Discrimination of different cell monolayers before and after exposure to nanosecond pulsed electric fields based on Cole–Cole and multivariate analysis

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Abstract
Normal and cancer cells, which were grown in monolayers, were investigated and discriminated by electrical bioimpedance spectroscopy (EBIS) before and after exposures to nanosecond pulsed electric fields (nsPEFs). Bioimpedance data were analysed with a Cole–Cole model and the principal component analysis (PCA). Normal and cancer cells could be clearly distinguished from each other either from Cole parameters ($R_0$, $\alpha$, $\tau$) or from two dominant principal components. The trend of changes for Cole parameters indicated distinctively different post-nsPEF-effects between normal and cancer cells. PCA was also able to distinguish characteristic impedance spectra 30 min after exposures. The first principal component suggested that post-nsPEF-effects for normal cells were revealed especially at lower frequencies. The results indicated further that the extracellular resistance, which is dominated by cell–cell connections, might be an important factor with respect to selective nsPEF-effects on cancer cells that are organized in a monolayer or a tissue, respectively. Accordingly, the results support the application of EBIS as an early, non-invasive, label-free, and time-saving approach for the classification of cells to provide in particular predictive information on the success of cancer treatments with nsPEFs.

Keywords: electrical bioimpedance spectroscopy, cancer, nanosecond pulsed electric fields, principal component analysis

Supplementary material for this article is available online
(Some figures may appear in colour only in the online journal)

1. Introduction
The investigation of effects on cancer and normal cells is of crucial importance for the development of novel anti-cancer strategies. Cancer and normal cells can be discriminated by their different electrical properties [1], which can be determined by electrical bioimpedance spectroscopy (EBIS) [2, 3]. EBIS measures the impedance by sending a series of very low voltage sinusoidal signals (typically of 10 mV) to the analyte and deriving the ratio of the output voltage and corresponding electrical currents [4]. Consequently, this constitutes an inherently non-invasive, real-time, sensitive and label-free
diagnosis of cell behavior, including cell–substrate interactions, cell–cell interactions and cell adhesion [5]. The method is already established for the diagnostic of cells that are grown and investigated under controlled conditions, such as monolayers on commercial EBIS-electrode arrays. Conversely, the challenge remains to further develop the approach into a viable method also for tissues in vivo. Therefore, reliable experimental and analytical methods and meaningful interpretations still need to be developed, which is one of the goals of this study.

The electrical properties of biological samples, including cells and tissues, determine also the primary effects of an applied stimulus, which is prone to change these characteristics as it is in particular relevant for currently pursued treatment of skin tumors by pulsed electric fields [6]. Especially the application of nanosecond pulsed electric fields (nsPEFs) is an attractive approach for the treatment of solid tumors [7, 8]. Conversely, electric field-induced changes of the electrical properties can be reflected in bioimpedance spectra, which can then be used for the evaluation of the respective treatments. Accordingly, impedance spectroscopy could offer a way to predict a patient-specific treatment success before any biological effects become obvious [9, 10].

With our previously reported work, we have established a method and procedures to use EBIS for the investigation of effects of pulsed electric fields with a duration of 100 ns on rat liver epithelial cells (WB-F344) that were grown in monolayers [11]. The information of a bioimpedance spectrum for the cell monolayer could be summarized by four descriptive parameters that were obtained by fitting spectra with a Cole model. The resistance-value at zero frequency, $R_0$, is commonly related to extracellular resistance and membrane integrity of individual cells. The dispersion width, $\alpha$, has been correlated with the morphology of the extracellular space and to some degree with the distribution of cell size [12]. A significant decrease of $R_0$ and $\alpha$ suggested the disruption of cell–cell junctions, in particular tight junctions. This effect was confirmed by the increase of another parameter, the characteristic time constant, $\tau$, which is, together with $R_0$, descriptive for the contribution of the capacity $C_m$ of the cell membranes to the overall capacitance of the monolayer. Moreover, a transient increase of $R_0$ within 1 min after exposures suggested cell swelling, probably due to electroporation. Altogether, the study proved the possibility to use EBIS to monitor nsPEF-treatments.

Compared to treatments with longer pulses of microseconds to milliseconds, exposures with nanosecond pulses are essentially non-thermal and are known to induce intracellular effects, especially apoptosis, without the need for chemotherapeutics [13, 14]. Interestingly, there has been some theoretical and experimental evidence for different sensitivities of cancer cells and normal cells towards nsPEF-exposures [15–17].

Regardless of the extensive research on the differences of nsPEF-effects on cancer and normal cells, underlying mechanisms and criteria that are responsible for the differences are not well described. Sensitivities are presumably determined by morphologies [18], mechanical properties [19] and also electrical properties [15]. The latter dominate electrical field distributions as well as current distributions and densities and will therefore determine electroporation mechanisms or any other mechanism of electrical manipulation [20–23].

