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Analysis of the role of elution buffers on the separation capabilities of dielectrophoretic devices

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ABSTRACT

Field flow fractionation dielectrophoretic (FFF-DEP) devices are currently used, among the others, for the separation of tumor cells from healthy blood cells. To this end specific suspension/elution buffers (EBs), with reduced conductivity (with respect to that of the cell cytoplasm) are generally used. In this paper we investigate the long-term alterations of the cells and elution buffers. We find that the EB conductivity is critically modified within few minutes after cells suspension. In turn, this modification results in a change the ideal separation frequency of the FFF-DEP device. On the other hand we prove that DEP manipulation is preserved for more than three hours for cells suspended in the considered EBs.

1. Introduction

Dielectrophoresis (DEP) is currently used to determine the electrical characteristics of the cells, which are then analyzed [1] and exploited for the manipulation [2,3] and the selection of target cells from a mixture in suspension [4]. Metastatic diseases, a major cause of poor prognosis, are caused by the detachment and dissemination, through the blood stream, of cancer cells from the primary tumor mass. Circulating Tumor Cells (CTC) are able to take root and continue to grow into new tissue districts very distant from the primary site, leading to a, sometimes more aggressive, new tumor development [5–7]. Moreover, the identification of the CTC and their counts in cancer patients cannot only be considered as a prognostic factor, but it can follow the trend of a certain treatment indicating possible changes and/or improvements from a simple analysis of the blood avoiding invasive biopsies [8].

The dielectrophoretic forces are generated in a non-homogeneous electric field, respectively, as positive-DEP (+DEP) (with respect to the gradient of the electric field) or negative-DEP (−DEP) cell movement under the action of this electric field is used to sort cells [9–11].

The direction of cells' movement for a given frequency of the electric field depends on the polarizability of the cells compared with that of the extracellular medium in which they are suspended. This parameter also depends on the specific dielectric constant of the cell membrane (in turn dependent on the radius of the cells), a factor known as Clausius–Mossotti factor [12].

The exposure to a high electric field, however, induces a condition of high stress for the cells, which results in a change of the inner biochemical and biophysical properties, leading in the extreme cases either to cell death via cell lysis or apoptosis [13]. For these reasons, the cells can be manipulated for DEP only in buffer with low conductivity.

The search for an optimal buffer for the manipulation of cells on a dielectrophoretic device was the purpose of our research. We analyzed the physical (in terms of conductivity, pH) and chemical (in terms of composition of the buffer and dissolved salts) properties to characterize the best elution buffer (EB). In particular, we have turned our attention to the relationship between the buffer type and the capacity of DEP induced movement of the cells when the device is in action. Moreover, we have correlated these evidences with the capability of survival of the cells in the specific buffers and the variation of the physical–chemical

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parameters of the buffer, following the time variation of different measurable quantities.

These preliminary analyses were conducted on different tumor cell lines, from solid and liquid tumors, to evaluate the existence of any specificity of the cell type with buffer type.

2. Materials and methods

MDA-MB-231(breast cancer triple negative) and K562 (human immortalized myelogenous leukemia) were obtained from American Type Culture Collection (ATCC; Manass, VA, USA), and were cultured under condition recommended by ATCC.

Both cell lines were grown in DMEM supplemented with 10% heat-inactivated FBS (Heat-Inactivated Fetal Bovine Serum, from Gibco) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin; Sigma-Aldrich). All cells were maintained at 37% with 95%/5% air/CO_2, and are grown to 70% confluence up to 48 h before being detached with trypsin (Trypsin-EDTA, Sigma-Aldrich) for MDA-MB-231 or to be re-suspended (K562). After neutralizing the trypsin with the complete medium, such type of cells was centrifuged for 5 min at 1200 rpm at 4 °C, re-suspended in specific extracellular medium to give a final concentration of 500,000 cells/ml and finally centrifuged again at the same condition.

