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Interdependence of initial cell density, drug concentration and exposure time revealed by real-time impedance spectroscopic cytotoxicity assay

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We investigated the combined effect between the initial cell density (12500, 35000, 75000, and 100000 cell/cm^2) and concentrations of the anti-cancer drug Doxorubicin on HeLa cells by performing time-dependent cytotoxicity assays using real-time electrochemical impedance spectroscopy. A correlation between the rate of cell death and the initial cell seeding density was found at 2.5 μM Doxorubicin concentration, whereas this was not observed at 5 or 10 μM. By sensing the changes in cell-substrate interaction using impedance spectroscopy under static condition, the onset of cytotoxicity was observed 5 h earlier than when using a standard colorimetric end-point assay (MTS) which measures changes in the mitochondrial metabolism. Furthermore, with MTS assay no cytotoxicity was observed after 15 h of incubation with 2.5 μM Doxorubicin, whereas impedance showed at this time point cell viability that was below 25%. These results indicate that impedance detection reveals cytotoxic events undetectable using the MTS assay, highlighting the importance of combining impedance detection with traditional drug toxicity assays towards a more in depth understanding of the effect of anti-cancer drugs in *in vitro* assays. Moreover the detection of Doxorubicin induced toxicity determined with impedance under static condition proved to be 6 times faster than under perfusion culture.

1. Introduction

Cancer is a pathological condition characterized by uncontrolled cell division of abnormal cells, invasion into the nearby tissue and ultimately spreading through the lymphatic system or bloodstream.[1] Chemotherapy is based on the administration of drugs that induce cell death in cancer cells. One of the major challenges in the development of anti-cancer therapies is finding the optimum dose of the drug that maximizes cancer cell death with minimum side effects.[2] In pre-clinical studies, several well established cell-based *in vitro* assays are regularly used for the evaluation of the effects of chemotherapeutic drugs on cell proliferation, viability and cytotoxicity.[3–7]

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The majority of the standard assays, e.g. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium),[5] do not provide kinetic information about the biological events occurring in real-time within the same cell population; moreover, they are invasive and labor intensive. When performing *in vitro* cytotoxicity assays, incubation time, drug concentration and initial cell density are important parameters to consider.[8] It has been observed using traditional cytotoxicity assays that the initial cell density influences the cytotoxic effect of certain anti-cancer drugs in various cell lines.[9–12]
However, there is a lack of studies evaluating the combined effect of initial cell density, drug concentration and exposure time in real-time using a label-free minimally-invasive method which provides information about changes in adhesion and morphological properties of the same cell population during the onset of cytotoxicity. Cell based electrochemical impedance spectroscopy (EIS) pioneered by Giaever and Keese in the 1980's[13] became a well-established label-free, minimally-invasive technique for real-time drug screening and toxicity

testing of anti-cancer drugs, detecting cell adhesion, morphological changes as well as cell death induced by cytotoxic compounds on the same cell population acting as its own control.[14–20]

Doxorubicin (DOX), an anthracycline-based antibiotic widely used in the treatment of a broad range of solid tumours as well as acute leukaemia and malignant lymphoma,[21] has been shown to have decreased cytotoxic activity with increased cell density, defined as 'positive inoculum effect',[9,11] EIS-studies are mainly focused on dose and/or time dependency of a drug on a specific cell density.[14,17,18] Therefore, the scope of this work was to investigate the time dependent effect of different concentrations of DOX, used as a model anti-cancer drug, on several initial cell densities of HeLa cells using EIS. The obtained EIS data were compared with the traditional end-point cell viability assay (MTS assay) which evaluates the toxic effect on mitochondrial functions and with data from experiments performed in perfusion culture given the increasing popularity of microfluidic lab-on-chip devices.[22–26]

2. Results and discussion

2.1. Optimization of cell density for EIS based cytotoxicity assay.

In order to perform successful cytotoxicity determinations, the initial cell density needs to be considered[8] since it has been shown to influence the action of certain drugs.[8,9,12,27] Moreover, when developing an EIS-based assay, adhesion properties and proliferation rate of the used cell line need to be studied in relation to the biological effect of interest (e.g. cytotoxicity) in order to define the optimum initial cell density. In EIS-based cytotoxicity assays, the initial cell density has to provide a control growth curve characterized by a stable steady state for the duration of the experiment dependent on the rate of drug-induced cell death. The initial cell density was optimized to provide enough space for the cells to steadily multiply during the incubation with the drug. We investigated the correlation between cell density and Cell Index (defined in section 3.5.) values by seeding different HeLa cell densities (1000, 12500, 35000, 75000, 100000 and 160000 cells/cm^2). The different cell densities were continuously monitored during 38 hours, while the control experiment was performed using only cell culture medium.

