562 cells, using a 1 MHz modulation wave.

Similar low-frequency effects were observed by Maier [18] using electrorotation to study carboxylated latex beads, indicating an inorganic origin to the effect. However, in both of these cases the low rate of electrorotation may mean this observed effect is attributable to polarisation of the electrical double layer that causes a significant increase in positive dielectrophoresis, an effect observed at much higher frequencies in nanospheres [12]. Notably, both the Maier and Hölzel studies only considered frequencies above about 100 Hz; Georgieva *et al*. [7] performed similar experiments using red blood cells down to 3 Hz and observed a rotation peak at between 10–100 Hz, though again no explanation was given. Due to the mathematical foundation of the relationship with ROT and DEP [13], we would anticipate this would correspond to the peak rate of change in the DEP spectrum below this frequency region, which is indeed observed.

EFFECTS OF DRUG TREATMENT

In general, the use of channel blockers produced a lowering of low-frequency DEP activity; a plot of the difference between treated and untreated cells after adjustment showed a reduction in relative intensity, indicating a reduction in the general membrane conductivity. However, several local peaks of the kind described in the literature [20] were observed in many of cases. The results of these analyses are shown in Figure 3a–d.

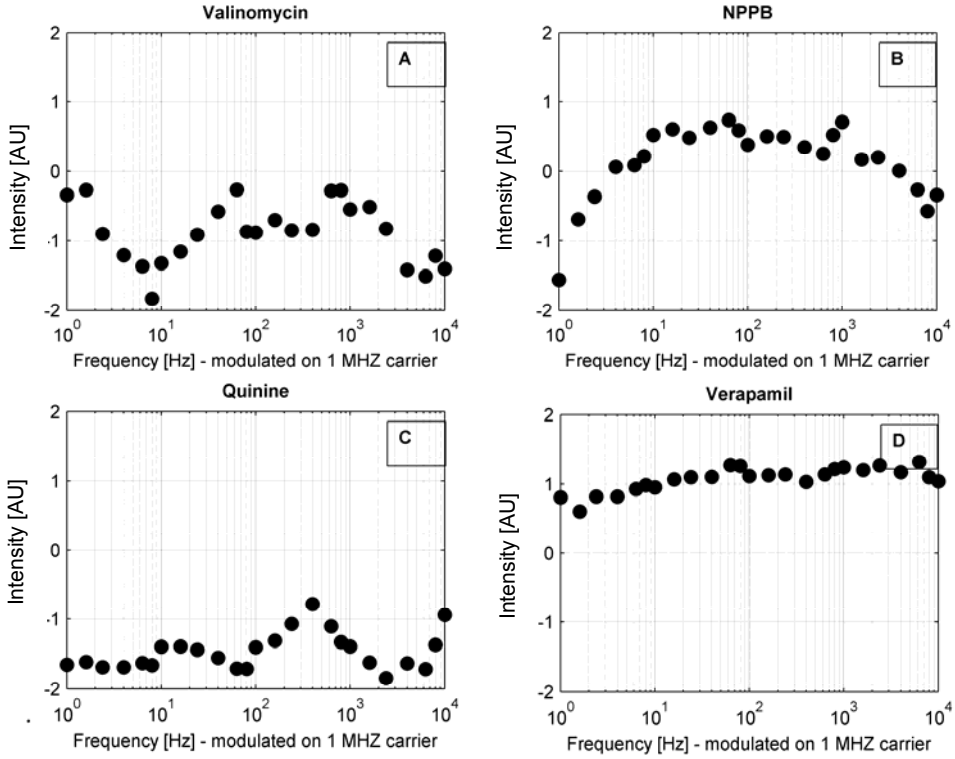

Fig. 3. Spectra of the differences between drug-treated and control cells for a range of different modulating agents, between 1 Hz – 10 kHz.

Valinomycin

Valinomycin is a potassium ionophore – that is, it allows passive transport of potassium ions across the membrane. Then, when we analyse the difference between spectra (Figure 3a) with and without the addition of the drug, we find that there is a reduction in the conduction across the membrane, indicating a general lowering of the transmembrane conductance. Notably, there is also a substantial increase in conduction below 5 Hz, possibly due to synchronised ion transport driven by the near-DC fields. There are also elevated conduction peaks centred at 50 Hz and 1 kHz.

NPPB

NPPB is a chloride channel blocker. When the cells were treated with NPPB prior to analysis and then compared against controls of the same cells (Figure 3b), the spectra indicated a small but broad increase in conduction at frequencies between 10–1000 Hz, but a substantial reduction in conduction below 10 Hz and a small reduction above 1 kHz.

