



# **Tomas Bata University in Zlín**

## **Centre of Polymer Systems**

Doctoral Thesis Summary

### **Interaction of stem cells with materials**

**Interakce kmenových buněk s materiály**

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## ABSTRACT

With the continuing progress in medicine, life expectancy is increasing. Today, due to more advanced technologies and a better understanding of biological systems, it is possible to treat even formerly incurable diseases. Requirements for the replacement of various organs and tissues that have been damaged are also increasing. Thus, in recent years, tissue engineering has primarily focused on the development of three-dimensional constructs – so-called scaffolds. To create such a construct, it is necessary to consider the material properties suitable for a given application, as well as important to select an appropriate cell line. Stem cells are often used in tissue engineering due to their ability to self-renew and differentiate into different cell types. Currently, material characteristics such as chemical composition and surface topography are known to influence stem cell behaviour. It is also necessary to pay attention to the presence of biochemical substances in the culture medium, which are added either purposefully (growth factors) or are released from the biomaterial. The subject of this research was to investigate the effect of the abovementioned properties of biomaterials on cell lines, such as mouse fibroblasts, embryonic stem cells, and induced pluripotent stem cells. All cell-material interactions were tested *in vitro* in cell culture laboratories.

Keywords: *tissue engineering, biomaterials, stem cells, interactions, topography*

## ABSTRAKT

S pokrokem medicíny je čím dál více prodlužovaná doba života. Dnes je díky novějším technologiím a lepšímu porozumění biologických systémů možno léčit i dříve nevléčitelná onemocnění. Požadavky na náhrady orgánů a tkání, které byli nějak poškozeny jsou stále vyšší. Tkáňové inženýrství je tedy v posledních letech primárně zameřeno na vývoj trojrozměrných konstruktů neboli scaffoldů. Při vytváření takového konstruktu je třeba zvážit materiálové vlastnosti vhodné pro danou aplikaci, stejně tak je důležité vybrat funkční buněčnou linii. V tkáňovém inženýrství se často uplatňují kmenové buňky díky své schopnosti sebeobnovy a diferenciaci do různých buněčných typů. Materiálové charakteristiky jako je chemické složení a topografie povrchu ovlivňují chování kmenových buněk. Stejně tak je třeba věnovat pozornost přítomnosti biochemických látek v kultivačním mediu, které jsou přidány buď účelně (růstové faktory) nebo jsou uvolňovány z biomateriálu. Předmětem této práce je zkoumání právě vlivu těchto vlastností na buněčné linie, jako jsou myší fibroblasty, embryonální kmenové buňky či indukované pluripotentní kmenové buňky. Veškeré interakce buněk s materiály byly testovány *in vitro* v laboratořích buněčných kultur.

Klíčová slova: *tkáňové inženýrství, biomateriály, kmenové buňky, interakce, topografie*

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# 1. INTRODUCTION

The field of biomaterials originated as a combination of the disciplines of medicine, chemical engineering, materials science, and mechanical engineering due to the joint efforts of individual researchers and physicians. Founded in 1954, the American Society for Artificial Internal Organs was probably the first society to focus on biomaterials<sup>1</sup>. Biomaterials are widely used in tissue engineering – for example, in cardiovascular medical devices (stents, grafts), orthopedic and dental applications (implants), bioelectrodes and biosensors, skin dressings and replacements, and many other areas<sup>2</sup>. Now, as a result of continual advances in the medical field, biomaterials are under intense scrutiny with respect to the fabrication of biological scaffolds for the replacement of damaged tissues and organs.

Biomaterials can be classified into several groups. One of the general divisions is into natural and synthetic biomaterials<sup>3</sup>. Both groups include widely used materials - polymers. The use of polymers offers advantages such as easy synthesis, biocompatible properties, relative flexibility, and also a wide range of mechanical, chemical, and electrical properties<sup>4</sup>. An interesting group of polymeric biomaterials are conductive polymers (CPs). CPs find their use in tissue engineering due to their conductivity, with which they can promote cell differentiation<sup>5,6</sup>. Polyaniline (PANI) and polypyrrole (PPy) are among the main representatives of biocompatible CPs<sup>7</sup>.

This thesis is mainly focused on testing the material properties of biopolymers. A large part is devoted to the study of CPs and their influence on the behavior of cell lines. The previously unexplored effect of low molecular weight products occurring in PPy extract on embryonic stem cell (ESCs) differentiation proved to be particularly interesting. In addition, hemocompatibility properties of the modified PANI were studied.

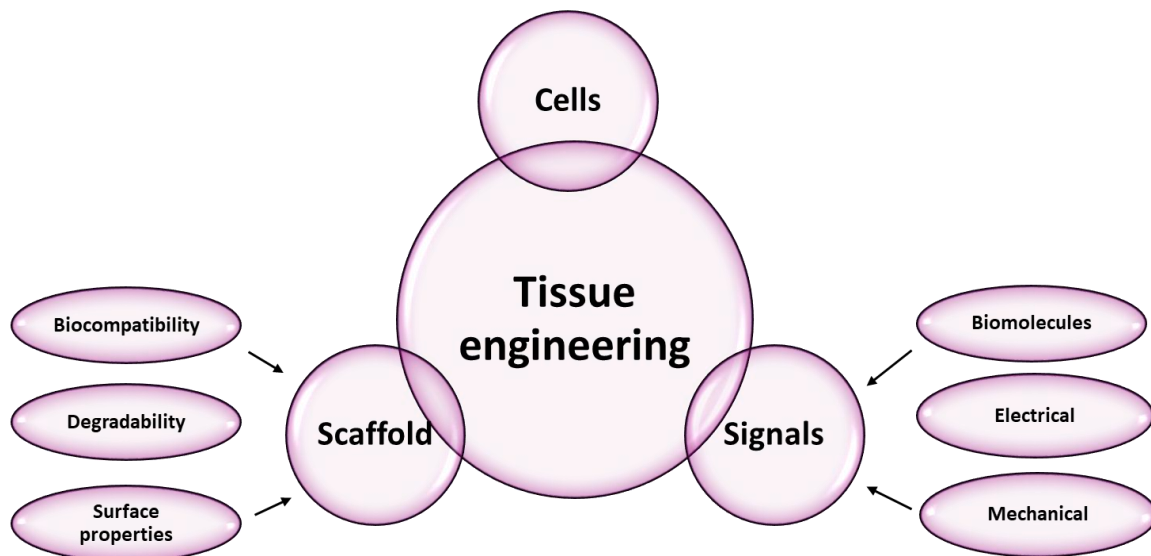
The thesis also focused on the influence of the topographic properties of materials on the behavior of different cell lines. Micro- and nanostructured polymer surfaces were tested using ESCs as well as induced pluripotent stem cells (iPSCs). Owing to their properties such as self-renewal and the ability to differentiate into different cell types, stem cells serve as a perfect tool for preparing scaffolds.



## 2. TISSUE ENGINEERING

In 1993, Joseph Vacanti and Robert Langer introduced tissue engineering to the general public as „an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ“<sup>8</sup>. Since then, the field has grown enormously in both science and industry<sup>9</sup>. One of the main goals of tissue engineering is to develop tissue and organ replacements and to design human Tissue Chips that can eliminate drug testing and disease modeling on animals<sup>10</sup>.

The most common organs that need to be transplanted due to disease or damage include the kidneys, heart, liver, lungs and pancreas<sup>11</sup>. For example, electrosensitive tissues such as nerve and heart are studied in conjunction with the use of CPs. The rate of organ damage is raising due to getting higher prevalence of obesity, high blood pressure, smoking, elevated blood glucose levels. In addition, life expectancy is still increasing<sup>12</sup>. The transplantation process is one of the most complex procedures in biomedicine. Organ transplantation is associated with the adverse immune response of the organism related to transplant rejection. The selection of donors and suitable patients often clashes with ethical and religious issues. Another big problem is that global demand exceeds supply<sup>11, 13</sup>. For these reasons, tissue engineering is largely focused on the fabrication of three-dimensional structures for cells - scaffolds. Tissue engineering follows the construction of a 3D construct by three principles called the tissue engineering triad (Figure 1). 1) When creating scaffold, it is important to take into account all the properties of the material used. Biocompatibility, suitable chemical composition, surface properties, degradation at the required rate and mechanical properties are very important. 2) It is substantial to select a suitable cell line for scaffold inoculation. 3) There is a need stimuli for cell proliferation or possible differentiation. This includes, for example, growth factors present in the culture medium or directly in the scaffold construct itself, as well as various electrical or mechanical stimulations<sup>9, 14</sup>. All these aspects are gradually discussed in more detail in this work.