Fig. 1A shows the Cell Index increase during 38-h impedance monitoring of adhesion and proliferation of HeLa cells initially seeded at different densities. The first hours provide a good indication of the cell adhesion and spreading process that takes place immediately after seeding. These processes induce a rapid Cell Index increase during the first 5 hours as can be seen in Fig. 1A.

In analogy to what has been reported earlier,[14] our results show that cell adhesion and spreading can be considered completed after approximately 5 h, i.e. the determined Cell Index value reflects the total number of seeded and adhered cells.

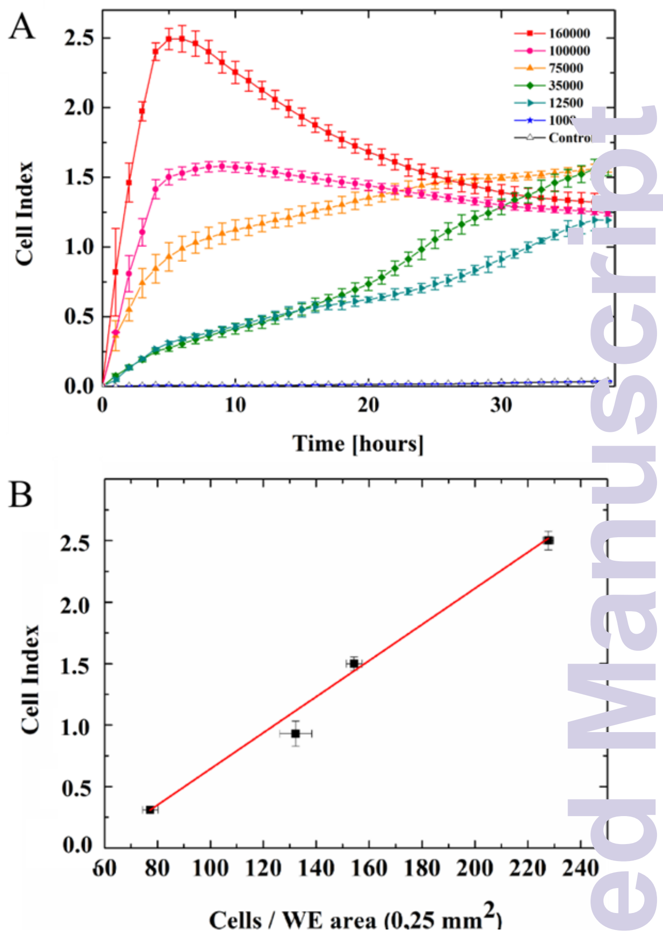

Fig 1. Optimisation of initial cell density. (A) Cell Index vs. time for real-time EIS monitoring of HeLa cell adhesion and proliferation over 38 h. Cells were initially seeded at different densities (1000, 12500, 35000, 75000, 100000, 160000 cells/cm^2) on laminin coated microelectrode chips. The increase in Cell Index during the first hours of the measurement indicates the adhesion and spreading of cells. At high cell densities (160000 cells/cm^2), a strong decrease in Cell Index (after 5 h) indicates the upper detection limit of the experimental setup due to full coverage of the electrode arrays/cell culture well. At low cell density (1000 cells/cm^2), the measurements are below the detection limit, the Cell Index remaining low even after 38 h. Between 12500 and 100000 cells/cm^2 cell density, cell proliferation could be followed up to 38 h. (B) Linear correlation between Cell Index and number of cells on the electrodes was found between 35000 and 160000 at 5h after seeding. Error bars represent s.e.m, n=6.