A cell monolayer expresses an extracellular matrix and cell–cell connections similar to tissues while it reduces the overall complexity. Two normal epithelial cell lines, WB-F344 and human keratinocytes (HaCat) were cultivated, which express more compact cell–cell connections compared with cancer cells. A possible reason (or consequence) for the degradation of cell–cell connections of cancer cells is the facilitation of invasion and metastasis [24, 25]. The liver epithelial cells were compared to a syngenic counterpart, WB-ras, which was derived from WB-F344 cells by transfection with the oncogene ras. Since such a direct comparison was not possible for HaCat-cells, these were compared to a human melanoma cell line (Sk-Mel-28).

Since the primary goal of the study was the investigation of differences with respect to electrical characteristics and exposures, preferably non-fatal conditions were chosen for exposures. Cell death and extensive electroporation would dominate the bioimpedance spectra regardless of the cell line. Such unselective nsPEF-effects were observed in our previous work and were likewise reported for example for the response of normal skin and melanoma after exposure to 100 pulses of 300 ns PEFs with 40 kV cm$^{-1}$ [6].

Due to its simplicity, popularity and explanatory power, the first choice for the interpretation of bioimpedance spectra is the analysis by a Cole model and a corresponding equivalent circuit. However, there are limitations to such a parameterized approach [26]. Fitting errors are inevitable and Cole parameters extracted from repetitive measurements for the same specimen are prone to large errors [27, 28]. Typical errors are due to the implementation of different algorithms for the analysis together with statistical variations of measurements. Even errors of only a few percents might prevent discrimination of cell types and assessment of therapeutic results.

Therefore, in addition, a multivariate model, i.e. principal component analysis (PCA), was applied as a non-parametric representation. PCA is commonly implemented to reduce the dimensionality of a raw spectrum without ab initio assumptions on the physiological or physical meaning of the parameters (principal components) that are extracted for a description. PCA has been widely applied for the classification of phenomena and for process monitoring [29–31]. Linearly uncorrelated eigenvectors are derived from an observed dataset by an orthogonal transformation. The eigenvector with the highest eigenvalue is chosen as the first principal component (PC) of the dataset, representing the most salient information. The inherent error of the approach is determined by the number of principal components that are included in the analysis in addition to the variation of results for individual measurements.

2. Methodology

2.1. Cell culture

The electrical properties of normal and cancer cells with either the same origin, i.e. syngeneic liver cells, or cells that can be
found together in the same organ, i.e., skin, were investigated. The WB-F344 cell line was derived from a normal adult male Fischer 344 rat liver by Grisham et al [32]. The cancerous cell line WB-ras was derived by transfecting WB-F344 cells with the HRAS oncogene. As a result, these cells are characterized by the absence of contact inhibition, a spindle shape, and tumorigenicity in vivo [33]. Both cell lines were obtained from Prof J E Trosko, Michigan State University, East Lansing, MI, USA. In addition, cells that are typically found in skin tissue, i.e., Sk-Mel-28 melanoma cells and non-tumorogenic HaCat cells (both from ATCC, LGC Standards GmbH, Germany) were compared. The pairing is typical also for other studies on melanoma cells with normal cells [34, 35].

All cell lines were cultivated in DMEM with 1 g l⁻¹ glucose supplemented with 2 mM L-glutamine, 5% fetal calf serum (FCS) and 1% penicillin/streptomycin (all purchased from PAN-Biotech GmbH, Aidenbach, Germany). The osmolality of the medium was determined to be 300 mOsmol kg⁻¹ based on the freezing point depression (OSMOMAT 3000, Gnotec GmbH, Berlin, Germany).

2.2. Bioimpedance analysis

A detailed description of setup and procedures for the conducted bioimpedance measurements has been presented previously [9]. Basic steps and approaches are summarized in figure 1.

In brief, ECIS-8W20idf-plates (Applied Biophysics, Inc., NY, USA) with an integrated interdigitated electrode array were pre-treated with 10 mM L-cysteine 15 min before seeding cells to obtain a stable impedance [36]. Each well was filled with 300 µl cell culture medium and a cell seeding density of 50 000 cells/well. The monolayer was considered confluent when the impedance at 20 kHz became stable for two consecutive days. The impedance at 20 kHz is close to the largest increment of the impedance module when the cell is grown on electrodes and avoids ambiguous contributions from electrode polarization. Impedance measurements as well as nsPEF-exposures were conducted with the electrode arrays embedded in the wells. The impedance was measured from 100 Hz to 10 MHz using an impedance analyser (Agilent 4294A, Keysight Technologies, Inc., USA) together with the appropriate test fixture (Agilent 16047E, Keysight Technologies, Inc., USA). Recorded data were analysed on the one hand by two individual Cole-models for the respective electrical components, i.e., describing electrode processes or monolayers, connected in series and on the other hand by a PCA. The information of impedance spectra was summarized by four Cole parameters (R∞, R0, α, and τ) and score and loading plots for the PCA, respectively. The impedance for the Cole-models in series can be expressed by:

\[ Z = Z_{\text{ep}} + Z_{\text{cells}} = \left( \frac{R_{\text{ep}} - R_{\text{cell}}}{1 + (i\omega \tau_{\text{cell}})^\alpha} \right) + \left( \frac{R_{\infty}}{1 + (i\omega \tau_{\infty})^\alpha} \right). \]