*Figure 1: The tissue engineering triad.*

### 3. STEM CELLS

Currently, stem cells are receiving attention in various contexts. They are used in research, in the testing of new biomaterials but also in biomedicine for the treatment of Parkinson's disease, Alzheimer's disease, stroke, arthritis. Stem cells are primary undifferentiated cells that can transform into another cell type based on their determination. Due to this ability, it participates in the regeneration of worn or damaged parts of organs and tissues in the body, as well as they can renew themselves<sup>15</sup>.

Stem cells can be divided from several points of view. One of the possibilities is their distribution in terms of differentiation potential. In this case, the cells are divided into totipotent, pluripotent, multipotent and unipotent. Totipotent cells give rise to the whole organism. During the development of the organism, they occur only in the zygote stage, it means about 4-5 days. Then the zygote enters the state of the blastocyst and the cells become pluripotent. Pluripotency is maintained throughout the embryonic phase and cells are able to differentiate into all cell types. The next stage is multipotent cells, which can differentiate into other cell types but to a limited extent. Their advantage is that they can be isolated from the tissues of adult mammals. The last, unipotent stem cells have a limited ability to differentiate compared to other stem cells. As the name implies, they give rise to only one type of cell<sup>16, 17</sup>.

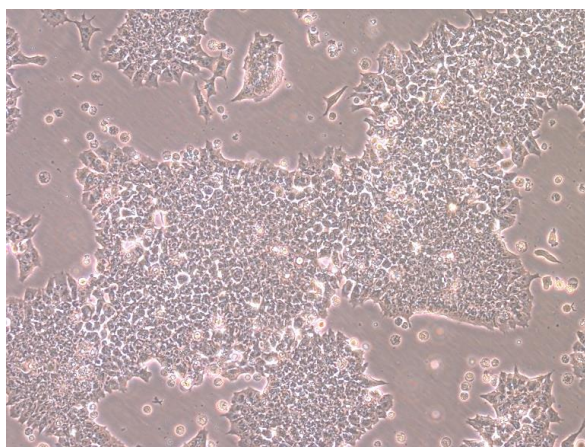
Furthermore, we can divide stem cells based on their source. In this way, the cells are partitioned into ESCs, iPSCs and adult stem cells (ASCs). For ESCs is the source of the early embryo and they are also classified as totipotent and pluripotent. The source for ASCs are various tissues. The ASCs are tissue-

specific, thus being able to differentiate into cells corresponding to their tissue origin. They are especially important because tissues in adults such as the intestines, blood, lungs, and skin have to be constantly renewed. However, it is now known that stem cells also occur in tissues that have been considered non-regenerating, for example, the brain or heart. This group includes mesenchymal, hematopoietic, neural, epithelial, hepatic stem cells. ASCs are also classified as multipotent<sup>18-20</sup>.

### 3.1 Pluripotent stem cells

For the production of artificial tissue *in vitro*, it is necessary to use a suitable cell type to fill a scaffold. One of the cell types is pluripotent stem cells<sup>21</sup>. Stem cells generally have the ability to regenerate themselves by cell division and also differentiate into a variety of specialized cell types<sup>22, 23</sup>.

Among the PSCs belong also ESCs, which are several types: embryonic carcinoma, embryonic germ, embryonic stem cells<sup>24</sup>. Initial ESCs research was performed on mouse models. In 1981, Martin successfully isolated the first ESCs from blastocyst mice. These isolated cells were cultured in the medium used for embryonic carcinoma. Due to this, he induced culture of cells having the characteristics of murine teratocarcinoma stem cells (such as morphology, pluripotency and the ability to form a teratocarcinomas)<sup>25</sup>. Later, research was also performed on human ESCs. J. A. Thomson is a well-known American biologist, mainly because of the first human ESCs derivation in 1998. He and his group cultivated human embryos up to the blastocyst stage and 5 cell lines were derived. Undifferentiated proliferation took place over a period of 5 to 8 months (depending on the cell line). The derived cell lines retained the potential to form all three germ layers (ectoderm, endoderm, and mesoderm), as evidenced by the formation of teratomas after injection into the mouse. These embryonic lines also showed a high level of telomerase activity<sup>26</sup>. In culture, then, they could be cultivated almost indefinitely.



**Figure 2:** Morphology of mouse ESCs (cell line ESR1). Magnification: 100x

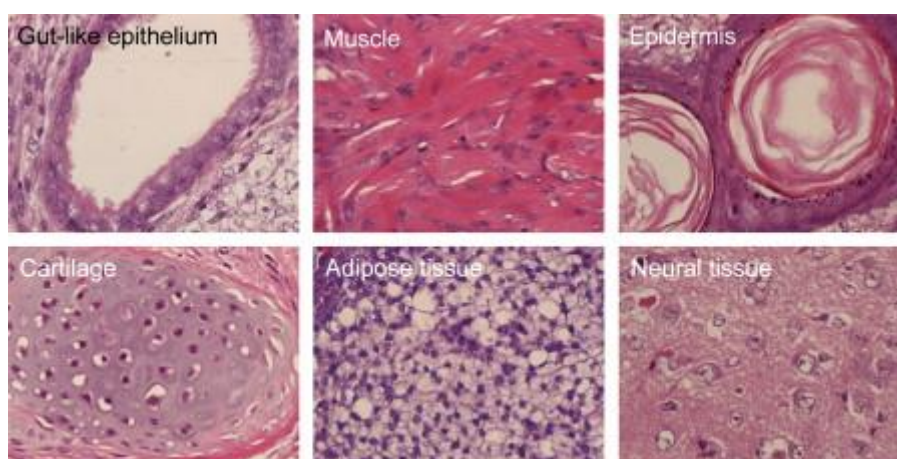
### 3.1.1 Induced pluripotent stem cells

From an ethical point of view, it is difficult to work with human ESCs. For this reason, other options have been sought. A major breakthrough in tissue engineering is the induction of pluripotent somatic cells<sup>27</sup>. iPSCs have similar properties to ESCs, namely cell morphology, growth characteristics, expression of stem cell markers, methylation of DNA, a profile of genetic expres<sup>28</sup>. Reprogramming of an adult cell into an embryonic state can be accomplished, for example, by transferring the nuclear content to oocytes or by cell fusions<sup>29,30</sup>. The problematics of cell reprogramming will be discussed in a separate chapter (3.2.2 Reprogramming of cells). The following paragraphs will describe the discovery of murine iPSCs from 2006 through the shift to human iPSCs in 2007 to studies describing the function of factors.

Takahashi and Yamanaka in 2006 created iPSCs from mouse somatic cells. By introducing the four pluripotent genes into mouse skin cells, they created immortal cells with the ability to form all the cells in the adult mouse's body<sup>30,31</sup>. Specifically, they studied the factors that are responsible for totipotency and pluripotency. One murine such factor is Nanog mRNA, which occurs in pluripotent human and mouse cell lines but absent in differentiated cells. Nanog is considered a homeobox gene because it is also expressed in the founder cells of the early embryo. For this reason, scientists were named the gene after the mythological Celtic land of the ever-young Tir nan Og<sup>32</sup>. An additional homeobox factor is Oct4 (also known as POU), which is involved too in activating or suppressing gene expression in the ESCs<sup>33</sup>. This factor is found in the blastocyst in the inner cell mass (ICM). *In vitro*, Oct4 can only be found in ESCs, undifferentiated embryonic carcinoma and embryonic germ cells<sup>34</sup>. Oct4 and Nanog interact with other factors. One of them is Sox2, which is also responsible for keeping stemness<sup>35</sup>. Nanog, Oct4 and Sox2 share an important part of the target genes and actually in mouse and human ESCs they cooccupy genes. In the human genome, these genes occupy approximately 10 % of the promoters. About half of the promoter regions bound by Oct4 were also bound by SOX2 and 90 % of these doubly bound genes were in turn bound by NANOG<sup>36</sup>. On regulation of ESCs are also involved factors as Stat3, c-Myc, n-Myc, Smad1, Klf4, Zfx, etc.<sup>32,35,37</sup>. Takahashi and Yamanaka induced iPSC from mouse fibroblasts by factors Oct4, Sox2, c-Myc and Klf4. Surprisingly, the Nanog factor was not needed. In their study, cellular retroviral transduction was used to introduce factors into the cells. To verify pluripotency, they created teratomas under the skin of the mouse. Teratomas contained different tissues of all three germ layers, even neural tissues, cartilage, and columnar epithelium<sup>30</sup>. Their results were reproducible, as demonstrated, for example, in the studies Maherali et al. 2007<sup>38</sup> or Okita, Ichisaka and Yamanaka 2007<sup>39</sup>.