It can be seen that at high initial cell densities (160000 cells/cm^2), the Cell Index strongly decreases after having reached its maximum value at about 5 hours from cell seeding. This behaviour suggests that at higher cell densities, yielding a confluent cell layer (complete cell coverage on the WEs and/or in the entire culture well), the cell adherence (cell-substrate interaction) is weaker, leading to lower measured impedance. In other words, under such conditions the upper detection limit of a device is reached, eliminating the possibility to continue monitoring of cell proliferation. We determined the optimal cell density range for this assay to be between 12500 and 100000 cells/cm^2. Under such conditions, after reaching steady-state Cell Index, proliferation could be monitored for a sufficiently long period to facilitate a reliable performance (Fig. 1A). In addition, the number of cells adhering on the IDEs was directly quantified and a linear correlation between

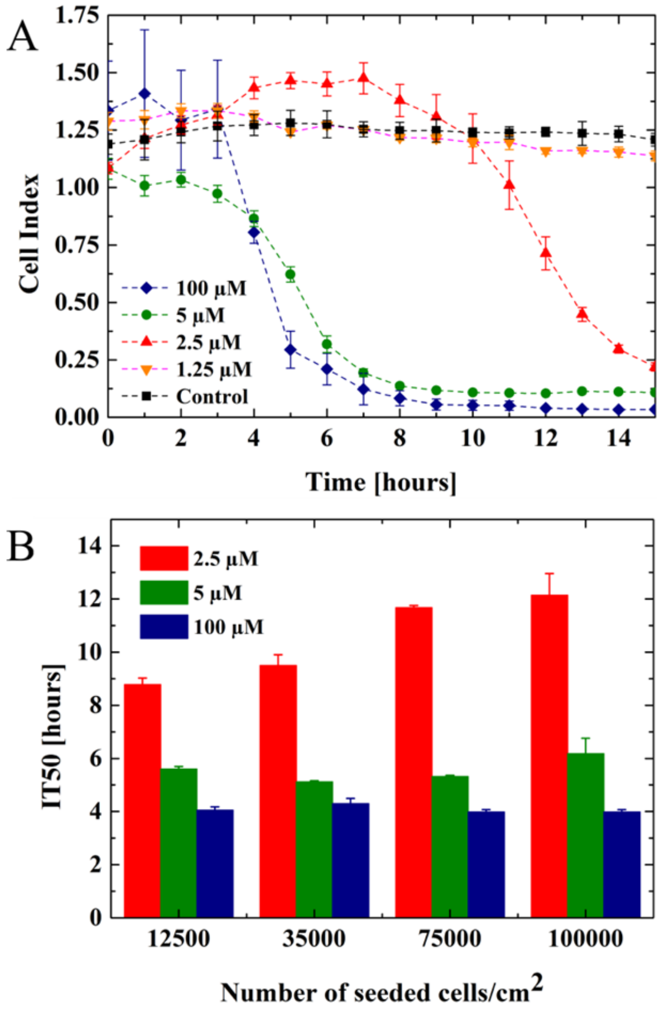

Fig 2. Effect of cell density and DOX concentration on cytotoxicity. (A) The Cell Index decreases after addition of DOX showing the concentration dependent cytotoxic effect of the drug. Cell Index profiles of DOX-induced cell death: HeLa cells (75000 cells/cm^2) were seeded on the microelectrode chips and DOX was added 10 hours after cell seeding (t = 0) to achieve a final concentration of 1.25, 2.5 5.0 and 100 µM. (B) IT50 values for four different HeLa cell densities (12500, 35000, 75000, 100000 cells/cm^2) exposed to 2.5, 5.0 and 100 µM DOX. The rate of cell death correlated with cell density at 2.5 µM DOX while this effect cannot be observed at 5 and 100 µM DOX. Error bars represent s.e.m, n=6.

the number of cells on the IDE area and the Cell Index value 5 h after cell seeding was found (Fig. 1B).

2.2. Relationship between initial cell density and rate of cell death

Since traditional endpoint assays have shown that the initial seeding density has an important role when studying the effect of DOX on various cell lines,[9,11,28] we studied the cytotoxicity of DOX concentrations on several HeLa cell densities using real-time EIS. Based on the set of growth curves presented in Fig. 1A, four different densities of HeLa cells (12500, 35000, 75000 and 100000 cells/cm^2) were seeded on the microelectrode chip to evaluate the effect of the initial cell density on the rate of cell death at different drug concentrations. Ten hours after cell seeding, different volumes of the DOX stock solution (prepared in 0.1 % NaCl) were added into the culture medium to obtain the final concentrations of 1.25, 2.5 and 5 µM chosen based on MTS assays (Supporting Information, Fig. S1). Simultaneously, control experiments were performed by exposing the cell populations to culture medium only containing 0.1 % NaCl. Fig. 2A shows cytotoxicity profiles at different DOX concentrations presented as relative Cell Index at an initial cell density of 75000 cells/cm^2. The observed decrease in Cell Index is related to both changes in cellular morphology and adhesion, which at a later stage of the cytotoxic effect result in total detachment of cells (Supporting Information S2).

