Effect of electric field on cell behaviour

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(projektu, uměleckého díla, uměleckého výkonu)

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Zásady pro vypracování

Student zpracuje literární rešerši popisující vliv elektrického pole na chování buněk, fyziologické a patologické procesy v tkáních.
V praktické části student provede sérii experimentů popisujících vliv vnějšího elektrického pole různého nastavení na chování a životaschopnost buněk.
Dále student připraví a prozkoumá vliv přítomnosti vodivého polymerního substrátu.

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[1] Aguilar, A., Ho, M., Chang, E., Carlson, K., Natarajan, A., Marciano, T., Bomzon, Z., Patel, C., 2021. Permeabilizing Cell Membranes with Electric Fields. Cancers 13, 2283. https://doi.org/10.3390/cancers13092283.
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ABSTRAKT

Elektřina hraje důležitou roli v organismu, jelikož různé funkce jsou na ni částečně nebo zcela závislé. Toto vedlo k myšlence využití externí elektřiny pro lékařské účely. Tato práce se zaměřuje na různé buněčné funkce, které je možno ovlivnit vnějším elektrickým polem a možné využití těchto elektrických polí v medicíně. V praktické části, buněčná linie myších fibroblastů a buněčná linie myších kmenových buněk byly vystaveny vlivu elektrického pole o různých nastaveních. Experimenty s elektřinou byly provedeny také na buňkách kultivovaných na vodivém polypyrrolovém substrátu. Výsledky práce přispívají k hlubšímu pochopení vlivu exogenní elektrické stimulace na buněčnou morfologii a životaschopnost.

Klíčová slova: exogenní elektrické pole, morfologie buněk, životaschopnost buněk, napětí, polypyrrol

ABSTRACT

Electricity plays an important role in an organism, as various functions are partially or totally dependent on it. This led to the idea of using external electricity for medical uses. This thesis focuses on various cell functions that can be influenced by exogenous electric fields and possible uses of these electric fields in medicine. In the practical part, mouse fibroblast cell lines and mouse stem cell lines were put under the influence of electric fields with different settings. The experiments with electricity were also performed on cells cultivated on conductive polypyrrole substrate. The results of the study contribute to a deeper understanding of the influence of exogenous electrical stimulation on cell morphology and viability.

Keywords: exogenous electric field, cell morphology, cell viability, voltage, polypyrrole

I would like to thank Ing. Leona Mahelová for her help and patience as the supervisor of my Bachelor’s thesis. I would also like to thank my family and friends who supported me during my studies.

I hereby declare that the print version of my Bachelor's thesis and the electronic version of my thesis deposited in the IS/STAG system are identical.

CONTENTS

INTRODUCTION

Electricity is a natural part of organisms, including humans. This electricity is produced in the organism and takes part in physiological and pathological processes, among which can include the presence of electricity in the nervous system or heart, but also some less-known ones, such as endogenous electricity in natural wound healing.

Since endogenous electricity is clearly necessary for life, efforts to use exogenous electricity emerged. It has been researched how cells react to electrical stimulation and how we can benefit from it. Examples of the usages that are studied or particularly already in use are the already mentioned wound healing or curing severe diseases such as cancer. With all the possible settings of electricity that have different influences on certain cells and the ways that can be used for delivering the electricity in required areas, there is still a lot that needs to be uncovered.

This thesis goes over types of electric fields that can affect cell functions and how this knowledge can be used in medicine and tissue engineering. In the practical part, the effects of different settings of electric fields on the mouse fibroblast cell line and the mouse stem cell line are determined. In addition, the impact of a conductive substrate on the effect of electricity on cells is also characterized. The results show a set voltage and exposure time that is not harmful to cells and has the potential to encourage cell migration and increase cell viability.

I. THEORY

1 ELECTRIC FIELD

Every particle or object that is electrically charged exerts an electric force on others through an electric field. It is possible for them to be either positively charged when there is an excess of protons in atoms or negatively charged when there is an excess of electrons in the atoms. The original terms were glass and resin electricity before they were renamed into positive and negative. It applies that the same charges (positive-positive or negative-negative) repel each other, and different charges (positive-negative) attract each other. (Gussow, 2007; Etkin, 2017)

Through time, several physical quantities were formulated for the use of the description of electric fields and electricity.

1.1 Coulomb's law

To define the electric force produced between two charged particles, we use Coulomb's law. This law is an analogue to the modified Newton's law of gravity. For simplification, we consider those two particles as charged mathematical points placed in a vacuum. We can use this if the dimension of the particles is much smaller than the distance between them. (Shadowitz, 1975; Etkin, 2017) Coulomb's law was confirmed by many experiments and was summarised as:

$$F_{12} = K\frac{q_1 \cdot q_2}{r_{12}^2}\widehat{R}_{12}, \tag{1}$$

where $\vec{F}_{12}$ is an electric force in newtons (N) between two charged particles q_1 and q_2 in coulombs (C), r_{12} is a distance in metres (m) between particles, K is a constant of proportionality which depends on the system of units used, and $\widehat{R}_{12}$ is a unit vector in a direction from q_1 to q_2. (Shadowitz, 1975)

According to the International System of Units, or SI, the constant of proportionality is:

$$K = \frac{1}{4\pi\epsilon_0}, \tag{2}$$

where $\epsilon_0 = 8.85 \cdot 10^{-12}$ s^4 $A^2 \cdot m^{-3} \cdot kg^{-1}$ is the permittivity of vacuum. (Guru and Hiziroglu, 2004)

Connecting formulas (1) and (2) then gives us the final form of Coulomb's law for two charged particles: (Shadowitz, 1975)

$$F_{12} = \frac{1}{4\pi\epsilon_0}\frac{q_1 \cdot q_2}{r_{12}^2}\hat{R}_{12} \tag{3}$$

1.2 Electric field intensity

To simplify the expression of the electric field even more, we get the electric force obtained from Coulomb's law and define it per unit charge to get the electric field intensity:

$$E = \frac{F_{12}}{q_2}, \tag{4}$$

where q_2 is a test charge. The units for electric field intensity, or just electric intensity, are $N \cdot C^{-1}$ or $m \cdot kg \cdot s^{-1}\ A^{-1}$. (Shadowitz, 1975)

The formula for electric intensity (4) then can be rewritten according to the formula (3) as:

$$E = \frac{1}{4\pi\epsilon_0}\frac{q_1}{r_{12}^2}\hat{R}_{12}, \tag{5}$$

where q_1 is the source charge which produces the intensity and is independent of the q_2 as the test charge. This leads to the thought that the electric force produced by the source charge would exist even without the presence of the test charge. (Shadowitz, 1975)

1.3 Electric potential

An electric charge can do work due to the electric force. The work is happening by moving another charge via attraction or repulsion. This ability is called electric potential. Different charges have different potentials between them, and the sum of these potential differences is called electromotive force. The difference in potentials is called voltage (*U*) because of its unit, which is volt (V). (Gussow, 2007)

1.4 Current

In the presence of potential difference, the electrons are moved, which causes a flow of electrons called current (*I*). The unit of current was defined as the movement of one C past any point of a conductor during one second (s) and is called an ampere (A). (Gussow, 2007) Current then can be formulated as:

$$I = \frac{Q}{T}, \tag{6}$$

where I is current in units of A, Q is charge in units of C and T is time in units of s. (Gussow, 2007)

Current can be divided based on the direction of its movement. If the current is moving in one direction, it is called direct current (DC). The polarity of the voltage source in this case keeps the same polarity and the supplied voltage is called direct-current voltage. If the current periodically changes the direction of its movement, as depicted in Figure 1, it is called alternating current (AC). The polarity of the voltage source is periodically alternated, and the supplied voltage is called alternating-current voltage. (Gussow, 2007)

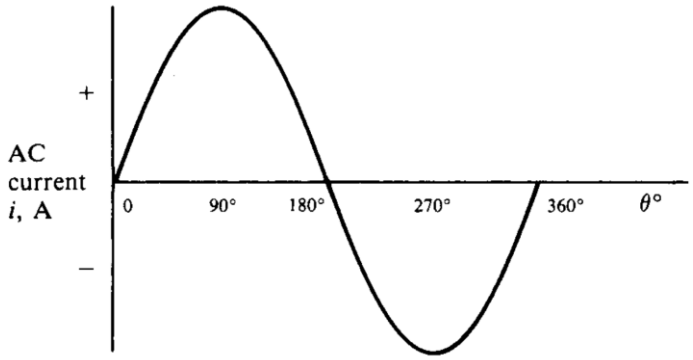

Figure 1 One cycle of alternating current (Gussow, 2007)

One parameter that can be used to define an alternating current is its frequency (f). The unit of frequency is hertz (Hz), and it is one cycle of the current per second. The more cycles per second the alternating current makes, the higher the frequency. (Gussow, 2007)

A frequency can be converted to the period:

$$T = \frac{1}{f}, \tag{7}$$

where f is the frequency in units of hertz (Hz), and T is the period in units of s and defines the amount of time to complete one cycle. (Gussow, 2007)

Material properties that limit the current is resistance (R) with unit ohm (Ω). (Gussow, 2007)

1.5 Ohm's law

With the knowledge of U, I, and R, Ohm's law can be formulated as it establishes a relationship between those three quantities. (Gussow, 2007) Ohm's law can be formulated in three ways:

$$I = \frac{V}{R}; \quad \text{or} \qquad R = \frac{V}{I}; \quad \text{or} \qquad V = IR \tag{8}$$

With Ohm's law, it is possible to calculate one quantity if we know the other two. (Gussow, 2007)

2 ELECTRICITY IN BIOLOGY

Electricity that plays a role in the biological functions of any living organism is called bioelectricity. This term covers both the electricity that is naturally presented in the organism (endogenous) and the electricity that is externally applied to the organism or its part (exogenous). (Martinses and Heiskanen, 2023)

The tissue can be put into two groups depending on how it conducts electricity, one being a conductor and the other being dielectric. There is no direct separation of which group which tissue belongs to since it can change according to the frequency of the electricity. Most tissues act as electrolyte conductors at frequencies around 100 kHz. At lower frequencies, around 50 kHz, tissues mainly act as dielectrics. (Martinses and Heiskanen, 2023)

2.1 Endogenous electric field

The presence of electricity in humans or any other organisms is not anything new. Various tissues' functions depend on producing and receiving electromechanical signals through an endogenous bioelectric system. The nervous system or muscles belong among the most known tissues that are strongly affected by electricity. (Kamalov et al., 2022)

Electricity is an inherent part of every living organism and survival would not be possible without it. This endogenous electricity comes from the electric field within the membranes of the cells. It is possible for cells to control the conductivity of membranes via ionic channels. (Pliquet et al., 2008)

Besides cell membranes, another source of endogenous electric field is the epithelium surrounding organs and organisms as a whole. These power sources put ionic currents in motion and allow them to travel through cells, tissues, and organisms, which generate the endogenous electric field. (Nuccitelli, 2007)

The natural electric fields are DC electric fields. Their potential is measured between the epidermis and the dermis and is called transepithelial potential. (Sun, 2017)

2.2 Exogenous electric field

As the name suggests, exogenous electric stimulation does not originate in an organism but is artificially supplied into organisms, tissues, or cells in order to alter their behaviour, morphology, and other characteristics.

Several studies have been carried out on the effect of exogenous electric stimulation on cell viability or its behaviour. Results suggest it affects cell proliferation and apoptosis or possibly necrosis as well as adhesion to surfaces, speed and direction of migration, and differentiation. (Kamalov et al., 2022; Mycielska and Djamgoz, 2004; Titushkin and Cho, 2009)

The state of the cell's membrane, both biochemical and metabolic, can determine the effect that exogenous electric fields have on the cell. One of the determining factors is the resting potential of the membrane. It is clear that different cells might have different sensitivity to the field, which defines their responses. Also, a healthy cell might react differently from a damaged cell. This is helpful, for example, when a treatment targets only affected areas. (Muehsam and Pilla, 1999)

It was also found that the sensitivity to the electric field depends on the number of the cell. A single, isolated cell showed a lower sensitivity to the electric field compared to a linear array of cells connected through gap junctions. (Muehsam and Pilla, 1999)

The topic of the exogenous electric field is discussed more in the following chapter. While Chapter 3 focuses on possible ways of delivering electricity to cell cultures, together with their advantages and disadvantages, Chapter 4 focuses on different cell functions that can be affected by exogenous electric field.

