Dielectrophoretic characterization and separation of metastatic variants of small cell lung cancer cells

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ABSTRACT

In this study, the ability of Dielectrophoresis (DEP) to recognize phenotypic variants of small cell lung cancer (SCLC) cells has been tested. The phenotypic variations of the SCLC cells are associated with the cell-surface decorations, which in turn, provide the cells with anti-adhesive properties allowing detachment from the primary tumour and the subsequent metastatic spread of such tumours. Dielectrophoretic separation chambers have been used to sort the phenotypes and so form a cytometric tool for the analysis, identification and monitoring of tumour heterogeneity.

Keywords: Dielectrophoresis, Electrokinetics, Cancer Cells, Cell Membrane Capacitance

1 INTRODUCTION

Dielectrophoresis (DEP) has been shown to be a useful technique for the manipulation of particulate matter and is also being widely used to understand the dielectric properties of cell suspensions. DEP has been commonly used as a cell sorter and one of the main advantages that it provides, is the ability to manipulate hundreds of particles in parallel without the need to fluorescently label them. DEP based flow through systems allow us to discriminate between particles with very subtle differences in their dielectric properties. Several studies have been undertaken to determine a role for DEP-based devices in cancer research [1]. There is accumulating evidence that changes in the physicochemical properties of the cell surface, including charge, could contribute to the efficiency of the metastatic process. More recently DEP has been used to differentiate drug accumulation phenotypes in breast cancer cell lines using cytoplasmic conductivity [2], so opening possibilities in the area of drug discovery where DEP devices may be used to determine the functional impact of drug candidates.

Changes in the expression of polysialic acid (PSA) polyanionic decorated cell surface molecules have been increasingly associated with the dissemination of cells from the primary tumour mass [3]. By increasing expression of PSA and its associated negative charge, the adhesiveness of the cell is reduced, which promotes detachment from the primary tumour and hence metastatic spread. The pleiotropic effects of the neural cell adhesion molecule NCAM, a major target molecule for PSA decoration, appear to reflect the ability of NCAM to regulate membrane-membrane contact required to initiate specific interactions between other molecules. Indeed when NCAM with a low PSA content is expressed, adhesion is increased and contact-dependent events are triggered. Our biological hypothesis is that the phenotypic variations of the SCLC cells are associated with the extent of cell-surface decoration provided by the α-2, 8-linked polysialic acid (PSA) and the neural cell adhesion molecule (NCAM). Here we have used dielectric properties to investigate changes in DEP properties associated with phenotypic variants of small cell lung cancer cells (NCI H69) which we call H69-adherent and H69-suspension variants.

2 THEORY AND SIMULATIONS

Dielectrophoresis can be defined as the movement of polarisable particles in the presence of non uniform electric fields. Taking the simplistic view of a cell as a spherical homogeneous particle, the DEP force on the cell is given by

$$F_{DEP} = 2\pi\varepsilon_m r^3 Re\,(CM)\,\nabla E^2 \qquad (1)$$

Where r is the radius of the cell, ε_m is the absolute permittivity of the medium, E is the electric field acting on a cell and CM is the Clausius-Mossotti factor, which describes the Maxwell-Wagner relaxation that occurs at the interfaces between dissimilar dielectrics such as the particle and medium and is given by

$$CM = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \qquad (2)$$

Where ε_p^* and ε_m^* are the effective complex permittivities of the cell and the medium respectively.

The Clausius-Mossotti (CM) factor is a measure of the effective polarisability of the cell and is distinctive of a particle being dependent on the cell contents and morphology. CM is a frequency dependent variable and by changing the frequency of the electric field it can exhibit shifts from a negative to a positive value. This, in turn, causes the direction of the dielectrophoretic force

experienced by a particle to change direction. If the real part of *CM* is positive then particles move towards the regions of high electric field experiencing what is termed positive DEP. In negative DEP, particles move way from high field regions towards regions of low field intensity.