Each curve provides real-time kinetic information related to the specific response of the cells to the different concentrations of drug. While 5 and 100 µM of DOX induce fast cell death, 2.5 µM leads to a response that can be divided into two phases. Initially, a significant increase in the Cell Index is observed, which after 8 hours is followed by a rapid decrease. This initial increase in impedance also observed by others[18,29] might reflect the intensified metabolic activity, increased adhesion properties and/or changes in cell morphology in response to the stress when trying to overcome the apoptosis induced by the accumulation of the drug. The second phase represents cell death. In contrast, the Cell Index profile related to 1.25 µM DOX does not significantly deviate from the control experiment, where no relevant variations are recorded. Therefore, further experiments were conducted using 2.5, 5 and 100 µM DOX.

In Fig. 2B, it can be observed that 5 and 100 µM DOX give similar IT50 values (defined in section 3.5.) for all the cell densities under investigation (from 12500 up to 100000 cells/cm^2). However, when incubating the cells with 2.5 µM DOX, the IT50 value increases with increasing cell density. In the case of DOX, there are a number of proposed mechanisms of action, such as intercalation into DNA during cell division and induction of oxidative stress through free radical formation.[30] For 2.5 µM DOX, the cell death might be predominantly induced by intercalation of the drug into DNA,[30,31] which ultimately results in growth inhibition and apoptosis. On the other hand, the faster and cell density independent cell death induced by 5 and 100 µM DOX may be explained by the fact that at this concentration other mechanisms are involved, possibly related to free radical formation.[32] Since one of the proposed mechanisms leading to DOX-induced cell death is dependent on DNA intercalation during cell division,[30] cell proliferation during an assay could play a significant role in the kinetics of cytotoxicity induced by the drug. Considering this, the faster kinetic response at 2.5 µM (about 30 % lower IT50 value) for lowest cell density (12500 cell/cm^2) compared with the highest cell density (100000 cell/cm^2), could be related to the fact that at the low cell densities the cells had sufficient space for effective proliferation resulting in faster cell death (Fig. 2B). Another possible explanation of this effect could be the decreased amount of DOX accumulation at higher cell densities.[11]

2.3. Comparison between MTS assay and EIS performed under static and perfusion conditions

Standard spectrophotometric cell viability assays performed in a multiwell format are widely implemented in drug screening and cytotoxicity studies,[33,34] and have thus frequently been used for correlation with real-time impedance data.[35,36] In this work, MTS assays were performed to compare the time-dependent cell death kinetics determined using EIS (Supporting Information, Fig. S3). MTS time-dependency studies were performed for the four cell densities at different time points (2, 4, 6, 8, 10, 13, 15 and 24 h) after exposure to 5 µM DOX and the results were compared with those obtained with EIS monitoring for the same cell densities (Supporting Information, Fig. S3). Under the same experimental conditions (cell density, DOX concentration, incubation time) the two methods (EIS and MTS) show similar trends for the different cell densities (Supporting Information, Fig. S3A and B). However, there is a significant difference between the IT50 values determined by the two methods. The IT50 values for the MTS assay were over 11 h while those for the EIS monitoring were below 6 h, i.e. the response time for the MTS assay is 5 h longer (Supporting Information, Fig. S3C). Moreover, the kinetic response for 2.5 µM DOX at a cell density of 12500 cell/cm^2 was 54% faster for EIS monitoring. The IT50 values for higher cell densities (35000, 75000, 100000), obtained using the MTS assay upon exposure to 2.5 µM DOX, could not be compared with EIS data since they indicated 80% cell viability even after 79 h (Supporting Information, Fig. S4).

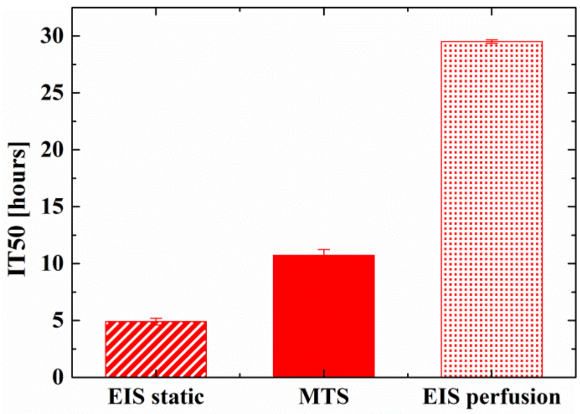

Fig 3. Cytotoxicity determined with the MTS assay and EIS performed in static and perfusion culture. IT50 values were calculated for 75000 cells/cm^2 density exposed to 5 µM DOX. Error bars represent s.e.m, n=6.

