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Reflection Coefficient S11 Related Measurement System for Label-Free Cell Seeding Analysis and Drug Testing in a Three-Dimensional (3D) Cell Culture Model

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Abstract

Context: With regard to two-dimensional (2D) cell cultures in flat dishes, most of the three-dimensional (3D) cell cultures have advantages and gain importance in life sciences. But, their higher complexity requires adaptation of existing methods or different measurement systems in order to achieve accurate results. Bio-Impedance spectroscopy (Bio-IS) is a label- free and non-destructive method that is considered for analysis of 3D cell models.

Purpose: We design a new Bio-IS measurement system for a 3D liver cell model to assess cell seeding and drug effects.

Experiment Methods: We measured reflection coefficient S11 with differently (disc- and needle-like) shaped electrodes, which are in defined contact with the 3D liver cell model, metabolic activity (MTT test), and cytotoxicity (LDH assay). Results: We demonstrate that reflection coefficient S11 may be utilized to assess the 3D liver cell system in the frequency range of β-dispersion at 73 MHz only by means of the needle-like electrodes. This enables a reliable label-free and non-destructive determination of cell seeding and drug effects in the 3D liver cell culture model. The physical results have been verified by destructive biochemical methods (LDH and MTT assay).

Conclusion: Our Bio-IS system for 3D liver cell models using needle-like electrodes enables label-ree and non-destructive determination of cell seeding and drug effects.

Abbreviations: Acetaminophen (APAP) or N-acetyl-p-aminophenol or Paracetamol DMSO: Dimethyl Sulfoxide; Bio-IS: Bio-Impedance Spectroscopy; f: Frequency; FBS: Fetal Bovine Serum; LDH: Lactate Dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide PBS: Phosphate Buffer Saline; PS: Polystyrene; 3D: Three Dimensional

Introduction

Bio-IS measures the electrical properties of a biological test object when current flows through it. It is an emerging tool for biomedical research and medical diagnosis with a broad range of applications. For example, Bio-IS is used to estimate body composition, determine total body water levels and calculate the fat/fat-free mass [1]. Bio-IS can be used for tissue classification, as in breast cancer screening [2]. On the cellular level, Bio-IS is used in commercial blood analyzers to determine hematocrit levels [3]. Various cell-based impedimetric sensing systems are currently commercially available [4-6]. Several other devices and systems that study cell-substrate interactions in vitro have been reported in scientific literature [7].

The application spectrum of Bio-IS is expanding towards advanced cell culture techniques and complex cell systems that are used for tissue engineering and pharmacological and toxicological *in-vitro* testing. This analysis technique is expected to provide informative results about tissue structure, physiological states, and cell functions.

Determination and counting of cells or analysis of cell properties and cell behavior as a response to a drug in 3D cell culture models is a challenge. Established analytical methods for this purpose fail or can only partially be transferred from conventional 2D cell cultures in petri dishes [8]. For instance, when using disc-like polystyrene scaffolds, it is difficult to view and determine seeded cells using the light microscope

because towards 3D direction extended material is 1mm thick and often not translucent. In addition, the conventional cell detachment and digestion procedure for counting cells is more difficult and will not always give reliable results.

Some cells may stay attached in scaffold niches after trypsinization and may undergo prolonged digestion. An attempt to detach all cells from the carrier material may destroy and resolve cells.

The use of sophisticated valuable cells, stem cells or progenitor cells, such as HepaRG cells coming from human liver, in a 3D cell culture model or/and in bioreactors makes it necessary to follow long proliferating or/and differentiation protocols that bear the risk of contaminating cell culture, and may produce non-replicable effects, which increases working time and costs.

For advanced cell cultures, such as 3D cell culture, it is important to monitor cell viability, proliferation, differentiation states or contaminations, the best time to start an experiment, meanwhile and after its termination. In contrast, these evaluations can be easily performed in conventional, label- free 2D cell culture models, because the cells are generally visible by means of a light microscope.

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The aim of this study was to explore the application of Bio-IS in a human 3D liver cell culture for label-free and non-destructive determination of cell seeding and drug effects. Therefore, differently shaped electrodes were designed and tested for their applicability and it was assessed whether the Bio-IS at specific frequencies can be linked to cell seeding or drug effects in the human 3D liver cell culture. We investigated APAP, an over-the-counter drug, that is considered a safe drug, but is often abused or overdosed and many hepatotoxic APAP effects on cellular level have been enlightened [9]. Therefore, APAP is a reference substance for hepatotoxicity. Following toxic doses of APAP, intracellular glutathione pools are depleted, leading to oxidative damage and binding of reactive metabolites covalently to liver proteins. Subsequently, mitochondrial dysfunction leads to necrosis [9].

Material and Methods

Materials for electrode construction were provided from the Institute for Microelectronics and Microsensors (JKU, Linz, Austria). Materials for cell culture were obtained from Greiner Bio-one (Austria) and Lonza (Belgium). Cell culture media and all other chemicals were purchased from Sigma-Aldrich (Austria). The Cytotoxicity Detection Kit (LDH) was obtained from Roche Diagnostics (Germany). The polystyrene (PS) scaffolds were purchased from Gesellschaft für Werkstoffprüfung mbH (GWP), Zorneding, Germany.

3D Human liver cell model

Human *in vitro* models such as human liver 3D cell models give rise to more reliable species-specific testing results, are easier and cheaper to perform and are ethically better accepted than animal experiments [10]. HepaRG cells were obtained from BioPredic (France) and were grown and kept in a permanent proliferating state in conventional culture flasks (Greiner-bio one) in a humidified atmosphere at 37°C and 5% CO2. The HepaRG cells grow adherently on the flask bottom (2D cell culture).

