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CIRCULATING TUMOR CELLS ISOLATION USING ON-CHIP DIELECTROPHORETIC PLATFORMS

Florina S. ILIESCU[1], Ciprian ILIESCU[2]

Abstract. *Understanding the specific and intriguing properties of cellular subpopulations is one essential aspect of the future development of biomedical research. It relies however on the isolation of the cells to be analyzed. The existing downstream assays permited step-by-step identification and separation of the cells under scrutiny via specific immunologic labeling. Dielectrophoresis (DEP), which developed as one alternative and complementary approach to the immune assays, employed differential electrical proprierties of the cellular subpopulations to be identified and characterized. DEP belongs to the family of label-free technologies besides the biomechanical-based procedures. DEP proved itself succsefully in the detection and isolation of the cells and it showed real clinical potential when applied to the study of circulating tumor cells (CTC). The present work highlights the technological advances in the field of detection, isolation and characterization of the scarcely noticeable malignant cells in the blood of patients with various malignancies. Since the moment the body of research evidenced the role of CTC in the malignant progress towards metastasis, CTC transformed into a prognostic element of the deadly disease cancer is. However, the cells have to differentiate from the blood cells and this is possible if the various intrinsic physical proprierties are used by the label-free technologies like DEP. Such technologies are ideal in the process of isolation of CTCs which lost or transformed their antigenic expressions during the malignant transformation. The validity of the procedure is analysed in the current review to highlight the clinical applicability of DEP as label-free isolation technique in oncology.*

Keywords: dielectrophoresis, circulating tumor cells, microfluidic

1. Introduction

Microfabrication offers an excellent platform for chemical and biological applications due to cost-effectiveness, controllability, low volume, high resolution, and sensitivity. The efforts concentrate upon one of the deadly diseases which, according to statistics, remains a principal cause of death worldwide: if in 2008, 7.6 million lives were lost to cancer (~13% of all human deaths), more than 13 million are expected in 2030.[1,2] However, the World Health Organization estimates that at least 30% of the deaths and 90% of cancer-related mortalities due to metastatic cancer are preventable with a development of new therapies to

[1]Senior Lecturer, PhD, School of Applied Science, Republic Polytechnic, Singapore. (florina_iliescu@rp.edu.sg).
[2]Senior Scientist, Ph.D., Department of Cell and Tissue Engineering, Institute of Bioengineering and Nanotechnology, Singapore, (ciliescu@ibn.a-star.edu.sg), Member of the Academy of Romanian Scientist.

prevent recurrent malignant diseases. As a result a relevant number of applications were developed: MEMS based cell culture platforms,[3,4] applications in gene therapy,[5-7] transdermal drug delivery,[8-10,100] microfluidic driven drug screening systems.[11,101] The study of circulating tumor cells (CTCs) stands out and may allow achieving these important goals if the applications develop into sensitive and specific screening and diagnsotic devices. To date, the immune-affinity and separation using antibody coated particles,[12-16] demonstrated CTCs in the most common solid malignancies.[17-25] If translational cancer medicine emploies detection methods to analyze these rare CTCs[26,27] morphologically, it will contribute essentially to the the better understanding of the key events underlying the progression of this deadly disease. Moreover it will contribute essentially to the detection of CTCs of various origins and to the isolation of the sub-populations.

In order to succeed in depicting a comprehensive phenotypic profile of the CTCs, these methods must also consider the inter- and intra-individual variability in CTC count overtime,[26] the average CTC frequency across specific malignancies,[28] and most importantly the methods of CTC detection.[29] The newly proposed technologies which developed and were validated[30] are the label-free procedures based upon the biomechanical and electrical proprieties of CTCs. They emerged as alternatives to the biological markers-based tests[12, 13] to complement them and to contribute to early diagnosis and accurate prognosis of malignancies.