The first term in brackets represents electrode polarization; the term in the second bracket describes the contribution of the cell monolayer. Parameters R∞ and R0 are describing the respective resistance at infinite frequency and at very low frequency. The expression \((i\omega \tau)^\alpha\) is known as constant phase element (CPE) to describe non-ideal capacitance, \(Z_{\text{CPE}}\), with \(\alpha\) a dimensionless dispersion factor \((0 < \alpha < 1)\) and \(\tau\) the characteristic time constant. The latter is commonly related to the average cell capacitance, for example, according to Trainito et al [37]:

\[ C_{\text{m}} = \frac{\tau^\alpha}{R_0 - R_{\infty}}. \]

Parameters with a subscript ‘ep’ represent parameters associated with electrode polarization. The parameter \(R_{\text{cell}}\) was omitted in the fitting process since it should be much smaller than \(R_{\text{ep}}\) and \(R_{\infty}\).

2.3. Pulsed electric field exposures

A series of rectangular 100 ns pulses were produced by an in-house built Blumlein transmission line square wave pulse generator [38]. The high voltage pulses were delivered to the ECIS-chip with a pair of copper clamps. A high voltage probe (P5100A, Tektronix, Beaverton, OR) was connected to a fast oscilloscope (TDS3054, Tektronix, Beaverton, OR) to monitor the amplitude and the shape of pulses. The advantage of providing nsPEFs by the interdigitated electrode array is that only cells close to the electrodes are affected. Consequently, these cells determine changes of the impedance spectra. A lethal stimulation would kill cells and let dead cells float away, resulting in gaps with similar changes to the spectrum that are determined by the medium regardless of the particular cell line. Therefore especially interesting for comparison are non-fatal exposures to pulses with a voltage of 330 V and pulse numbers of 8, 16 and 24 that were applied with a repetition rate of 1 Hz. Corresponding to the voltage is an average
Table 1. Mean values for Cole parameters together with their standard deviations, δ, of untreated WB-F344 and WB-ras cell monolayers (*p < 0.05, **p < 0.01).

| Cole parameter | WB-F344 | δ   | WB-ras | δ    | p-value |
|----------------|---------|------|--------|------|---------|
| $R_\infty$ (Ω·cm$^2$) | 255.21  | 38.81 | 234.82 | 24.84 |         |
| $R_0 \times 10^3$ (Ω·cm$^2$) | 1.81   | 0.32 | 1.07   | 0.20  | **      |
| $\alpha$          | 0.71   | 0.02 | 0.73   | 0.01  |         |
| $\tau \times 10^{-6}$ (µs) | 7.42   | 2.03 | 7.91   | 1.71  |         |
| $C_m$ (µF·cm$^{-2}$)  | 0.15   | 0.05 | 0.24   | 0.04  | **      |

Table 2. Mean values for Cole parameters together with their standard deviations, δ, of HaCat and Sk-Mel-28 cell monolayers (*p < 0.05, **p < 0.01).

| Cole parameter | HaCat  | δ    | Sk-Mel-28 | δ    | p-value |
|----------------|--------|------|-----------|------|---------|
| $R_\infty$ (Ω·cm$^2$) | 224.70 | 31.94 | 212.41    | 31.46 |         |
| $R_0 \times 10^3$ (Ω·cm$^2$) | 0.82   | 0.04 | 0.62      | 0.06  | **      |
| $\alpha$          | 0.72   | 0.01 | 0.49      | 0.04  | **      |
| $\tau \times 10^{-6}$ (µs) | 3.54   | 0.14 | 13.196    | 3.71  | **      |
| $C_m$ (µF·cm$^{-2}$)  | 0.19   | 0.01 | 11.929    | 7.32  | **      |

2.4. Principal component analysis

The magnitude |θ| and phase angle θ as a function of frequency of nine samples for each untreated cell line and three samples for each treated cell line were computed for PCA with Origin 2017 (OriginLab, Northampton, MA) and PCA score plots and loading spectra were derived accordingly.

2.5. Statistical analysis

Impedance measurements for all exposure conditions were conducted at least in triplicates and mean values and standard deviations, δ, for each Cole parameter were calculated correspondingly. For monolayers exposed to pulsed electric fields, three independent monolayers were analysed. Nine samples were investigated for each cell line for the comparison between different untreated cell lines. For this case, the statistical significance (p-value) of each Cole parameter was evaluated by a paired-sample t-test with MATLAB (MathWorks, Natick, MA, USA).