Cells were detached from the surface using trypsin and separation through pipetting up and down, and the cell suspension was centrifuged. After discarding the supernatant, the cell pellet was diluted with fresh medium. An aliquot was used to determine the cell number using CASY® Cell counter [11].

To construct the liver 3D cell model, 1×10^6 HepaRG cells were seeded on porous plasma activated PS scaffolds and cultivated in Williams' E medium supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine, and 0.5 μM hydrocortisone hemisuccinate. Differentiation of the HepaRG cells was induced after a one week proliferating period with a 1% DMSO- containing medium and took three weeks.

Electrical properties of cells and tissues

Electrical Impedance Spectroscopy (IS) of a test object is performed by measuring the complex electrical impedance (Z) and its phase angle (Φ) at different frequency points within a specified frequency band, from the measured potentials (V) developed by injecting a constant sinusoidal current signal (I) to the object [12].

Bio-IS measures the passive electrical properties of complex tissues or single cells. Parameters that influence the bio-impedance of a tissue or a cell cluster are the resistance of the extracellular medium and the Intracellular cytoplasma, and the resistance and capacitance of the cellular membrane. The extracellular space acts as a conductive medium. This can be described by a simplified electrical circuit [13,14]. In the simplest case, single cells may be modeled as a group of the three electronic components, comprising two resistances representing the intra- and extracellular conduction, and a cell- membrane capacitance [15].

Cells possess specific dielectric properties. Three different frequency-dependent main dispersions can be classified, termed α, β and γ dispersions. α dispersion, the low- frequency dielectric cell behavior up to 10 kHz is assumed to be dominated by current flow through membrane ion channels and counter-ion polarization along cell membranes. β-dispersion (10 kHz to 100MHz) is associated with the dielectric properties of the cytoplasm-membrane interface, intracellular membrane structures, and the polarization of proteins and other organic macromolecules. In this frequency range, membrane capacitance becomes electrically conducting: it short-circuits the membrane resistance, and the electric field begins to penetrate the interior of the cell. γ-dispersion (100MHz to 100 GHz) is determined by the polarization of free and bound water molecules, protein-protein interactions, and the dissociation/association relaxation of small charged groups [15].

Reflection coefficient S11 measurement

Bio-impedance is assessed by measuring the reflection coefficient S11 (dB), defined as $S11 = (Z-Z_0)/(Z+Z_0)$, where Z_0 and Z denote the characteristic line impedance and impedance of the sensor, respectively.

The dielectric properties of the 3D liver cell model between two electrodes (Figure 1) are measured by emitting electromagnetic waves, and measuring amplitude and phase of the reflected wave. In this configuration, the cells of the 3D liver cell model act as a network of resistors and capacitors, which affect the recorded reflection coefficient S11.

Reflection coefficient S11 measurements were done with a commercial network analyzer (Agilent E5061B, Agilent Technologies Inc., Palo Alto, CA, USA), which allows the analysis of the reflection coefficient in the frequency range between 5 Hz and 3 GHz. The two electrodes were connected to the network analyzer through a coaxial wire. The experimental setup with needle-like electrodes is shown in Figure1B.

After the reflection coefficient S11 measurement and data analysis, impedance was calculated at 73 MHz.
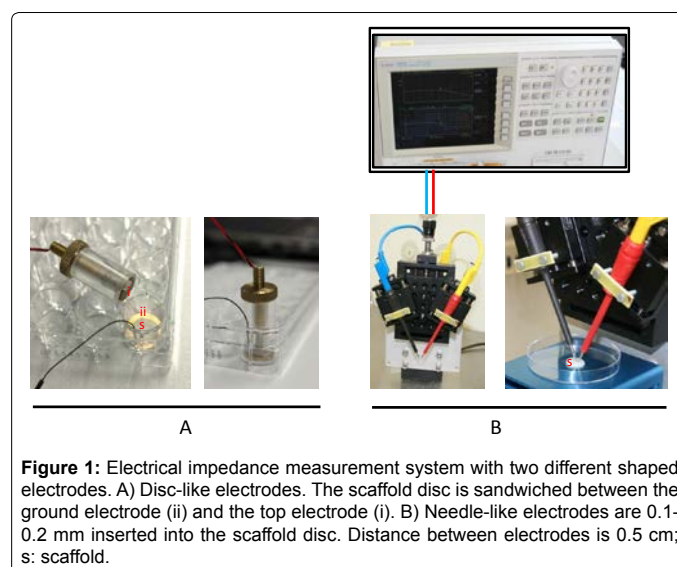

Figure 1: Electrical impedance measurement system with two different shaped electrodes. A) Disc-like electrodes. The scaffold disc is sandwiched between the ground electrode (ii) and the top electrode (i). B) Needle-like electrodes are 0.1-0.2 mm inserted into the scaffold disc. Distance between electrodes is 0.5 cm; s: scaffold.

Prior to the experiments, the measurement instrument was calibrated. For this purpose, predetermined loads were connected to the sensor (open, short and 50 Ω), thereby correcting for the influence of the cables and the electrodes on the measurement signal. In all experiments the reflection coefficient S11 was recorded in the frequency range between 5Hz and 3GHz. To measure S11, the 3D liver cell models were removed from the incubator for a short time to the sterile hood. They were taken out of the culture medium using forceps and were placed on a cell culture plate. In case of the cell number determination experiment, scaffolds without cells were used as control. In case of the analysis of drug effect, scaffolds without cells, but treated with drug were used as control.

Electrode configurations

Two electrode designs were studied: disc-like electrodes with 1 cm in diameter, and needle-like electrodes with adjustable distance between the electrode tips. The disc-like electrodes was covered with gold and the needle-like electrodes were covered with stainless steel to improve bio-compatibility. Figure 1 shows examples of the electrode designs.