Fig. 3 shows a comparison of the calculated IT50 values obtained in the MTS assay and EIS monitoring under static and perfusion culture condition based on our previously published study[29] using 75000 cell/cm^2 cell density. The difference in the DOX response time for the two methods is related to the type of biological/chemical/physical event that is being monitored, as previously pointed out in other toxicological evaluations.[35] EIS and MTS assay provide different information about the effect of the drug and measure two different parameters related to cellular functions. While the MTS assay assesses the cell viability based on changes in mitochondrial activity at later time points, the EIS method responds in real-time to the early changes in the cell-substrate interaction. In the light of the presented results, the two methods provide complementary information for cytotoxicity assessment. On the other hand, the difference in the IT50 values between perfusion culture and static condition, monitoring of cell-substrate interaction reveals the effect of perfusion condition in cell based assay.[37,38] The onset of cytotoxicity is significantly delayed under perfusion condition as indicated by the IT50 value of close to 30 h. This observation indicates the significance of comparative studies in the light of the increasing popularity of microfluidic cell based assays.

3. Experimental

3.1. Chemicals

Sodium hydroxide, potassium hydroxide, hydrogen peroxide, cell culture tested phosphate buffered saline (PBS), sodium chloride, laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane and doxorubicin hydrochloride were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Trypsin-EDTA (0.05 %) and penicillin/streptomycin (P/S) were purchased from Life Technologies Ltd. (Paisley, UK). CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) was purchased from Promega Corporation (Madison, WI, USA).

3.2. Instrumentation, cell culture and experimental setup

The impedance measurement setup is composed of a plastic cell culture unit (Fig. 4A), having a microelectrode chip with an array of 12 interdigitated electrodes (IDE) (Fig. 4B and C), fabricated based on a previously published lithographic process including e-beam evaporation of 150 nm of Au on a 10-nm Ti adhesion layer[39], a tailor-made 12-channel bipotentiostat with miniaturized PCB and data acquisition software[40] (Fig. 4D). A schematic view of the measurement setup is shown in Supporting Information S5). Aside from the array of 12 IDEs each of the independently addressable measurement site of the microelectrode chip has an additional large counter and reference electrode that have been used in other cell based applications.[41,42]

As schematically presented in Fig. 4A, two 5 mm thick micromilled poly(methyl methacrylate) (PMMA) layers are assembled on top of each other. The lower one is used as a holder for the microelectrode chip, while the upper one, having an opening in the middle, defines a 600-µL well for cell culturing.