3 SUPPLY OF ELECTRIC FIELD INTO THE CELL CULTURE

Testing *in vitro* is one of the first steps whenever we want to find out if the examined factor would benefit living organisms or if it would harm them. An electric field is no exception to this rule.

When we want to examine what kind of effect the electric field has on cells, the first thing we need to consider is which method of delivering electrical stimulation to cell culture is going to be used. Every method has its own advantages and disadvantages that can influence how cells react to electrical stimulation or, in other ways, can influence the results. (Meng et al., 2022)

3.1 Electrode-based method

This method is particularly popular because of its simplicity. On the other hand, it has its disadvantage in the fact that it can acquire several forms. Not only the material used for the fabrication of electrodes can be various but also the way of their connection to cell culture. For simplification of this problem, the methods can be summarized into three categories, which are also depicted in Figure 2. (Meng et al., 2022)

3.1.1 Usage of electrodes inserted in culture medium

In a system consisting of two immersed electrodes, the electric field is formed in the culture medium, working as an electrolyte, between the cathode and the anode, which are connected to the power source. (Meng et al., 2022)

Since the electrodes are inserted directly into the culture medium of the cells, it's necessary to prevent their degradation because of erosion. This can be accomplished, for example, by using electrodes made of noble metals such as silver, gold, titanium, and platinum or made of graphite. Another problem may be caused by the present Faradaic current when redox reactions are happening since even a low concentration of products from these reactions might be cytotoxic for the cells. (Meng et al., 2022)

The biggest advantage of this method is its simplicity of delivering electric stimulation into the cell culture. (Meng et al., 2022)

3.1.2 Usage of salt bridges between electrodes

Salt bridges are used, so we prevent possible contamination of the culture medium by the redox products. KCl or KNO_3 in high concentration can be used as an inert electrolyte, which is together with gelation or agar, working as gelling agents, placed in a glass tube in the shape of "U". For creating an electric field, there are two of these tubes needed. One end of them is immersed in the culture medium, and the other is placed in a container with their respective electrode. (Meng et al., 2022)

While the culture medium is separated from electrodes, contamination from the bridge's content is still possible. With a larger diameter of the tube, which helps with a better flow of the inert electrolytes, there is a danger of the electrolyte leaking into the medium. (Barry et al., 2013)

Another disadvantage of the salt bridges is the size of the experiment setup. Unlike the method using the electrodes in the medium, this method requires more equipment in the form of additional containers with electrodes and salt bridges themself. There is also a risk of the salt bridges, which are made from glass, breaking while placed in the incubator. (Meng et al., 2022)

3.1.3 Usage of electrodes as a substrate for cell growth

If the electrodes used for delivering electric fields have the right qualities, such as adhesion, they can be used as substrates for cells. The cells are adhered to the working electrode with equipotential under the influence of a uniform electric field. (Meng et al., 2022)

This method uses a three-electrode electrochemical cell. There is a working electrode with adhered cells, a reference electrode, and an auxiliary electrode. Working electrodes, used as a substrate for the cells, can be made from various metals such as titanium or stainless steel, but also from conductive polymers. As was previously mentioned, the range of materials used in different experiments causes difficulties with comparison of the results. (Meng et al., 2022)

The usage of working electrodes made from metal has the advantage of easy fabrication into various configurations, which allows a larger variety of requirements to be met. The disadvantage is the direct exposure of the cells to the electric field and potential redox reactions. These factors might cause some interferences in results. (Meng et al., 2022)

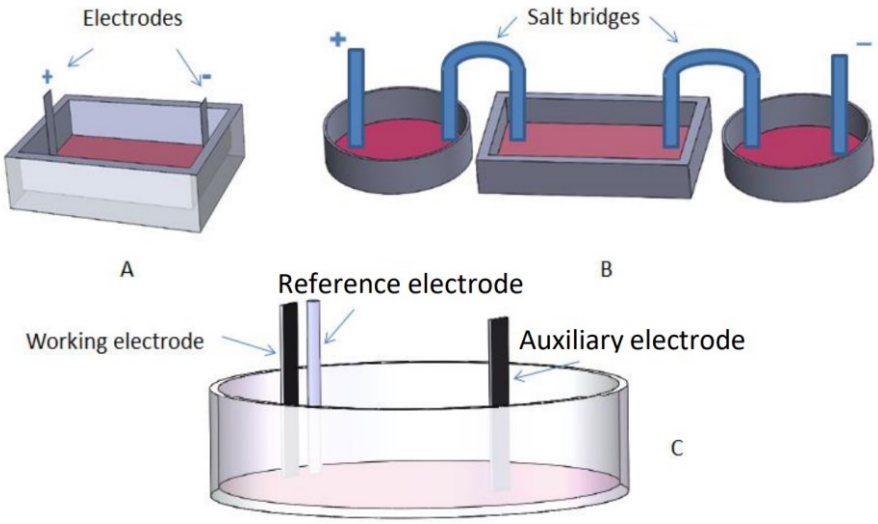

Figure 2 (A) Electrodes inserted in culture medium; (B) Salt bridges between electrodes; (C) Electrodes as a substrate for cell growth (Meng et al., 2022)

3.2 Conductive substrate

Contrary to the electrode-based method, in this case, a semiconductor not only provides an electric stimulation but also works as a substrate for cell growth, as shown in Figure 3. Wired in a complete circuit, the substrate does not generate an electrode reaction. Cells attached to the surface interact with a potential gradient formed along the substrate. While using an alternative current, the electric field is not the only one affecting the cells but also the electromagnetic field. (Meng et al., 2022)

The conductivity of the substrate also must be kept in mind. If it happened to be too high, it could increase the temperature above the physiological one, and if it was too low, then it would not create a sufficient potential gradient to be physiologically significant. (Meng et al., 2022)

Conductive substrate can be combined with electrodes. When only electrode-substrate contact is desired, separating the electrodes from the culture medium is necessary. One of the possibilities for achieving the isolation is coating the electrodes with medical-grade silicone grease. With the isolation, there is no measurable current between the electrodes without the presence of the conductive substrate. (Zhang et al. 2007)

Carbon and its allotropic substances or conductive polymers can be used as materials for the fabrication of conductive substrates. These materials can be fabricated not only in 2D structures but also in 3D structures such as tubes or porous scaffolds. This is useful

as cells might show different behaviour in 2D and 3D systems while under the influence of the electric field. (Meng et al., 2022)

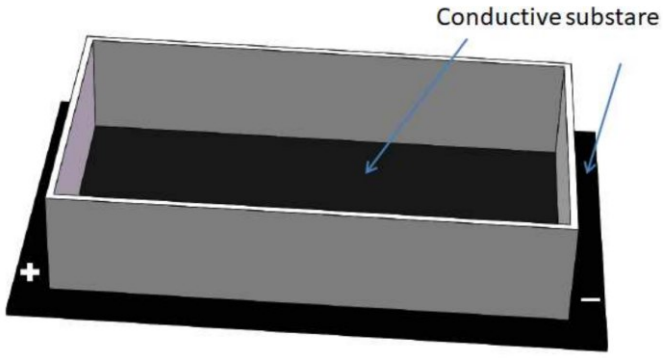

Figure 3 Conductive substrate (Meng et al., 2022)

3.2.1 Polypyrrole

Recently, conductive polymers have attracted interest in chemical and physical fields with polypyrrole (PPy) being among the most studied polymers for that matter. It has been applied as a solid-state electronic material since it has good physical and electrical properties.

The conductivity of PPy depends on several aspects. For example, the method used for its fabrication, the final shape of the polymer, or the used oxidant. In general, the conductivity can range from as little as 20 $\mu S \cdot cm^{-1}$ to over 60 $S \cdot cm^{-1}$. Thin films of PPy are mostly used as substrates, and these have conductivity lower than 1 $S \cdot cm^{-1}$. (Pang et al., 2021)

The downside of PPy is in its mechanical properties which prevent wider use of the polymer. This problem has been tried to be solved by choosing specific synthetic conditions, preparing composites of PPy with other materials in the form of dopant ions, and post-synthesis modification induced by electric stimulation. While PPy ensures the conductivity of the composite, the additional steps are supposed to enhance its mechanical properties such as topography or surface chemistry. (Migahed et al., 2004)

PPy is a non-toxic, biocompatible material, which means it is suitable for direct contact with cells and tissues. The cytotoxicity can be increased, for example, due to synthetic procedure residuals of precursors or solvents. Because of this, these factors must be closely monitored. Otherwise, they might distort the results. (Gniadek et al., 2020)

The polymer can be prepared in two ways. One is chemical synthesis by polymerization of pyrrole as a monomer with the use of a strong oxidizing agent. As the agent, iron(III) chloride ($FeCl_3$) is typically used. The reaction of pyrrole with $FeCl_3$ is shown in Figure 4. This method is suitable for the synthesis of larger quantities. The second method is electrochemical synthesis, which has advantages in the simplicity of this method, better control of material characteristics, and others. The electrochemical reaction is usually used for synthesis in biomedical applications. (Ateh et al., 2006)

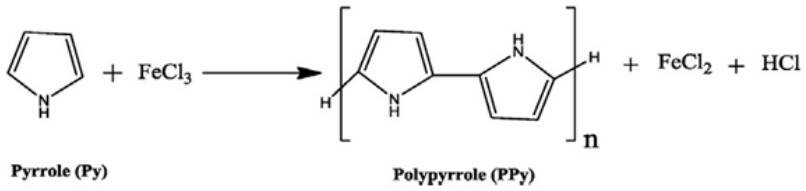

Figure 4 Polymerization of polypyrrole with FeCl3 used as the agent (Gahlout and Coudhary, 2016)

Polypyrrole does not only have to be a flat film but can also be fabricated into 3D scaffolds. *In vivo*, cells rarely grow and live on a flat surface. A 3D porous scaffold represents a cell's natural environment, and in the best case, it mimics the configuration of the extracellular matrix. One way to fabricate such a scaffold is electrospinning. Electrospinning is a fabrication process of fibres where an electric force is used to form and control a jet. This method can create fibres with a diameter in a typical range from 100 nm to 1 μm. (Jin et al., 2012; Rutledge and Fridrikh, 2007)

3.3 Source without direct contact

While using this method, the source of electricity is not in any contact with cells or culture medium but is only surrounding the cell culture. This way, some issues connected to electrode usage, such as tissue compatibility, cytotoxicity, or corrosion, can be easily avoided, and the potential risk can be reduced. (Meng et al., 2022)

Electric stimulation can be delivered, for example, by an electromagnetic field generated by a Helmholtz coil. The cell culture can be placed either inside one coil or between two coils, as is depicted in Figure 5. The electromagnetic field generated by a Helmholtz coil is pulsed. In one coil, the cell culture might be placed parallel to the magnetic field, and between two coils might be placed perpendicularly to the magnetic field. (Meng et al., 2022)

While using the electromagnetic field, it must be remembered that electric fields and magnetic fields do not have the same effect on the cells. Then it might cause a problem of identifying if the cells were stimulated by the electric field, magnetic field, or potentially by both. (Meng et al., 2022)

Besides the electromagnetic field, the other option for indirect delivery is through capacitive coupling, where a container with culture medium and cells is placed between two capacitor plates, as is shown in Figure 5. Parallel plates are mostly used to ensure a uniform electric field. The intensity of the electric field can then be regulated by using materials with specific dielectric constants between capacitor plates. However, a potential issue arises with the required usage of unsafely high voltage, which can rise over 1000 V. (Meng et al., 2022)