Media whit the same conductivity, osmolarity and pH were prepared mainly by varying the type of salts. Our DEP-buffer consisted of an aqueous solution of sugar with the following percentage: 9.5% sucrose (S7903, Sigma-Aldrich), 0.1 mg/ml dextrose (D9559, Sigma-Aldrich), 0,1% pluronic F68 (Pluronic F68 non-ionic surfactant 100×, Gibco).

This buffer has a conductivity equal to 5 μS/cm and represents our elution buffer, which is then brought to the desired conductivity with solution 1 M of KCl (Sigma-Aldrich) or DMEM (11965, High glucose, GIBCO). The composition of the buffers used in comparison to the total composition of the culture medium is shown in Table 1.

The conductivity and pH of the media was calibrated using a conductivity/pHmeters (SG23 Seven Duo pH/conductivity, Metter Toledo).

2.1. Design, fabrication on the devices DEP

The electrode design used to study the kinetics of the cells is based on a polynomial schema: the electrodes' shapes can be described by the following parametric system:

$$\begin{aligned} &D \le x \le L \\ &y = \pm\sqrt{x^2 + D^2} \end{aligned} \tag{1}$$

for each electrode, rotated of 90° one with respect to the other. In Eq. (1), D represents half the distance of opposing electrodes whereas L is related to the electrode width. The polynomial electrode design is known to produce well defined 3D non-uniform electric fields and it is used for the study of negative and positive DEP. This electrodes schema has been fabricated by deposition of a very thin multilayer of 20 nm of Chrome (to improve adhesion), 100 nm of Gold on a standard microscope glass. The electrodes were delineated by lithographic methods followed by wet etching.

2.2. Experimental set-up for experiments DEP

80 μl of each type of suspension was put over the devices DEP, at the intersection between the 4 electrodes. To generate the out-of phase AC voltage for the DEP operation on the devices, a waveform generator (Agilent 33500B Series) was employed. A sinusoidal signal at fixed frequencies was applied to adjacent electrodes while two opposed electrodes were maintained at the same electrical potential. Electrical input signals were checked using a digital oscilloscope (Agilent Infini Vision DSO-X 2014A). The final distribution was observed with a standard contrast inverted microscope (Zeiss Axiovert 40 C). Movies and images were captured and recorded with a CANON camera connected to a microscopy. Data analysis was conducted with Excel, through the analysis of the t-test and ANOVA test, and program MATLAB.

3. Results and discussion

3.1. Effect of different cell types on the chemical–physical characteristics of the elution buffer and effect on the mobility of the cells on the device DEP

Once prepared the colloidal solution, we monitored in time the trend of the conductivity of the medium with the various cell types in suspension. Two different types of behavior have been recovered, related to the two types of cells, despite that they are re-suspended in the same type of buffer (EB-KCl 300 μS/cm) (Fig. 1).

This variation can be attributed to the different chemical, physical and biological properties, due to the different origin of the two tumor cell lines. Moreover, it also depends on the ability of cells to adapt in different ways to changes in the external environment: i.e. expressing different classes of receptor or ionic channels and pumps, express adhesion complex [13], deregulating the PI3K-AKT-mTOR pathway trough modulation of PTEN a p53 expression [14], fitting the area of the cell membrane to changes in metabolic activity [15,16].

In addition, there is another factor that should not be underestimated. Indeed, it is well known that the electrical properties of cell membranes that are in suspension depend on the morphology that they have before being detached and removed from their normal site of growth.

Microscopy investigations show that the change in the size of cells suspended in a medium derived from the loss of cytoplasm.

Table 1
Composition of different buffers used in our experiments.