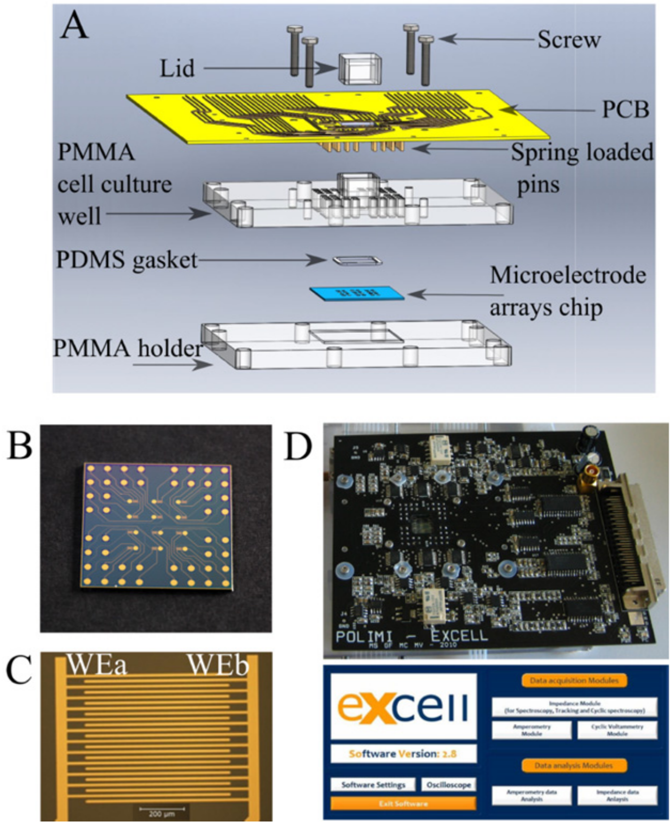

Figure 4. Impedance measurement setup. (A) Schematic diagram of the cell culture device, with microelectrode chip integrated between the PMMA holder and upper layer defining the cell culture well. The PDMS gasket placed between the chip and the upper PMMA layer ensures a liquid tight sealing of the well. The custom-made PCB potentiostat is connected to the microelectrode arrays through spring loaded pins. The PMMA lid minimizes medium evaporation and maintains sterile conditions. (B) Photo of the microelectrode chip comprising 12 gold electrode arrays. (C) Microscopic image of one IDE used for coplanar impedance measurements (WEa vs. WEb). (D) Photo of the PCB potentiostat and acquisition software.

A polydimethylsiloxane (PDMS) gasket is placed between the microelectrode chip and the upper PMMA layer to form a liquid tight sealing of the vial. Each of the two combs of the IDEs (shown as working electrode WEa and WEb in Fig. 4C) is independently addressable and composed of 12 digits (length: 500 µm; width and gap: 10 µm).

3.3. Cell culture

HeLa (cat. No. 85060701) cells were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). In preparation for experiments, cells were cultured in standard T25 cm^2 flasks with medium exchange regularly every 2 days. Prior to seeding cells on the microelectrode chips, cell suspensions were prepared by standard trypsinization using Trypsin-EDTA solution. Cells were centrifuged for 5 min at 900 rpm and 20 °C followed by resuspension in cell culture medium. The cell number was determined using a standard hemocytometer and the desired cell densities were prepared by diluting the initial cell suspension with fresh culture medium.

Prior to seeding cells in the cell culture device for EIS-based assays, each microelectrode chip was cleaned following the previously described method[43] including a chemical (10 min in the mixture of 25% H_2O_2/50 mM KOH) and electrochemical (potential sweep in 50 mM KOH between -200 mV and -1200 mV) step. Sterilization of the culture well was achieved by a 20-minute treatment with 500 mM NaOH followed by thorough rinsing with PBS. To promote cell adhesion, the chip surface was modified using laminin (20 µg/mL; 2 h, 37 °C). The applied cleaning procedure facilitated reusability of the microelectrode chips. Each chip was used for three experiments.

All cell preparations were kept in an ordinary humidified incubator at 37 °C in an atmosphere of 5% CO_2 in air. HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% P/S.

3.4. EIS monitoring protocol

EIS recordings were programmed to be performed continuously at time interval of 1 hour over the entire experimental period. The applied sinusoidal perturbation potential was set to 200 µV. Full spectra were acquired measuring 30 points in the frequency range from 100 Hz to 100 kHz 100 kHz was found to be the frequency corresponding to the most sensitive region of the spectra (Fig. 5). At this frequency, the impedance magnitude is influenced by extracellular resistance and membrane capacitance which are still primary contribution even if performing measurements up to 1 Mhz (Supporting Information Fig. S6).

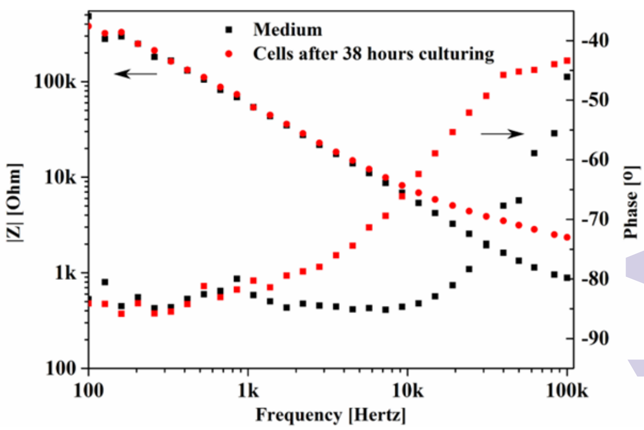

Figure 5. Impedance spectra recorded for cultured cells (seeded at a density of 75000 cells/cm²). Bode Plots acquired in the frequency range between 100 Hz and 100 kHz (10 data points per decade) using the miniaturized 12-channel bipotentiostat. The cells were cultured for 38 h (cell culture medium as control). To quantify changes in impedance, the Cell Index is calculated based on complete spectra using Eq. 1.