Quinine

Quinine is a potassium channel blocker. The effect of the use of Quinine on the DEP spectrum (Figure 3c) was interesting in that, when subtracted from the control spectrum, a double peak was observed. At low frequencies, conduction was seen to be reduced generally. There is a minor variation from the general reduction between 10 and 100 Hz which could be interpreted either as noise or a very minor peak, but a much more significant peak was observed between 100–1000 Hz (centre approximately 400 Hz).

Verapimil

Verapimil is a calcium channel blocker. The change in the DEP spectrum of cells thus treated (Figure 3d) was small, but significant in that the change in DEP spectrum showed a rise in the polarisability, and by implication conductivity, at all frequencies but it exhibited no peak-like features whatsoever. This may indicate a change in the membrane properties without any increased trafficking, due to the absence of any calcium in either the intracellular or extracellular environments.

DISCUSSION

The low-frequency behaviour of suspended particles in electric fields is a complex field that has been subject to a great deal of study in the past. As outlined in equation (2), the DEP behaviour is governed by the conductivity of the particles, which itself consists of several components; the transmembrane conductance (communicating charge between the interior and exterior of the cell), the tangential conductance (acting around the cell membrane), and the conductance of the electrical double layer that surrounds the cell to a depth of a few nanometres. All these factors can be affected in steady state by chemical agents that can, for example, alter the surface chemistry of the cell to increase or decrease surface charge, or change the morphology so as to alter the effective cell radius. The changes in steady-state conductance caused by the action of the drugs used here may be due to any of these effects; the most likely causes are the change in surface conduction due to alteration of surface chemistry and the decrease in transmembrane conduction due to the reduction of charge carriers (either the lack of cytoplasmic ions, or the dysfunction of ion channels or transporters) to conduct charge across the membrane, but other mechanisms cannot be ruled out. Interestingly, those agents which act to reduce the concentration of ion in the cytoplasm (quinine and valinomycin, both of which exclude potassium from the cytosol) acted to reduce the effective particle conductance whilst those that blocked channels used to exclude ions from the cytosol, resulting in elevated levels in the cytosol (for example, chloride levels in medium are typically 30× higher than in the

cytosol, and similar to cytosolic levels of potassium in untreated cells), both showed elevated particle conductance, potentially indicating the model based on charge diffusion across the membrane may hold. Contributions from double layer conduction can certainly not be ruled out, though this effect may be partially minimised by the effects of the carrier frequency in reducing significant charge accumulation in the electrical double layer.

It has been noted by researchers past that there are changes in the electrokinetic behaviour of cells in low-frequency regimes that cannot be explained using interfacial polarisation theory. These had only been reported when using electrorotation, and had not been studied in detail. Using modulated DEP, we have shown a similar pattern of behaviour, with rising DEP spectrum below 100 Hz indicative of changes in the membrane conductivity. As with previous studies using ROT, we have observed anomalous activity – namely, a rise in effective membrane conductivity – at low frequencies (in particular, below 100 Hz). We suggest that this may be indicative of direct conduction of ions through the ion channels themselves. Peaks occur due to resonance conditions where particular channels are strongly affected. This is supported by the fact that this is reduced in all three cases of channel blocker treatment, and most notable in the case of the potassium channel blocker (potassium being the majority cytoplasm ion).

We have also examined the changes to the DEP spectrum caused by using ion channel blockers and an ionophore. The compounds that affect potassium concentrating in the cytoplasm both resulted in a reduction in membrane conduction, possibly due to the reduction in potassium diffusion from the potassium-rich cytoplasm of normal cells to the potassium-depleted treated cells. Notably, there were two conduction "peaks" where the cytoplasmic conductivity rose visible in the spectra, though not at the same frequencies; valinomycin produced peaks at about 50 Hz and 1 kHz, whereas Quinine produced spikes at approximately 20 Hz and 500 Hz. Channel modulation of calcium and chloride produced a much less peak-heavy activity, with verapamil showing a simple elevation of conductivity and NPPB producing elevated activity between 10 Hz and 3 kHz.

CONCLUSIONS

The low-frequency component of the DEP spectrum has the potential to offer new insights in low-cost, label-free ion channel study, but it has thus far been limited due to electrode effects at low frequencies. By modulating the electric field with a 1 MHz carrier wave we have revealed the low-frequency spectrum by DEP for the first time, showing effects previously observed by ROT and indicating that ion channel conduction can be observed at very low frequencies. Further studies in the sub-10 Hz regime may allow the development of DEP as a low-cost alternative to patch clamp techniques for ion channel screening.

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