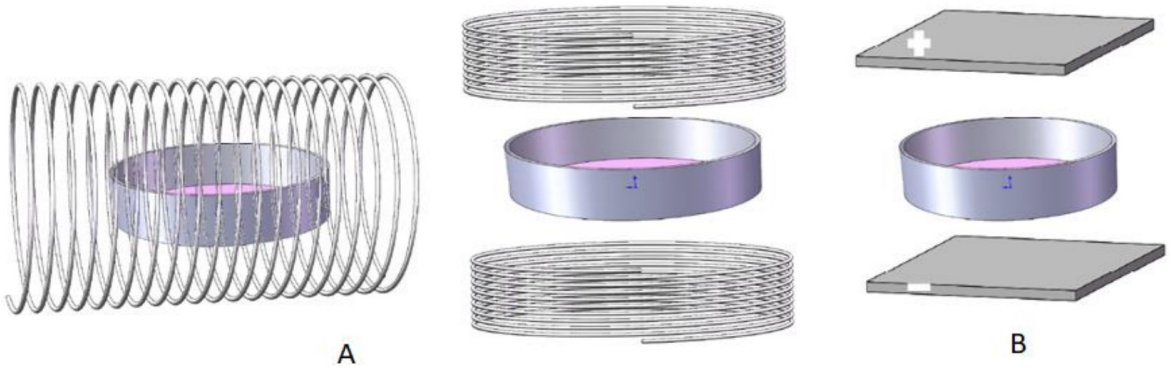

Figure 5 (A) Cell culture placed inside one Helmholtz coil, or place between two Helmholtz coils; (B) Cell culture placed between two paralleled plates (Meng et al., 2022)

4 EFFECTS OF ELECTRIC FIELD

Types of electric fields can be various since there are several aspects that can be set up. From the field with direct or alternating current to voltage and amper range. With the time the cells are electrically stimulated, the effect that the electric field has on cells is not uniform. Also, not all types of cells react to the same electric field in the same way. (Love et al., 2018)

With a combination of different settings and cell types, we can get many different results of how cells are affected by the field. There is mainly a focus on its effect on cell viability. The right type of electric field can help with proliferation or migration but just a slightly too high voltage or time of exposure can be enough to lead to cell death. But not always is the death of cells a bad result. Controlled death of specific types of cells caused by electric stimulation without harming surrounding cells could have its usage, for example, in medicine to treat diseases. (Love et al., 2018)

4.1 Permeability of cell membranes

In a normal state, the cell membrane is impenetrable to ions except for a pathway through specialised channel proteins. The radius for resting potential for these membranes is from 30 mV to 300 mV in the case of a few plant cells. Increasing transmembrane voltage until it reaches a critical level can be used to make the membrane more permeable. Then, small enough ions can pass through the membrane without the need for the channel proteins. (Pliquett et al., 2006)

The effect of an electric field creating a way for ions by rearranging lipids in the membrane is called electroporation. Another term to describe the temporary permeabilization of the membrane of a cell caused by the electric field is electropermeabilization. (Pliquett et al., 2006; Golzio et al., 2003)

There are two desired ways of implementing electropermeabilization. The first one is supposed to leave affected cells alive and the second aims to kill cells in an efficient way. The usage of an electric field with pulses in a range of nanoseconds is a preferred way to achieve greater permeability. (Sweeney et al., 2018)

Once the permeability of a cell is increased, temperature plays a great role, as the speed of the membrane reseal depends on it. The higher the temperature is, while still in a range

of cell viability, the faster the resealing. A balance between the speed of the resealing and a temperature that would not affect the cells has to be found. (Hofmann et al., 1999)

With the possibility of getting external molecules inside a cell due to the permeabilization of a membrane, this method is tested to have a use in medicine to help with drug delivery. Most of drugs contain a hydrophilic compound that cannot go through a membrane on its own but needs some kind of a transporter. Electropermeabilization can erase this problem and enable drugs to enter a cell without any problem. One of the examples of drug delivery is rapidly developing electrochemotherapy. (Teissié et al., 1999)

4.2 Cell adhesion

Adhesion is an important ability for anchorage-dependent cells. Only after adhesion other cell processes, which include for example proliferation or migration, follow. Besides these two processes, cell adhesion greatly influences the cell's final morphology, especially when cells are seated on a surface in a monolayer culture. If the adhesion does not occur, cells usually die. (Cai et al., 2020; Sun et al., 2006)

Cells are able to adhere through integrins. The extracellular matrix (ECM), which surrounds cells *in vivo*, provides proteins to which the integrins and other cellular receptors respond and bind. Cell adhesion *in vitro* works on the same principle, cells adhere to proteins adsorbed to the surface of a substrate. (Cai et al., 2020)

Research is largely focused on cell adhesion because of tissue engineering. Some injuries or health issues require or benefit from the growth of new cells on an implant. A bone replacement that allows bone to grow through and regenerate can be one of the examples. For this purpose, the biomaterial is often coated with specific molecules to help with the adhesion and then the proliferation of targeted cells. (Cai et al., 2020)

The ECM uses an endogenous electric field to provide cells with signals, which then can control cell functions. Cell adhesion is not an exception, so there is an effort to exploit an electric field as another way how to influence it.

When an exogenous electric field is applied, adsorbed proteins' quantity, spatial confirmation and orientation on the surface are influenced, which might change their biofunctionality. (Pehlivanova et al., 2013)

The use of a low-frequency AC electric field can cause a connected process of redistribution of the receptors and microfilament structure reorganization.

These changes are most likely related to an increased value of Ca^{+2}. The frequency of the electric field plays the main role in inducing such effects. (Pehlivanova et al., 2013)

When an AC electric field was applied, the results of an experiment on cell adhesion on polymer structures showed that cells exhibited a higher adhesion to the surface than without the influence of the field. Another observation was that the adhesion was better visible in 2D structures than in 3D ones. However, in 3D structures, more factors might impact the adhesion, probably the most important being the porosity of the structure. (Pehlivanova et al., 2013)

Cell adhesion is also no exception to the fact that various cells react differently to the same electric field. Fibroblasts and mesenchymal stem cells, which were placed in a 3D collagen-based scaffold, were put under the influence of a DC electric field, and the orientation of the adhered cells was observed. While fibroblasts were fully reoriented, stem cells showed only limited reorientation. (Sun et al., 2006)

The type of cell adhesion is also diverse among the cells. Under an electric field influence, some prefer cell-substrate adhesion, while others prefer cell-cell adhesion. (Pehlivanova et al., 2012)

Another parameter of the electric field that has an influence on cell adhesion is intensity. Breast cancer cells had their adhesion increased when a low-intensity biphasic electrical field was applied and decreased when a higher-intensity one was applied. (Pehlivanova et al., 2012)

4.3 Cell migration

The importance of cell migration starts from the beginning of embryonic development, where it determines the final morphology and function of cells, and follows us for the rest of our lives. Examples of migration during adulthood can be the migration of leukocytes in immune response or the migration of cells needed for proper wound healing. (Lauffenburger and Horwith, 1996)

Cells, both *in vitro* and *in vivo*, need some kind of guidance to be able to migrate in a specific direction. There are various stimuli, confirmed by both theoretical studies and experiments, which can do this. Migrating cells do not have to be under the influence of just one cue but also under a combination of them. Examples of cues are illustrated

in Figure 6. The most known and commonly used *in vitro* experiment is chemotaxis, where cells orient according to chemicals attached to the surface. (Cortese et al., 2014)

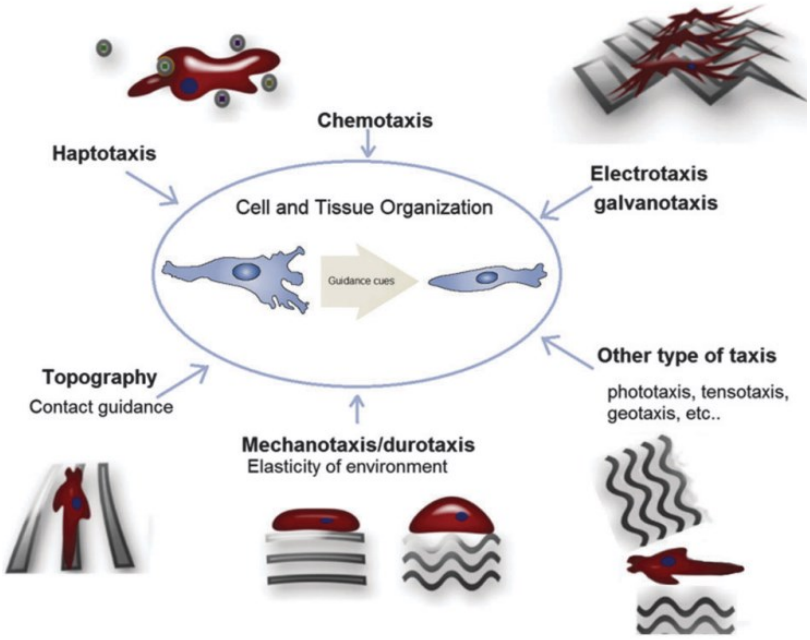

Figure 6 Cues controlling the direction of cell migration (Cortese et al., 2014)

When cells are guided by electric stimulation, it is called electrotaxis or galvanotaxis. Electrotaxis occurs in both physiological and pathological conditions. It is present in processes such as embryonic development or wound healing. It is an important cue, and cells can sometimes prioritise it over chemotaxis and other cues. (Cortese et al., 2014; Funk, 2015)

When applied, the exogenous electric field can either override or enhance the already existing endogenous electric field. The latter then has the potential to speed up the regeneration of damaged tissue. This might be useful in cases where the natural migration of the cells is weakened, as might, for example, occur in chronic wounds of elderly people. (Zhao, 2009)

Cells placed in an electric field, formed between two electrodes, orient themselves or directly migrate to one of the electrodes. Not all cells migrate to the same electrode. For example, from human somatic cells, keratinocytes migrate towards the anode, while corneal epithelial cells migrate towards the cathode. When it comes to stem cells, the number of passages can also be an influencing factor. With a higher number of passages, the stem cells can differentiate into cells with a preference for the second electrode. This can be observed on mesenchymal stem cells, which migrate towards

the anode, differencing into a chondrogenic phenotype, which migrates towards the cathode. (Iwasa et al., 2017)

4.4 Proliferation

Proliferation, or the ability of cells to rapidly multiply, belongs to fundamental biological processes. The process is still present in an adult organism, but when the proliferation rate increases without any natural causes, it might be a signal that the specific cells are cancerous, as high proliferation is a typical sign of the cancer phenotype. (Bading and Shields, 2007)

When it comes to the effect of electric stimulation on the proliferation of cells, the results mostly consist of increased proliferation. However, there are also cases where there was no significant effect or when the proliferation was decreased after cells were affected by the electric field. (Love et al., 2018)

The membrane of cells has many ion channels that can be influenced by an electric field. These channels take part in the control of the cell cycle. DC electric field with high enough intensity can create cytotoxic molecules and heat, but when an AC electric field with low frequency and low intensity is applied, it affects the proliferation of the cells without harming them in other ways. (Cucullo et al., 2005)

The effect of 50 Hz AC and DC electric fields was observed in a study with the use of osteosarcoma cell lines, which is a type of bone cancer. For this line, it was found that at certain voltages, both fields had a positive impact on cells' proliferation. Other voltages showed more neutral results but no negative ones. (Fidan et al., 2019)

On the other hand, it was also proved that the use of a low-intensity AC electric field can be used to alter the cell cycle and decrease proliferation without affecting the viability of cells. This process was also reversible, and after the cells were no longer under the influence of the electric field, the ability to proliferate at a normal rate was restored. (Cucullo et al., 2005)

4.5 Cells death

Death of cells can happen through several processes or their combinations, depending on the cause of the death and other factors, and the most common ones are apoptosis and necrosis, which are shown in Figure 7. Despite the original knowledge that these two have

completely different mechanisms, it has been shown that they share many biochemical intermediates that regulate the process. The levels of Ca^{2+} and cellular ATP belong among these intermediates. It is possible for cells going through apoptosis to change the process into necrosis if certain changes occur in modulators. (McConkey, 1998)