For cells that exhibits a change from negative to positive DEP as frequency of the electric field is increased, the change in direction occurs at a single frequency known as the cross-over frequency (f_{cr}). At f_{cr}, the dielectric properties of the cell equal those of the medium and so the cell appears transparent to the externally applied electric field and experiences zero DEP force. *fcr* can be approximated to [4]:

$$f_{cr} = \frac{1}{2\pi}\sqrt{\frac{(\sigma_p - \sigma_m)}{(\varepsilon_p - \varepsilon_m)}\frac{(\sigma_p + 2\sigma_m)}{(\varepsilon_p + 2\varepsilon_m)}} \qquad (3)$$

Where σ_p, σ_m and ε_p, ε_m are the conductivities and permittivities of the particle and the medium respectively. When DEP is used in combination with fluid flow, additional forces of drag, buoyancy have to be considered. The drag on a spherical particle is given by:

$$F_{Drag} = 6\pi\eta r(u - v) \qquad (4)$$

Where η is the viscosity of the medium, u is the fluid velocity and v is the particle velocity. Since cells are denser than their aqueous suspending media, the buoyancy force is given by:

$$F_{By} = \frac{4}{3}\pi r^3(\rho_p - \rho_m)g \qquad (5)$$

Where ρ_p and ρ_m are the densities of the particle and medium respectively and g is the gravitational constant.

Considering a two dimensional model with stable forces for computational simplicity, the steady state velocity in the horizontal (x) and vertical (y) directions are given by [5]:

$$v_x = \frac{F_{DEPx}}{6\pi\eta r} + u \ , \quad v_y = \frac{F_{DEPy}}{6\pi\eta r} - \frac{2r^2}{9\eta}(\rho_p - \rho_m)g \qquad (6)$$

To obtain a better understanding of the influence of the influence of fluid velocity on the dielectrophoretic capture of cells on microelectrodes, two dimensional simulations of the fluid and particle velocities within the microfabricated dielectrophoresis chambers used in this work were created using Comsol Multiphysics 3.2. Within the devices the fluid flow channel had a height of 150µm with interdigitated electrodes laid out on the bottom surface of the chamber with a width and interelectrode separation each of 20µm. The electrodes were energised with sinusoidal voltages such that the potential difference between adjacent electrodes had a peak value of 6V. Within the simulations *CM* was set to a value of 0.5, allowing particles to experience positive DEP towards the high electric field regions around the edges of the electrodes. Electric field simulations, an example of which is shown in figure 1, predict very localized high field intensities at the electrode edges with a rapid reduction in intensity with increasing distance from the electrode edge such a field pattern gives rise to a strong dielectrophoretic force towards the electrode edges.

The arrows in figure 1 indicate the direction and magnitude of fluid flow along the channel and exhibit a classic laminar-type flow profile. Particle trajectories are indicated by lines starting on the left hand side of the figures and have been calculated using equation 6. The simulations show that for a low fluid velocity of 1µm/s particles up to approximately 75µm above the electrodes experience a net DEP-dominated vertical force towards the electrodes. Simulations at 10µm/s, shown in figure 1, reveal the DEP-dominated vertical force extends only to a height of 60µm with particles above this height typically unaffected by the electric field. The observations from these and similar simulations have been used to optimize the experimental parameters such as fluid velocity, electrode geometry and energising voltage for the selective capture and enrichment of cells.

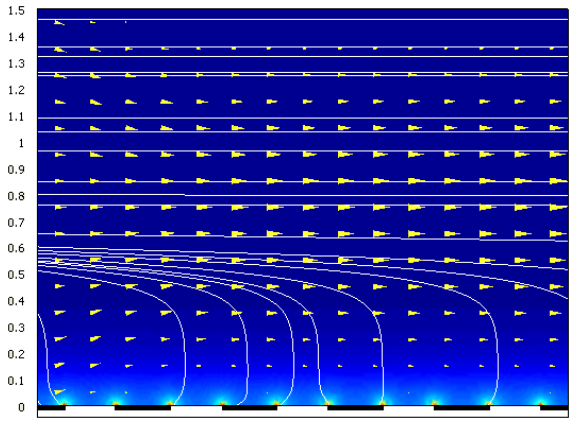

Figure 1: Particle trajectories at a fluid flow rate of 10 microns per second

3 EXPERIMENTAL

3.1 Cell Preparation

Small cell lung cancer (SCLC) cells (NCI-H69) [6] were grown in RPMI-1640 media (GIBCO) with 10% FBS under standard culture conditions at 37°C, 5% CO_2 in a humidified incubator. NCI-H69 cells grow as loose aggregates in suspension culture, but consistently express a sub-population (< 5%) of variants that show substrate adherence. A simple enrichment protocol for substrate adherence was used to generate an adherent phenotypic variant (H69-adherent).