3. Results

Cole parameters were extracted for cell monolayers before and after exposures from the equivalent circuit model that is shown in figure 1. Mean values and standard deviations for untreated cell lines, as well as the associated statistical significance, are presented in tables 1 and 2. Data for normal and cancer cell monolayers were considered significantly different for $p < 0.05$ (*) or $p < 0.01$ (**), respectively.

The PCA score plot is presented in the subsequent section. Values, in general, were normalized against values obtained for untreated monolayers for the analysis of the temporal evolution of each Cole parameter.

3.1. Cole parameters for untreated normal and cancer cell monolayers

Cole parameters of WB-F344 and WB-ras cell monolayers are summarized in Table 1. The value of $R_\infty$ for a WB-F344 cell monolayer was 255.21 ± 39.81 Ω · cm$^2$. Hence, the mean value was about 8.7% higher than for corresponding tumorigenic WB-ras cell monolayers (234.82 ± 24.84 Ω · cm$^2$). Overall the difference was not statistically significant ($p > 0.05$). Likewise, no significant differences were revealed for the dispersion width, $\alpha$, between WB-F344-monolayers (0.71 ± 0.02) and WB-ras cell monolayers (0.73 ± 0.01) or for the time constant, $\tau$, of WB-F344 cells (7.42 ± 2.03 µs) in comparison to WB-ras cells (7.91 ± 1.71 µs).

However, a significant difference was found for $R_0$ and the cell membrane capacitance, $C_m$. The value of $R_0$ for WB-F344 monolayers was 1806 ± 317 Ω · cm$^2$, and therefore almost twice that of $R_0$ for WB-ras cell monolayers, i.e. 1065 ± 200 Ω · cm$^2$. The cell membrane capacitance, $C_m$, of WB-F344 and WB-ras cells was also significantly different, with 0.15 ± 0.05 µF · m$^{-2}$ and 0.24 ± 0.04 µF · m$^{-2}$, respectively. Hence, $C_m$ for WB-ras cells was 1.6 times larger than for WB-F344 cells. It should be noted that $C_m$ is not a completely independently determined parameter, unlike $R_0$, $\alpha$, and $\tau$, and hence respective changes are in particular associated with $R_0$ and together with $R_0$ also with $\tau$.

Table 2 shows the mean values and standard deviations of the Cole parameters $R_\infty$, $R_0$, $\alpha$, and $\tau$ for monolayers of HaCat cells and Sk-Mel-28 cells. The values for $R_\infty$ were again similar for cancer cells and normal cells but still significantly different. For HaCat cells, $R_\infty$ is with 224.69 ± 31.94 Ω · cm$^2$ about 6% larger than for Sk-Mel-28 cells (212.41 ± 31.46 Ω · cm$^2$). Values for $R_\infty$ were not much different from WB-F344 cells and WB-ras cells.

Differences were more pronounced for other Cole parameters. Values of $R_0$ for HaCat cell monolayers (816 ± 37 Ω · cm$^2$) were about 32% higher than for Sk-Mel-28 cell monolayers (620 ± 60 Ω · cm$^2$). The dispersion width $\alpha$, of a
HaCat cell monolayer is $0.72 \pm 0.01$, which is about 1.5 times larger than for a Sk-Mel-28 cell monolayer ($0.49 \pm 0.04$). On the contrary, $\tau$ for HaCat cells is almost 4.7 times smaller than $\tau$ obtained for Sk-Mel-28 cells, i.e. $3.54 \pm 0.14 \mu s$ versus $13.20 \pm 3.71 \mu s$. Values of $C_m$ for HaCat cells and Sk-Mel-28 cells were again significantly different, with $0.19 \pm 0.01 \mu F m^{-2}$ and $11.93 \pm 7.32 \mu F m^{-2}$, respectively.

Cole parameters for electrode polarization showed no significant differences regardless of the cell line, as shown in tables S1 and S2 of the supplementary information (SI) (stacks.iop.org/JPhysD/52/495401/mmedia).

### 3.2. PCA for untreated normal and cancer cell monolayers

Figure 2 displays the PCA score plot of phase spectra for untreated cell monolayers for the four investigated cell lines. A score plot describes the transformation of original variables into new coordinates (principal components) with the goal to give an alternative representation of a data set. The approach offers a better visual differentiation of the measurements. The first three principal components contributed with a variance of 98.45% to the phase spectra. In the 3D-space, which is defined by the principal components (PC1, PC2, PC3), every sphere represents an individual EBIS-measurement for a sample. In addition, the ellipsoidal envelopes describe the 95% confidence regions for each cell line. The distinct separation of each ellipsoid indicates a clear and significant differentiation between cell lines. (Additional views, showing the separation with respect to pairs of PCs, are provided with the supplementary information for this manuscript in figure S1.)