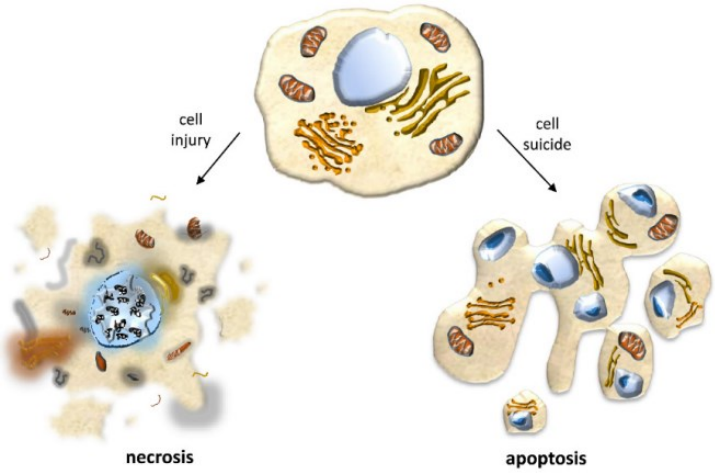

Figure 7 Illustration of cell death by apoptosis and necrosis (Priante et al., 2019)

4.5.1 Apoptosis

Apoptosis is a programmed type of cell death. During this process there are apoptotic bodies being formed inside a cell, containing cellular content. After the cell bursts due to the disintegration of cell junctions in the plasma membrane, the variously sized apoptotic bodies spill into the surrounding of the cell. Since apoptotic bodies prevent the leaking of the cellular content itself, it usually does not lead to inflammation. (Lawen, 2003)

Apoptosis is a physiological type of cell death and is necessary for the right function of an organism. It then becomes a problem if apoptosis is happening on too large or too small scale, which can result in diseases such as Alzheimer's or cancer. (Lawen, 2003)

It is not exactly clear in which way the electric stimulation affects apoptosis of cells, as it was already mentioned, it highly depends on specifics of the used electric field. There are studies ranging from results of increased rates of apoptosis to ones where there was a reduction of apoptosis but also where it didn’t have any effect at all. (Love et al., 2018)

One of the ways of inducing apoptosis in cells is the use of a nanosecond pulsed electric field (nsPEF) with a high intensity. The field can then induce apoptosis in both cells *in vitro* and *in vivo* while targeting the cell interior. The effect on cell viability does not

depend on temperature or energy. Pulses which did not produce any heat still induced apoptosis and pulses that provided identical energy input but at different pulse duration caused apoptosis markers with the same intensity. (Beebe et al., 2002)

4.5.2 Necrosis

The most distinct difference of necrosis from apoptosis is the lack of ability of a cell to control the process of its own death. This happens in situations when the cell is under extreme influences from its environment or when there is a defect in its genetic material. (Syntichaki and Tavernarakis, 2002)

The aforementioned study on the impact of nsPEF on cells by Beebe et al., 2002 focused more on death by apoptosis but that did not mean that death by necrosis did not occur. It was considered that only secondary necrosis, which followed the apoptotic process, was present. However, there were signs that showed that necrosis might be more important than it seemed. One of the signs that cells went through a necrotic path and not apoptotic was the time between the application of nsPEF and the death of the cells. This period was observed to be too short for the cells to enter apoptosis. There were also reports of swelling of the cells, which is typical for necrotic cells. (Pakhomova et al., 2013)

In the end, it was revealed that necrosis might be more common than apoptosis when cells are exposed to nsPEF. It, of course, still depends on other factors. The necrosis happens through a water uptake caused by a permeabilised membrane, which leads to swelling of a cell and, eventually, rupture of the membrane. This means that if the cell does not have a lot of spare membrane, it will swell to its maximum faster and will not have enough time to repair the membrane before it bursts. (Pakhomova et al., 2013)

4.6 Usage of electricity in medicine

The previously mentioned effects of the electric field are considered to have a practical use in the medical field. With the specific mechanisms of interaction between cells and the exogenous electric field being still largely unknown, these uses are still mainly experimental, but in the future, they might become a common treatment method.

4.6.1 Wound healing

Skin is the largest organ and the main barrier preventing harmful external factors from entering an organism. Damaged skin causing a disruption of the barrier represents a danger for the organism and needs to be healed as soon as possible. (Luo et al., 2021)

Any wound healing is not a simple process and involves several steps, including proliferation, migration, and differentiation of cells. If the process is disturbed, it can cause a prolongation of the healing or even prevent it from healing at all. Like this, chronic and nonhealing wounds are formed. (Jia et al., 2021; Chen et al., 2021)

When the skin is damaged, an endogenous electric field appears. The generation of the endogenous field is illustrated in Figure 8. Through electrotaxis, this field directs the migration of keratinocytes and helps with the healing. It was detected that this field appears immediately after creating a wound, is present through the healing process and disappears again when the wound is fully healed. (Nuccitelli et al, 2008)

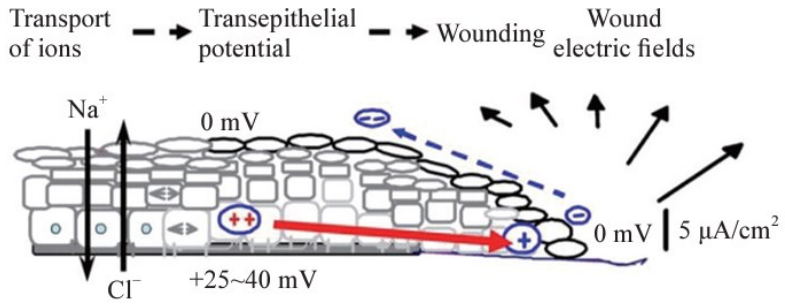

Figure 8 Schematic diagram of endogenous electric field generation (Jia et al., 2021)

With the existence of an endogenous electric field in a healing wound, the idea of using an exogenous field to promote healing was brought up. (Jia et al., 2021)

One study on the effect of electric fields on wound healing was conducted with the use of a DC electric field delivered through electrode configuration. The best results were obtained with the negative electrode covering the wound and the positive electrode covering the unaffected skin around. When it comes to other factors, it was observed that moisture surroundings have a more useful impact on this method than dry surroundings. (Sun, 2017)

While the wound is healing, the organism is prone to infection in this area since it is exposed to external influences. Because of this, wound dressings are used. Not only can they prevent infection, but they can alleviate pain or prevent future scarring as well.

There is an effort to speed up the healing process or help with ineffective healing by using a special wound dressing. (Yu et al., 2022)

Healing mainly depends on the migration of keratinocytes, which is directed by the endogenous field. Negatively affected migration can then lead to failure of re-epithelialization. A combination of negative pressure wound therapy and exogenous electric fields is one way that can help overcome this issue and accelerate healing. To combine these methods, a flexible conducting dressing is used. The dressing can be made from conductive polymers, graphene, carbon nanotubes or other electrically conductive materials. (Chen et al., 2021)

4.6.2 Cancer treatment

Cancer can be treated in few ways: by surgery, radiation therapy, chemotherapy, or still-improving biological therapy. It becomes a problem when the tumour is situated in more sensitive areas, such as glioblastoma, which is a very deadly type of cancer located in the brain. In these cases, the influence of an electric field can be useful. (Aguilar et al., 2021; Schirrmacher, 2018)

It was tested to see if the alternating fields with an intermediate frequency of 200 kHz, named tumour treating field (TTF), would benefit cancer treatment. The TTF was added to temozolomide chemotherapy for patients suffering from glioblastoma who had just ended their radiochemotherapy. (Stupp et al., 2017)

The TTF was delivered into the tumour by electrodes placed directly on the scalp. The treatment prevented cell division of cancer cells by interrupting the mitotic phase, causing apoptosis of the dividing cells, and making the cells more sensitive to chemotherapy. In the end, the study showed a higher survival rate of patients who had TTF added to their primary temozolomide chemotherapy. (Stupp et al., 2017)

While TTF induces apoptosis of cells, an electric field in cancer treatment does not have to cause direct death of cells. In electrochemotherapy, short but intense square-wave electric pulses, which are on their own harmless to the cancer cells, can reversibly increase their membrane permeability and allow hydrophilic nonpermeant drugs to enter. It is the drugs that are cytotoxic and cause damage or death of the cells. (Mir and Orlowski, 1999)

Electrochemotherapy increases the amount of drug that can get inside the targeted cell, and the effectiveness of the method is largely based on the combination of the type of electric pulses and the type of cytotoxic drug. Not all combinations are going to get the desired results. One of the drugs that can be combined with electric pulses is bleomycin. (Mir and Orlowski, 1999)

II. ANALYSIS

5 THE AIM OF THE WORK

The main aim of the analysis was to observe changes in the morphology and viability of the mouse embryonic fibroblast cell line (NIH/3T3) and the mouse embryonic stem cell line (ES R1) exposed to external electric stimulation. To extend current knowledge on the impact of electrical stimuli on cell behaviour, two different types of electrodes and various voltage settings ranging from tens of V lasting for minutes to tens of mV lasting for hours were applied to the cells. Furthermore, conductive cell substrates in the form of thin polypyrrole films were prepared and used to determine the influence of the surface conductivity on the morphology of both cell lines, both with and without the application of external electrical stimulation.

6 MATERIALS AND METHODS

6.1 Chemicals

- 2-Mercaptoethanol (Serva, Germany)
- 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; Duchefa Biochemie, Netherlands)
- ActinRedTM 555 (InvitrogenTM ThermoFisher Scientific, USA)
- Ammonium persulfate (APS; Sigma-Aldrich, Austria)
- Calf Serum (Biosera, France)
- Dimethyl sulfoxide (DMSO; Sigma Aldrich, USA)
- Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA)
- Dulbecco's Modified Eagle Medium High Glucose (DMEM High Glucose; Biosera, France)
- Formaldehyde (Penta s. r. o., Czech Republic)
- Hoechst 33258 (InvitrogenTM ThermoFisher Scientific, USA)
- Hydrochloric acid (HCl; Penta s. r. o., Czech Republic)
- Iron(III) chloride ($FeCl_3$; Sigma-Aldrich, Austria)
- Leukemia inhibitory factor (LIF; Chemicon, USA)
- MEM non-essential amino acid (MEM NEAA; Gibco, USA)
- Methanol (Penta s. r. o., Czech Republic)
- Penicillin-streptomycin (Biosera, France)
- Phosphate-buffered saline (PBS; Carl Roth, Germany)
- Pyrrole (Sigma-Aldrich, Austria)
- Sodium hydrogen carbonate powder (PAA, Austria)
- TritonTM X-100 (Sigma Aldrich, USA)
- Trypsin (Biosera, France)

6.2 Equipment

- Incubator HeraCell 150 i (Thermo Scientific, USA)
- Inverted fluorescence phase-contrast microscope IX81 (Olympus, Japan)
- Platinum/Iridium wire (Pt90/Ir10, Goodfellow, Germany)
- Spectrophotometer Infinite M200 PRO (Teca, Switzerland)
- TC-3 bioreactor (Ebers, Spain)
- Tenma Power Supply 72-10480 (Newark, USA)
- Tissue culture plastic Petri dishes, flasks, and 96-wellplates (TPP, Switzerland)
- Vortex GENIE 2 (Scientific Industries, USA)

6.3 Biological material

Two cell lines were used for testing: a mouse embryonic fibroblast cell line NIH/3T3 (ECACC 93061524, England) and a mouse embryonic stem cell line ES R1 (Nagy et al., 1993). NIH/3T3 cell line was cultivated in DMEM high glucose cultivating medium with added sodium hydrogen carbonate powder, calf serum (10 vol. %), and penicillin-streptomycin (1 vol. % of 100 µg/mL solution). ES R1 cell line was cultivated in DMEM cultivating medium with added calf serum (16.5 vol. %), penicillin-streptomycin (1 vol. % of 100 µg/mL solution), MEM NEAA (100 mM), 2-mercaptoethanol (0.05 mM), and LIF. Both cell lines were cultivated in tissue culture dishes in the incubator at 37 °C, in humidified air, and in 5% CO_2. Viable cells were observed under the phase-contrast microscope.

6.4 Electricity supply

The electricity was delivered to cells through a pair of electrodes situated in the lid of a Petri dish. Wires used as electrodes were stripped of insulation on both ends. One of the ends of the electrode was then threaded through a created hole in the lid and secured with glue. The end of the electrode inside a Petri dish was inserted into the culture medium, and the second end was connected to a source of electrical voltage. For the experiments, two types of electrodes were used – ones made of copper wire and the other made of platinum and iridium wire in a 90:10 ratio.