	Elution buffer	Buffer KCl (low cond.)	Buffer DMEM (low cond.)	Buffer KCl (high cond.)	Buffer DMEM (high cond.)	DMEM (mg/L)
Sucrose	9.5%	9.5%	9.5%	9.5%	9.5%	–
Dextrose	01 mg/ml	0.1 mg/ml	0.1 mg/ml	0.1 mg/ml	0.1 mg/ml -	–
KCL	–	0.02%	–	0.04%	–	400
$Fe(NO_3)_3.9H_2O$	–	–	–	–	–	0.1
$MgSO_4$ (anhyd)	–	–	–	–	–	97.67
NaCl	–	–	–	–	–	6400
NaH_2PO_4	–	–	–	–	–	125
Sodium pyruvate	–	–	–	–	–	110
$CaCl_2$	–	–	–	–	–	200
Amino acids	–	–	–	–	–	100% as per catalog
Vitamins	–	–	–	–	–	100% as per catalog
DMEM	–	–	15%	–	30%	
Conductivity	5 μS/cm	300 μS/cm	300 μS/cm	600 μS/cm	600 μS/cm	1856 mS/m
pH	6.5	6.8	7.20	6.8	7.40	

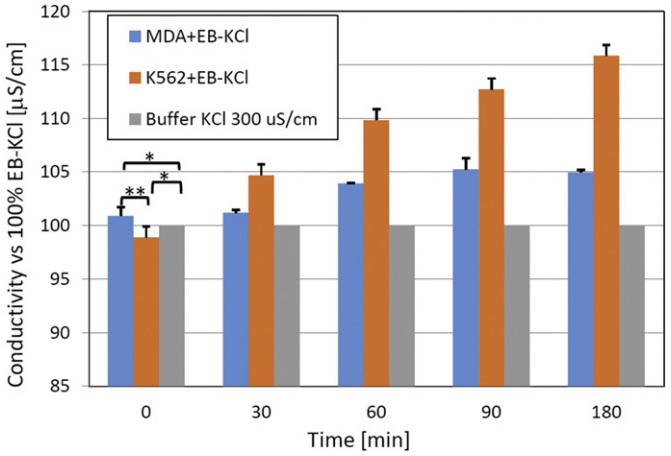

Fig. 1. *Conductivity over t time of MDA-MB-231 and K562 in the Buffer-KCl 300 µS/cm*: It is measured the conductivity of the cells (concentration of 500,000 cells/ml) immediately after they are suspended in the medium. All values are expressed as percentage of buffer without cells, normalized as 100%. EB-KCL = elution buffer with KCL 300 µS/cm, EB-DMEM = elution buffer with DMEM 300 µS/cm. Data represent the mean ± SEM (bars) of the least 3 independent experiments. T-test and one-way ANOVA have been performed, $^{*}p < 0.05$, $^{**}p > 0.05$. These values are constant for each time intervals.

Immediately after the suspension procedure in the medium, they show a ruffles rich morphology of the membrane. Subsequently, over the course of several hours in the buffer there is a progressive loss of cytoplasmic volume [17,18]. If on one hand the cell size, related to the dielectric properties, does not influence strongly the goodness of the technique in a short time; whilst at longer times, because of this great morphological variability, there are major complications for the success of the sorting with the DEP.

Our results demonstrate that, in accordance with the previous discussion, there is a remarkable difference in the variation the conductivity of the extracellular medium, with a more marked increase of the 17% for K562 while only 5% for MDA-MB-231(Fig. 1). This different behavior is consistent with the mechanism of loss of cytoplasm and the shrinking of the cell: MDA-MB-231 could lose a larger quantity of channels and ion pumps with the consequent decrease of the ion exchange with the external environment. This process is confirmed by the fact that only breast cancer cells from the solid line, growing adherent and needing a detachment from the solid support, incur to the physiological mechanism described above. Anyhow, for both lines linear increase with the time of the medium conductivity is observed.

3.2. Effect of the buffer on the mobility in time of the cells on the device DEP

There is a correlation between the membrane's capacity and its morphology, which could translate into a different frequency of cross-over. Typically this frequency could also to be varied in tumor cells from the same tissue [19]. Due to the increased size and complexity of the cell membrane, the cells of solid tumors demonstrate an average cross-over frequency which is much lower compared to one of the blood's cells. We note that, if the cells have healthy membranes and if they are suspended in a medium with a lower conductivity than the cytoplasm, the DEP crossover frequency should depend on the product of the cell radius R, the membrane folding factor ϕ and the capacitance