In 2007, Takahashi et al.<sup>40</sup> moved further and induced iPSCs from human somatic cells. For this experiment, the same factors and methods (retroviral

transduction) were used as in the case of mouse iPSCs. Mouse fibroblasts were replaced by a human. However, they introduced the mouse retrovirus receptor into human cells, increasing the transduction rate from 20 % to 60 %. For comparison of properties with ESCs, they focused on morphological studies, epigenetic status, monitoring of *in vitro* embryoid bodies formation, controlled differentiation to nerve cells and cardiomyocytes, and teratoma formation. The tumor again contained the tissues of all three germ layers<sup>40-42</sup>. At the same time, another study from the same field was published by Yu et al.<sup>43</sup> However, this research group used a different combination of transcription factors, namely Oct4, Sox2, Nanog and Lin28. Their induction was successful as well because reprogrammed human somatic cells showed similar properties to ESCs.



**Figure 3:** Types of tissue contained in the tumor former by human iPS<sup>40</sup>

## 4. BIOMATERIALS

In tissue engineering, it is not only important to select the appropriate cells type but also to choose the suitable material. The materials used in tissue engineering is called biomaterials. The definition of biomaterial has changed and evolved over the years due to the development of new applications in medicine<sup>44</sup>. According to the National Institutes of Health, biomaterial can be defined as: „any substance (other than a drug) or combination of substances synthetic or natural in origin, which can be used for any period of time, as a whole or part of a system which treats, augments, or replaces tissue, organ, or function of the body.“<sup>45</sup>.

### 4.1 Classification of biomaterials

Biomaterials can be made from a variety of materials, including solids, liquids, and gels<sup>46</sup>. Biomaterials are divided in several ways. If we divide them based on the source from which the material comes, we divide them into natural and synthetic. Based on degradation, we then introduce a division into biodegradable and non-biodegradable. Alternative, materials can be divided in terms of

interatomic bonding forces into polymers, metals and ceramics<sup>45</sup>.

#### 4.1.1 Polymers

Polymers are very widely used today and humanity can no longer imagine life without them. It could be said that their use can be found in any industry. The term polymer comes from the composition of two Greek words, the word poly meaning „many“ and meros meaning „parts“. As the name suggests, a polymer molecule is composed of several repeating units (monomers), which are often linked by covalent bonds<sup>47</sup>. The choice of a suitable polymer for application is related to the knowledge of their properties, such as molecular weight, structure of polymers, crystallinity, thermal and electrical properties.

Polymers usually have a high **molecular weight** (between 10000 - 100000 g / mol)<sup>47</sup>. However, the polymers do not have a uniform molecular weight value because their chains are a mixture of macromolecules of different lengths. For this reason, a distribution M is introduced from which the average molecular weight is determined. There are two types of average molecular weight: weight ( $M_w$ ) and number ( $M_n$ )<sup>48</sup>.

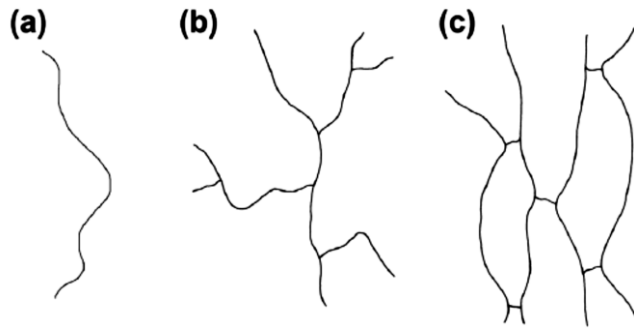
$$M_w \text{ is defined: } \quad M_w = \frac{\sum_i N_i M_i^2}{\sum_i N_i} \quad (1)$$

$$M_n \text{ is defined: } \quad M_n = \frac{\sum_i N_i M_i}{\sum_i N_i} \quad (2)$$

where:  $i$  – the number of polymer molecules

$N_i$  – the number of molecules that have molecular weight  $M_i$ <sup>49</sup>

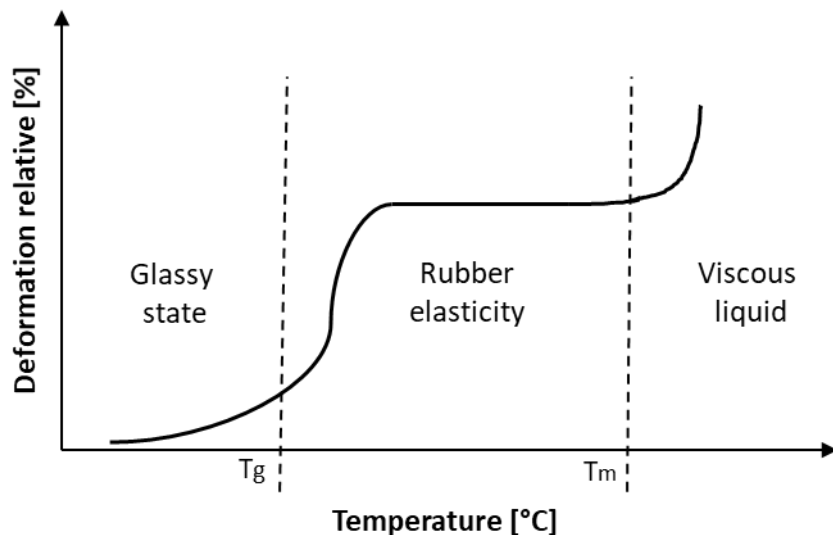
If we are interested in the **structure of polymers**, we divide them into linear, branched and crosslinked (Figure 7). In a linear structure, the monomer is associated with only two others. For example, polystyrene (PS) or poly(methylmethacrylate) (PMMA) are also considered linear, even though they contain short branches in their monomer. Branched polymers are formed from monomers that have a functionality greater than 2 and also by the choice of the polymerization process. Due to this, mentioned PS can be both linear and branched (the same goes for polyethylene and others). If the monomers have a functionality higher than 2, they can give rise to crosslinked polymers. They are also prepared by chemical bonding of linear or branched polymers. Known rubbers can be included in this group of crosslinked<sup>50-52</sup>.



**Figure 4:** Polymer structure: a) linear, b) branched, c) crosslinked<sup>49</sup>

Another factor influencing the properties of a polymer is the degree of its **crystallinity**. The degree of crystallinity indicates the weight or volume fraction of the crystalline part. Theoretically, this value ranges from 0 (amorphous polymer) to 1 (completely crystalline polymer). In reality, however, the proportion of the crystalline phase is in the range of 10% – 80%, for such polymers, the classification as semicrystalline is then introduced<sup>51</sup>. The polymer is able to crystallize if it has a regular structure. The cooling time during preparation is also important, sometimes long times are required to allow the chains to group into a crystalline structure<sup>53</sup>.

When choosing a suitable polymer, it is also useful to know its **thermal properties**. These play a role in selecting the type of processing and application. At normal room temperatures, most thermoplastics are solid because the molecular chains lack the energy to move them. By heating the plastic material, we supply the chains with the energy needed to move them and the material begins to expand<sup>54</sup>. The thermal behavior of a polymer can be characterized by two temperatures:  $T_g$  - glass transition temperature for an amorphous polymer and  $T_m$  - melting point for a crystalline polymer<sup>54, 55</sup>. The exact temperature  $T_g$  for material does not exist, as it depends on many factors, such as the rate of cooling and heating, the rate of deformation, the method of measurement or the molecular weight.  $T_m$  depends on the presence of double bonds in the chain, aromatic groups, side groups<sup>56, 57</sup>. Amorphous polymers do not have a temperature  $T_m$ , but all polymers have a  $T_g$  because, as already mentioned, a purely crystalline polymer is only a theoretical concept<sup>57</sup>. At  $T_g$  the amorphous part of the polymer, changes from a steady state to a more elastic state and the polymers begin to soften. The liquid behavior of semicrystalline polymers occurs when the  $T_m$  is reached<sup>54</sup>. The  $T_g$  value is always lower than the  $T_m$ . The  $T_g$  to  $T_m$  ratio is lower for polymers with a symmetrically arranged structure<sup>55</sup>.



**Figure 5:** Thermomechanical curve of semicrystalline polymer.  $T_g$  - glass transition temperature;  $T_m$  - melting point.

An attractive aspect for the application of polymers in tissue engineering is their **electrical properties**. Most polymers are dielectric due to their low electrical conductivity. An increase in conductivity can be achieved by doping the polymeric material with acceptors or donors of electrons<sup>58</sup>. By forming a polymer composite together with metal, it is thus possible to prepare a material that has electrical properties close to metal conductors, but mechanical properties and a method of processing as a polymer. For instance, nickel, copper and iron powders are used as fillers<sup>59</sup>. The resulting conductivity of such composites then depends on the size and shape of the filler particles, the interactions between the surface of the particles and the polymer matrix, and the spatial arrangement of the particles<sup>60</sup>. However, some polymers are conductive without fillers due to their structure contains conjugated double bonds<sup>61</sup>.