To observe the effect of higher voltage on cells in real-time, a Petri dish was placed under a microscope, as depicted in Figure 9. A Petri dish affected by lower voltage for a longer time was placed in an incubator together with a reference Petri dish which was not under the influence of electricity. That was necessary to avoid external influences such as a lower temperature or non-sterile environment. This installation is depicted in Figure 10.

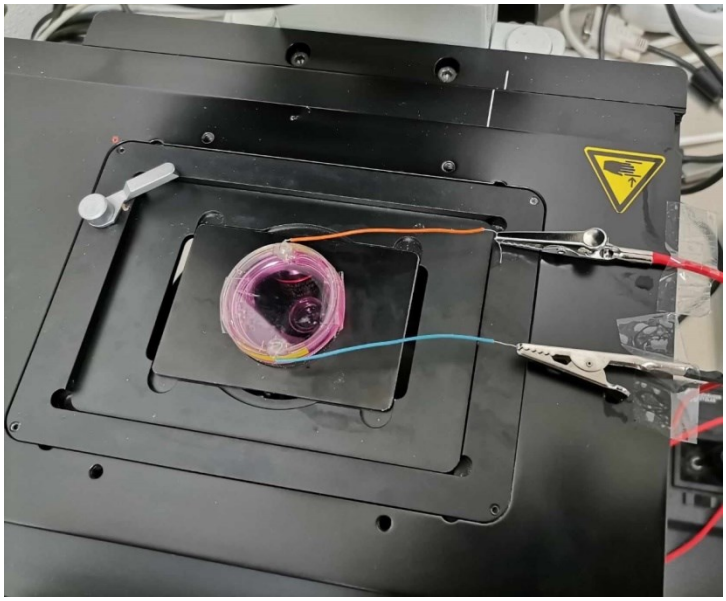

Figure 9 Petri dish with connected electrodes under a microscope

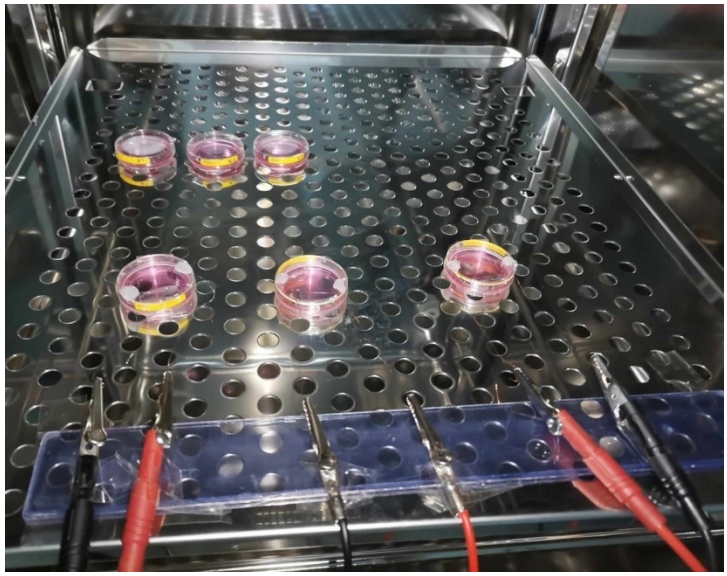

Figure 10 Petri dishes with connected electrodes and Petri dishes with reference samples inside of an incubator

6.5 Polypyrrole preparation

The PPy was prepared into a thin film on the bottom of a Petri dish, using solutions in deionised water (dH_2O). First, a 50 mL solution of pyrrole in a 0.2 mol PPy/ 1 L dH_2O ratio was prepared, and then two 25mL solutions of APS and $FeCl_3$ in dH_2O in 0.25 mol APS/ 1 L dH_2O and 0.25 mol $FeCl_3$/ 1 L dH_2O ratios. The APS solution and the $FeCl_3$ solution were then mixed with the pyrrole solution. Using a micropipette, 1 mL of both solutions was added to a Petri dish. The solutions were only left to react for a maximum of 30 s to prevent the formation of a powder, and then the Petri dish was immediately washed with 0.2 M HCl and methanol.

6.6 MTT assay

MTT is a yellow-coloured tetrazolium dye which can be solubilised in water. This dye can enter a cell and its mitochondria. There, MTT is reduced through a process of dehydrogenases. The product of the reduction reaction is formazan, formed in mostly insoluble purple crystals. The reduction of MTT can only occur in living cells, which led to its use in cell viability tests. The formed formazan is solubilised and can then be spectrophotometrically measured. The darker the solution and higher the absorbance, the greater the number of living cells. (Blumenthal, 2005)

The MTT assay was carried out in accordance with international standard ISO 10993-5:2009 - Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity. The assay was performed in a Petri dish instead of directly in a microtitration plate. The microtitration plate with 96 wells was only used to measure absorbance.

A solution for the MTT assay was prepared by dissolving MTT in ultrapure water (UPW) in a 5 mg MTT/ 1 mL UPW ratio. 0.2 mL of the solution with 1.8 mL of culture medium was then added to the Petri dish, from which the old medium was previously aspired and was washed with 1 mL of PBS. The Petri dish with the solution and cells was placed in the incubator for the next 4 hr. After 4 hr, the solution was removed and DMSO was added to dissolve the formed crystals of formazan. The formazan solution was pipetted into a microtitration plate, 100 µL into each well. The absorbance was measured using a spectrophotometer at wavelength 570 nm, with the reference wavelength being 690 nm.

6.7 Cell fixation and staining

Cells were stained with fluorescent dyes to highlight nuclei and cytoskeletons in fluorescent microscopy. Hoechst dye binds to the cell's cytoskeleton, while ActinRed or ActinGreen binds to the cell's nucleus. After removing the old culture medium from a Petri dish and washing it with 1 mL of PBS, 1 mL of 4% formaldehyde in UPW was added to the cells. The solution was left in a Petri dish for 15 minutes, after which it was washed with 1 mL of PBS. Then, 1 mL of 0.5% Triton in PBS was added for another 5 minutes. After washing a Petri dish three times with 1 mL of PBS, the cells could be stained. While working in low light, 1 mL of PBS, 20 μL of Hoechst and one drop of ActinRed or ActinGreen were added. The Petri dish with dyes was left in a dark place for 20 minutes. Once the dyes were replaced with 1 mL of pure PBS, the cells could be observed under the inverted fluorescence phase-contrast microscope.

7 RESULTS AND DISCUSSION

7.1 Voltage settings

The experiments were started with the use of higher voltage. Petri dishes, which were placed under a microscope, were supplied with a 30 V constant DC. An effort was made to have the cells outside of an incubator for the shortest time possible so it would not have an impact on the results. The observation under a microscope was also time-limited as, due to high voltage, the cultivating medium eventually started to decompose, as depicted in Figure 11. Not only would the redox reaction affect the viability and functions of cells, but it would also make the optical observation of cells impossible.

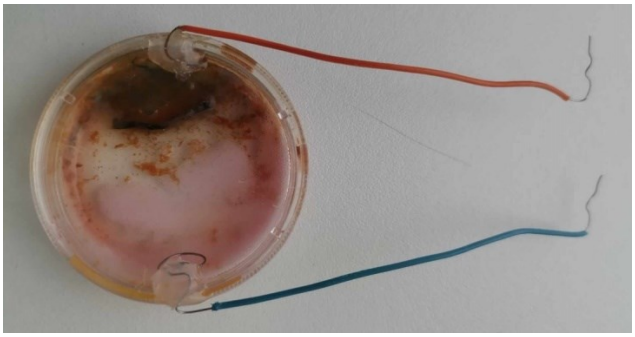

Figure 11 Degraded culture medium

Therefore, all experiments with high voltage setting were done under the maximum of 5 minutes. When NIH/3T3 cells were put under the influence of external electricity, most of the cells did not react to the stimulation. The ones, that did react, initiated a cell death by apoptosis. Figure 12 shows segments from a video record of the cells, where a highlighted cell is increasing its volume and forming apoptotic bodies. The process was relatively gradual. ES R1 cells then proved themself to be more sensitive to electric stimulation. With the same settings, instead of going through apoptosis, the cells committed cell death by necrosis. Also, in contrast to the case of fibroblasts, where only a part of the cells was affected, almost every observable stem cell went through necrosis. Segments from a video record of ES R1 are depicted in Figure 13. The cell death was sudden and faster.

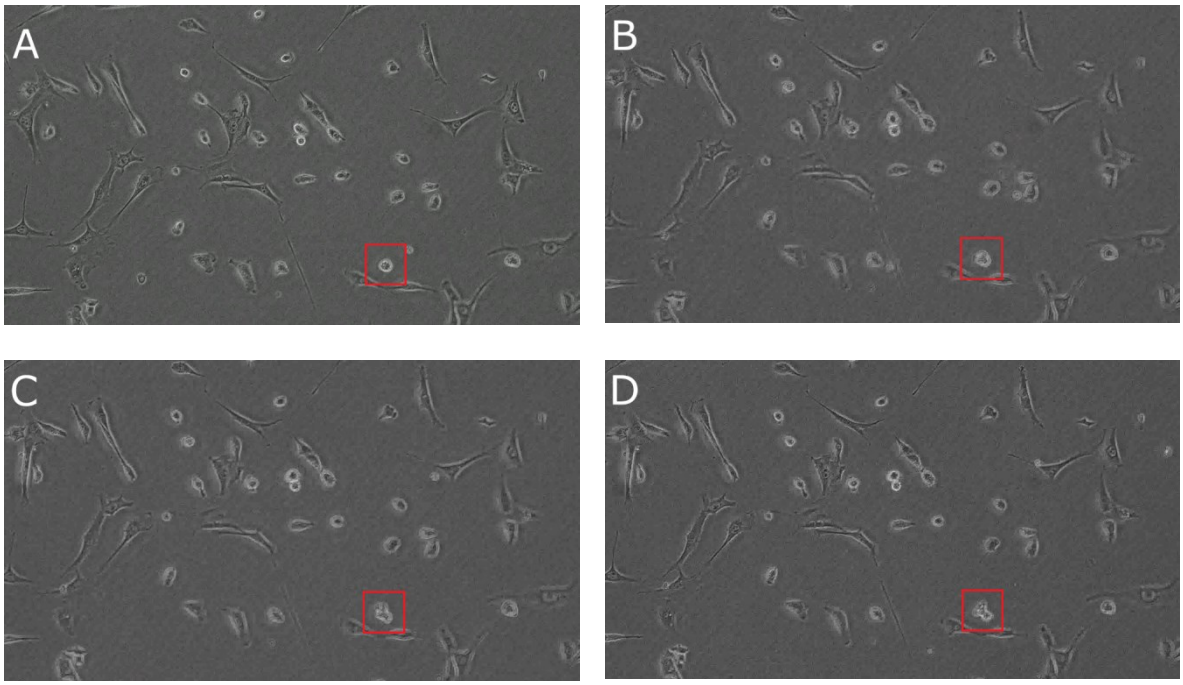

Figure 12 Process of death by apoptosis of NIH/3T3 at 30 V in times: (A) 0:00, (B) 2:30, (C) 3:30, (D) 4:00 (magnification 100×)

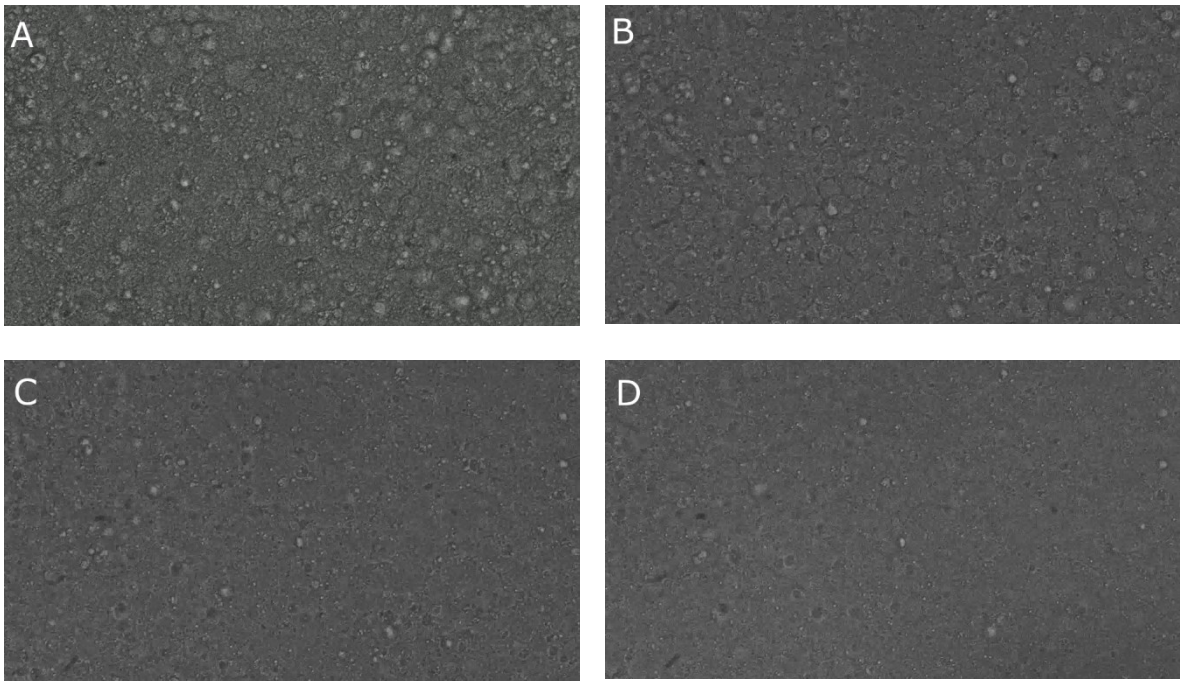

Figure 13 Process of death by necrosis of ES R1 at 30 V in times: (A) 0:00, (B) 2:20, (C) 2:30, (D) 2:40 (magnification 400×)

Since the observation of both cell lines under a microscope was recorded on video, the final pictures in Figure 12 and Figure 13 are of lower quality. After this set of tests, the supplied voltage was decreased, and experiments were moved inside an incubator so the time of exposure could be extended.