The label-free procedures are based upon the biomechanical and electrical proprieties of CTCs and their aim is to provide a rigorous, timely and accurate identification of the malignant cells through their evaluation, isolation and enrichment in blood. Presently, comprehensive research studies have to bring in solid evidence to transform the newly emerged label-free-modus-based technologies into approved sensitive and specific diagnostic modalities. Several label-free technologies, used both alone or in combination with biological and immunological assays, have recently been reported and related in particular to the these steps and to the possible analysis of CTCs.[31-35] Moreover, various methods for the detection and isolation of CTCs have been established recently, with strong links between the CTC count and the survival rate or the disease progression after therapy.[36, 37] Additional benefit stems from the use of CTCs count as a possible monitoring tool of the intermediate response to therapy[30,38] or of the presence of residual tumoral activity post-surgery, inclusive of the novel changes in cancer cells.[39,40]

The current focus of the research in the field is on validation of CTCs as biomarkers[30] via several aspects: the development of highly sensitive and specific new devices to analyze the CTCs, the affordability of such devices and the capability to manipulate the tumor cells towards the better definition of CTCs

(genetic mapping, gene transcription or biological behavior of CTCs).[41] Various reviews analyzed either the CTCs isolation[31,42-45] or the potential clinical applications of the CTCs subpopulations based on their analysis.[29] The conclusions highlighted the potential utility of CTCs analysis in solid cancers serving multiple critical functions from being a liquid biopsy to avoid the disadvantages of repeated solid biopsies, to contributing as prognostic markers, as affordable predictive and intermediate endpoints biomarkers of therapeutic response or as Pharmacodynamics endpoints in the monitoring of novel therapeutics. Other aspects were highlighted as desirable features of the label-free technologies: to provide a reliable, repeatable, rapid, cost-efficient procedure [11] with a high sensitivity, sufficient specificity with the capability to process clinically relevant blood samples[46-48] if possible in an automated process. Last but not least they have to isolate viable and as less as possible disturbed CTCs for further analysis.

Majority of the label-free technologies employed microfluidic systems as results of intensive development of Micro and Nano-technologies [10, 31, 49]. Two major directions can be observed in the detection and enrichment the CTCs with the help of microfluidics devices related to the development of: techniques based on the differences between biomechanical proprieties such as cell size, density, and deformability and techniques that exploit the differences in the cellular electrical proprieties – dielectrophoresis[50] and impedance spectroscopy[51]. Apart from these two major aspects stands the magnetophoretic separation of red blood cells as a modality of CTCs pre-enrichment with subsequent applications.[52-54] The various approaches that exploit the differences in the cellular electrical proprieties will be presented below. The scope is to highlight again specific label-free modus which gained stronger support as promising minimal-invasive and real-time investigation modalities, with clinical, pharmacological, toxicological and genetic applications towards the personalized therapeutic strategies.

The future belongs to the Micro- and Nano-technologies which will contribute with sophisticated bio-medical devices and laboratories-on-a-chip to confer advantages such as small dimensions, transportable, easy-to-use and small-amount-of-sample-required type of analysis.

2. Cell trapping and separation using dielectrophoresis

Dielectrophoresis presents a particular interest in separation of cell population. The teminology was lauch by Pohl[55], dielectrophoresis (DEP) being define as the movement of the dielectric particles due to the polarization efect in an unhomogenous electric field. A neutral particle becme electrical polarized in an electrid field. The presence of a gradient of the electric field, made this polarization of the particle to be non-uniform, generating a net DEP force that

moves the particle towards the higher electric field gradient region (so called „positive DEP"-pDEP) or moves the particle towards the lower electric field gradient region (negative DEP-nDEP). (Figure 1).