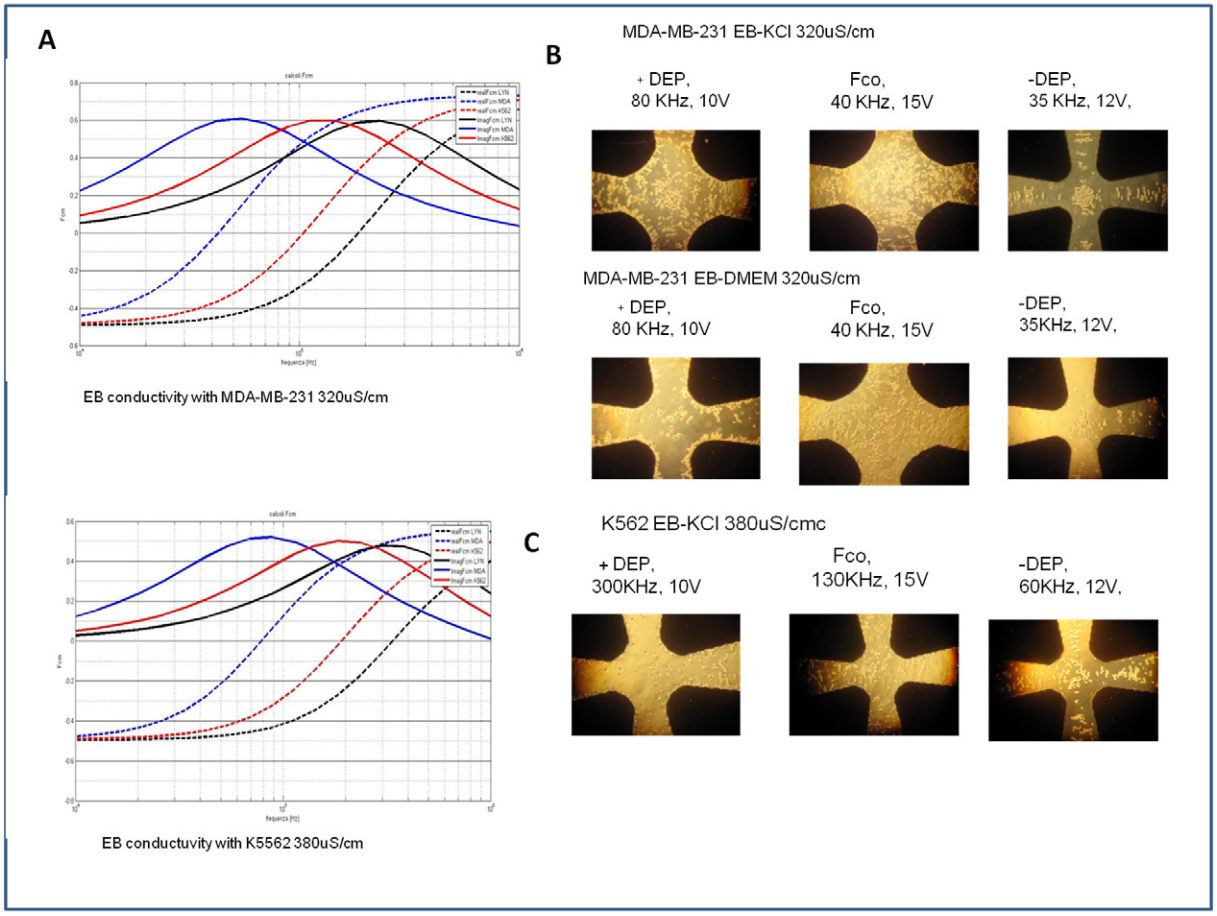

Fig. 2. *Cross-over frequencies for MDA-MB-231 and K562 in buffer at 300 µS/cm*: In A represent the cross-over frequencies theoretical (calculated with MatLab) of MDA-MB-231. In B we represent the experimental cross-over frequencies of MDA-MB-231, in the buffer of 300 µS/cm titrated respectively with KCl and DMEM. In Fig. C the theoretical (calculated with MatLab) and experimental cross-over frequency of K562, in the buffer with KCL 300 µS/cm are reported. In all the data the cell's concentration is 500,000 cells/ml.

