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Conductivity of cell membrane investigated by a novel dielectrophoretic technique

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ABSTRACT

This article succeeded to introduce a new method, that mimics the radio broadcasting, used amplitude modulation to deliver the low range frequencies to the cell membrane. Consequently, avoiding the ACEO which prevents the measurement of DEP spectrum. In addition to the use of quinine and NBBP as ion channel blockers along with the modulated dielectrophoresis technique enabled the measurement of membrane conductivity providing a low cost method. © 2013 Trade Science Inc. - INDIA

INTRODUCTION

Electrokinetics is the movement of particles and/or cells under the effect of the electric field. The main interest of this study is 1- 10⁴Hz, range of frequencies. Dielectrophoresis (DEP), as a term was the first used by Pohl in 1951^[1] to describe the motion of electrically neutral matter caused by its interaction with non-uniform electric fields. Also, it is known as the lateral motion imparted on uncharged particles as a result of polarization induced by non-uniform electric fields. DEP: the motion of suspensoid particles, with permanent and/or induced dipoles, relative to that of solvent resulting from polarisation forces produced from inhomogeneous electric field, AC or DC. It depends on the asymmetrical charge distribution induced by the electric field within the particle. Eventually, results in variation of motion between the particles and fluid. Which is more polarisable will move towards the regions of high elec-

tric field for both AC and DC^[1]. Unfortunately, Dielectrophoresis is the weak force within this frequency range acquiring the particle a velocity of 1-10 μm/s^[2]. This motion may be towards or away from the regions of electric field maxima, depending on the electrical properties of the particles, the suspending medium^[3] and the frequency of the electric field. N. G. Green and H. Morgan^[4] proved that it is possible to use the DEP to separate nano-particles by using the proper frequency. This induces each particle type to behave differently according to the differences between them in their dielectric properties then using the proper flow rate to remove the particle type which is in the low field area away from the electrodes without affecting those particles experiencing positive DEP.

The dynamics of particles in the DEP process were studied by many researchers^[5-8] showed that the implementation of AC-DEP using extremely low frequency led to high control of different particles or groups of

them. In addition to the shape of AC voltage that proved to have an effect on the direction of particle's motion, together they could be used to improve the efficiency of particle separation and fractionation of heterogeneous particle mixtures. The cell shape, geometry, differs within different stages of cell cycle which implies a dielectric change in the cell spectrum. This makes the dielectric spectrum, dielectrophoresis, a good tool to monitor cell cycle changes^[9]. Dielectrophoresis (DEP) describes the change in the polarizational distribution of charges of a particle. Whether internal or on its surface depending on the frequency of the applied field, with respect to its surrounding medium in the form of movement which velocity depends on the volume of the particle, the square of the applied field and the permittivity of both the particle and its medium. DEP can be inserted safely to accurately describe the steps of any process that involves a change in, or a movement of, ion concentration within or during this process. DEP is used in a number of other experimental settings using cancer cell line models^[10-17]. Further DEP applications in biotechnology can be found in the review article of (Ronald Pethig and Gerard H. Markx^[18]). Dielectrophoresis as a label free tool could solve the problem of the lack of specific surface markers for human neural stem/progenitor cell (huNSPC) populations that mostly generate neurons and could identify, separate from other types and quantitate the cells based on their degree of differentiation^[19].

Also, DEP was successfully used to detect early stages of oral squamous cell carcinoma (OSCC), based upon the changes of cytoplasmic conductivity and the cell membrane capacitance^[20].

Antonio Ramos et al.^[21] observed a strong frequency dependant fluid flow during AC electrokinetic experiments on particles in suspension. The flow is below the charge relaxation time. They termed the phenomenon as AC electroosmosis. Electro-hydrodynamics, movement of fluids under the effect of electric field, is the main problem that we need to overcome, suppress and/or use in a way that serves our goal. It is the major force in our region of interest and causes the fluid to move with a velocity of 40-500 $\mu\text{m/s}$ ^[22]. Electro hydrodynamics is strongly represented in this low frequency region by the newly discovered phenomenon termed ACEO as it is responsible of this massive velocity mentioned earlier.

The existence and dominance of ACEO makes impossible to extend the dielectrophoretic spectrum within the frequency range of interest, 1 Hz – 10 kHz.

This means the interplay between the Electrohydrodynamics and Electrokinetics is one of the main interests of this study. This paper will show the ability of ACEO reduction, through amplitude modulation, which enables the DEP measurement.