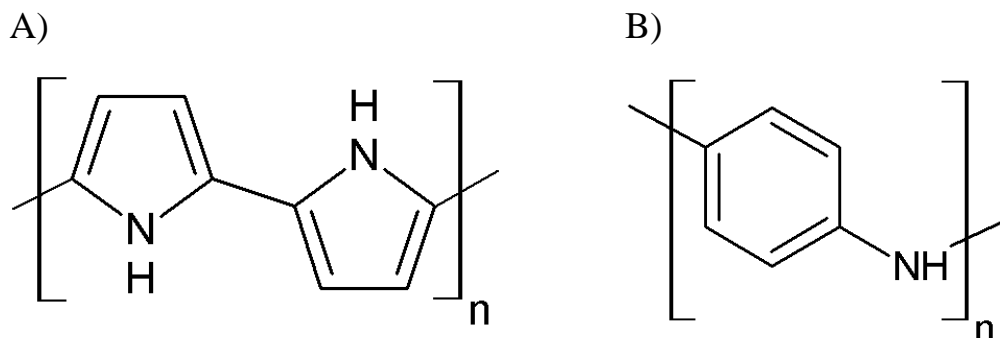
### ***Conductive polymers***

CPs as biomaterials find use in tissue engineering mainly due to their low cost, ease of synthesis, biocompatibility and conductivity<sup>62-64</sup>. In the structure of CPs, there are conjugated carbon-carbon double bonds<sup>61, 65</sup> (conjugated  $\pi$ -electrons, respectively)<sup>63</sup>. The conjugated structure is responsible for electrical properties such as high electron affinities, low energy optical transmission and low ionization potential<sup>65</sup>. Thus, CPs can be easily oxidized or reduced by doping, which can significantly increase their conductivity (conductivity in the neutral state:  $10^{-6}$  S/cm, after doping:  $10^5$  S/cm). Due to their ability to convert biochemical information into electrical signals, CPs are an attractive material for biosensors<sup>64</sup>. Another preferred aspect of utilizing CPs is that they exhibit both electron conductivity and ionic conductivity. As a result, they are able to convert electrical signals to ionic (and vice versa), that has the advantage of exchanging signals and stimuli between cells and material. Mentioned properties have a



crucial role in creating an active biointerface<sup>66</sup>.

Today, there are just over 25 types of CPs. Polyacetylene has the simplest structure of them, but it is unstable in air and the synthesis is relatively difficult. However, CPs containing heterocycles in their structure demonstrate good stability. Polypyrrole (PPy) and polyaniline (PANI) (Figure 6) could be mentioned as two main representatives of this family of polyheterocycles<sup>5</sup>.



**Figure 6:** Structural formulas of conductive polymers: A) polypyrrole; B) polyaniline.

#### 4.1.2 Ceramics

Ceramics are an inorganic non-metallic material. The ceramic is very hard, heat-resistant and has excellent compressive strength. On the other hand, have very poor mechanical properties and are brittle<sup>44, 52</sup>. It might seem that ceramics are not a suitable candidate for use in tissue engineering, but there are applications where they are appreciated. For example, it is completely unsuitable for soft tissue regeneration, but due to its chemical and structural similarity to the mineral phase of bone, it is a suitable candidate for bone regeneration<sup>14</sup>. Ceramics are also used in dental implants or hip replacements<sup>45</sup>.

#### 4.1.3 Metals

Metal are inorganic materials that are interesting for biomedical applications due to their electrical and thermal conductivity and the possibility of forming into various shapes<sup>52, 67</sup>. The complication with using metals as biomaterials is that they are subject to corrosion. During corrosion, the condition of the material deteriorates and substances are released into the environment. This can cause adverse reactions in the organism. Metals such as gold, silver and platinum are inert. Other metals (titanium, chrome) have the ability to form an inert layer, thus preventing interaction between the metal and the biological system<sup>52</sup>. The use of metal is frequently encountered in joint replacements, dental implants, orthopedic fixations, stents<sup>68</sup>.

## 5. INTERACTION OF CELLS WITH MATERIALS

In the previous chapters, the properties of stem cells and the properties of the used biomaterials were described. The next step in creating a biomaterial is to understand the principles of the interaction between cells and material. Generally, we are interested in whether the cell will adhere to the material, subsequently, if it will be able to migrate, proliferate or differentiate<sup>69</sup>.

### 5.1 Control of cells by biochemical properties

#### Biochemical properties of the surface

The biochemical properties of the surface are involved in the control of cell-material adhesive interactions<sup>70</sup>. In the natural environment, the ECM gives biochemical information to cells<sup>71</sup>. Earlier, the ECM was considered only as a matrix that provides primarily cell mechanical support, now it is known to play a very important role in other cellular processes<sup>72</sup>. Collagen, fibronectin, laminin and elastin are one of the major proteins in the ECM<sup>73</sup>. 2D cell culture is often performed on substrates coated with collagen, laminin or Matrigel<sup>1 75</sup>. Flanagan et al.<sup>76</sup> investigated the effect of laminin, fibronectin and collagen on the differentiation of murine and human neural stem/precursor cells (NSPCs). Laminin-coated surfaces led to the formation of more neurons compared to other types of proteins. During osteogenic differentiation, cells produce an ECM composed predominantly of collagen type I<sup>77</sup>. Not surprisingly, collagen promotes differentiation towards bone cells. Mizuno et al.<sup>78</sup> cultured rat bone marrow MSCs in Petri dishes in the presence of collagen type I. When compared to culturing on untreated plastic, collagen has been shown to induce cell differentiation into osteoblasts. These naturally occurring ECM polymers undergo rapid degradation. Therefore, it is appropriate to form substrates by combining natural and synthetic polymers<sup>79</sup>. Such a material can be prepared, for example, by combining synthetic polycaprolactone (PCL) with covalently immobilized collagen on its surface. Collagen was grafted onto the surface via surface-initiated sodium methacrylate. The substrate improves cell proliferation due to collagen and improves mechanical properties due to PCL<sup>80</sup>.

#### Cytotoxicity of extracts of material

The presence of chemical compounds in the medium clearly affects the behavior of cells. However, the medium can contain biomaterial release leaching compounds that are undesirable for the cells. An extract exposure method is

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<sup>1</sup> *Matrigel - a mixture of proteins (mainly laminin, collagen, perlecan) produced by Engelbreth-Holm-Swarm mouse tumor cells, used as a model of the basement membrane* (74. Funaki, M.; Janmey, P. A., Chapter 23 - Technologies to Engineer Cell Substrate Mechanics in Hydrogels. In *Biology and Engineering of Stem Cell Niches*, Vishwakarma, A.; Karp, J. M., Eds. Academic Press: Boston, 2017; pp 363-373. Doi: <https://doi.org/10.1016/B978-0-12-802734-9.00023-8>.

frequently used to assess the toxicity of materials, detecting toxins from leached surfaces of materials present in the medium<sup>81</sup>. Materials can hold residues of substances with which they have been sterilized. In healthcare, ethylene oxide (EO) is used for sterilization. The residual amount of EO released from the substance to the extracts then affects the viability of the cells. The amount of captured EO depends on the type of material<sup>82</sup>. An interesting study on substances released from biomaterials was performed by Onnekink et al. They investigated the effect of organosiloxane compounds on the morphology and apoptosis of HeLa and Jurkat<sup>2</sup> cells. Humans are exposed to these substances on a daily basis when using cosmetics (deodorants, hair and skincare products, etc.). Silicones are also released from gel implants, although they were originally considered inert. Specifically, the effect of octamethylcyclotetrasiloxane (D4) was investigated in mentioned study. The release of silicones from implants creates microdroplets that migrate through the body and induce cell apoptosis in certain tissues<sup>84</sup>. Impurities and by-products extracted from the material not only have an adverse effect but some forms of impurities are involved in differentiation cellular processes. Skopalová et al.<sup>85</sup> examined that conductive PPy extract contains residues that induce neurogenesis in mouse ESCs. Neurogenesis was stimulated by the presence of low molecular weight products such as PPy oligomers in the extract, which were determined by mass spectrometry.