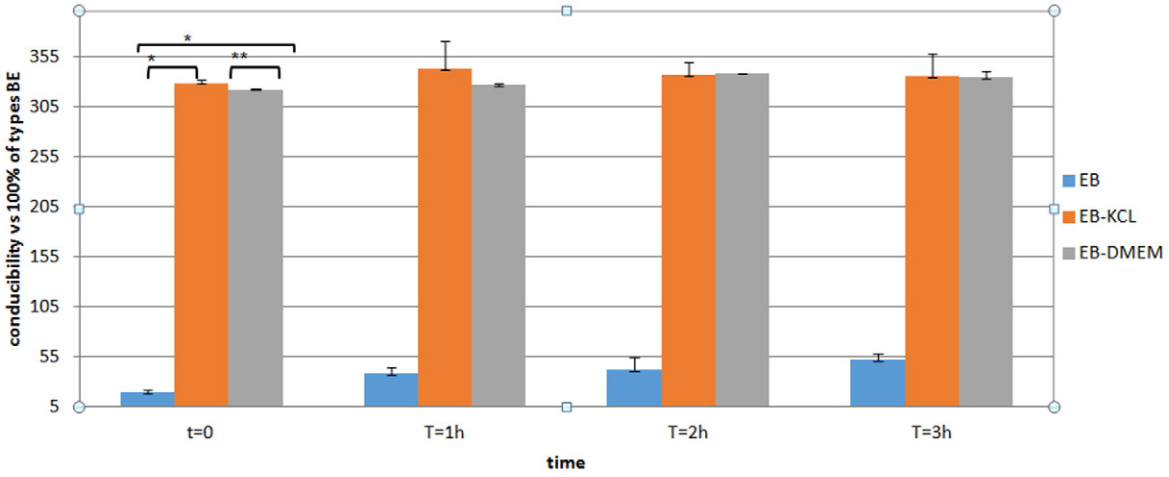

Fig. 3. *Conductivity of MDA-MB-231 cells immediately after they are suspended in the different media.* In all the data the cell's concentration is 500,000 cells/ml. All values are expressed as percentage of the buffer without cells, normalized as 100%. EB = elution buffer, EB-KCL = elution buffer with KCL 300 μS/cm, EB-DMEM = elution buffer with DMEM 300 μS/cm Data represent the mean ± SEM (bars) of the least 3 independent experiments. T-test and one-way ANOVA have been performed, *$p < 0.05$, **$p > 0.05$. These values are constant for each time intervals.

per unit area of smooth membrane, which are the major factors influencing their dielectrophoretic response [20].

To verify the sorting behavior of the different cells in these types of buffers, over time, we have proceeded to the analysis of the cell's response in our DEP device. The study was conducted on our device taking into account the parameters already known in the literature [21,22], calculating the Claussius–Mossotti factor and comparing the experimental feature with the ideal one.

Claussius–Mossotti factor has been calculated from the measured value of the conductivity of the buffer with the cells already suspended, in order to have a value as near as possible to the real change conductivity due to the presence of cells. Indeed, from an initial titration of 300 μS/cm with KCL, the presence of MDA-MB-231 cells increases the conductivity to 320 μS/cm while in the K562 case to 380 μS/cm (see the data reported in the previous paragraph).

Again our results demonstrate the marked difference between the frequency of cross-over of the MDA-MB231 cells and that found in the K562 case (Fig. 2 A,B,C). We also analyzed the behavior of the cells in a medium with high conductivity (initial value of 600 μS/cm). Also in this case the cells retain the values in the frequencies of the cross-over very differently (see Fig. 5 A,B,D,E), even if their mobility on the device in time appears to be significantly reduced (data not shown).

We can derive that this discrepancy in the different frequencies reported does not dependent on the composition of the medium or its conductivity, but it is correlated to the intrinsic morphological properties of the cells.