The power supply was set to 1 V, and only NIH/3T3 cells were used for the test. Two tests were performed. During the first one, cells were under the influence of constant DC for 19.5 hr, and in the second one, were under the influence of pulses for 22.5 hr with 3000 s of exposure and 600 s of pause. The pulses were made by connecting a power supply to a bioreactor. Photos taken before the experiment and after constant DC application did not show any difference in morphology as showed in Figure 14.

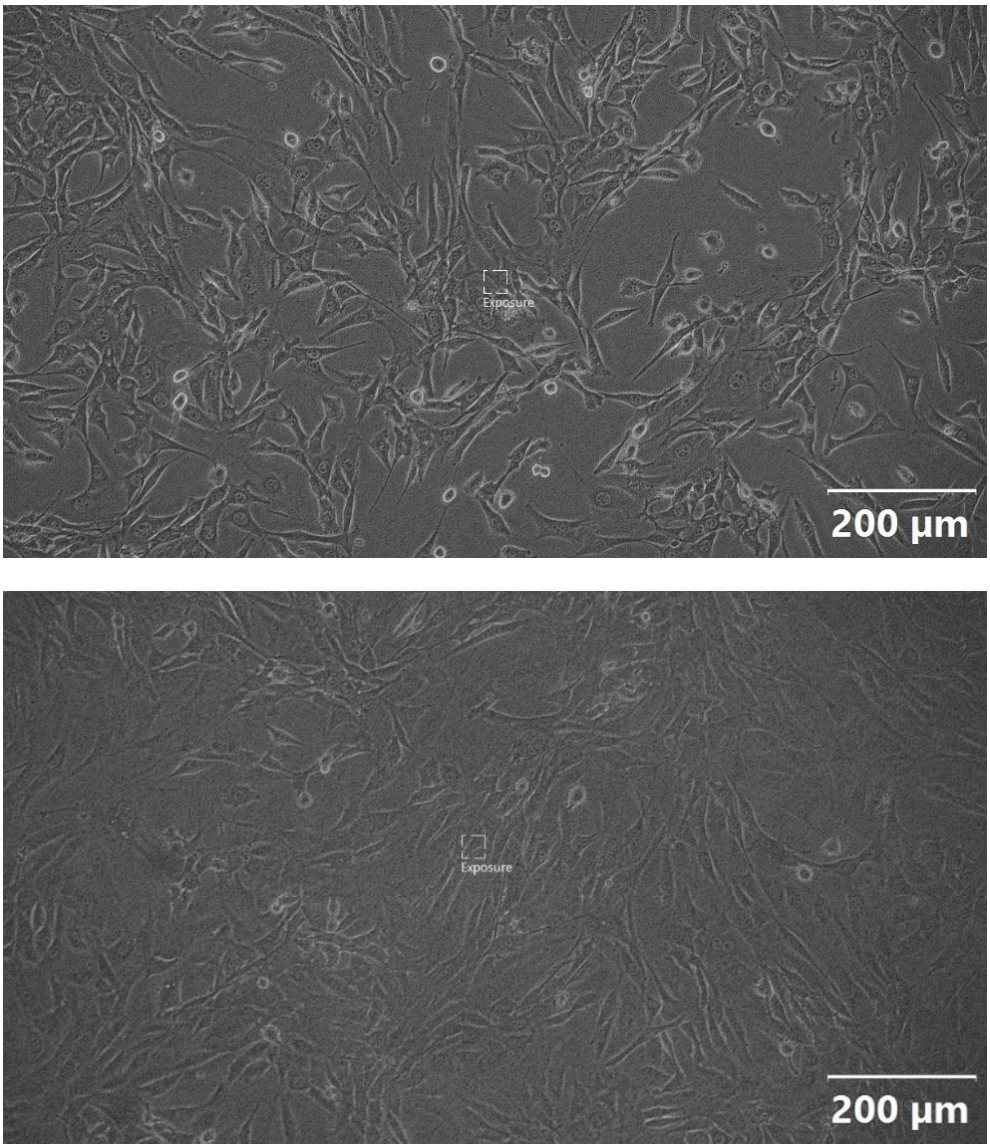

Figure 14 (up) NIH/3T3 before the experiment, (down) NIH/3T3 influenced by constant DC at 1 V for 19.5 hr (magnification 100×)

On the other hand, when the electric stimulation was delivered through pulses, the cells initiated cell death by apoptosis, which did not occur in the reference sample. In Figure 15, a few cells with formed apoptotic bodies are shown.

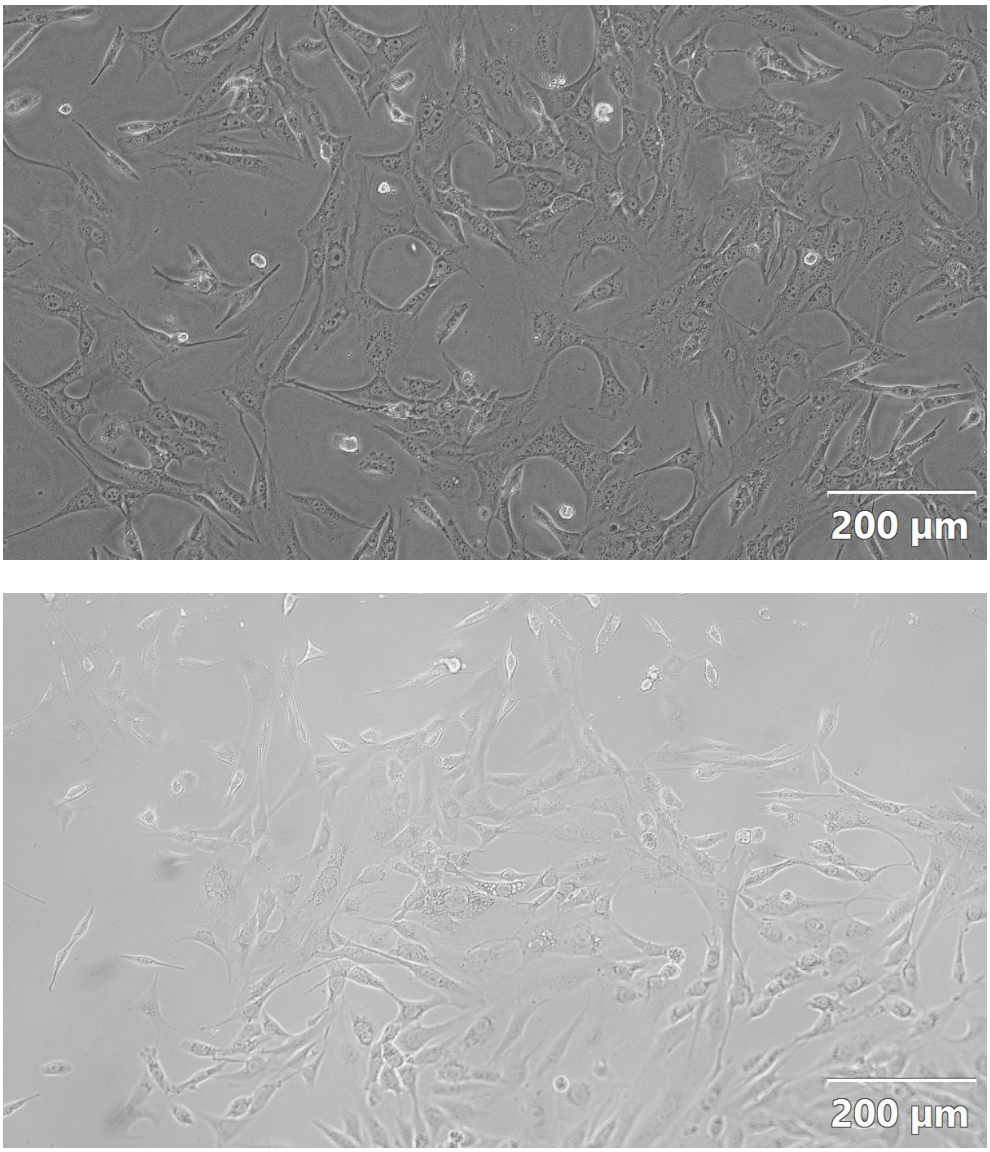

Figure 15 (up) reference NIH/3T3, (down) NIH/3T3 influenced by pulses at 1 V for 22.5 hr with 3000 s exposure and 600 s pause (magnification 100×)

The final chosen voltage used in the following tests was 50 mV. This setting was used for both NIH/3T3 cells and ES R1 cells with and without PPy film used as conductive substrate. 50 mV was chosen since studies showed that a range of tens of mV is not harmful to the cells. In contrast, endogenous electric fields are within this range. Besides constant DC, experiments with pulses were also continued. The negative effect that the previous experiment showed was eliminated by an adjusted setting. It was appropriate to continue with the pulses as they are present in organisms such as the nervous system and also because of their potential healing and regenerative ability. (Chen et al., 2019; Ghasemi-Mobarakeh et al., 2011; Messerli and Graham, 2011)

7.2 Changes in morphology

NIH/3T3 cells were put under the influence of electric stimulation at the setting of 50 mV. One sample was supplied with a constant DC and another with pulses, where stimulation lasted for 3000 s every 600 s. Both samples were left under the influence for 4 hr. The results are shown in Figure 16. While cells influenced by constant DC did not show any changes in morphology, there are visible changes in cells influenced by pulses. The cells have a larger cytoskeleton and oval shape, almost without protrusions. There are also more cells than in the two other samples. This could be caused by using a spot overgrown with more cells in a Petri dish, but there is also a possibility that the electrical stimulation promoted proliferation.