The PCA score plot for the magnitude $|Z|$ can be found in figure S2 of the supplementary information (SI). In this case, PC1 and PC2 contribute with more than 99.97% variance.
and the ellipsoidal envelopes for different cell lines are not well-differentiated.

3.3. Temporal evolution of cole parameters for WB-F344 and WB-ras cell monolayers after nsPEF-exposures

The change with time of normalized Cole parameters ($R_\infty$, $R_0$, $\alpha$, $\tau$) was pulse number-dependent for both normal and cancer cell monolayers. The results are shown in figure 3.

The temporal evolution of $R_0$ for WB-F344 cell monolayers is shown in figure 3(a). A transient increase was observed within 1 min after exposure to either 8, 16, or 24 pulses with values that were 20%, 40%, and 28% higher than the initial values, respectively (see inset of figure 3(a)). The data confirm a transient increase that was also observed in our previous study for the same exposure conditions [9], although values were actually lower. A medium with lower glucose concentration was used in the present investigation which is presumably responsible for the difference. After the brief increase, $R_0$ gradually decreased before it recovered again. The decrease was more pronounced for higher pulse numbers and also took longer. For eight pulses, $R_0$ went down to 76.5% of the initial value and recovery started at about 30 min after exposure. The same temporal development was observed for 16 pulses although with slightly larger changes. A larger decrease of $R_0$ of 67.2%, compared to the initial value, was observed 1 h after exposure to 24 pulses. Values for $R_0$ even increased above the initial values 2 h after exposure with 11.6% and 18.7%, respectively, during the recovery after the application of 8 and 16 pulses.

Figure 3(b) shows the changes of Cole parameter $\alpha$ with time. There was scarcely any change for exposures to eight pulses and only a moderate decrease for 16 and 24 pulses. Within 1 h after the respective exposures, $\alpha$ was still reduced at most by about 12% before the values had recovered again after 2 h.

Another Cole parameter, $\tau$, which generally exhibited a tentative increase, is depicted in figure 3(c). The largest increases for $\tau$ were observed 1 h after exposure to either 8, 16 and 24 pulses, with values about 1.3, 2.8, and 6 times larger than observed for untreated cell monolayers. Eventually, values for $\tau$ had recovered for both cell types 3 h after exposures.

In contrast, $R_0$ decreased for WB-ras cell monolayers already within 1 min after exposure to 100 ns pulsed electric fields: (a) low frequency resistance $R_0$, (b) dispersion width $\alpha$ and (c) time constant $\tau$.

Figure 4. Temporal evolution of normalized Cole parameters for WB-ras cell monolayers before and after exposure to 100 ns pulsed electric fields: (a) low frequency resistance $R_0$, (b) dispersion width $\alpha$ and (c) time constant $\tau$. 

Figure 4(b) describes the time course of changes of $\alpha$ for WB-ras cell monolayers, with the biggest decrease observed within 30 min after exposures to 8, 16 and 24 pulses.
pulses, with 5%, 7% and 27% in comparison to untreated cells, respectively.

The characteristic time constant, $\tau$, that is shown in figure 4(c) increased slightly within 1 min after exposures followed by a decrease. Values for $\tau$ went down to 80%, 71% or 88% in comparison to untreated cells within 10 min after exposures to 8, 16 or 24 pulses. Overall, the changes are not distinct, in particular taking error bars into account.

3.4. Temporal evolution of Cole parameters for HaCat and Sk-Mel-28 cell monolayers after nsPEF-exposures

Changes of normalized Cole parameters with time are displayed in figure 5 for HaCat cell monolayers and for Sk-Mel-28 cell monolayers in figure 6. Figure 5(a) describes a fast drop of $R_0$ for HaCat cell monolayers within 1 min after exposure to eight pulses followed by a recovery eventually even exceeding initial values at 1 h. Conversely, for 24 pulses, $R_0$ jumped to values 35% above values obtained for untreated cells in 1 min before values decreased again for the next hour. The response for the application of 16 pulses followed development between the results for exposures to more or fewer pulses. After a slight ascent for about 20 min, values were not changing by much anymore. After 1 h, values about 15% higher in comparison to untreated cell monolayers, were observed for all exposure regimens.

The dispersion width, $\alpha$, as shown in figure 5(b) changed only slightly after exposures. For eight pulses, $\alpha$ decreased by about 3% after 1 min and recovered again towards values similar to initial values within 30 min. An opposite trend, i.e. a small increase at the beginning with the highest values that are 2.5% and 6% above the initial values after 10 min, was observed for 16 or 24 pulses.

The development of the characteristic time constant, $\tau$, which is presented in figure 5(c), was very similar to the changes that were recorded for $R_0$. For the smallest number of pulses, i.e. eight, values dropped by 16% by 1 min before increasing again. In contrast, $\tau$ increased to values about 1.6 times larger than for untreated cells right after exposure to 24 pulses before values declined afterward. For 16 pulses, a slow initial increase within 10 min could be observed to values 20% higher in comparison to untreated cells. Similar to the development of $R_0$, values for $\tau$ were eventually very similar again for all exposure conditions and about 15% lower than the values that were determined for the untreated monolayers.