Indeed, only the small differences found between the known theoretical values and our experimental setting may depend on the type of device used and its geometry or to different conditions of current and parameters applied.

3.3. Effect of different added salts to the medium on the physical–chemical properties of the buffer and on the mobility cell

Due to the ability of MDA-MB-231 lines to differentiate better on the DEP device than the K562 ones, we decided to use them to further study the properties of the buffer. The type of the buffer used up to now, contains nothing but sugars and salts as the KCl.

To improve the cells' vitality and their ability of mobilization, we have thus compared the previous buffer with a second buffer, in

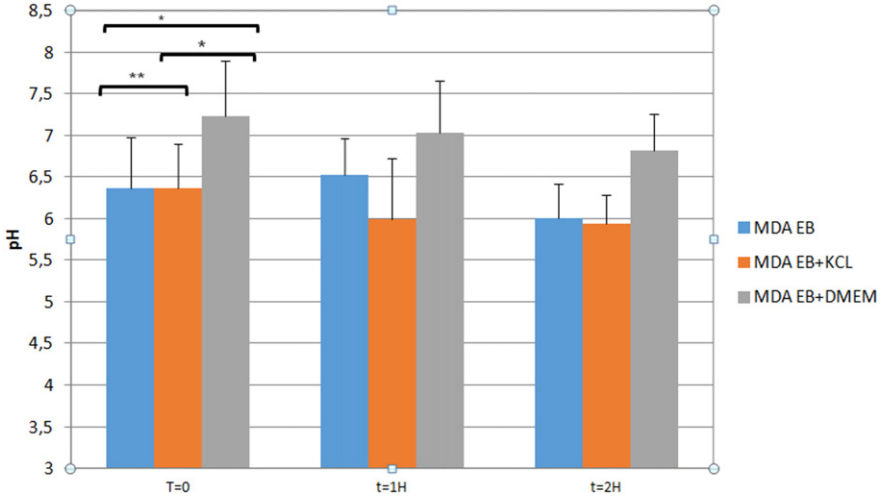

Fig. 4. *Trend of the pH in time of the MDA-MB-231 in the different types of buffers.* It is measured the pH of the cells once suspended in the different medium. All are the concentration of 500,000 cells/ml. B = elution buffer not entitled, KCL = elution buffer with KCL 300 μS/cm, DMEM = elution buffer with DMEM 300 μS/cm. T-test and one-way ANOVA have been performed, *$p < 0.05$, **$p > 0.05$. These values are constant for each time intervals.