Prior Bio-IS measurement at sterile condition under the bench, the scaffolds were transferred wet on a culture plate. The measuring apparatus was fitted with adjustable electrodes.

The disc-like electrodes match the diameter of the scaffold (Figure 1A). During the measurement, the scaffold is sandwiched between the electrodes, so that the averaged reflection coefficient S11 (dB) was measured over the whole scaffold.

In contrast, the needle-like electrodes mainly capture the small volume between the electrode tips (Figure 1B). To keep measuring conditions for the 3D liver cell models constant, the two needle-like electrodes were inserted into the scaffolds at a constant inter-electrode distance (5 mm) and penetration depth. Three measurements took place at different scaffold areas.

Cell determination in porous scaffolds using reflection coefficient S11 measurement

Liver cell cultures on polystyrene (PS) scaffolds are a new advanced 3D cell culture technique in basic science, pharmacology and tissue engineering. It promises more reliable results than conventional cell culture techniques using flat petri dishes. We have already established a human 3D liver cell model that reflected better the physiological parameters than the corresponding 2D cell culture system [16]. When adherent cells grow on the bottom of the flask (2D cell culture model), they are to large extent in contact with the plastic material. However, when cells grow in a 3D environment, the different cell-cell and cell-matrix interactions better resemble complex tissue structures, normal proliferation, differentiation, polarization, cell behavior and intercellular interactions. The majority of 3D cell cultures display a more realistic biofunctionality [17]. In a cell monolayer the growth area is limited to the bottom of the culture flask, thus high cell numbers cannot be obtained, and moreover cell function is limited.

For the cell seeding experiment, the HepaRG cells, kept as proliferating cells, were seeded in the porous scaffolds and cultivated for 48 h with different HepaRG cell numbers with a constant seeding volume of the medium. After that, reflection coefficient S11 was measured followed by metabolic activity using MTT assay.

Determination of drug effect in the 3D human liver cell model using reflection coefficient S11 measurement

After 21 days of differentiation time, the 3D liver cell models were treated with Acetaminophen (APAP), a reference substance for hepatotoxicity, for 24 hours. Reflection coefficient S11 was then measured at various APAP concentrations (0, 5, 10, 20 mM).

Cytostaining

The visualization of liver cells within the PS scaffolds was performed by Hematoxylin staining (Sigma Aldrich) according to the manufacturer's protocol, followed by light microscopy analysis (Zeiss Axiovert 40). Cell nuclei are stained blue.

MTT assay

Metabolic activity of all cell models was determined by reduction of MTT [18]. The MTT reagent is taken up into cells by endocytosis or protein-facilitated mechanisms. It is reduced to yield a purple formazan product that is largely impermeable to cell membranes, thus resulting in its accumulation within living cells. The samples were incubated for 2h with an MTT reagent containing medium. The medium was then removed, cells were lysed and the dye was solubilized. Absorbance was quantified by measuring at 590nm wavelength with a spectrophotometer (Infinite M200 Tecan, Austria). The amount of purple formazan depends on the amount of living cells in the 3D liver cell model. After treatment with APAP the amount of formazan produced by living cells was compared with the amount of formazan produced by the control (0 mM APAP).

Lactate Dehydrogenase (LDH) is an intracellular localized oxidoreductase which catalyses the interconversion of lactate and pyruvate. It is often used as a marker of cell and tissue breakdown, since LDH is a ubiquitous enzyme. LDH is indicative of tissue or cell damage. The release of LDH from cells into the medium increases when plasma membrane leakage occurs. Spectrophotometric measurements from the 3D liver cell model drug testing samples were performed using the LDH cytotoxicity detection kit from Roche Diagnostics according to the manufacturer's protocol. Supernatants from the cell culture samples were collected 24h after APAP treatment.

Results and Discussion

The cultivation of human cells has great economic potential due to its important applications for human health, such as being a source for adult stem cells in leukemia treatment and tissue engineering, or drug discovery, and pharmacological or toxicological drug testing.

For our study, we opt for S11 measurement because it can characterize the 3D liver cell model as a "black box". The 3D liver cell model is a complex living system and at defined frequency S11 it is possible to predict its external behavior without any regard for the detailed influencing contents. The S11 measurement, using our developed apparatus, may reflect hepatic behavior to estimate cells, and pharam- and toxicological relevant doses and time points for humans health and safety. We demonstrated that reflection coefficient S11 and subsequent determination of bio-impedance Z is particularly interesting for the 3D liver cell model, as this is a label-free non-destructive technique that allows repeatable and low-invasive analysis. After the S11 measurement of living HepaRG cells, the cells can be kept in their original state. The 3D liver cell models can be reused for further investigation, which facilitates the study of cell growth or treatment with drugs or toxic substances over time. This will save time and minimizes costs. Especially valuable, the slowly growing human cells are increasingly used for screening, pharmacological or toxicological testing or for biomedical applications such as tissue engineering.

The reflection coefficient S11 is a parameter originated from high frequency electronics and is used to characterize the behavior of linear electrical networks when undergoing various steady state stimuli electrical signals. Although applicable at any frequency, S- parameters are mostly used for networks operating at Radio Frequency (RF) and microwave frequencies where signal power and energy considerations are more easily quantified than currents and voltages [19]. Here, S11 (dB) is a new relevant parameter in testing 3D cell culture models. As a function of frequency the dielectric properties of the 3D liver cell model can be measured based on the interaction of an external field with the electric dipole moment of the sample. Differences in dielectric frequency-dependent behavior can be used to determine in the 3D liver models cell numbers or alterations in cell membrane integrity or various physiological changes induced in cells following drug treatment. The dielectric properties of biological samples display extremely high dielectric constants at low frequencies, falling off in more or less distinct steps as the excitation frequency is increased. The general frequency-dependence of permittivity is referred to as dispersion. According to Schwan, there are three major dispersion regions termed α, β and γ, as discussed in section 2.2 [20].