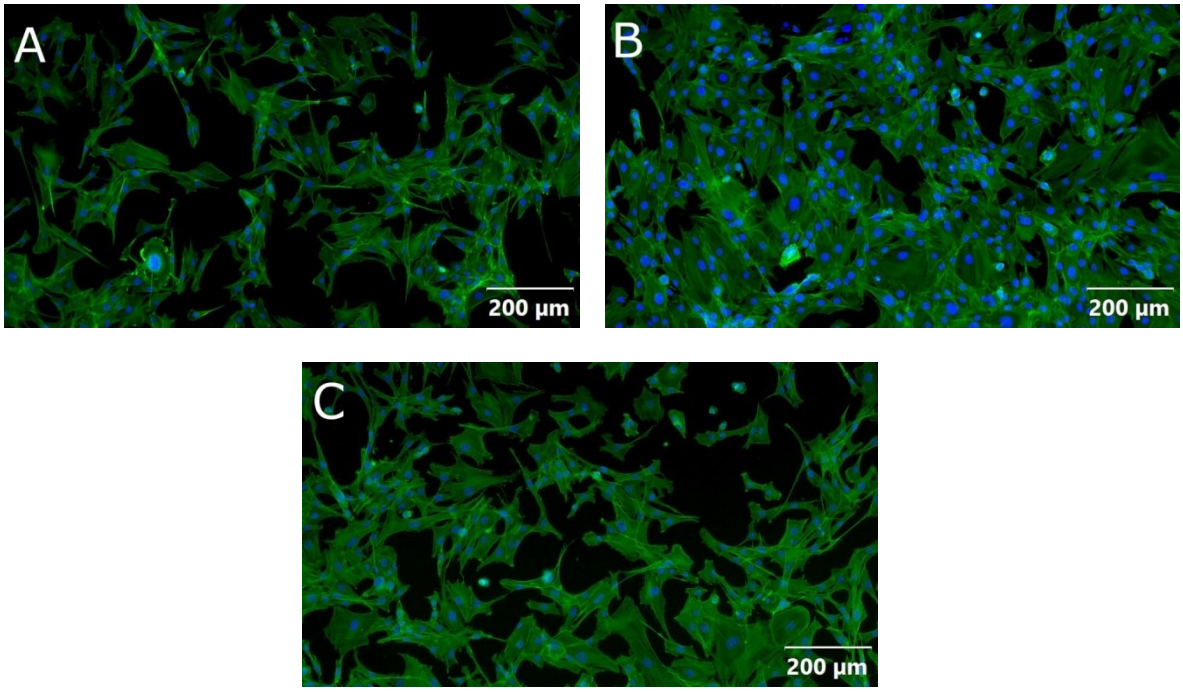

Figure 16 (A) NIH/3T3 influenced by constant DC at 50 mV for 4 hr, (B) NIH/3T3 influenced by pulses at 50 mV for 4 hr with 3000s exposure and 600s pause, (C) reference NIH/3T3 (magnification 100×)

The identical settings were also used for two samples of ES R1 cells with one reference sample. The results are shown in Figure 17. Even with their higher sensitivity to the influence of electricity, as with fibroblasts, the electrical stimulation did not cause any visible cell deaths, proving the absence of negative effects of this setting. The observation of individual cells is not possible because their size was more difficult because of their size and proximity to each other, but whole clusters of cells are better visible. The clusters in samples influenced by electric stimulation did not show any changes and had the same morphology as the reference sample.

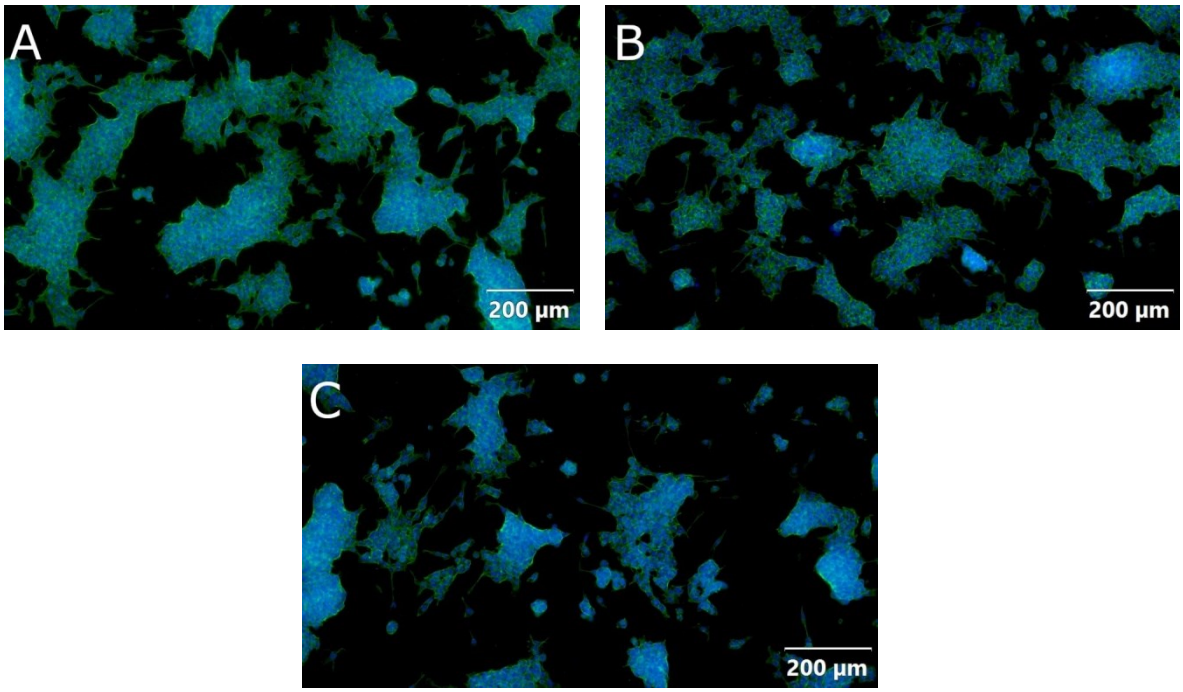

Figure 17 (A) ES R1 influenced by constant DC at 50 mV for 4 hr, (B) ES R1 influenced by pulses at 50 mV for 4 hr with 3000s exposure and 600s pause, (C) reference ES R1 (magnification 100×)

Visible changes in morphology occurred in cases when conductive PPy substrate was used. Figure 18 shows that NIH/3T3 cells, seated on PPy substrate, had slightly alternated morphology from normal. Their protrusions thinned and were elongated, spread over the surface of the substrate, in both the reference sample and the sample stimulated by electricity, though under the influence of 50 mV for 4 hours, delivered in pulses with 3000 s of exposure and 600 s of pause, the extension was more prominent than in the reference sample. In neither of the samples, the elongation had a visibly negative effect on cell viability.

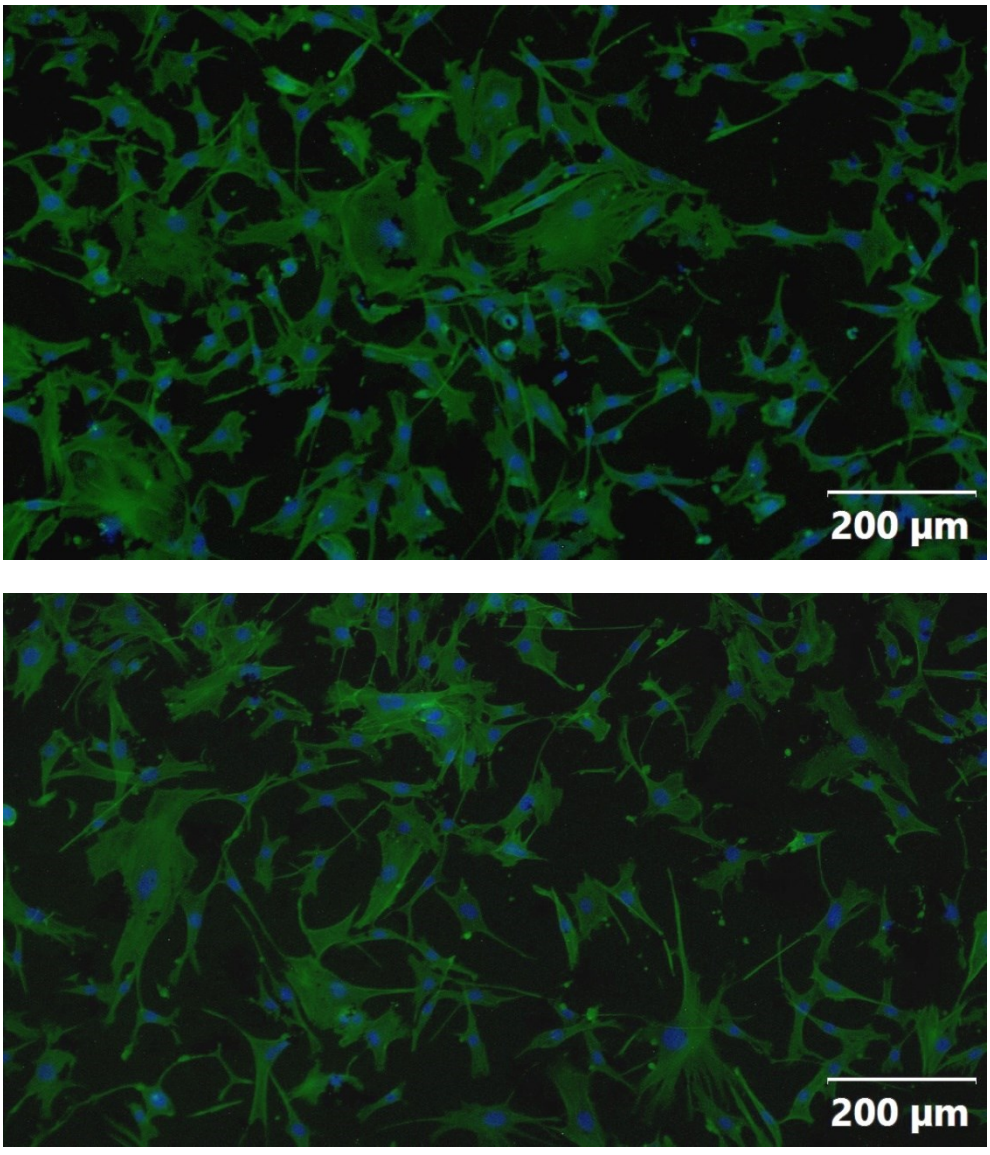

Figure 18 (up) reference NIH/3T3 with PPy substrate, (down) NIH/3T3 stimulated by pulses with PPy substrate at 50 mV for 4 hr with 3000s exposure and 600s pause (magnification 100×)

The morphology of ES R1 cells did not change when using a PPy substrate without any electric stimulation. However, a difference could be seen when the cells were supplied with 50 mV for 4 hours, delivered in pulses with 3000 s of exposure and 600 s of pause. Then, the clusters of stem cells were more branched and not as crammed together as in the reference sample, which is shown in Figure 19. This can be a sign of cells moving away from each other and the beginning of cell migration. However, from the picture, it cannot be told if cells are migrating in more directions or one specific direction, which would be more desired as influencing cells to move in a set direction is beneficial to navigate them in a place where they are needed.