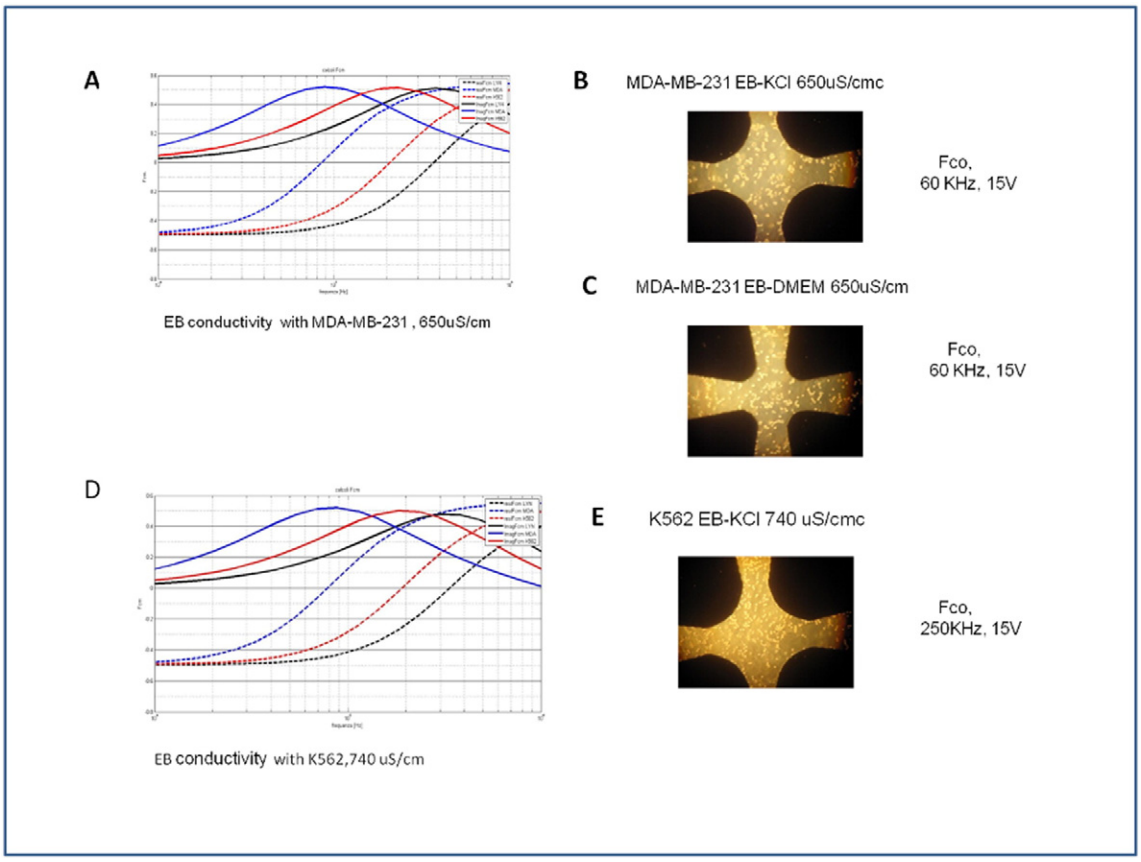

Fig. 5. *Cross-over frequencies for MDA-MB-231 and K562 in buffer at 600 μS/cm:* In A we represent the cross-over frequencies theoretical (calculated with MatLab) of MDA-MB-231. In B and C we represent the cross-over experimental frequencies of MDA-MB-231, in the elution buffer of 300 μS/cm titrated respectively with KCl and DMEM. In Fig. D and E the theoretical (calculated with MatLab) and experimental cross-over frequencies of K562 in the elution buffer with KCL 300 μS/cm are presented. In all the data the cell's concentration is 500,000 cells/ml.