Cells were washed and resuspended three times in conductivity buffer prior to use for DEP. The conductivity buffer (8.6% sucrose, 0.3% dextrose and 0.1% BSA in

water) was calibrated at room temperature against a 1413uS/cm standard solution (Hanna instruments, Hungary), to 150µS/cm by adding PBS.

3.2 Devices and Methods

Quadrupole electrodes were used to dielectrophoretically characterise the two phenotypic variants of SCLC cells used in this work. These electrodes, patterned in an 80nm gold and 5nm chromium film on a glass substrate using photolithography, consisted of 4 electrodes with semicircular tips arranged at 90° to each other with an interelectrode separation of 150µm between opposite electrodes..

Flow experiments were undertaken in a dielectrophoretic separation chamber similar to that shown in figure 2, consisting of a glass substrate onto which approx 2cm^2 array of interdigitated microelectrodes with a 20µm width and spacing was photolithographically fabricated. The channel structure was fabricated from a 150µm thick adhesive backed polymer cut to shape using femtosecond laser micromachining [6]. An upper polycarbonate plate was used to encapsulate the channels. After assembling the substrate, channel forming layer and upper plate the device was passed through a heated roller to ensure good bonding between the layers. Additionally, the upper plate contained 4 drilled holes over which NanoPort (Upchurch Scientific) fluidic interconnect ports were bonded to form microfluidic inlet and outlets to the separation chamber. Fluid flow through the chamber was controlled using a peristaltic pump (Gilson Minipuls 3).

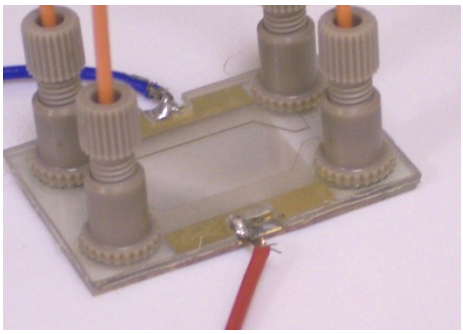

Figure 2: Dielectrophoretic fluidic separation chamber.

3.3 Dielectrophoresis Experiments

SCLC Cells of each phenotype were characterised using the quadrupole electrodes. The electrodes were energised using sinusoidal voltages of 3Vpp with a 180° phase difference between adjacent electrodes. Cells were characterised to identify the crossover frequency for each phenotype by observing the direction of dielectrophoresis for electric field frequencies ranging from 1kHz to 10MHz. The crossover frequency was measured by finding the frequency at which the cells exhibited zero dielectrophoretic motion.

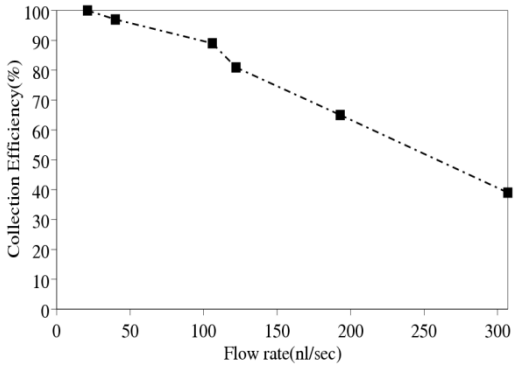

Figure 3: Effect of flow rate on collection efficiency.

Experiments to selectively collect and enrich samples of mixed phenotype were undertaken using the dielectrophoretic separation chambers. Prior to these experiments, the chambers were characterised for collection efficiency at different fluid flow velocities. The flow rate to achieve a desired level of cell collection was determined by fixing the electrode voltage and frequency and adjusting the fluid velocity to achieve good capture of a sample of adherent phenotype cells on the electrodes. Figure 3 shows the influence of fluid flow rate on the collection efficiency of the chambers.

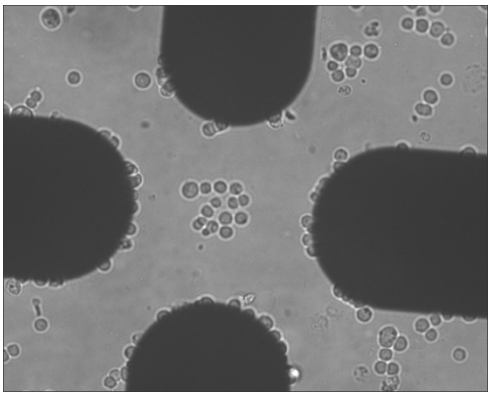

Figure 4: Differential DEP separation of adherent and suspension cells from a mixed population of SCLC cells.