A comparison of the resulting characteristic curve plots of reflection coefficient S11 (dB) versus a broad range of frequencies was used by us to easily define the particular frequency where changes in the 3D cell culture model occur most. Furthermore, the comparison of curves helped to identify the frequency that show maximal differences in reflection coefficient S11 (dB) values in the context of each experimental setting. At this localized frequency, we were sub sequentially able to carry out more complex analysis, such as impedance Z analysis.

Design of electrodes for IS adapted for 3D liver cell model

The usage of differently shaped electrodes for our study revealed that needle- like electrodes (Figure 1B) better capture the cellular changes of the 3D liver cell model than the disc-like electrodes. The disc-like electrodes (Figure 1A) that represent a coplanar waveguide structure turned out to be inappropriate, since it was practically impossible to bring them into defined contact with the 3D liver cell model. As a result, there was strong variation in the measurement signal.

During the needle-like electrodes measurement, the scaffold was penetrated by the electrodes, which means that only a small volume fraction of the cell carrier contributed to the measurement signal (Figure 1B). To proof the measurements, each 3D liver cell model was measured three times at three different points. It has been shown that the data were broadly uniform and thus it was reliable to calculate the mean values. Hence, we opted for S11 measurement by the needle-like electrode for determination of cell seeding (Figure 3) and drug testing (Figure 5A).

3D liver cell model

The 3D liver cell model is structurally more complex due to HepaRG cells, that grow within the pores of the PS-scaffolds (Figure 2C) than the conventional cell culture where cells grow flat on the cell culture plates (Figure 2C). The unique characteristics of the HepaRG cells [21,22] together with their improved biofunctionality in the scaffolds [16] make this 3D liver cell model a reliable tool for the development of new low-invasive and label-free electronic detection techniques for pharmacology and biomedicine.

Determination of cell seeding in the 3D liver cell model

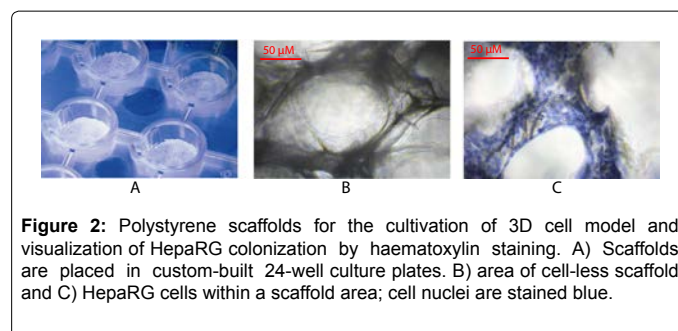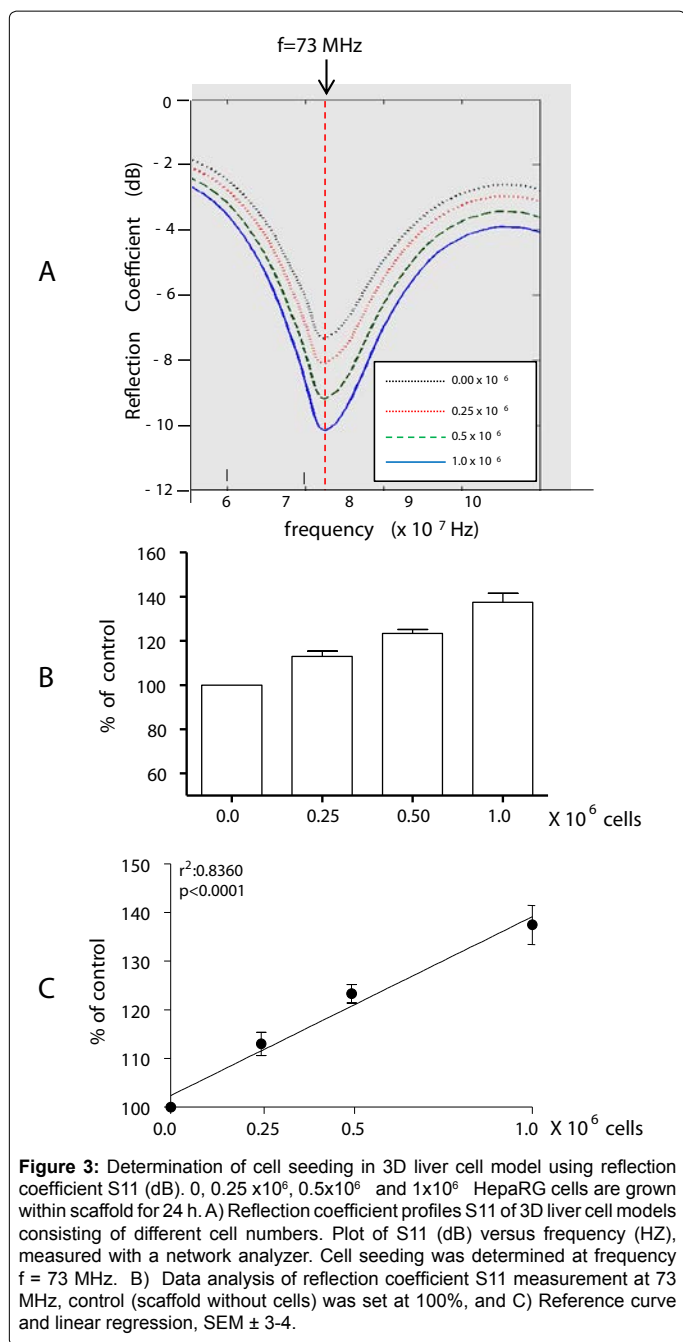Critical steps in culturing cells are the cell seeding at the beginning