### **Biochemical properties and hemocompatibility**

The biochemical properties of the materials are further important in terms of hemocompatibility. Hemocompatible material should not interact adversely with blood. It should not cause clots (thrombosis) or inflammatory immune response<sup>86</sup>. Thus, it is highly desirable to control interactions between materials and individual blood components such as erythrocytes, platelets, leukocytes and proteins<sup>87, 88</sup>. One of the greatest known anticoagulants is a polysaccharide called heparin<sup>89</sup>. Polymeric biomaterials is possible to functionalize with heparin to improve hemocompatible properties<sup>90</sup>. Li et al.<sup>91</sup> in their research covalently immobilized heparin on the surface of PPy. The material thus formed retained its electrical properties and heparin increased hemocompatibility as indicated by prolonged plasma recalcification time and inhibition of platelet adhesion. Copolymers containing sulfate and carboxyl groups (as well as heparin) have anticoagulant effects<sup>92</sup>. Humpolíček et al.<sup>93</sup> functionalized the surface of PANI films with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA), thus significantly improving the anticoagulant properties of the material. Following these studies, it can be stated that dodecylbenzenesulfonic acid sodium salt (SDBS), 2-aminoethane-1-sulfonic acid (taurine), and N-(2-acetamido)-2-

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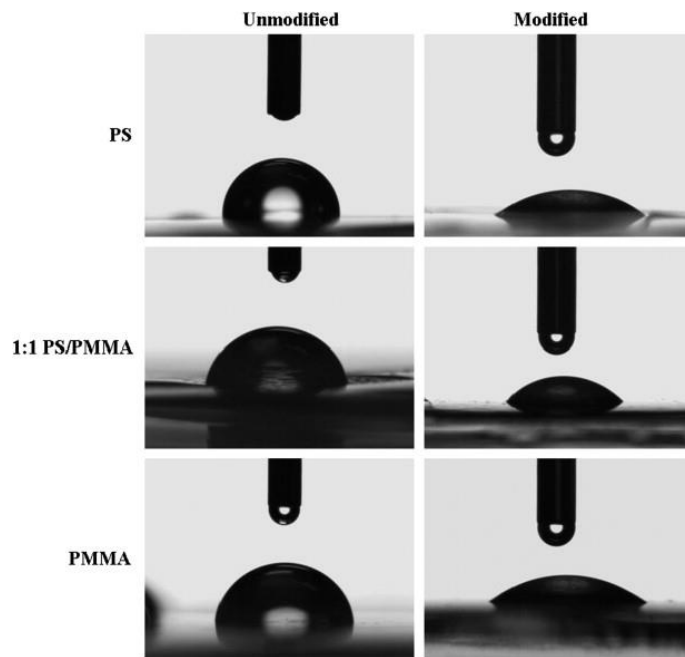
<sup>2</sup> Jurkat cells - human T-lymphoblast line (83). Gottlieb, R. A.; Nordberg, J.; Skowronski, E.; Babior, B. M., Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. *Proceedings of the National Academy of Sciences of the United States of America* **1996**, 93 (2), 654-658. Doi:10.1073/pnas.93.2.654.)

aminoethanesulfonic acid (ACES) will also have the potential as anticoagulants. Nevertheless, PANI films treated with these substances did not show the expected anticoagulant activity, which was probably due to their low molecular weight compared to heparin<sup>94</sup>.

## **5.2 Control of cells by geometrical properties**

To create a natural environment for cells, it is necessary to know the connection between cell differentiation and geometric properties of the material. The most important geometric feature that is completely absent in conventional 2D cultures is the three-dimensional ECM. The cells in the body also take on a characteristic shape according to their function and location. The shape of the cells affects the structure of the ECM and vice versa<sup>79</sup>. Management of the shape and fate of cells in 2D cell cultures can be controlled by surface treatment of substrates to create the so-called pseudo-3D models<sup>95</sup>. To achieve such models, the surface topography of the substrates can be modified.

Surface topography together with chemical properties affects the wettability of the substrate, which is an important factor for cell adhesion. Modification of the topography and related hydrophobicity can be achieved by plasma treatment of the material<sup>96</sup>. Keshel et al.<sup>97</sup> changed the surface of the polyurethane (PUR) substrate using oxygen and argon plasma. Oxygen plasma provided higher material roughness than argon. The contact angle of PUR was also significantly reduced after oxygen treatment, which is connected to the increase in the hydrophilicity of the material. Adhesion of unrestricted somatic SCs from human cord blood was increased on substrates treated with both types of plasma against the reference sample. Similarly, Liu et al.<sup>98</sup> investigated the effect of plasma Dielectric Barrier Discharge (DBD) on the surface of PMMA films. Plasmating the material changed the topographic properties, wettability and increase adsorption of bovine serum albumin. The same method was used in Borges et al.<sup>99</sup> Using DBD, they modified the substrate surface from PS and PMMA. Topographic changes were observed on PMMA, reducing the contact angle and increasing hydrophilicity (see Figure 10). In the case of PS, the reduction in contact angle was due to a chemical modification.



**Figure 7:** Photographs of water drops on surfaces modified and unmodified by DBD plasma<sup>99</sup>.

In addition to the scale of the topography, the geometrical arrangement of the structure also plays a role in cell behaviour. For example, substrates made by the electrostatic spinning of Poly(lactide-*co*-glycolide) (PLGA) promoted cell migration when the fibers were oriented. On the contrary, randomly arranged fibers prevented migration<sup>100</sup>. Similar results were obtained in the work Wang et al.<sup>101</sup> By electrospinning, they formed a substrate with oriented and non-oriented fibers of poly( $\epsilon$ -caprolactone) and gelatine. Oriented fibers certainly promoted migration. Furthermore, oriented fibers increased adhesion and osteogenic differentiation in MSCs. Another factor influencing the fate of cells is the geometric arrangement of the nanopillars. Some studies have shown that the disordered symmetry of the nanopillars promotes osteogenesis compared to a flat surface or a symmetrical square pattern. Dalby et al.<sup>102</sup>, tested this fact on a PMMA surface, using hexagonal, square, slightly disordered square and random fields as the geometry of the nanopillar distribution. Higher production of osteopontin and osteocalcin (an indication of osteogenesis) occurred when a slightly irregular square pattern was used. This finding was also proved by Tsimbouri et al.<sup>103</sup>, when a slightly disordered geometry promoted osteogenesis, in the case of a symmetrical square pattern there was a decrease in metabolic signaling activity, which is behind the phenotypic changes in cells. Thus, the square symmetry kept the cells in a differentiated state. In both studies, testing was performed on human MSCs.

## **6. AIMS OF DOCTORAL THESIS**

Briefly, this work deals with the interactions between biomaterials and stem cells. The main content of the research can be divided into 4 points:

1. Introduction of stem cell culture methodology at the Center for Polymer Systems
2. Use of stem cell lines in practice for testing material-cell interactions
3. Testing the effect of conductive polymers on stem cell differentiation
4. Determination of impact of chemical and geometrical properties of biomaterials on adhesion, proliferation and differentiation of stem cells

## 7. EXPERIMENTAL PART

The experimental part of the dissertation was focused on testing the interactions between material and cells. In cell biology laboratories, routine assays were performed to evaluate the cytotoxicity of the material using the NIH/3T3 mouse fibroblast cell line. However, it was more appropriate to use stem cells for experiments to evaluate how the material affects cellular behavior or differentiation. Specifically, culture of mouse ESCs has been established. The fate of the cell is, as follows from the theoretical part of the work, influenced by many properties of materials. A large part of the study was devoted to testing conductive polymers, as they can not only stimulate cells to differentiate but also monitor changes taking place in the culture environment using reverse electrical signals. It is already known that cells can be controlled by electrical signals, but the influence of low molecular weight products present in extracts of CPs on differentiation has not been given much importance. Interesting results have been obtained in testing PPy extracts in interactions with ESCs. Furthermore, CPs can be modified with various chemical dopants to improve their properties for certain applications. Investigating the effect of dopants in combination with PANI were tested to enhance hemocompatible properties. Another direction of author research was to investigate the topographic properties of materials. In cooperation with the Faculty of Science of Masaryk University in Brno, for example, PS micro and nano structured surfaces with iPSCs were tested. The following chapters describe all the methodology that was needed to evaluate the experiment and discuss all the results achieved during the doctoral study.

### 7.1 Sample preparations

Most of the tested samples were prepared in collaboration with other faculties and colleagues. Here, the methodology is discussed in more detail only for samples that the author was able to prepare himself. Other methodological procedures are given in individual articles.

#### 7.1.1 Preparation of PPy powders

PPy-S was synthesized by oxidizing 0.2M pyrrole (Sigma-Aldrich, USA) with 0.5 M iron-(III) chloride hexahydrate (Sigma-Aldrich, USA) in an aqueous environment. The oxidant-to-pyrrole mole ratio was 2.5. The mixture was left to polymerize at room temperature for 12 h. The precipitated black PPy was collected on a filter, rinsed with 0.2 M hydrochloric acid followed by acetone, and dried at room temperature over silica gel<sup>7</sup>. The deprotonated form (PPy-B) was prepared by immersing the powder in 1 M aqueous ammonium hydroxide<sup>104</sup>.