Michael P. Hughes et al.^[23] designed a multilayered well shaped measuring cell to use the DEP phenomenon as a physical tool to study differences among different suspensions. FDEP forces particles to move with the aforementioned velocity depending on the permittivities, conductivities of the particles and suspending medium as well as the the square of the applied field gradient, frequency and the particle's volume. By capturing particles' motion sequentially through certain time duration, while applying FDEP, then subtracting these images from a control image taken before applying the electric field, a density profile, for the cells or particles under study, will be obtained. the latter will provide an estimation to the reaction of the suspension towards the applied FDEP showing a net positive or negative reaction. Using an arbitrary unit, the software that subtracts images, will provides a frequency versus intensity relation for the particles under study. According to Lionel B. et al.^[24] the frequency range of interest here expresses the membrane and medium conductivities.

An inhomogeneous electric field, E , exerts a force $F = P \cdot \nabla E$ on the electric dipole P . The field gradient pulls (both permanent and field induced) dipoles toward regions of high or low electric field strength, depending on the frequency of the electric field. The time average DEP force, F_{DEP} , acting on a polarisable particle of radius " r " exposed to an alternating and inhomogeneous electric field, E , is expressed as

$$F_{\text{DEP}} = 2\pi r^3 \epsilon_m \text{Re}[K(\omega)] \nabla E^2$$

Where, ω is the angular frequency of the applied field and $\text{Re}(\omega) = (\epsilon_p - \epsilon_m) / (\epsilon_p + 2\epsilon_m)$, is the real part of Clausius-Mossotti factor, ϵ_m , ϵ_p are medium and particle permittivities, respectively.

The Clausius-Mossotti factor determines the sign of the DEP force, as its value varies from -0.5 to $+1$.

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The particle is said to experience positive DEP when $K(w) > 0$ (high field seeker) and negative DEP when $K(w) < 0$ (low field seeker) Dielectrophoretic force, F_{DEP} , can be used as non-invasive and label free tool that selectively manipulate particles having sizes in range of 60 μm down to less than 1 μm ^[25-28]. The use of F_{DEP} is considered to be a useful tool that characterise and differentiate cells, bacteria, viri and bioparticles. It was also used to differentiate between viable and dead yeast, mutant bacteria^[29-34] and normal from cancer cells^[35].

MATERIALS AND METHOD

DEP system

Figure 1 represents a schematic diagram of the DEP system which is composed of:

Optical microscope (Nikon type), digital camera (USB2.0, 1.3 Megapixel, colour Supplied by Brunel Microscopes), oscilloscope, two function generators (Thandar 2002 TG sweep function generator (FG) and Grundig/digimess FG100 signal generator) the former is used for Amplitude Modulation of the frequency and PC.

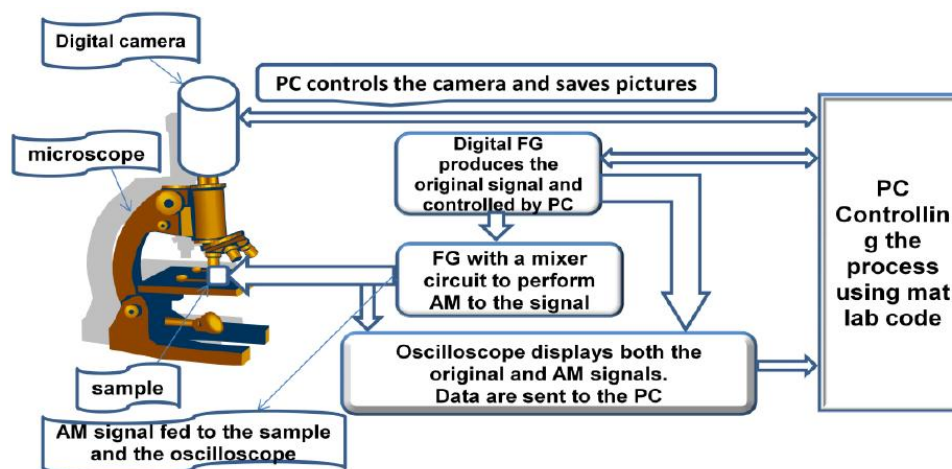


Figure 1 : Schematic diagram illustrating the DEP system.

The strength of FDEP differs with distance. Looking at the well as a cylinder and dividing its upper surface into 10 circles starting from the centre forming 10 equi-thickness cylinders. Generally, the figures in the paper will show the F_{DEP} effect on the cells located within the outer regions of 6-8, 6-9 and 7-9 where; the F_{DEP} has its strongest effect. So, the cells will be denoted as K568, K569, and K579, respectively. In summary, DEP-well instrument, is described in detail elsewhere^[36] and well electrodes in^[37].