Figure 2: Polystyrene scaffolds for the cultivation of 3D cell model and visualization of HepaRG colonization by haematoxylin staining. A) Scaffolds are placed in custom-built 24-well culture plates. B) area of cell-less scaffold and C) HepaRG cells within a scaffold area; cell nuclei are stained blue.

and the cellular state determination prior to an experiment. Both need to be strictly controlled to avoid underproductive results. Accurate assessment of the growth of cells cultured in scaffold is a based assay. Inhomogeneous cell seeding and growth in those 3D cell models can lead to unreliable results.

To visualize cell seeding in the scaffold, we used cell nuclei staining by means of hematoxylin. Cell staining followed by light microscopy analysis allows just viewing of distinct scaffold regions of the polystyrene matrix structure (Figure 2B and 2C) and of the settled HepaRG cells within the scaffold. After cell nuclei have been stained blue, cell seeding was visible on the level of single cells (Figure 2C), as control an area of the unseeded scaffold did not stain blue (Figure 2B). Hematoxylin staining is a destructive method and therefore the 3D liver cell models cannot be used again.

To validate the cell seeding data assessed from reflection coefficient S11 measurement, the same 3D liver cell models were used for metabolic activity determination using MTT assay. Our results suggest that the assessment of the cell seeding at our established 3D liver cell model by means of the physical method, reflection coefficient S11, and the biochemical method, metabolic activity (MTT assay), has been achievable. We explored if seeding of different numbers of HepaRG cells into scaffolds can be determined quantitatively due to the reflection coefficient S11 (Figure 3A). Changes of the reflection coefficient S11 at the frequency range from 67-to-83 MHz, where S11 value was lower than -6dB, were associated with different cell numbers in the 3D liver cell models (Figure 3B). The reflection coefficient S11 profile revealed a resonance frequency of 73 MHz at S11=-10dB, where the 3D liver cell model reflection was negligible. At the frequency of 73 MHz, the lowest points were located and the curves differ mostly from the other spectra depicted at different cell numbers (Figure 3A). The decrease of the reflection coefficient S11 values at 73 MHz coincided with the increase of the number of cells seeded onto the scaffold (Figure 3B and 3C). In the radio frequency range cells typically exhibit a significant β-dispersion due to the Maxwell-Wagner effect at the interface between the intra- or extracellular solution and the phospholipid membrane. In addition, water molecules will cause a gamma- dispersion and any protein or macromolecule will produce dispersions at frequencies ranging from the- through the γ-dispersion-range, depending on the size and charge of the molecules. Water bound to proteins will also cause a δ-dispersion [23]. 73 MHz is primarily within the frequency range of β-dispersion. Therefore, we figure that quantitative measurements of cell numbers in the PS-scaffolds can be explained mainly through β-dispersion. β-dispersion is associated with polarization of cell membranes, proteins and other organic macromolecules [24]. The biological response to electromagnetic waves depends on one hand on the external power density, and on the other hand on the interaction with electric charges and dipoles inside of the

Figure 3: Determination of cell seeding in 3D liver cell model using reflection coefficient S11 (dB). 0, 0.25 x10^6, 0.5x10^6 and 1x10^6 HepaRG cells are grown within scaffold for 24 h. A) Reflection coefficient profiles S11 of 3D liver cell models consisting of different cell numbers. Plot of S11 (dB) versus frequency (HZ), measured with a network analyzer. Cell seeding was determined at frequency f = 73 MHz. B) Data analysis of reflection coefficient S11 measurement at 73 MHz, control (scaffold without cells) was set at 100%, and C) Reference curve and linear regression, SEM ± 3-4.

3D liver model. The external power density was held constant during the experiment. But the interacting dipoles and charges in the 3D liver model increased due to the increase of cell number within the scaffolds, because more ions are captured in cellular membrane compartments leading to higher ionic content and ionic mobility.

Thus, as the cell number increased in the 3D liver models, the number of interacting charges and dipoles increased and the intensity of the reflection coefficient S11 decreased (Figure 3A). As a consequence, due to this relationship precise recording and quantification of cell numbers in the PS- scaffolds is possible.

We figure that quantitative measurements of cell numbers in the PS-scaffolds can be determined through β-dispersion. We described the relationship between reflection coefficient S11 (dB) and cell

number to be at 73 MHz in linear range (p<0.0001; r^2=0.8360) (Figure 3C). In a similar manner was the relationship between the absorption of blue formazan and the number of seeded cells which was determined using the biochemically method by means of MTT assay (p<0.0001; r2= 0.9858) (Figure 4C).

The S11-dependency on the cell number was used to setup a standard curve for the determination of cells. A linear calibration curve (Figure 3c) is applicable for the determination of the cell numbers in unknown 3D liver model samples.