#### 7.1.2 Preparation of PANI powders and films

Polyaniline salt (PANI-S) was prepared according IUPAC technical report<sup>105</sup>. Specifically, a 0.2 M aqueous solution of AnH (Penta, Czech Republic) was

oxidized with 0.25 M APS (Penta, Czech Republic). Polymerization was carried out at room temperature for 12 h. The resulting green solids of polyaniline salt were collected on a filter, rinsed with 0.2 M hydrochloric acid, and similarly with acetone, and dried at room temperature over silica gel. The deprotonated form (PANI-B) was prepared by immersing the powder in 1 M aqueous ammonium hydroxide.

The PANI films were formed *in situ* directly on the tissue culture plates (TPP, Trasadingen, Switzerland).

### **7.1.3 Preparation of PANI films functionalized with dopants**

PANI films containing dodecylbenzenesulfonic acid sodium salt (SDBS), 2-aminoethane-1-sulfonic acid (taurine), and N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) were prepared. The modification was performed in two ways: 1) By modification of the surfaces of neat PANI films, PANI-S, or PANI-B with SDBS, taurine or ACES, and 2) by adding the respective substance directly into a reaction mixture of aniline hydrochloride and ammonium peroxydisulfate used for the preparation of PANI.

1) PANI-S films were prepared according to the standard procedure given above<sup>105</sup>. A part of the samples was deprotonated by immersion in 1 M ammonium hydroxide for 12 h to convert to the blue form of PANI-B. For reprotonation of pure PANI-B, samples were exposed to 2 wt% solutions of SDBS, taurine or ACES (all from Sigma-Aldrich, USA). Successful reprotonation results in a change of color from blue back to green. This process was slow and took several weeks (for ACES even 3 months). Then, the solutions were removed, and the films were rinsed with methanol and left to dry in air. Modified PANI-S films were prepared by simple pouring each of the 2% solutions of SDBS, taurine or ACES onto the neat film. After 24 h, the solutions of dopants substances were removed, and the films rinsed with methanol and dried in air.

2) The second type of PANI film was prepared by adding the SDBS or taurine (concentration 40 gL<sup>-1</sup>) into a mixture of AnH and APS. The reaction mixture was poured directly onto the sample. The reaction was left to continue for 1 h. The films of PANI salts formed on the substrates containing respective dopants were then rinsed with 0.2 M HCl and were left to dry in the air.

## **7.2 Cell lines**

Here are described individually the cell lines that were used to evaluate the biological properties of the materials.

### **7.2.1 Culture of mouse embryonic fibroblast cell line NIH/3T3**

Cytotoxicity and proliferation assays of the materials were performed using a mouse embryonic fibroblast cell line (ATCC CRL-1658 NIH/3T3, USA). ATCC-



formulated Dulbecco's Modified Eagle's Medium (PAA Laboratories GmbH, Austria) containing 10% bovine calf serum (BioSera, France) and 100 U mL<sup>-1</sup> of Penicillin/Streptomycin (GE Healthcare HyClone, United Kingdom) was used as the cultivation medium.

### **7.2.2 Culture of ESCs**

The embryonic stem cell ES R1 line<sup>106</sup> was propagated in an undifferentiated state by culturing on gelatinized tissue culture dishes in complete media. The gelatinization was performed using 0.1% porcine gelatin in water. Complete medium with the following composition was used for the cultivation: Dulbecco's Modified Eagle's Medium, 15 % fetal calf serum, 100 U mL<sup>-1</sup> penicillin, 0.1 mg mL<sup>-1</sup> streptomycin, 100 mM non-essential amino acids solution (all from Thermo Fisher, Waltham, MA, USA), 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA) and 1000 U mL<sup>-1</sup> of LIF (Gibco, MA, USA)<sup>85</sup>.

### **7.2.3 Culture of iPSCs**

Experiments with iPSCs were performed at the Faculty of Science of Masaryk University in Brno. The cytocompatibilities of the materials were determined using two hiPSC lines of different origins, namely, the M67 line (derived from fibroblast from a 67-year-old male that was reprogrammed by episomal vector Epi5 Epi5 (ThermoFisher) and the Neo d1 line (derived from neonatal fibroblast (Lonza) and reprogrammed by Sendai virus using Cytotune kit 1.0 (ThermoFisher). HiPSC were derived and cultured according to the protocol of the European Bank for induced pluripotent Stem Cells.

## **7.3 Biological properties**

The majority of the presented work is devoted to the evaluation of biological properties of materials. Sample preparations for biological evaluation were often performed in accordance with ISO standard 10 993 Biological evaluation of medical devices.

### **7.3.1 Cytotoxicity of extracts of materials**

#### **Extracts preparation**

Sample extracts were prepared according to ISO 10993-12. The extraction was conducted in chemically inert closed containers using aseptic techniques at 37 ± 1 °C under stirring for 24 h. Subsequently, the extract was separated from the polymer powder by centrifugation at 1000 g for 15 min followed by the second centrifugation of supernatant under the same conditions. The parent extracts (100 %) were used for the testing of cytotoxicity according to the ISO 10 993-5 protocol. The parent 100% extracts were diluted in complete medium to obtain a series of dilutions. All extracts were used within 24 h. Prior to *in vitro* testing, the extracts were sterilized by sterile filtration through a 0.22 µm Millex GV filter (Merck, Darmstadt, Germany). All tests were performed in four separate sets.

## **MTT assay**

The MTT assay is widely used to quantitatively evaluate cell viability<sup>107</sup>. This test is based on the reduction of the yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple crystals of formazan<sup>108</sup>. MTT (Invitrogen Corporation, USA) is added to the cells as a solution, usually in a concentration 0.2 - 0.5 mg / ml, then the MTT cells are cultured for at least 4 hours. Viable cells with active metabolism are capable of using mitochondrial enzymes convert MTT to purple formazan. The amount of formazan is directly proportional to the number of viable cells and is measured by changing the absorbance at 570 nm using a spectrophotometer (Infinite M200 Pro NanoQuant (Tecan, Switzerland)). Formazan accumulates inside the cells in the form of crystals, before measuring the absorbance it is necessary to dissolve it using dimethylsulfoxide (DMSO)<sup>107</sup>.

## **ATP assay**

Another option to quantify cell viability is to use an assay of adenosine triphosphate (ATP). This method of ATP analysis is based on the linear luminescence response of firefly extracts to ATP<sup>109</sup>. The two-step oxidation of firefly luciferin in the presence of ATP, Mg<sup>2+</sup> and oxygen is catalyzed by firefly luciferase accompanied by visible light emissions<sup>110</sup>. If cells lose membrane integrity, they also lose the ability to synthesize ATP and the remaining ATP from cytoplasm is rapidly depleted by endogenous ATPases<sup>107</sup>. In the presented work, Cellular ATP Kit HTS (Invitrogen Corporation, USA) was used for ATP evaluation and luminescence was measured on an Infinite Lumi luminometer (Tecan, Switzerland).

### **7.3.2 Cell adhesion and proliferation**

Cell adhesion and proliferation are ones of the factors determining the biocompatibility of a material. In the case of adhesion, the cells were seeded on the samples in the concentration of  $1 \cdot 10^6$  cells mL<sup>-1</sup>. After one hour, the cells were stained with Hoechst 33258 (Molecular Probes, Carlsbad, CA, USA). To determine the ability of cells to proliferate on the surfaces, the cells were seeded at an initial concentration of  $1 \cdot 10^5$  cells mL<sup>-1</sup> and cultivated for 48 h (or longer, as needed). After 48 h the cells were fixed and stained with Hoechst 33258 and ActinRed 555 (Thermo Fisher Scientific, Waltham, MA, USA). Micrographs were taken using the fluorescence microscope Olympus IX 81 (Olympus, Tokyo, Japan).

### **7.3.3 Differentiation of cells**

The ESC differentiation was induced through the formation of embryoid bodies (EBs) by hanging drop techniques (400 cells per one 35  $\mu$ L drop) in LIF-free complete medium. The 5-day-old EBs were transferred to a gelatinized 24-well plate (one EB per well) and cultivated in serum-free DMEM-F12 media (1:1)

containing 100 U mL<sup>-1</sup> penicillin, 0.1 mg mL<sup>-1</sup> streptomycin and insulin–transferrin–selen (ITS) supplement (all from Thermo Fisher, Waltham, MA, USA) for the next 11 days. The medium was replaced with fresh medium every 2 days of cell culture. Differentiating cells were observed using an inverted Olympus phase contrast microscope (Olympus IX51, Tokyo, Japan) and documented with a digital camera (Olympus E-450, Tokyo, Japan). All test were performed in four separate sets.