Tissue culture methodology for K562 cells

The cells were centrifuged at room temperature at $190 \times g$ 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^[38]. 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 2.5×10^6

cells/ mL ($\pm 15\%$) 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 modelling.

Viability and cell counting

Viability is the measure of the proportion of live, metabolically active cells in a culture, as indicated by the ability of cells to divide or to perform normal metabolism. The index is usually expressed as a percentage of viable cells in a population:

$$\text{Viability index} = \left(\frac{\text{number of viable cells}}{\text{total number of cells}} \right) * 100$$

The number of viable cells counted using haemocytometer then the total number of cells/ ml is determined.

Drugs used in this work

The different drugs used and their concentrations are given in the following table:

Drug	Function	type	Concentration
Quinine	K ion channel blocker	generic	20 μ M
NPPB(5-Nitro-2-(3-phenylpropylamino)benzoic acid)	Cl ion channel blocker	generic	15 μ M

Modulation, moving average and carrier frequency correction

The Amplitude modulation of the frequency is used in this work for the first time to reduce the velocity of ACEO phenomenon dominating the low frequency range. The idea is to move the testing frequency to higher range where the strength of ACEO phenomenon is reduced and that of the F_{DEP} is increased. Thus, allowing safe and reproducible results of the membrane conductivity that is less affected with the fluid motion produced by the ACEO. Two frequency spot points were used for modulation in this work namely, 1 MHz and 5 MHz. No significant difference between the two carrier frequencies is observed. The applied frequency produced from the FG100 function generator is fed to the Thandar 2002. Second FG, which produces the higher frequency, 1 or 5 MHz, in addition the second function generator has a mixer circuit.

The second stage is the capture of the cells motion resulting under the effect of the DEP signal, whether modulated or not, within the well. This motion is captured through successive shots (pictures that are taken by a digital camera fixed upon an optical microscope). The group of pictures taken are then analyzed by a homemade mat lab software at the centre of biomedical engineering, Surrey university, using subtraction mode.

In order to reduce noise,

Central moving average was used to filter out noise, smooth the data, and identify the direction in the region of interest. Data of this work does not suffer the lag caused by the moving average as we used an odd number of points to preserve the position of peaks within the spectrum.

Carrier frequency correction is applied on each frequency point. This decreased the difference among the control and the treated data. Data points were divided by four to compensate for the peak-to-peak voltage and the gradient of the squared field. Then the data of the control cells were subtracted from the drug treated cells to give the final spectrum.

RESULTS AND DISCUSSION

Amplitude modulation

From Figure (2- a, b) it is clear that the modulation has a net positive effect on the DEP measurement. Noting that the points in Figure 2b indicate the cells are still alive during the measuring process. As dead cells will cause a negative intensities for the whole DEP spectrum.

Effect of 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB)

In a medium conductivity of 0.41 mS/m, NPPB has similar behaviour as that with quinine as shown in Figure 3. The membrane conductivity decreased leaving a wide hump above the zero level upon the blockage of Cl ion channels and keeping Cl ions at the interior of the cell. Consequently, the potassium ions were locked in as well, to preserve the membrane potential. On the other hand, the cytoplasmic conductivity decreased which indicates that Cl ions migrate from the cytoplasm towards the cell membrane at the internal side of the Cl ion channels and/or the Ca ions flows outside the cell through the Ca ion channels in the membrane by diffusion. The hump on the membrane conductivity represents the Cl ion channels with their ions blocked at their internal surface. The movement of Ca ions through its channels in the cell membrane may have raised the hump level.

In a medium of a slightly higher conductivity, 10 mS/m, NPPB addition is affected by the higher conductivity of the medium that increases the complexity of the situation, the membrane conductivity is generally reduced leaving a crest with increased conductivity. Using a 5 MHz as a carrier frequency, the crest's height is increased as seen in Figure 4. The cytoplasmic conductivity remains positive due to the blockage of K and Cl ions in the cell and due to the presence of free ions in the outer medium reduced the amount of Ca ions moving out.