The MTT assay is well established in characterizing cell proliferation in 2D monolayer cultures of low cell densities, but has the disadvantage of being invasive and destructive. It is a biochemical method to determine cell seeding, indirectly. It should be kept in mind that using the MTT method, dying or dead cells are not detected. But, prior to the extracting step with MTT lysis reagent, it gave information about the localization of the cell seeding within the PS-scaffold, because viable HepaRG cells were able to metabolize the yellow MTT reagent into blue formazan (Figure 4A) and the blue color localizes viable HepaRG cells of the 3D liver cell model. The more cells were seeded, the more blue color was located within the scaffolds (Figure 4A). The bar chart (Figure 4B) displays indirectly the increase of cell number
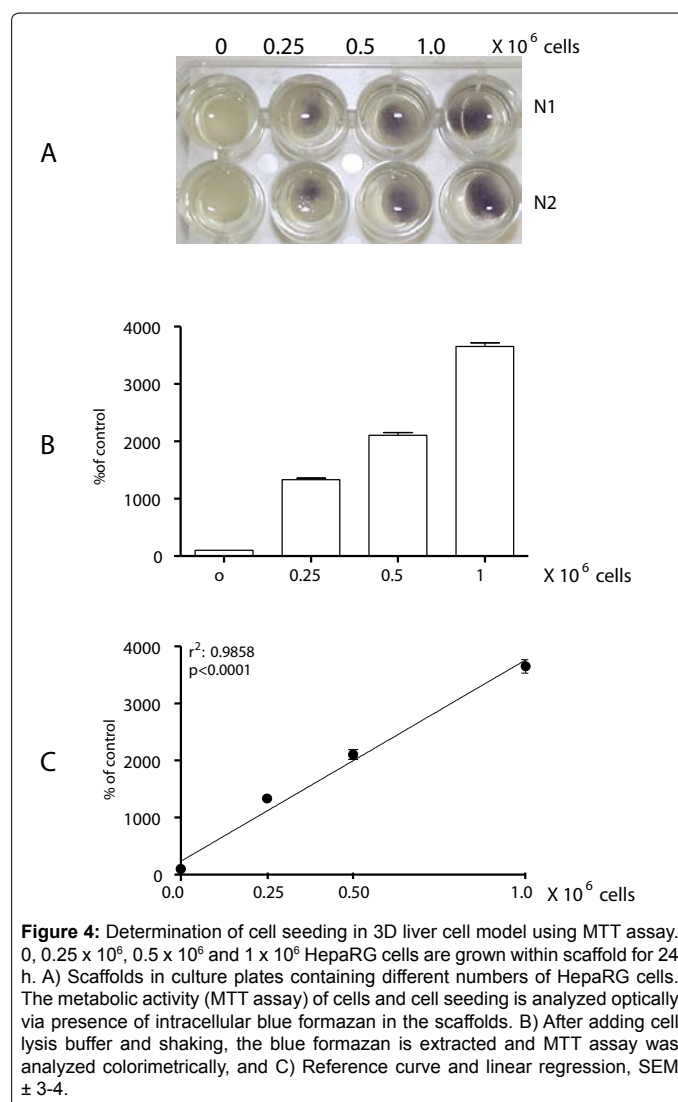

Figure 4: Determination of cell seeding in 3D liver cell model using MTT assay. 0, 0.25 x 10^6, 0.5 x 10^6 and 1 x 10^6 HepaRG cells are grown within scaffold for 24 h. A) Scaffolds in culture plates containing different numbers of HepaRG cells. The metabolic activity (MTT assay) of cells and cell seeding is analyzed optically via presence of intracellular blue formazan in the scaffolds. B) After adding cell lysis buffer and shaking, the blue formazan is extracted and MTT assay was analyzed colorimetrically, and C) Reference curve and linear regression, SEM ± 3-4.

after the colorimetrical analysis of the MTT assay after extraction of the blue color ((Figure 4B). In addition, the dependency of metabolic activity on the number of viable cells from the

3D liver models were used to setup a reference curve for cell determination of unknown 3D samples (Figure 4c).

We have already tested the number of cells in scaffolds between 250.000-1.000.000 cells. These cell numbers are in a realistic range of cell culture effort to establish a 3D liver model. According the two-step time consuming cell culture protocol, the HepaRG cells need high cell density and special treatment to differentiate into hepatocyte-like cells [22]. But, using Bio-IS devices and electrodes that are appropriate adapted to the living system it is possible to detect less cell numbers in the scaffold. For instance, recent advances in microfabrication and lab-on-a-chip concept enabled Bio-IS-devices to detect and analyse a single cell. A single cell is placed inside an electric field that leads to a local distortion of the field enabling the measurement of the characteristic impedance signature of the cell [25].

The sensitivity of MTT assay depends on the cell type, their metabolic status and the appropriate solubilizing of the formazan crystals and was reported for a zebrafish liver cell line to led to low signals, at cell densities of ≤ 12.500 cells/well [26].

APAP effect on liver 3D cell model

The toxic effect of APAP on the human liver 3D cell model was investigated. In our liver 3D cell model in vitro experiment is 5 mM equivalent to 0.8 g APAP, 10 mM APAP to 1.5 g and 20 mM to 3.0 g. The usual adult minimal daily dose of APAP is 1g, and the maximal daily dose is 4 g [27]. If the bioavailability of APAP is assumed to be 100%, this means, that 1g APAP is equivalent to 6.6 mM and 4 g is equivalent to 26.5 mM after intake to the human body. The cytotoxicity (LDH release from cells as a valid marker of membrane damage) increased with higher APAP dose ((Figure 6A) starting to differ significantly more than two fold compared to control from the concentration of 20mM APAP(Figure 6B). Consistently with the cytotoxicity was the dose-dependent behavior of the impedance, calculated from reflection coefficient S11 data after measuring at 73 MHz (Figure 5). At the 3D liver cell model, S11 decreased and impedance at 73 MHz, as a percentage of the controls, increased significantly at 10 mM APAP and above (p<0.05 vs. 0 mM APAP=control). Figure 6 displays the dose-dependent APAP effect on cell viability in 3D liver cell model. In the case of the liver 3D model, the capacitance, or charge-storing characteristics were measured using Bio-IS and differ mostly at 73 MHz after drug treatment. Supposing equal liver cell numbers (number of capacitors) at the beginning of the treatment with APAP, the ability of charging via Maxwell-Wagner polarization, which occurs because of the non-conductive nature of cellular membranes, decreased after drug exposure. The dose-dependent hepatotoxic drug effects of APAP on the liver 3D cell model, such as increasing loss of HepaRG cell membrane integrity and cellular ion content because cells were dying, may have changed the extracellular increased, the dielectric permittivity of the extracellular medium increased and the intensity of the reflection coefficient S11 decreased.