### **Evaluation of differentiation Quantitative RT-PCR**

RT-qPCR allows direct quantification of cDNA during a reaction obtained by reverse transcription of RNA. The principle of the RT-qPCR method is the recording of PCR products in real time, ie. immediately after their formation and in each individual reaction cycle. Total RNA was extracted by Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized according to the manufacturer' instructions for RevertAid Reverse Transcriptase reverse transcriptase kit (Thermo Fisher, Waltham, MA, USA). Real-time PCR of neural cell phenotype markers was performed in a Roche 480 Light-Cycler using Light Cycler®480 DNA SYBR Green I Master (Roche, Basel, Switzerland). Primers and reference genes were selected according to current experiments. Data are presented as the differences between reference and samples treated with PPy extracts after normalization to the reference gene by the  $2^{-(Cq(\text{target})-Cq(\text{reference}))}$  method<sup>85</sup>.

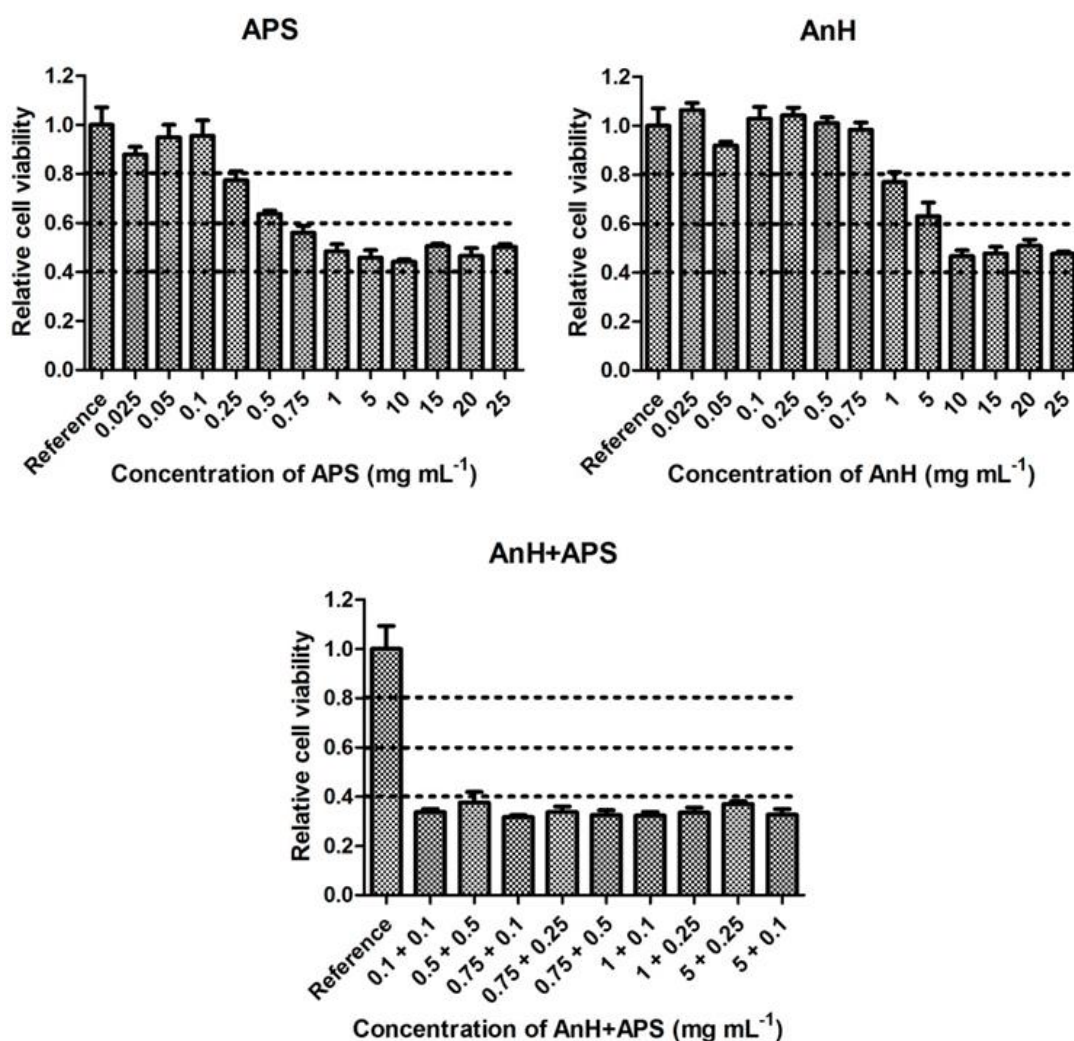
## 8. SUMMARY OF RESULTS

The author's main focus during doctoral studies was to deepen current knowledge about the interactions between materials, especially polymeric materials, and eukaryotic cells. Material that is placed into contact with human or animal tissue as part of a medical or cosmetic procedure is generally referred to as a biomaterial. One of the first conventional definitions of a biomaterial was established by Williams in 1987, this stating that a biomaterial is "a nonviable material used in a medical device, intended to interact with biological systems."<sup>44, 111</sup>. Biomaterials can be classified in several ways; for example, according to whether they are ceramics, metals, polymers, or composites<sup>52</sup>. The following discussion of the results will concern mainly polymeric materials.

Biomaterials applied in tissue engineering must meet several conditions with respect to their properties. One of them is biocompatibility. For example, the biomaterial must be non-toxic to the organism, biocompatible with blood, non-inflammatory, non-allergenic, and non-carcinogenic<sup>112</sup>. Concrete properties depend on the place of application or the duration of interaction. One of the basic ways to determine whether a biomaterial fulfils such conditions is to determine its cytotoxicity, either by direct contact or by monitoring the effect of the extract of the material on a cell population. However, negative responses of an organism to an applied material are not the only outcome. The material may also induce positive reactions, such as the targeted modulation of signaling pathways of differentiating cells. Other important material properties influencing cell behaviour are described in the theoretical part (chapters 5.1.1 and 5.1.2). On the basis of all results achieved in this research, my experimental work could be divided into three comprehensive activities: 1) testing the biocompatibility of PANI; 2) testing the bioactive properties of PPy; and 3) testing the influence of material topography on cell behavior.

1) *Testing the biocompatibility of PANI.* The application of CPs in tissue engineering is mainly connected to their easy synthesis, their good biocompatibility, and their conductivity<sup>113</sup>. In addition, CPs have the advantage over other conductive materials such as platinum or gold as they can be prepared in tissue-mimicking form, especially in the context of their mechanical properties. Unlike unyielding metals, CPs prepared in such a form can conform to tissues without causing adverse immune responses<sup>114, 115</sup>. In the literature, PANI is considered as one of the representatives of CPs with good biocompatibility suitable for clinical use. PANI salt is mostly prepared by the oxidative