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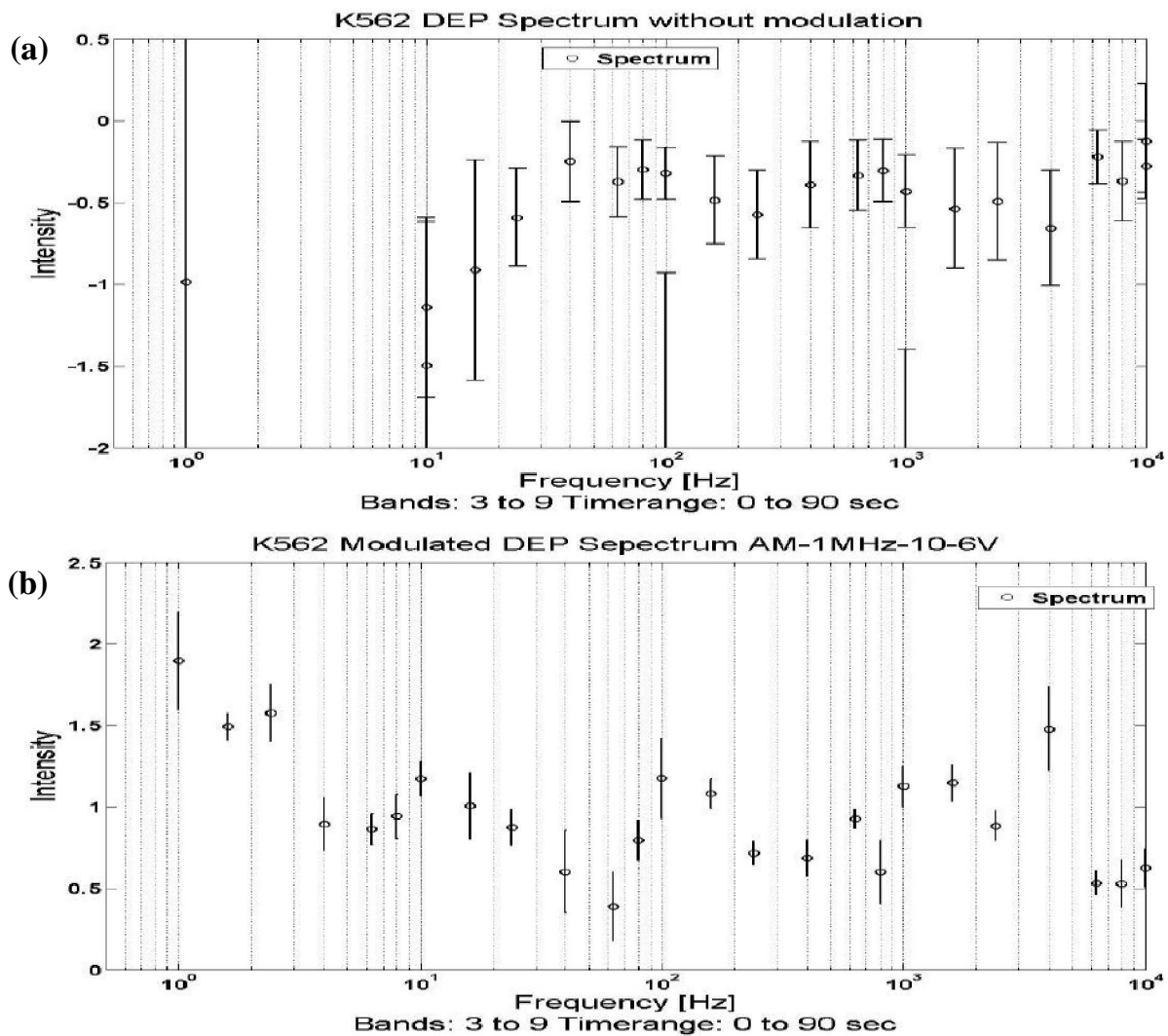


Figure 2 : F_{DEP} normal spectrum for the range 1- 10^4 Hz, (a) without modulation and (b) AM modulated.