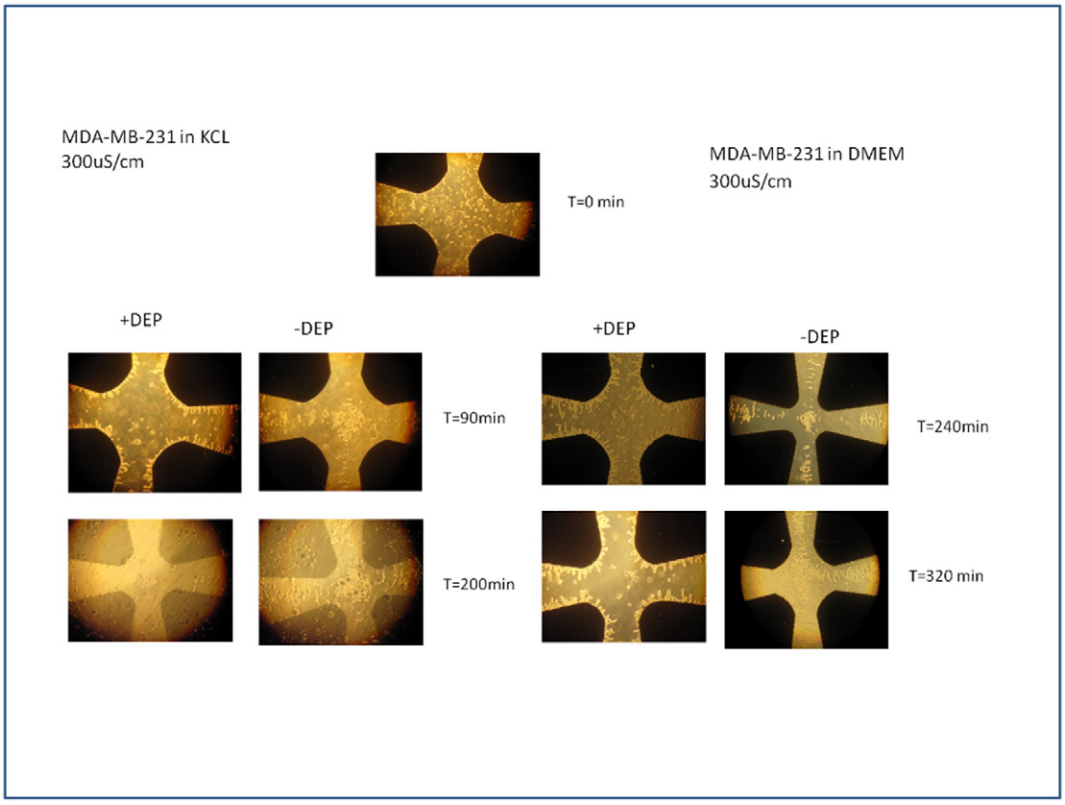

Fig. 6. *Motility of MDA-MB-231 in DEP-devices, over time, in different buffers with the same conductivity*. After the suspension preparation in the different buffers the cells are maintained in it up to five hours. Every 30 min we study the handling capacity of the DEP device using a portion of the prepared suspension. In all the data the cell's concentration is 500,000 cells/ml.

which the complete culture medium (DMEM) was added always maintaining the conductivity to 300 μS/cm. The culture medium contains many mineral salts, amino acids and vitamins which allow the cells to survive.

It was thoroughly studied to see whether the presence of different salts added, varies the response of the cells into the extracellular medium and the relative cross-over frequency (Figs. 2B,3,5B,C), we evaluated the performance of pH over time of the solution of the cells in suspension (Fig. 4), followed by the tests on the device mobility DEP over time (Fig. 6).

These results make evident that the presence of different salts affect the conductivity of the extracellular medium in the same way (Fig. 3). This is also confirmed by the stability of the cross-over frequencies, demonstrating that the mobility of MDA-MB-231 and K562 remained unchanged in time (Fig. 2B,C).

However, there is a substantial difference in the buffer titrated with DMEM: the pH of the solution is in fact maintained for much longer time to a value close to neutrality pH (Fig. 4), while the percentage of viability of the cells appears to be very similar in the other two types of buffers to 300 μS/cm (data not show).

We have also demonstrated that all types of cells, both of solid and liquid tumor cell lines, are able to respond rather well after 3 h of suspension in buffer, albeit with a reduced mobility after the 3 h (Fig. 6). This data is discordant with the one presented in the literature, where it is stated that after 30–40 min the cells are in suspension, the cells show a lower mobility [23,24].

4. Conclusions

The identification of the CTC in recent years has taken on a growing role, since they can be isolated and identified (liquid biopsy) for both diagnostic and prognostic applications. The DEP technique allows to isolate the CTC of different tumor origin, without the use of specific markers. Furthermore, the construction of the device is very easy and economical, and the technique itself does not even require the presence of an experienced operator.

However, as shown by these data, it is very difficult to find the optimal conditions of the external medium to which cells respond uniquely to the frequency of the applied field.

Our work is based on the study of the characteristics of the buffers for the preparation of an optimal colloidal suspension with cells, which can maintain over time the viability , the morphological characteristics and the dielectric properties. From our analysis we note that for the same conductivity of the buffer, for example, the presence of the culture medium prolongs its vitality and does not alter the dielectric characteristics of the cell. Furthermore, for the mobility of cells on the dielectrophoretic device, the buffer with a lower conductivity gives the best results compared to that at higher conductivity.

Further analyses should be carried out to evaluate the different composition of the buffer. The concentration of sugars, for example, is one of the important factors to be taken into account, because they determine not only the viscosity of the medium, but also the main sustenance to the cells.

Acknowledgments

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