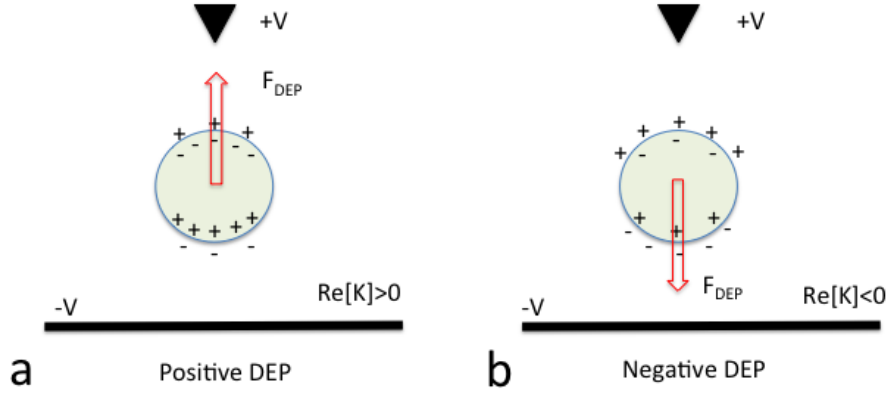

Fig. 1. Definition of positive (a) and negative (b) dielectrophoretic effect.

The DEP force can be define as: [56]

$$\left\langle \overline{F}(t) \right\rangle = 2\pi\varepsilon_1 R^3 \left\{ \mathrm{Re}[K]\nabla E_0^2 + 2\,\mathrm{Im}[K](E_{x0}^2\nabla\varphi_x + E_{y0}^2\nabla\varphi_y + E_{z0}^2\nabla\varphi_z \right\} \quad (1)$$

where E_{i0} and $\varphi_i (i = x; y; z)$ are the magnitude and phase, respectively, of the electric field components in the principal axis directions. The *real* component of the force induced dipole moment in the particle. The sign of this force is depending on the sign of the real part of the Clausius–Mossotti (CM) factor - $\mathrm{Re}[K]$. As a result, in a "classical way" the DEP force is:

$$F_{DEP} = 2\pi R^3 \varepsilon_1 \,\mathrm{Re}[K]\nabla E_0^2 \quad (2)$$

The *imaginary* part of the expresion (1) induced dipole moment and to spatial non-uniformity of the field phase. This force directs the particle against or along the direction of travel of the electric field depending on whether the phases of the field components is positive $(\mathrm{Im}[K] > 0)$ or negative $(\mathrm{Im}[K] < 0)$. The phenomenon is known as travelling wave dielectrophoresis (TWD). The expression can be given by:[57]

$$F_{TWD} = \frac{4\pi^2 \varepsilon_m r^3 \,\mathrm{Im}[K(\omega)]E^2}{\lambda} \quad (3)$$

At the frequency where $\mathrm{Re}[K] = 0$ or $\mathrm{Im}[K] = 0$, the particle experiences no positive or negative DEP force. This specific value is called crossover frequency. and depends on dielectric properties of particle and medium.
The CM factor can be define as:

$$K = \frac{\varepsilon_2^* - \varepsilon_1^*}{\varepsilon_2^* + 2\varepsilon_1^*},\ \varepsilon^* = \varepsilon - j\frac{\sigma}{\omega} \quad (4)$$

where ε_2^* and ε_1^* are the complex permittivity of the particle and medium respectively. The complex permittivity for a dielectric material can be described by its permittivity ε, conductivity σ, and angular frequency ω of the applied electrical field E_0. Taking the example of conventional DEP item, the DEP force acting on a spherical particle is a function of Clausius–Mossotti factor Re (K), which determines the direction of the DEP force. The CM factor is a function of electric field frequency.

At a range of frequencies, the particle experiences positive DEP while it shows negative DEP at another range of frequencies. The frequency where the particle shows no positive and negative DEP is called crossover frequency. The change of the permittivity and conductivity of the particles and medium will cause the shift of the crossover frequency. Thus at a selected frequency range, one population of particles experience positive DEP while the other population exhibit negative DEP. This provides a possible basis to separate the mixture of different particles.

A particle suspended in a solution is subjected also to hydrodynamic force, gravitational force, and electro-hydrodynamic force. A careful analysis of these forces is presented in [58]. The movement of the particle in a fluid is determined by the resultant force of many factors. Considering the influence of all kinds of forces, different separation mechanism can be performed based on different microfabricated DEP devices.