For Sk-Mel-28 cell monolayers, $R_0$ increased by 18%, in comparison to untreated controls, within 1 h after exposure to eight pulses, which is followed by a gradual decrease towards initial values during the next couple of hours (figure 6(a)). Values fluctuated for about 5 min after the application of 16 and 24 pulses before a maximum increase was observed for $R_0$ half an hour later with values that were 22% and 30% above control values. For later times, values did not quite drop back again to values that were determined for unexposed cell monolayers.

Figure 5. Temporal evolution of normalized Cole parameters for HaCat cell monolayer before and after exposure to 100 ns pulsed electric fields: (a) low frequency resistance $R_0$, (b) dispersion width $\alpha$ and (c) time constant $\tau$. 
Comparative characteristics were also observed for $\alpha$ and $\tau$ as shown in figures 6(b) and (c). After a hardly noticeable increase, $\alpha$ slightly decreased within 1 min after exposures. Values for $\alpha$ reached maxima of about 5%, 12% and 20% above values obtained for untreated cell monolayers 1 h after exposures to 8, 16 and 24 pulses, respectively. The dispersion width, $\alpha$, was similar again to untreated monolayers 24 h after exposures. The time constant $\tau$ did hardly change for the application of eight pulses. A fast increase, of about 50% was recorded for exposures to 16 and 24 pulses, which was succeeded by a decrease towards initial values that was almost as fast as the increase until 4 min later. Notably, values then increased again to maxima of about 29% and 43% above values obtained for untreated cell monolayers 30 min after exposure to 16 and 24 pulses.

4. Discussion

4.1. Discrimination between normal and cancer cells from Cole parameter $R_0$

The electrical properties of the cell monolayers can be described according to an equivalent circuit model by the Cole parameters $R_0$, $\alpha$, and $\tau$ [9] and in addition by the derived quantity $C_m$. The results that are presented in tables 1 and 2 demonstrate significant differences at least for one and often for several of these parameters for all of the cell lines that were investigated. Notable in particular are the differences that were observed for similar cell lines or respectively normal and cancer cells that can be found together in the same tissue, such as HaCat cells and Sk-Mel-28 cells or WB-F344 cells and WB-ras cells. The comparison of the latter is in particular of interest since it allows to distinguish normal from cancer cells of the same 'type'.

The low frequency resistance $R_0$ can describe in particular the organization of cells in monolayers and hence presumably also in tissues. With our previous investigation, we could already show that $R_0$ represents the extracellular resistance, which is dominated by cell–cell junctions, and especially adhesion junctions and tight junctions [9]. The largest value for $R_0$ was recorded for WB-F344 cells, followed by WB-ras, HaCat and eventually Sk-Mel-28 cells. Hence values for untreated normal cells were indeed consistently larger than for their respective tumorigenic counterpart, indicating tighter cell–cell connections for normal cells. The results further show that such a comparison cannot be arbitrary but has to take into account the ‘primary’ tissue where cancer cells develop from similar normal cells, which is, in this case, either liver tissue or skin. The characteristic differences for $R_0$ are in agreement with other reports that cancer cells suppress the expression of tight junctions as well as of adherens junctions which is a prerequisite for the ability to metastasize [24, 25].

The smallest $R_0$ for Sk-Mel-28 cells corresponds to the loss of the connection between each cell in the monolayer, where a lot of gaps between cells could be found visually (data not shown).

After exposure to nsPEFs, changes of $R_0$ can be ascribed to the disruption of cell–cell connections. Cell monolayers of normal cells with more or/and stronger cell–cell connections (figures 3(a) and 5(a)) were accordingly more significantly affected compared to cancer cell monolayers with less or weaker connections, resulting in bigger changes of $R_0$ (figures 4(a) and 6(a)). This effect on cell–cell junctions was further confirmed by values for $R_0$ that were 30 min after exposures similar to the value determined for untreated Sk-Mel-28 cells. The cell line is generally known to rarely exhibit cell–cell connections [39]. The associated higher extracellular permeability of cell monolayers with lacking dysfunctional or degraded junctions resulted in $R_0$ to decrease to values equal to a value that is not determined by cell–cell connections.

For shorter times after exposures, i.e. within the first 10 min, two other phenomena are likely also affecting $R_0$ in addition to the disruption of cell–cell connections. During this time, some reversible electroporation of cell membranes might still provide another pathway for ions to pass through the monolayer and consequently reduce values for $R_0$. Due to the limitations of the present experimental procedures and limitations of the Cole model, respective changes of $R_0$ cannot be distinguished in the impedance spectra from the effect on cell–cell junctions. To separate electroporation-effects, an EBIS measurement system with a higher temporal resolution and an improved equivalent circuit model need to be developed.