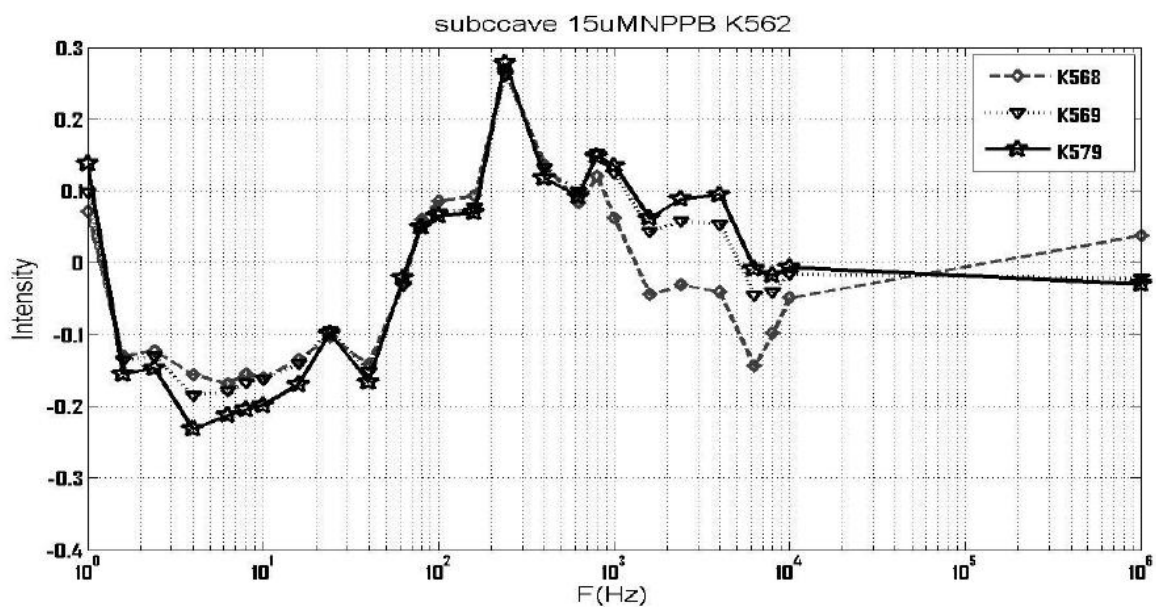


Figure 3 : NPPB channel blocker in a low conductive medium.

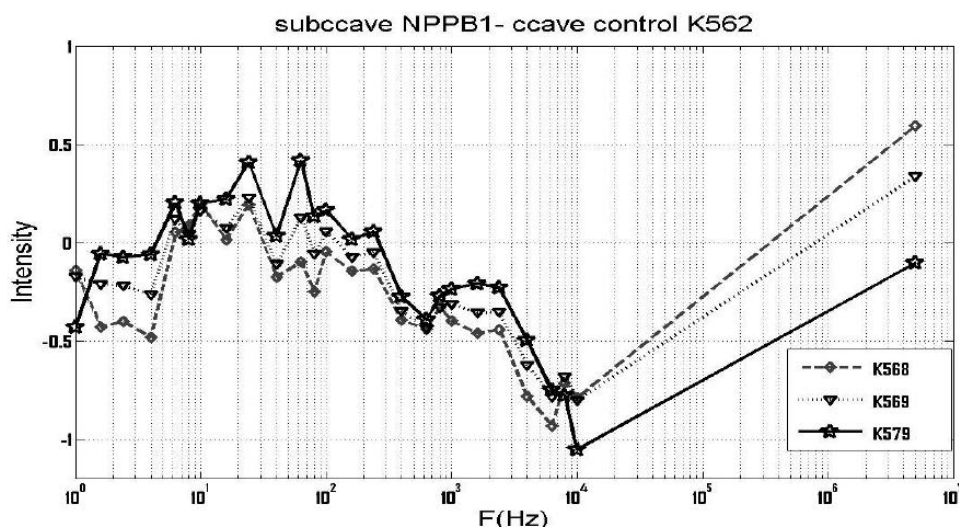


Figure 4 : 5 MHz carrier frequency.

In case of NPPB use, the situation is the same as that of quinine, decrease in membrane conductivity is noticed. The presence of the hump is also pronounced but covering a wider frequency range in our scale than that occupied by the quinine's peak. Unlike the peak appearing with quinine, occupies about one decade, this one takes up about two decades. It's appearance is greatly enhanced with the increase in medium conductivity and is reduced with the decrease of suspending medium conductivity. Again, this crest appears when using 1 MHz as a modulating frequency and changes its position mostly for the same reason as that of quinine. The use of 5 MHz for modulation, this height is increased in the positive part of the spectrum.

In short, the increase of ionic content of the medium causes a stronger crest. While increasing the modulation frequency transfers this crest up the conductivity axis to the positive part leaving it intact with no width reduction.

Effect of quinine

Quinine is added to the cells in culture medium and incubated at 37 C for two hours. The cells are centrifuged and washed in the measuring medium that contains the same concentration of quinine then the DEP spectrum is carried out. Cell concentration is 2.35×10^6 cells/ml and concentration of Quinine is 20 μ M. Using a medium conductivity of 10 mS/m, along with the quinine results in a decrease on membrane conductivity showing a small hump that hardly emerges above the zero level on the conductivity scale (Figure 5). This

hump is due to the accumulation of K ions at the interior side of the membrane at the K ion channels. The flow of Ca ions through Ca ion channels may have a role in raising the level of the hump. The Cl ions are bound to the K ions to preserve the membrane potential of the cell, so the movement of Cl ions through the membrane is limited and there are too many Cl ions in the outer medium. While that of Ca is not, actually Ca ions are forced to move by diffusion until balance is achieved. As for the cytoplasmic conductivity it remained positive due to the K and Cl ions kept in the cell.