The measurement of more negative S11 values (lower reflection) coincided with decreasing metabolic changes, cell health and viability. It was reported that the frequency region around 100 MHz could be caused by the dipole moment of large molecules, such as proteins [28]. Therefore, the dose-dependent liver cell response to APAP at 73 MHz might also be caused by changes on the dipole moment of proteins.
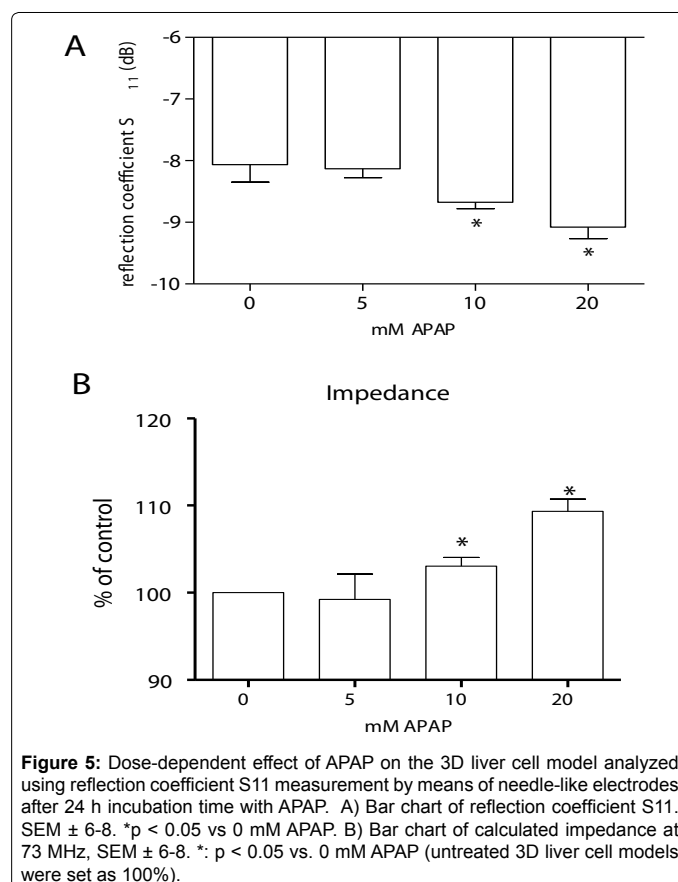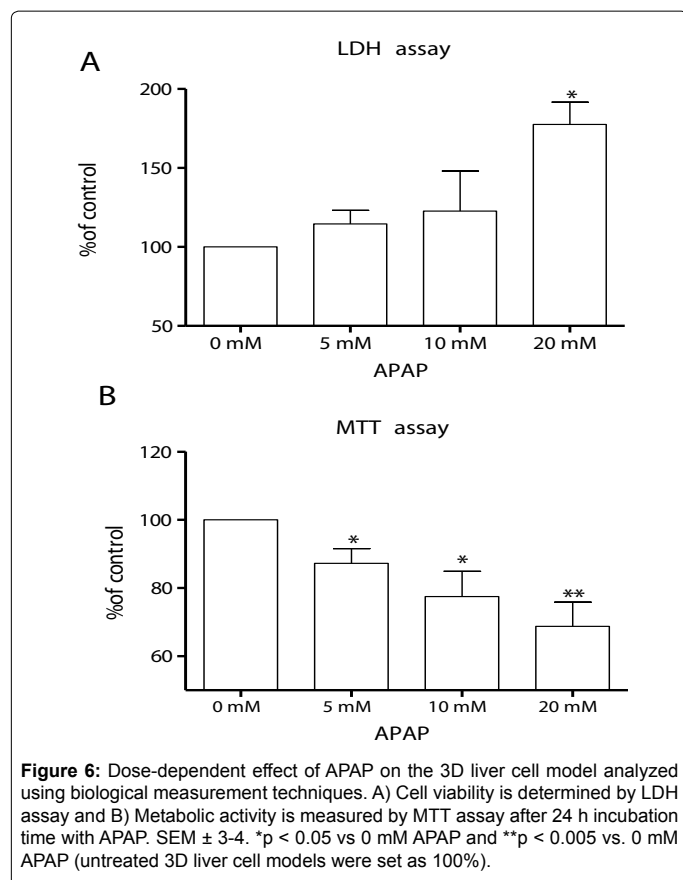
Figure 5: Dose-dependent effect of APAP on the 3D liver cell model analyzed using reflection coefficient S11 measurement by means of needle-like electrodes after 24 h incubation time with APAP. A) Bar chart of reflection coefficient S11. SEM ± 6-8. *p < 0.05 vs 0 mM APAP. B) Bar chart of calculated impedance at 73 MHz, SEM ± 6-8. *: p < 0.05 vs. 0 mM APAP (untreated 3D liver cell models were set as 100%).