Based on microfabrication and MEMS technologies, a lot of DEP devices have been developed for a broad range of particle manipulation and separation. According to the solution for generation of electric field gradients, the devices can be classified into:

1) Change of phase of the applied electric field (Travelling wave DEP);

2) Change of dielectric media (insulating DEP);

3) Change of electrode shapes (conventional DEP);

4) Change of electric field gradient by optical image (image DEP);

5) Others.

3. Dielectrophoresis in CTC isolation

The electrical proprieties of CTCs may be exploited to discriminate them from blood cells by either applying a non-uniform electric field through a phenomenon of dielectrophoresis (DEP) or by analyzing their electrical impedance through Electrical Impedance Spectroscopy (EIS). Dielectrophoresis (DEP) is considered an attractive method for cell sorting because it offers label-free detection, and requires small sample volume.

DEP is defined as the movement of a neutral but polarizable particle in a non-uniform electric field due to the interaction of the particle's dipole and spatial gradient of the electric field. The term DEP was introduced by Pohl [59] and subsequently analytical modeling of separation of particle populations using DEP was presented by Gascoyne and Vykoukal.[60] The phenomenon is described by DEP force. There are two types of DEP forces, positive (p-DEP) and negative (n-DEP). According to the electrical properties of the particles, medium and frequency of the electric field, the particles can move to high electric field strength (positive DEP) or to the low electric field strength (negative DEP)- Figure 3.

By manipulating the electric field within the microfluidic channels the separation of particles can be achieved.[46, 50, 61-63] It is worth mentioning that DEP forces depend on parameters like the volume, the dielectric proprieties of the cells, the surrounding medium and the applied electric field.[64] Two aspects are relevant for separation of two distinct cell populations in microfluidic devices. The dependence of the DEP force on the particle's volume of the, and on the gradient of the electric field. These variables allow separating and collecting populations of cells in different locations of the microfluidic device based on their complex permittivity, and the cell structure. The differentiation is possible due to the magnitude and direction of the dielectrophoretic force related to the intrinsic cell's properties, such as a change in cytoplasmic conductivity or the differential content of cellular organelles. Therefore the collection of the homogeneous groups is possible either using an external force (i.e. hydrodynamic force induced by the flow in the microfluidic channel- separation under continuous flow)[109] or the motion induced by DEP force only.[65]

Since the dielectric proprieties of cells both normal and tumoral vary significantly, the separation can be achieved using various methods.[66] There are five major DEP techniques, essentially classified based on the method used to generate the electric field gradient: conventional DEP - the gradient of the electric field is generated by micrometric-size electrodes having different geometries, isolating DEP (iDEP) - the electric field gradient is generated by a non-homogenous dielectric medium between the electrodes)[67, 68], travel wave DEP - based on phase changing of the applied electric field,[69] optical DEP – based on an optical image projected onto a photodiode surface which generates the gradient of the electric field,[70] and medium conductivity gradient DEP - based on the variation of the electrical conductivity of the medium between two parallel electrodes.[71] Each of these methods supported separation techniques of tumor cells conferring the DEP devices a large applicability for CTC separation. Such opportunity derived from the specificity of electrical properties of cancer cells remarked long time ago by Gascoyne and Becker. Their teams used interdigitated gold electrodes, to isolate

and collect with high efficiency leukemia [72] and breast cancer lines from spiked healthy donor blood.[73] During the early stages, CTCs isolation using DEP carried out with microelectrodes showed cell cleavage and low isolation efficiency and throughput.[74] Based on the success of the initial studies but willing to address the disadvantages, other studies were designed to implement and improve the conventional DEP method. Variation from conventional DEP method occurred along the way.