Increasing the carrier frequency to 5 MHz and the cell concentration to more than 5 million/ml at a medium conductivity of 10 mS/m, results in more dominance of the carrier frequency, as the frequencies ratio is about 0.2%, showing a complete reduction of the membrane conductivity and the small humps are immersed below the zero level (Figure 6).

The difference in polarisabilities between the cell membrane and the medium is decreased due to the presence of free ions in the outer medium, and then FDEP becomes of even more limited strength. The data revealed a net decrease in the membrane conductivity while the cytoplasmic one remains positive.

The use of quinine as a potassium channel blocker decreases the membrane conductivity. However, there is a small hump that may be due to the K ion channels themselves and the accumulated ions at their internal side or due to the aggregation of the ion channels together, as they're floating on the bilayer. This increases the size of the net dipole of such protein channels, thus

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changing the relaxation time according to the degree, shape of aggregation or amount of aggregates. The level of this hump or small peak clearly appears when using a 1 MHz as a modulating frequency and descends below zero as shown in the Figure 6 when using a 5 MHz. In spite of the descendance of the small peak's level below zero in latter case, it still exists in the negative part of the spectrum which indicates that it represents a dynamical process within the cell membrane that need to be unveiled. The changing position of the same hump

is also an interesting situation that requires further investigations. Another point to reconsider here is that the less the conductivity of the cell's measuring medium the more pronounced the peaks are as the difference in polarisability between the cell membrane and the suspending medium is large and vice versa. In other words, the degree of interaction between ionic flow and /or ionic retention and the electric field and dielectrophoretic force proportional to the difference in conductivity between the particles and suspending medium.

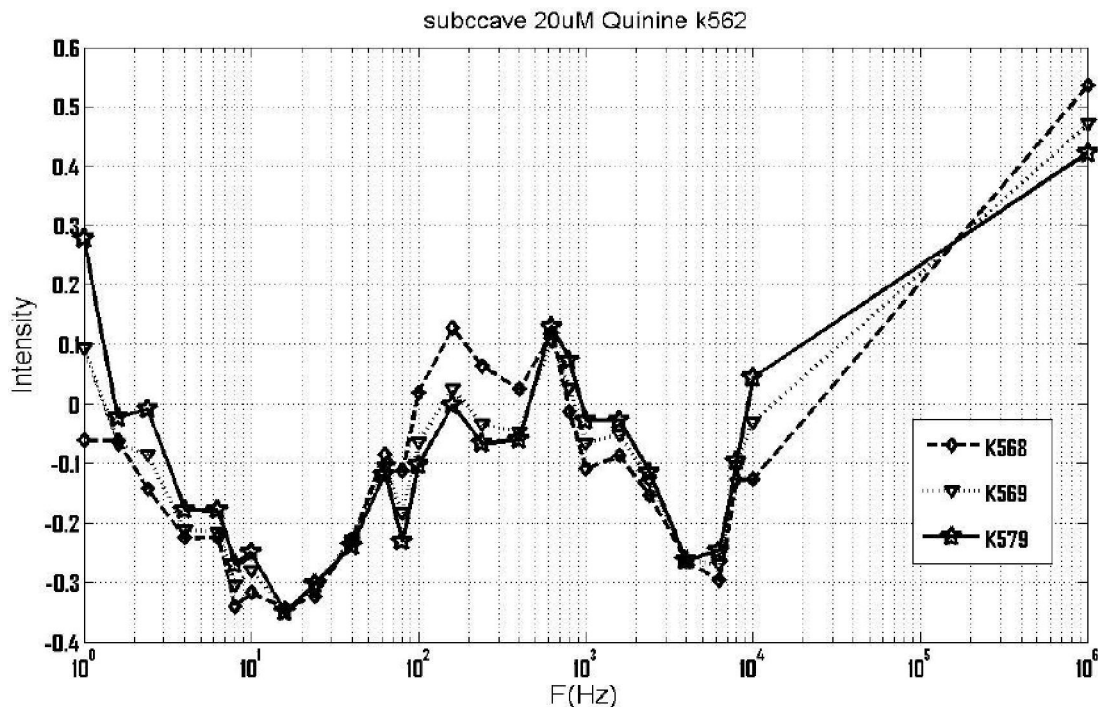


Figure 5 : Membrane conductivity versus frequency in 10 mS/m medium conductivity.