4 RESULTS AND DISCUSSION

Two SCLC cell phenotypes were studied, one an adherent line and the other a line showing predominately suspension characteristics. Using the quadrupole electrodes the distribution of crossover frequencies for each phenotype was measured. For the adherent line observations of >50 cells found the crossover frequency ranged from 30kHz to 40kHz. For the suspension phenotype a larger range of crossover frequencies was observed ranging from 30kHz to 60kHz. Additionally it was observed that a proportion of the suspension phenotype exhibited adherent-like properties and that within a population of suspension cells, choosing an electric field frequency of 40kHz it was possible to

selectively separate cells displaying a adherent-like properties. Such separation is shown in figure 4 where a sample of suspension cells is exposed to an electric field of 40kHz. Adherent-like cells are seen dielectrophoretically collected at the edge of the electrodes while suspension-like cells remain uncollected.

Using the separation chamber a mixed population of adherent and suspension cells were fractionated by flowing the cells through the chamber at a flow rate of 50nl/s while applying a 3Vpp 40kHz voltage to the chamber electrodes. At this frequency cells showing adherent characteristics would be expected to collect on the electrodes while suspension-like cells would experience a negative dielectrophoretic force and be carried through the chamber with the fluid flow. Cells exiting the chamber while the voltage was applied to the electrodes were collected. When the entire sample had been passed through the chamber the voltage was removed and additional suspending medium was passed through the chamber. This allowed cells trapped on the electrodes to be collected as a separate fraction. Both fractions were cultured in 96 well plates for 1 day. Post culture observation of the fractions revealed that the dielectrophoretically collected fraction exhibited adherent characteristics while the second fraction exhibited suspension characteristics. This experimental result supports the hypothesis that cells within the suspension population display phenotypic variations ranging from adherent-like to suspension like characteristics.

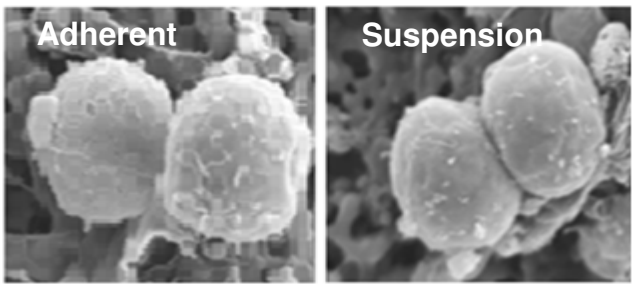

Figure 5: Cell Electron Micrographs shows greater membrane texturing in the adherent phenotype

For viable cells equation 3 can be approximated to [4]

$$f_{cr} = \frac{\sqrt{2}\sigma_m}{2\pi r C_{mem}} \qquad (7)$$

Where r is the cell radius and C_{mem} is the cell membrane capacitance per unit area. Microscope measurements give the radius of both cell types to be 10μm ± 1μm. Using the range of crossover frequencies measured for each cell gives membrane capacitances for the adherent cells of 17-22mF/m^2 while for the suspension cells the membrane capacitance is 11-22mF/m^2. Previous studies [7] have shown that membrane capacitance mainly depends on membrane surface topography. Extra surface features can increase the membrane surface area and hence the membrane capacitance. Scanning electron micrograph studies, similar to those shown in figure 5, of the cell phenotypes used in this work reveal that cells exhibiting adherent characteristics have a greater degree of surface decoration than those exhibiting suspension-like characteristics. This in turn leads to a larger membrane capacitance and subsequently a lower range of crossover frequencies. It is our biological hypothesis that the phenotypic variations of the SCLC cells studied here are associated with the cell surface decoration provided by the 2,8 linked polysialic acid (PSA) and the neural cell adhesion molecule (NCAM).

5 CONCLUSION

This study of H69 phenotypic variants has shown that dielectrophoresis has the ability to selectively sort cell types with subtle phenotypic differences. Electrokinetic studies have shown that within a single phenotype differences can be identified using dielectrophoretic examination leading to the possibility of cytometric devices for analysis, identification and monitoring of tumour heterogeneity.

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