To provide a sufficient noise reduction each point was measured with an averaging time of 2 s. The impedance measurements were performed using the coplanar (bipolar) sensing configuration (WEa vs. WEb) providing higher sensitivity as previously demonstrated.[44]

3.5. Cytotoxicity assays

For EIS monitoring of drug-induced cytotoxicity, four different HeLa cell densities (12500, 35000, 75000 and 100000 cells/cm^2) were seeded on the laminin modified microelectrode chips. 10 hours after cell seeding, upon obtaining adhering cell layer and a proper baseline for impedance measurements, the anti-cancer drug DOX was added (from 1 mg/mL sterile filtered stock solution prepared in 0.1% NaCl) to the cell culture well to obtain the final concentrations of 1.25, 2.5, 5 and 100 µM. These concentrations were chosen based on the dose-dependent values determined using MTS assay (Supplementary Material, Fig. S1). Control experiments were performed by adding 0.1% NaCl to the cell culture well in the absence of DOX.

Cell viability was measured and quantified by a standard colorimetric MTS assay (performed according to the protocol of the manufacturer) in 96 well plates[5] (covered with aluminium foil to protect from light) using the four different cell densities as during EIS monitoring. 10 hours after seeding, the cells were treated with 5 µM of DOX and incubated. After 2, 4, 6, 8, 10, 13, 15 and 24 h 20 µL of MTS solution were added followed by an additional incubation for 1 h at 37˚C and the absorbance was measured at 490 nm. Control experiments were performed under the same conditions as used during the EIS monitoring. The measured absorbance for each incubated cell population was normalized with respect to the absorbance of the control.

3.6. Data analysis and statistics

Changes in impedance were expressed using the dimensionless parameter Cell Index,[17] which represents the maximum value of normalized impedance based on Eq. 1,

$$\text{Cell Index}(t) = \max_{i=1,\ldots,N} \frac{|Z(t,f_i)|-|Z(0,f_i)|}{|Z(0,f_i)|} \qquad \text{(Eq. 1)}$$

where $|Z(t,f_i)|$ is the magnitude of the impedance at a given frequency and time point and $|Z(0,f_i)|$ is the magnitude of the impedance at the same frequency at the beginning of the experiment recorded in the absence of cells. In this work, for each time point, the Cell Index was calculated analysing the complete spectrum (N = 30). Matlab (R2013a) was used to create specific algorithms for data processing and analysis (for choice of frequency, see section 3.4).

In order to quantify the time dependency of cell death, we defined the half maximal inhibitory time (IT50) (Eq. 2), analogous to the IC50, as a quantitative measure to indicate how long time is required for the drug to cause 50 % decrease in cell viability. The sigmoidal fitting of the data and the IT50 values were calculated using the logistic 4-parameter function (Origin (version 9.0)),

$$y = \frac{A_1 - A_2}{1+\left(\frac{t}{IT50}\right)^p} + A_2 \qquad \text{(Eq. 2)}$$

where A_1 is the initial and A_2 the final Cell Index or Abs_{490}, t is time and p is the slope.

For each experiment, EIS data acquired on the electrodes were processed and averaged. Each experiment was repeated at least two times. Data are presented as average ± standard error of mean (s.e.m.).

4. Conclusions

Using EIS-based assays, we clearly demonstrate the interdependence between initial cell density, drug concentration and exposure time as key factors influencing DOX-induced cytotoxicity. The initial cell density, in combination with the concentration of the anti-cancer drug, determines the time dependent cytotoxicity. At low (2.5 µM) DOX concentration, a correlation between the initial seeding density and the rate of cell death was found, whereas this was not observed at higher (5µM) DOX concentration. Intercalation into DNA during cell division strongly depends on the proliferation rate of the cell population under investigation. Therefore, at low cell densities cells have sufficient space for proliferation, resulting in a faster cytotoxic response compared with higher cell densities. Moreover, our impedance based experimental data were compared with data obtained from MTS assays performed in parallel and found that the time-dependence of cytotoxicity determined with the two methods differs. EIS measurements detect the cellular response to DOX earlier, which is probably related to the type of event that is being monitored (cell-substrate interaction versus mitochondrial activity). This study demonstrates the importance of EIS assays providing additional insight in the cytotoxic activity of drugs unobtainable when only using standard toxicity assays and provided solid basis for the development of impedimetric cytotoxicity assays in perfusion culture.

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