A second phenomenon affecting $R_0$ after exposures may result from changes in the overall cell morphology, such as in particular cell swelling or other changes of cell shape or size. Cell swelling narrows the extracellular space and reduces the pathway for ionic transport, hence leading to an increase of $R_0$. An obvious increase of $R_0$ within 1 min after exposures was indeed observed for normal cell monolayers. For WB-F344 cells, $R_0$ experienced a transient increase within 1 min after exposures (figure 3(a)). Also, $R_0$ for HaCat cell monolayers (figure 5(a)) increased within 1 min after exposure to 16 and 24 pulses of nsPEFs. This increase was sustained much longer than for WB-F344 cells. A small transient increase of $R_0$ for Sk-Mel-28 cell monolayers within 1 min after exposure to 16 and 24 pulses (figure 6(a)) was also found. However, for this very small change, still measurement errors could be responsible. The presumed changes of cell morphologies and corresponding changes of $R_0$ are again superimposed in the impedance spectra. Similar mechanisms might also account for the second increase of $R_0$ that was observed for WB-F344 cells around 2 h after exposures.

4.2. Evaluation of morphologies of cells and extracellular space from Cole parameter $\alpha$

Information on morphologies of cells in the monolayers, in general, was reflected by another Cole parameter. The dispersion width, $\alpha$, is commonly believed to be associated with the heterogeneity of cell shapes and sizes and has accordingly been argued to be also related to the morphology of the extracellular space and in particular to be a measure of its tortuosity. A decrease of $\alpha$ hence indicates an increase of tortuosity of the extracellular space [12, 40]. The values of $\alpha$ for untreated WB-F344, WB-ras, and HaCat cell monolayers were similar and in the range from 0.71 to 0.72. The reason
is presumably their similar epithelial-like cell morphology. In contrast, Sk-Mel-28 cell monolayers had the smallest value of \( \alpha \) with 0.49, which reflects the extremely irregular polygonal morphology of the cells.

Changes of \( \alpha \) with time after nsPEF-exposures likewise exhibited clear differences for different cell monolayers (figures 3(b), 4(b), 5(b) and 6(b)). Notable is the second increase of \( \alpha \) that was observed for Sk-Mel-28 cells starting 5 min after exposures, indicating a significant change of the morphology of the extracellular spaces. This was probably due to the high mobility of Sk-Mel-28 cells during culture.

4.3. Derivation of membrane capacitance from Cole parameter \( \tau \)

The characteristic time constant, \( \tau \), is related to the membrane capacitance \( C_m \) as described by equation (2). A distinction between normal and cancer cells was also prominently observed for this parameter (tables 1 and 2). The capacitances were consistently higher for the cancer cell lines in comparison to their normal counterparts. The differences were small for WB-F344 and WB-ras cells, which is ostensibly explained by their very close relationship, considering that in principle differences are only due to a particular artificially selected genetic mutation. Conversely, the difference between the normal HaCat skin cells and the genuine Sk-Mel-28 melanoma cells was with a difference of two orders of magnitude much more pronounced.

Distinctively higher membrane capacitance for cancer cells was also reported by Mulhall et al [41], who compared the effective cell membrane capacitance \( C_{eff} \) for oral cancer cells, pre-cancer cells, and normal keratinocytes. However, it should be remembered, that in our study, cell membrane capacities were not determined directly and that the parameter \( C_m \) was derived from impedance spectra for the entire monolayer. Large gaps in the extracellular space of Sk-Mel-28 cell monolayers, that are filled with medium with higher dielectric constant, are expected to be contributing accordingly. Conversely, a highly irregular organization of cells is known as a specific characteristic for tumor tissues and therefore will likely be presented in the parameter \( C_m \) in a similar fashion.

An analogous contribution to the overall capacitance might also arise for a high degree of permeabilization by pulsed electric field exposures, which causes medium to be incorporated in conductive membrane channels at least temporarily and correspondingly change membrane permeabilities [37]. Consequently, a distinction of normal from cancer cells based on values for the capacitances should be considered very carefully at least for monolayers (or tissues) treated by pulsed electric fields. Altogether the temporal evolution of \( \tau \) appears to be a better and more direct accessible parameter for the evaluation of nsPEF-effects (figures 3(c), 4(c), 5(c) and 6(c)).

4.4. Discrimination between untreated normal and cancer cells by PCA

PCA provides a different way for the organization and analysis of the data that were obtained from impedance spectra.
The strength of the method is to exclude redundant information that is contained for different frequencies by deriving linearly independent variables (principal components) that might allow an alternative view to distinguish the most important criteria that are responsible for a particular result from less relevant contributions.

The PCA score plot for the phase spectra of untreated cell monolayers for different cell lines showed well-differentiated ellipsoidal envelopes indicating that each cell line can be discriminated. The separation between spheres inside of an ellipsoid illustrates the dispersion of the measurements for an individual cell line (figure 2).