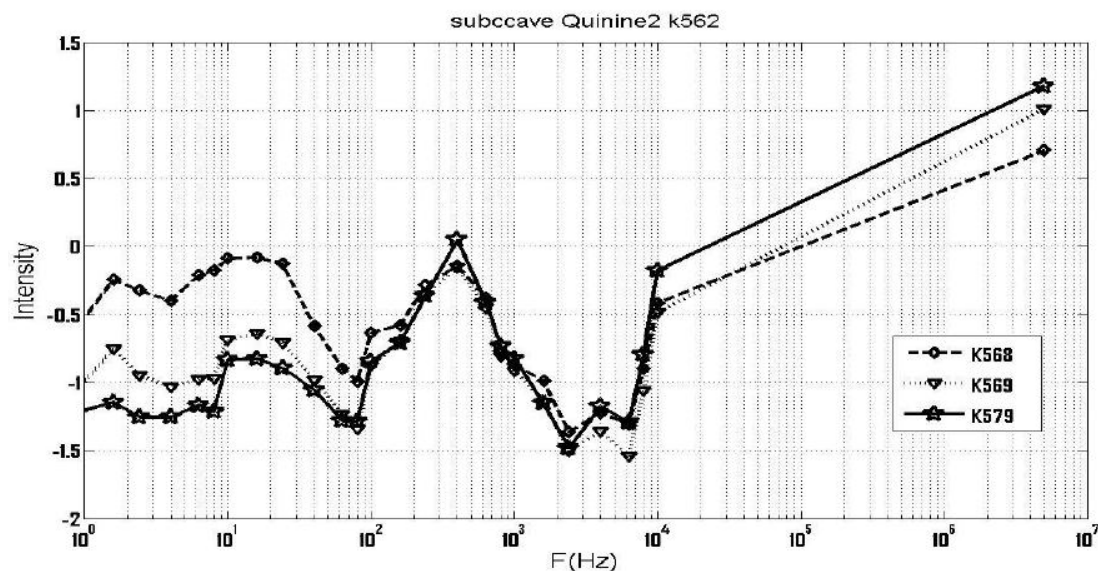


Figure 6 : 5 MHz as a carrier frequency and 10 mS/m as a medium conductivity.

Effect of NPPB and quinine

Figure 7 shows that there are two crests one taking just about one decade and the other takes more than one decade and is broader than the first pike. This indicates that the one peak occurs as a results of the blocking potassium ion channels and the other one represents

the blocked Cl ion channels.

There is something strange here, by using both K and Cl ion channel blockers together, results in two crests one is sharp like that appears with the blocking of K ion channels and the other is broader as that appears when using Cl ion channel blockers.

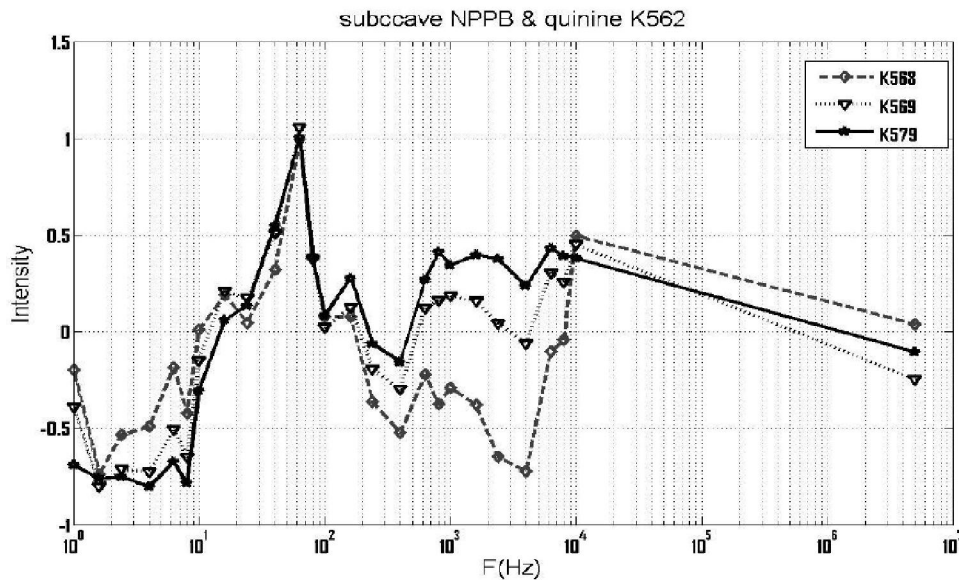


Figure 7 : Subtraction of control from treated data for both NPPB and Quinine in a 10mS/m conductive medium.