Cheng et al.[74] isolated HeLa cells from human peripheral blood using DEP, while Broche et al[75] showed the difference in electrical properties between oral squamous cell carcinoma (OSCS) and normal keratinocyte cell populations. In a similar study, An et al [76] demonstrated the specific dielectrophoretic properties of cancer cells using a DEP activated cell sorter. In their study, they separated malignant human breast cancer epithelial cells (MCF-7) and breast cells (MCF10A) based on cell's electrical properties. Another team designed a low-cost DEP microfluidic device for purification of colorectal cancer HCT116 cells from the mixture with human embryonic kidney 293 (HEK 293) and E. coli cells.[77]. Sabuncu et al. reported the use of DEP for differentiation between two malignant cell populations.[78] Jen et al. demonstrated the role of preconcentration of HeLa cells (metastatic human cervical carcinoma) in a device with concentric circular electrodes with the trapping efficiency of 76%.[48] Alazzam et al. proposed separation under continuous flow of MDA231 breast cancer cells spiked into blood. The device was a structure comprising interdigitated comb-like electrodes, with the electrode pairs positioned divergent and convergent with respect to the flow. This method effectively moved the target cells near the edge of the channel, while the blood cells were collected in the center.[79]

Recently, the planar design of electrode proposed by Das et al.[80] showed the advantage of not being a blockage or not acting as a barrier to cells. Consequently, the usage of lower voltage to isolate small particles/cells was possible. HeLa cells were isolated in the presence of non-uniform electric field with a continuous flow pattern. Chen et al.[81] reported improved isolation efficiency of HeLa and MCF-7 cell lines spiked with RBCs by adopting stepping electric field created by a spiral electrode. The concentration of cells on the central electrode was 80% and the survival rate of CTCs about 82% higher than the one of RBCs. A similar recovery rate for CTCs, a higher recovery of blood lymphocytes (90%) with a 94% cellular viability resulted from a study which used an interdigitated comb-like DEP device.[82] One 3D model of microfluidic device by Cheng et al[83] showed an outstanding throughput when tested to isolate PC14PE6/AS2-GFP (AS2-GFP) lung cancer cells. This device used lateral DEP (LDEP) and proved that the CTCs with different sizes, dielectric properties, and shapes showed different LDEP velocities resulted in their isolation. In this case, the recovery rate found was of

85%, with enrichment factor of 10^5 at the flow rate of 20μL/min with average frequency of 10 kHz. The study showed also that by increasing the channel length the throughput increased significantly. Other than conventional DEP, the other major DEP techniques have been investigated to evaluate their potential.

iDEP also had been the principle used to design microfluidic devices for CTCs isolation. For example, Kang et al.[84] in their attempt used DC DEP to separate WBC and breast cancer cells based on the cell size. In their application, the DC electric field facilitated the generation of iDEP effect and the electrokinetic transport of the particles. However, the iDEP-based method presented its limitations caused by the required high voltage and by the Joule heating effect. It is known that the increased temperature affects negatively the isolated cells' viability. In 2012, Salmanzadeh et al.[85] also used iDEP to observe the DEP response of different-stages ovarian cancer cells.

Another major technique evaluated was travel wave DEP. In this direction, Cen et al [86] combined travel wave DEP with electro-rotation for the manipulation and characterization of human malignant cells.

Optical DEP has been the principle used by Huang et al. in their study which isolated prostate cancer (PC-3) and human oral cancer (OEC-M1) cell lines. The results showed recover rates of almost 75% and 66% for the PC-3 and (OEC-M1) cell lines respectively. The cell viability was close to 94% and 95% for PC-3 and (OEC-M1) cell lines respectively.[87] Furthermore, Wu et al. studied the medium gradient conductivity DEP to demonstrate the capability of such microfluidic device. They evaluated the dielectric proprieties of colon cancer cells (HT-29) when dielectrophoretic capture voltage spectrum under various medium conductivities was used.[88] Since no technique was perfect for clinical application, combinations of DEP techniques contributed to a better understanding of the principle capability.

Huang et al.[89] proposed a DEP field flow fractionation (depFFF) approach to allow continuous processing of sample that did not require intermittent application of the electrical field for cell recovery. The depFFF method utilized the DEP force to position the cell at a defined level in a fluid velocity gradient. The cells with similar dielectrophoretic properties traveled with the same speed. Later on, Gascoyne et al [90] used a similar design for separation of three different cancer cell lines (MDA-MB-435, MDA-MB-468 and MDA-MB-231) from a mixture with peripheral blood mononuclear cells. The study reported recovery rate exceeding 90%. Another study used the depFFF method to characterize the membrane capacitance, density, and hydrodynamic properties in correlation with the morphology and size of cultured tumor cells.[91] A derivation from depFFF, the pinched flow isolation method, was presented by Bhagat et al. to demonstrate the