During the manifestation of hepatotoxic effects, an increase in protein dysfunction is observed due to the accumulation of APAP protein adducts [29]. However, it should be kept in mind that APAP can have also intracellular effects that could influence charge of other organic macromolecules, such as other proteins, DNA, RNA or on intracellular membrane surrounded structures, like vesicles, mitochondria, or nuclei in liver cells [9], which are in the range of β-dispersion. Opposed to the increased cytotoxicity at the 3D liver cell model (Figure 6A), metabolic activity of the cells decreased with higher APAP doses (Figure 6B). Using the MTT assay, depletion of metabolic activity of HepaRG cells occurred already at a concentration of 5 mM APAP and above (p<0.05 vs. 0 mM APAP (control). Therefore, the MTT test appeared to be most accurate, followed by S11-/Impedance technique, and subsequently the LDH assay.

The comparison of the three different techniques for analysis of the APAP effects on cell viability and cytotoxicity in the liver 3D model revealed that the effective concentration (EC) causing 15% loss of cell viability was about 5 mM APAP for MTT assay (Figure 6B) and LDH assay (Figure 6A). Thus, the estimated EC10 values for both standard biochemical assays were below 5 mM APAP. In contrast, the EC10 value was at a higher concentration at 20mM APAP for impedance analysis (Figure 5).

This implicates that the common biochemical assays were in case of APAP more sensitive than the new Bio-IS technique and our Bio-IS device needs further optimization. In summary, the label-free and non-destructive reflection coefficient S11 measurement and/or subsequently the determination of impedance might be suitable for monitoring drug

Figure 6: Dose-dependent effect of APAP on the 3D liver cell model analyzed using biological measurement techniques. A) Cell viability is determined by LDH assay and B) Metabolic activity is measured by MTT assay after 24 h incubation time with APAP. SEM ± 3-4. *p < 0.05 vs 0 mM APAP and **p < 0.005 vs. 0 mM APAP (untreated 3D liver cell models were set as 100%).

responses in 3D cell culture models under physiological conditions.

Conclusion

Electrical reflection coefficient S11 measurement is a new tool for advanced cell culture applications. Our Bio-IS test apparatus has many practical advantages over the international validated biochemical tests, that are gold-standard and international validated, such as MTT and LDH assay. One highlight is that it can measure the electrical passive properties of the 3D liver cell model for two different purposes, namely cell number determination and drug testing. Both purposes do have nothing in common at a first glance, but the change of the number of viable cells, differences in the amount of intact cellular membranes and ions that are separated in cellular compartments. Likely, the increase of the number of viable cells during cell proliferation and the decrease of the number of viable cells during drug testing at the 3D liver cell model was measured at one distinct frequency (73MHz).

Furthermore, it is non-destructive, requires low cost instrumentation, easy to applicable in practice and it gives ability of repeated assaying and on-line monitoring. Compared to the biochemical tests the used Bio-IS system is proposed to have broader applications and is not limited to cell viability/cytotoxicity testing. MTT and LDH assay are colorimetric tests, and interference with the test substance may occur [30-32].

The MTT test is an endpoint assay, while using LDH assay more than one measurement can be performed per experiment. Many methods of analysis exist for assaying for cell viability, cytotoxicity and other cellular responses (see elsewhere) and only few are here discussed. To overcome the two step procedure of the MTT assay,

incubation with the MTT reagent and thereafter solubilization of formazan, the colorimetric MTS (3-(4,5-dimethylthiazol-2- yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo- phenyl)-2Htetrazolium) in vitro assay is an updated version of the international validated MTT method, MTS assay has the advantage of being soluble and no solubilisation step is required. 4-[3-(4-iodophenyl)-2-(4-nitro- phenyl)-2H-5-tetrazolio]-1,3-benzene disul- fonate, is a water-soluble tetrazolium salt (WST-1) more stable, has broader linear range and enables more rapid color development [33].

Recently more and more used are Alamar Blue reagents, which are nontoxic, resazurin based, membrane permeable solutions that upon reduction form resorufin, a red fluorescent compound which can be quantitatively measured to determine viability [34]. Alamar Blue may circumvent many of the incompatibility issues described and offers many advantages over tetrazolium salts. To select an appropriate assay, different parameters like test substance, detection mechanism, specificity, and sensitivity have to be considered. Furthermore, the kind of biological material can influence test performance.

In summary, the evaluations obtained from disc-electrode measurements did not in practice produce satisfactory results and were therefore not appropriate. The disc-electrodes are bigger electrode structures, average over a bigger sample volume, but they may also sense much more parasitic current paths through the cell medium beyond the cells. Smaller electrodes, such as the needle-like electrodes, are more sensitive to changes in cell behavior in a 3D liver cell model, because they record from a more confined sample volume.

In addition, cell seeding and effects of drugs or chemicals on a 3D human cell culture system can be measured and analyzed with the described setup. Extracting the relevant information from reflection coefficient profiles obtained from the needle-like electrode measurement is a challenge in case of biological systems of high complexity and dynamics, because they can be described by a high number of parameters. The treatment of 3D liver cell models with increasing APAP doses reduced reflection coefficient dose-dependently (Figure 5A). Coincidently, there was a reduction of metabolic activity after treatment with increasing APAP doses. Because the reflection coefficient at 73 MHz involves the changes of transformation of energy and matter in the 3D liver cell model, this physical parameter may reflect the behavior of the 3D liver cell model due to the metabolic activity. As well as the reflection coefficient, refers the metabolic activity to the set of chemical reactions associated with ionic or charge transfers that maintain life in living systems. Future research is required to determine in detail which of the distinct intracellular changes in the 3D liver cell model has influence on the Bio-IS effects we measured.

Statistical analysis

Results are expressed as means ± SEM unless stated otherwise. Statistical differences between the two groups were assessed with the two-tailed unpaired t-test. The significance level was set at α=5% for all comparisons.

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