A principal component can be expressed by a linear combination of variables with respective coefficients that are called loadings. The magnitudes of the loadings represent the weight of each variable. Consequently, contributions for frequencies with larger loadings are more important. The loadings as a function of frequency for the first three PCs are shown in figure S3 (SI). PC1 has larger loadings for middle frequencies from 1.4 kHz to 562.3 kHz with a peak at 6 kHz. In contrast, peaks that were found for PC2 and PC3 at 3 kHz and 150 kHz were subsequently ignored due to their small contribution to the overall variance.

In general, PCA is difficult to link with physiological properties of the cells or physical characteristics. However, often an instructive interpretation can still be found for the principal components. For the most important principal component, PC1, the impedance spectrum for the respective frequency range was determined by properties of the cell monolayer. In particular, the extracellular resistance $R_{\text{ex}}$, which is dominated by cell–cell connections, was the determining salient feature when comparing cell lines. Moreover, the influence of electrode polarization has been inherently avoided since its impact on phase is generally decreasing as the frequency is increasing. Consequently, it can be omitted for frequencies higher than 1 kHz.

In contrast, the PCA score plot for the magnitude $|Z|$ for untreated cell lines did not discriminate variances between normal and cancer cell monolayers (figure S2 (SI)). This may be explained by the loading spectra shown in figure S4 (SI), i.e. the PCA loadings of the main two PCs as a function of frequency for the magnitude $|Z|$. Obviously, PC1 accounted for the lower frequencies ($<1$ kHz), where electrode polarization dominated the impedance spectra. As shown in tables S1 and S2 (SI), no significant differences could be found for the contributions due to electrode polarization for untreated cells. Consequently, electrode polarization may be the reason why the PCA score plot for $|Z|$ could not discriminate variances between normal and cancer cell monolayers.

4.5. Discrimination between nsPEF-treated normal and cancer cells by PCA

PCA score plots and loading spectra for the phase of impedance spectra for each cell line are presented in figure S5 (SI). The variances were calculated from the impedance spectra of cell monolayers for untreated, and for exposed monolayers, 1 min and 30 min after treatment with 24 pulses. (A number of 24 pulses had led to the most obvious changes of Cole parameters for these two time points.)

Similar to the results for changes of Cole parameters, the scores for WB-F344, WB-ras, and Sk-Mel-28 cells were distinctly different 30 min after treatments in comparison to results at 1 min after treatment and compared to untreated cells. For later times were the distributions too similar to be discriminated by PCA. A likewise more indistinguishable distribution was also observed for HaCat cells, however, scores for untreated cells and for cells 1 min after exposure are further apart than for the other cell lines. This confirms the findings for changes of Cole parameters for HaCat cells as shown in figure 5, with Cole parameters changing more obviously 1 min after exposures than for other cell lines.

The corresponding loading spectra for PC1, according to figure S4 (SI), suggests an important frequency range for the monolayer development after exposures from several kHz to hundreds of kHz for WB-F344, WB-ras and Sk-Mel-28 cell monolayers. The frequencies for the peaks are found at 2 kHz, 6 kHz, and 14 kHz, respectively. Conversely, peaks were found at 1 kHz and 60 kHz for HaCat cell monolayers. The peaks at lower frequency for normal cells suggest that PEF-effects are rather reflected by the response at lower frequencies in comparison to cancer cells.

In conclusion, the PCA confirms trends and observations that were already derived from changes of Cole parameters. In general, PCA requires large data sets and in this case the analysis of Cole parameters might be more convenient for an assessment of effects. However, PCA, in addition, offers the possibility to identify in particular outliers, which is helpful for any experimental design.

5. Conclusion

The investigation of cells in a monolayer is another step towards the conceivable application of impedance analysis on tissues in vivo. Normal cells could be clearly distinguished from cancer cells in laboratory experiments but more studies and developments are necessary before the method can be successful in clinical settings the effects of pulsed electric field treatments could already be evaluated from impedance spectra through Cole parameters and PCA. Different values and respective changes with time for electrical characteristics were not just found between inherently different cells from different organs but also for very similar normal and cancer cells from the same primary tissue, e.g. liver or skin. However, the overall analysis remains challenging, especially for exposures to pulsed electric fields, since different phenomena contribute simultaneously to the underlying impedance spectra and as a consequence are superimposed in the derived Cole parameters. To separate electroporation-effects, an EBIS measurement system with a higher temporal resolution and an improved equivalent circuit model need to be developed.

A PCA that is conducted in addition can, therefore, provide important additional information and decision criteria for therapies. Nevertheless, the study, in particular, suggests
that the extracellular resistance of cell monolayers does play a vital role for the discrimination of cell lines and monitoring of nsPEF-effects. In the future, this might present a way to predict the success of such treatments already within the first hours after exposures.

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