Analysis and explanation of the spectra over the frequency range of, 1 - 10^4 Hz, is very complicated and suffers from many problems such as:

1. Dominance of electroosmotic phenomenon. This problem has been overcome by using amplitude modulation of the frequency.
2. The intimacy between both membrane and medium conductivities in this frequency range.
3. The difficulty to use the reference frequency to compare and evaluate the changes in the region under investigation.
4. Unfortunately, the ion channel blockers used are generic and not specific.
5. Medium conductivity is a very important parameter as it affects the DEP spectrum in many regions and the cells' response changes under the effect of medium conductivity (this is due to the relation in Clausius-Mossotti factor). This point needs further investigations for the detailed effect of different medium conductivities for the type of cells studied.
6. FDEP does not have the same magnitude all over the well and that makes it difficult to get the same effect on two similar cells or particles located in different regions in the well. The reason of different responses when evaluating different regions in the well depending on the strength of FDEP in each region which affects the bio particle's polarisation compared to the medium polarisation.
7. FDEP forms a gradient that nearly vanishes at the well centre. We are convinced that a more advanced design, using an elliptical shaped well, would clarify the data and ease the experimental work. However, it will make the math work within the software more tedious, but it will only be done once.
8. The use of cell's velocity alone to determine the effect of FDEP is not enough since the force resulting from the electric field is in a gradient form and does not have a fixed magnitude. This makes the magnitude of the velocity gained by the particles under the effect of the field is a function of the position as well. In other words, the position as a new parameter to the measuring dilemma should be considered.
9. At the end, blocking one type of ion channels means that this ion type is blocked inside the cell. However, this does not affect any other ion type from

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moving freely inside and outside of the cell, unless this ion is using the same ion channel and we do not know this. In addition, the use of channel blocker will induce the cells to respond using different mechanisms that try to cancel the effect of the accumulation or loss of this specific ion inside or outside the cells.

Therefore, in spite of the progress made and the success of the experimental work in estimating the effect of two ion channel blockers upon the cell membrane, the notes mentioned above needs to be considered in the forth coming work to reduce the parameters and attain much more data.

Effect of modulation

The idea of modulation in which we used a carrier frequency that is high enough to carry the frequency range of interest. Modulation has four merits:

- i. It enables the use of higher voltages without worrying about burning electrodes.
- ii. Raised this part into the positive range of the spectrum, and gave us a logical reason to exclude odd data, which would only confuse the process of interpretation of the results.
- iii. Reduced the ACEO velocity to a limit that enabled the FDEP appearance leading to some sort of reproducibility as this phenomenon, ACEO, is reduced at these high frequencies.
- iv. Error bars became less in magnitude, which indicates better resolution for measuring data.
- v. The researcher can know the viability of its cells from the results of the experiment. As this part of the spectrum lies in the negative part of the spectrum, i.e. if the cells die during the experiment, one wouldn't know unless the viability test is repeated during the experiment. However, using the modulation rises the spectrum into the positive region. Consequently, having an unjustified negative point would indicate cell death.

CONCLUSION

The interplay of Claussius-Mossotti factor comprising the permittivities and conductivities of the particle and medium (as they control the net velocity of the cells towards or away from the high field regions) in addition

to the particle volume and the square of the applied electric field. The first four factors are affected simultaneously upon the use of ion channel blockers making the resulting effect on the DEP spectrum is both very complicated and difficult to interpret. The use of Modulation has helped a lot by the transfer of the axes into a known region where the researcher is able to identify the results and make use of them. In spite of involvement of too many parameters, ϵ_m , ϵ_p , σ_m , σ_p , Γ_p and E^2 that are involved and changed simultaneously, the FDEP spectrum for the cells in this frequency range succeeded in the addition of valuable information about the cell membrane conductivity and the movement of different ions through the ion channels in the cell membrane. In other words, the success of controlling the ACEO phenomenon using frequency modulation, In addition to the simple mathematical manipulation used in this work, enabled getting information about the cell membrane conductivity, the in- and out-flow of ions through the cell membrane and the ion channels within the membrane. This work made the examination of this low frequency range possible. Which would offer a potential 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^(39,40), in much cheaper way.

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