isolation of MCF-7 and MDA-MB-231 cell lines. In this case, the recovery rate was more than 80% with an enrichment of 3.5×10^5 fold and 1.2×10^4 fold for RBCs and peripheral leukocytes at a flow rate of about 10^8 cells/min.[92] Moon et al. [93] proposed a two-steps process for the separation of CTCs. The team combined inertial separation (multi-orifice flow fractionation MOFF) in a microfluidic device with dielectrophoresis. The study utilized physical proprieties of CTCs to successfully isolate the MCF-7 breast cancer cells from spiked blood. The first step of the combination (MOFF) presented high-throughput (10-100µl/min) but low specificity. The second step, a DEP sorter consisted of two zones. In the first zone, all the cells (CTCs, WBC and RBCs) were pushed through the channel walls, while in the second zone the CTCs migrated to the center of the microfluidic channel. The DEP separator used improved the selectivity of the whole process. Therefore, the results showed an isolation efficiency of 99% for MCF-7 breast cancer epithelial cells spiked in blood.

Another separation method derived from DEP field-flow-fractionation method (depFFF) is the support for a recently launched commercial microfluidic system, ApoStream™.(30) The mechanism of this label-free microfluidic device consisted of a field flow separator with interdigitated electrodes over which the mixed cell population flows. Since the CTC population expresses positive DEP it is pulled along the floor, flowing near the electrode plane. The other cells in the blood sample, which express nDEP, are levitated and pass the electrodes. The validation study for this device used two different cell lines, with a different levels of EpCAM: SKOV3 (high level) and MDA-MB-231 (low level). The results showed the average cell recovery of 75% and the viability of the MDA-MB-231 cells of 97% after a seven days culture. Unfortunately, apart from this commercialized system, only preliminary efforts towards the clinical application of the DEP have been reported.[94] In conclusion, DEP separation techniques may certainly achieve a good purification. However, the process presents disadvantages, which need to be addressed further to ensure the clinical applicability of the techniques.

It is known that DEP is a time-consuming process. Besides this time-related disadvantage, some other parameters require optimization in order to increase the efficiency of DEP-based microfluidic devices: frequency, voltage, flow rate, buffer composition, and the thermal effect. Several studies addressed these disadvantage-related parameters to understand better how to counterbalance them for an optimized process.

Since the buffer solution is critical for a good separation process,[34] the DEP buffer should ideally satisfy several conditions in order to fulfill the roles efficiently. The first condition is to maintain the cell viability during the separation process.[95] Secondly, it must maintain the physiological conditions by having a pH close to 7 and an osmolality of 200-400 mOsm/kg).

Moreover, the DEP buffer is desirable to have a low conductivity for a reduced Joule thermal effect during the process of cells isolation.[96] The last point is also one of the critical aspects to be discussed since the cells are sensitive to high temperature. It is known that the conductivity of the buffer solution and the large power generated around the electrode contribute actively to the intensity of the Joule thermal effect. Consequently, this effect has to be controlled carefully in DEP devices to avoid the negative effect on cell's viability.[97] Studies showed that DEP techniques, which require high voltage, are more likely to produce an increase in temperature and from this point of view, the iDEP is not recommended. [84] One more reason for not recommeding iDEP is the interaction of the high voltage field with cellular membrane with consequences on the cellular physiology due to changes in the membrane' electrical potential.[98] In comparison, the nDEP method which uses lower voltage field to trap CTCs may avoid such exposure and may protect the isolated cells. Moreover, the continuous flow-based-DEP separation methods, which are associated with good heat convection, are more recommended than the sequential methods.[65, 90, 93] Also recommended are the DEP methods which use 3D electrodes with good thermal conductivity (such as silicon) and low thermal effect.[99]

In conclusion, DEP has the potential to achieve a high purification rate for the CTCs in the blood samples examined despite the low throughput. [93]

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