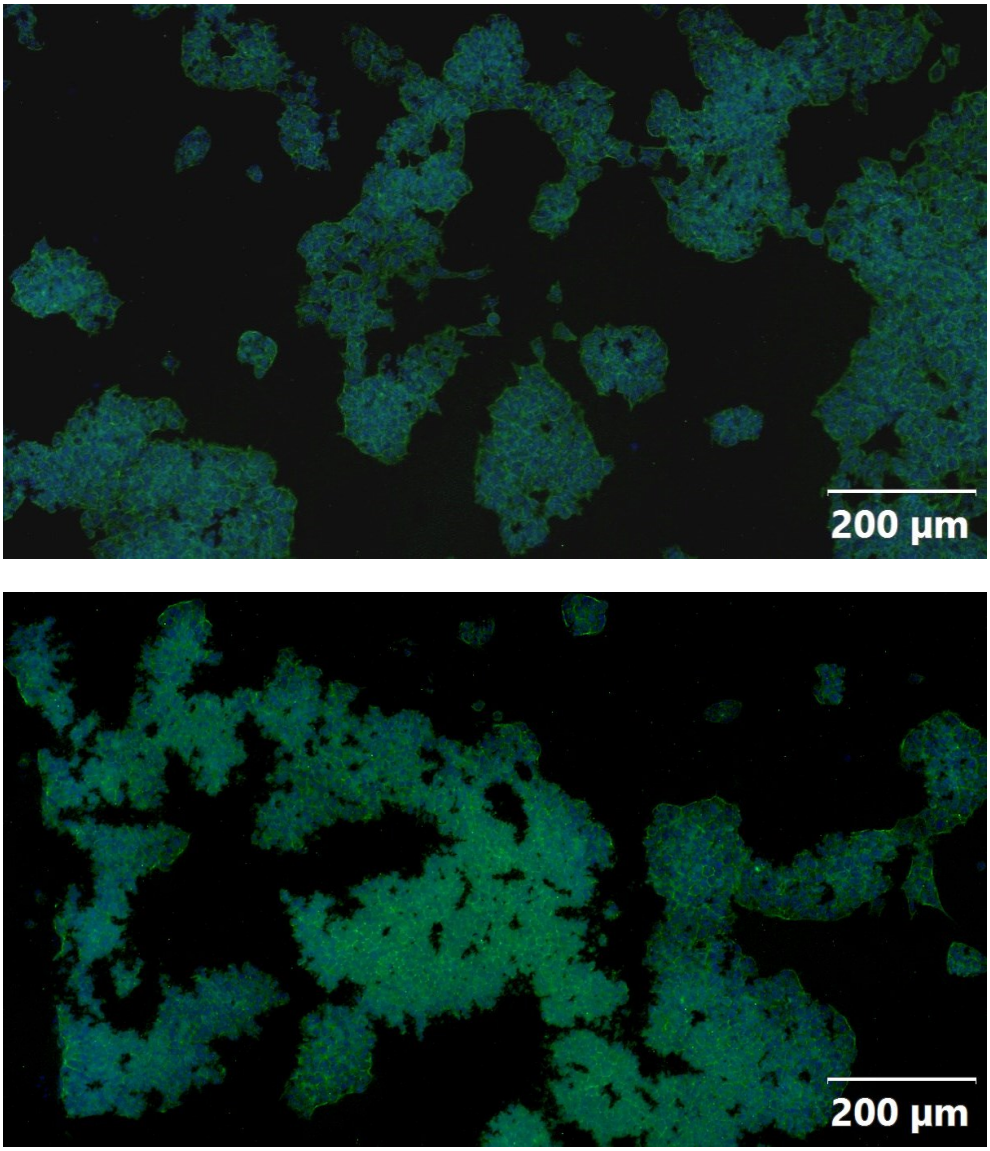

Figure 19 (up) reference ES R1 with PPy substrate, (down) ES R1 stimulated by pulses with PPy substrate at 50 mV for 4 hr with 3000s exposure and 600s pause (magnification 100×)

7.3 Cell viability

The influence of the chosen setting of the electrical field on the cell viability of the NIH/3T3 cell line was evaluated by MTT assay. For all the experiments described above, mainly wires made of platinum and iridium were used as electrodes. However, only one pair of electrodes was prepared due to the limited length of this wire. Therefore, two pairs of copper electrodes were also made. These copper electrodes were first tested in a culture medium without cells for potential medium oxidation. After 16 hr and 50 mV settings, no visible signs of oxidation were observed. The following test of cell viability was based on six NIH/3T3 cell samples. Three references which were not under the influence of electricity, one sample with electricity delivered by platinum/iridium electrodes and two samples with electricity delivered by copper electrodes. Samples were left in an incubator for 16 hr at a setting of constant 50 mV. After taking the samples out of the incubator, they were first observed under a microscope. From this observation alone, it was clear that copper is inappropriate for electrodes that come in contact with cell cultures. It can be seen that the majority of the cells in the sample that used copper electrodes did not survive. In Figure 20, the difference between the samples, which were under the effect of electric stimulation, is shown.

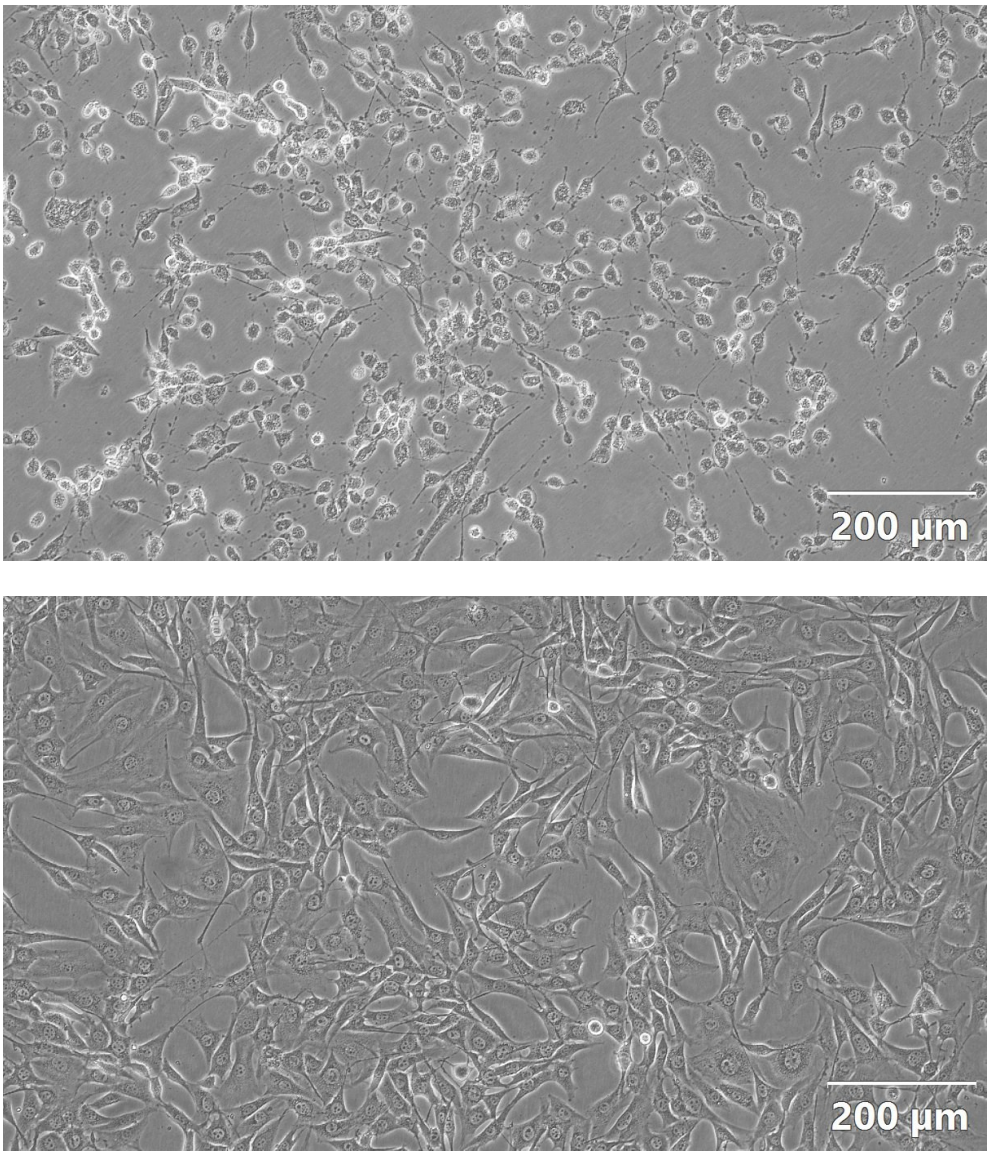

Figure 20 NIH/3T3 cells influenced by constant DC at 50 mV for 16 hr (up) samples using copper electrodes, (down) samples using platinum/iridium electrodes (magnification 100×)

In all 3 electrically stimulated samples and all 3 reference samples, an MTT assay was performed. The results of the assay were summarised in a graph pictured in Figure 21. As expected, samples with copper electrodes showed minimal cell viability. It is way lower than the value of 0.7 for relative cell viability, marking the highest value for a substance to be labelled as cytotoxic as established by the international standard. On the other hand, the cell viability of the sample with platinum/iridium electrodes slightly exceeded the viability of reference samples. The material has been proven suitable for electrodes, and the electricity setting has positively affected cell viability. The results

of this experiment also correspond with the categorisation of copper into toxic metal electrodes and platinum/iridium into non-toxic metal electrodes. (Im and Seo, 2016)

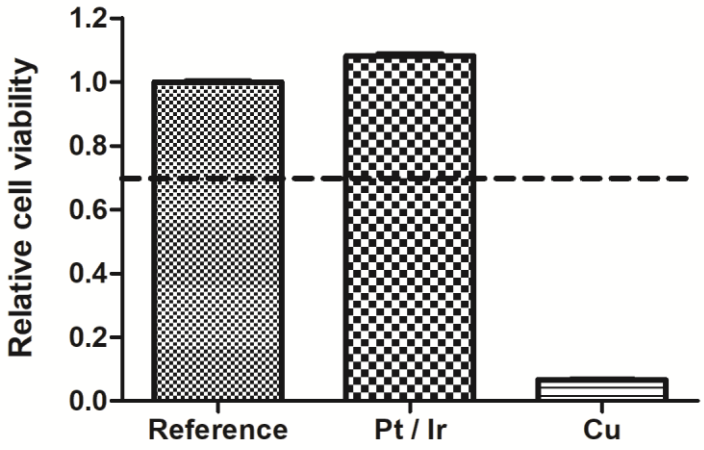

Figure 21 Relative cell viability of NIH/3T3 cell samples using copper electrodes, platinum/iridium electrodes, and reference samples

The graph in Figure 21 has almost no deviation. This is caused by a small number of samples. Three for reference, two for copper electrodes and one for platinum/iridium electrodes. The lack of samples was caused by an insufficient quantity of power supply devices. This means the results are not completely statistically proven. It would be appropriate, if the opportunity arises, to repeat the test with more samples to gain more reliable results.

Samples with PPy substrate were only observed under a microscope without performing an MTT assay. The assay could not be used since the DMSO solvent would dissolve the PPy substrate, affecting the measured absorbance and the final results of cell viability. (Song et al., 2000)

CONCLUSION

The theoretical part focused on ways to deliver electricity to cells, their respective advantages and disadvantages, and electricity's various effects on different cell functions. Because of the diversity of possible settings of electric stimulation, time of exposure, and types of affected cells, the range of effects is large. The alternate cell functions mainly lead to an increase or decrease in cell viability. Another area that was reviewed was the use of electrical stimulation in medicine, where influencing cell functions such as migration or viability can help with healing and curing diseases. This part shows that while many studies were made, there are still aspects of the influence of electric stimulation, and its potential uses are still unclear or unexplored, which offers opportunities for further research.

In the analysis part, monolayers of NIH/3T3 cell line and ES R1 cell line cultivated in tissue plastic Petri dishes were put under the influence of electric stimulation. It was also observed how the stimulation and the possible presence of conductive substrate in the form of PPy thin layer affected the morphology of cells and their viability. Cells exposed to higher voltages in a range of tens of V started to die; NIH/3T3 cells predominantly underwent apoptosis, while ES R1 cells underwent necrosis. When higher voltages were used, a problem occurred that after a few minutes the cultivating medium began to degrade and became unusable. When put under the influence of lower voltage in a range of tens of mV, only NIH/3T3 influenced by pulses changed their morphology and gained a more oval shape. However, the MTT assay showed that a sample influenced by constant DC had increased viability of NIH/3T3 cells compared to reference samples. During the test, results also showed the importance of the correct choice of material for electrodes with copper wire leading to cell death. Changes in morphology were observed in samples with PPy substrate. NIH/3T3 cells had elongated protrusions, which were present in both the reference sample and the sample with electricity. Elongation was more prominent in samples with electricity. ES R1 cells did not show any changes in the reference sample, but in a sample with electricity, clusters of cells were not that close to each other, which can be a hint of migration. The results showed electricity settings that can have positive effects on cells. This opens the way for following studies, where some possibilities are to focus on testing more samples to confirm obtained results or continue adjusting the setting to promote cell functions such as viability and migration.

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LIST OF ABBREVIATIONS

AC	alternating current
APS	ammonium persulfate
ATP	adenosine triphosphate
DC	direct current
dH_2O	deionised water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ES R1	mouse embryonic stem cell line
$FeCl_3$	iron(III) chloride
HCl	hydrochloric acid
hr	hour
I	current
MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NIH/3T3	mouse embryonic fibroblast cell line
nsPEF	nanosecond pulsed electric field
PBS	Phosphate-buffered saline
PPy	polypyrrole
R	resistance
s	second
TTF	tumour treating field
U	voltage
UPW	ultrapure water

LIST OF EQUATIONS

LIST OF FIGURES