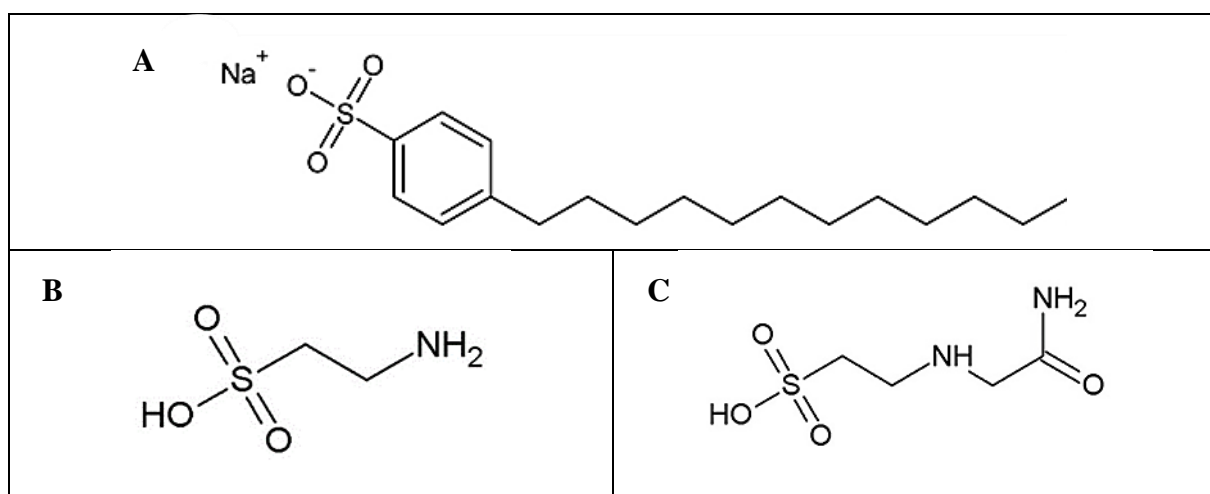
polymerization of Aniline (An) or aniline hydrochloride (AnH) with ammonium persulfate (APS) in an acidic environment<sup>116</sup>. Since the cytotoxicity of PANI is associated with precursors and impurities released from the material, it is important to know the cytotoxic effects of the individual reagents used for its synthesis. An assessment of the toxicities of individual reagents can be found in the literature. The study by Zhang et al.<sup>117</sup> deals with the cellular response of the NIH/3T3 line to aniline dimers, trimers, and tetramers. The aniline trimer exhibited the highest cytotoxicity. Further, Firoze et al.<sup>118</sup> investigated the subchronic toxicity of AnH in rats. The rats were given drinking water containing 600 ppm AnH. After AnH exposure, a decrease in erythrocyte, haemoglobin, and hematocrit count, and an increase in white blood cell count were observed. In addition, increasing iron content was noted in the spleen of rats with increasing exposure time. APS toxicity is summarized in a report by Pang and Fiume<sup>119</sup>. However, the precursors (AnH, An, APS) could be released into the external environment together and thus synergistically affect cell viability. No study has been published on the combination of individual precursors and its effect on cytotoxicity. The work by *Kašpárková V.; Humpolíček P.; Stejskal J.; Capáková Z.; Bober P.; Skopalová K.; Lehocký M. Exploring the Critical Factors Limiting Polyaniline Biocompatibility. Polymers. 2019, 11(2):362* is among the first to deal with this issue. Initially, the cytotoxic effects of individual precursors on the NIH/3T3 line were determined and subsequently their combined effect was investigated. As shown in Figure 12, APS loses its cytotoxicity at a concentration of 0.1 mg mL<sup>-1</sup>, while AnH does not even affect cell viability up to a concentration of 0.75 mg mL<sup>-1</sup>. The combination of concentrations of AnH and APS, which did not cause toxicity alone (for APS 0.1 and for AnH 0.75), considerably reduced cell viability to only 30% compared to the reference. Similar results were obtained with a combination of APS and An. To improve the biological properties of PANI, it is thus crucial to pay attention to the preparation in order to minimize the amount of releasable precursors. Therefore, in addition, the study modified PANI synthesis by employing various dopant acids, namely sulfuric, nitric, phosphoric, hydrochloric, and methanesulfonic. Phosphoric acid-doped PANI (PANI-P) exhibited the lowest toxicity. This finding also correlated with the determination of impurities for individual modifications. The lowest concentrations of AnH and APS were present in PANI-P extracts. The results presented in this study significantly improve our knowledge of the cytocompatibility of PANI and especially demonstrate how PANI synthesis can be modified to prepare a non-cytotoxic material.



**Figure 8:** Cytotoxicity of ammonium persulfate, aniline hydrochloride and their combinations

Another component of PANI biocompatibility is hemocompatibility. It is necessary that no excessive platelet adhesion or leukocyte activation leading to inflammation and coagulation should occur due to material<sup>87</sup>. Heparin is one of the best known and already commercially available anticoagulants<sup>120</sup>. Its anticoagulant properties are mainly associated with the presence of sulfate, sulfamide, and carboxylic groups and their conformation and steric order along the polymer chain<sup>92</sup>. In their work, Humpolíček et al.<sup>93</sup> modified PANI films using PAMPSA. Such modified films were shown to exhibit a significant reduction in platelet adhesion and also had an impact on coagulation parameters such as thrombin clotting time (TCT), activated partial thromboplastin time (aPTT), and prothrombin time (PT). Blood clotting on PANI-PAMPSA was prevented. In the present study, following on from the work of Humpolíček et al., the PANI was

modified with dodecylbenzenesulfonic acid sodium salt (SDBS), 2-aminoethane-1-sulfonic acid (taurine), and N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) (Figure 13). The modifications were made in two ways: 1) by modifying the surface of the PANI salt and the PANI base with the mentioned dopants, 2) by adding the respective substances to the reaction mixture of AnH and APS. As the substances contain similar functional groups to heparin and PAMPSA, a change in haemocompatible properties was expected. When determining the coagulation parameters (aPTT, TCT, PT), however, no differences were found between the modified samples or in comparison with the reference. This is probably due to the fact that SDBS, taurine and ACES are low molecular weight substances, while heparin and PAMPSA are high molecular weight substances. More detailed results are published in by *Skopalová K.; Capáková Z.; Bober P.; Pelková J.; Stejskal J.; Kašpárková V.; Lehocký M.; Junkar I.; Mozetič M.; Humpolíček P. In-Vitro Hemocompatibility of Polyaniline Functionalized by Bioactive Molecules. Polymers. 2019, 11, 1861.*



**Figure 9:** The formula of A) SDBS, B) taurine, and C) ACES

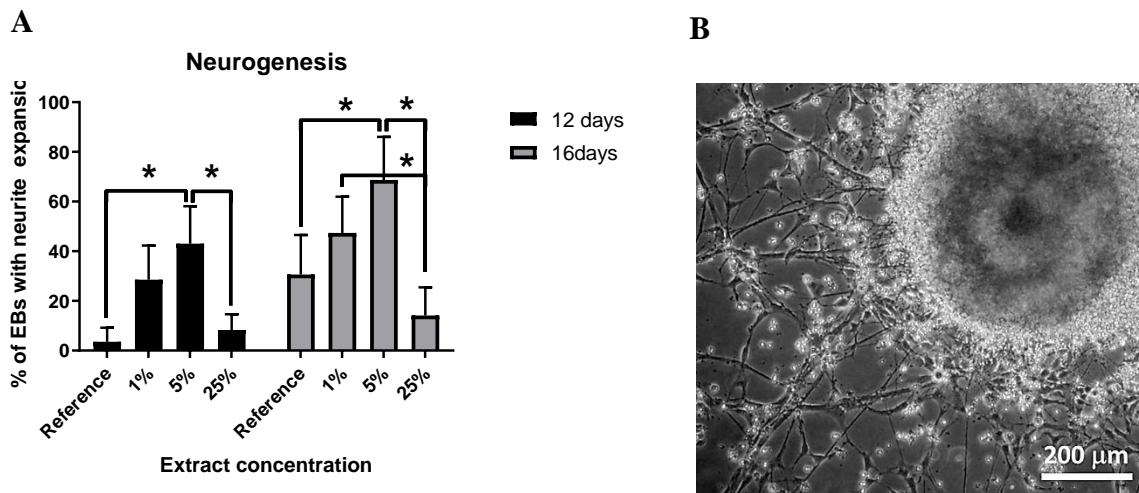
2) *Testing the bioactive properties of PPy.* As already mentioned, the elimination of a negative cellular response during interaction with materials is a prerequisite for modern biomaterials. Conversely, the material may also induce a targeted positive response and affect, for instance, signaling cascades in cells leading to differentiation. Polypyrrole, another representative of CPs considered for applications in tissue engineering, exhibits, like PANI, good biocompatibility and conductivity, and can easily be synthesised<sup>121</sup>. Due to its conductivity, it is used mainly in relation to electrosensitive tissues, such as heart or nerve. Gomez et al.<sup>122</sup>, for example, studied the effect of PPy with immobilized growth factor NGF on the surface of the material on the rat PC12 cell line. The combination of

PPy and NGF promoted neurite outgrowth. Further, when an electric field was applied, even greater neurite growth of up to 50 % was observed compared to samples without stimulation. Zhang et al.<sup>123</sup> investigated the behavior of PC12 cells on a biodegradable PPy-coated polymeric membrane. They applied direct currents of various intensities to the membranes. The intensity of the current affected the number of emerging neurites. Composite materials containing PPy influenced the differentiation of the cells into neural phenotypes. From the abovementioned studies, it is therefore clear that PPy has a potential for application in the tissue engineering of neural tissue.

As with PANI, low molecular weight by-products and impurities are formed during the synthesis of PPy. Nevertheless, the starting material for the synthesis of PPy is pyrrole, which, together with its derivatives, serves as a source of pharmacologically active substances. For example, 2-Substituted-1,4,5,6-tetrahydrocyclopenta [b]pyrrole or 1H-pyrrole-2,5-dione derivatives have anti-inflammatory effects. Furthermore, pyrrole 3-carboxamide derivatives show anti-depressant stimulation. Pyrrole derivatives are also used as anticancer, anti-malarial, insecticidal, and anti-hypertensive agents<sup>124</sup>.

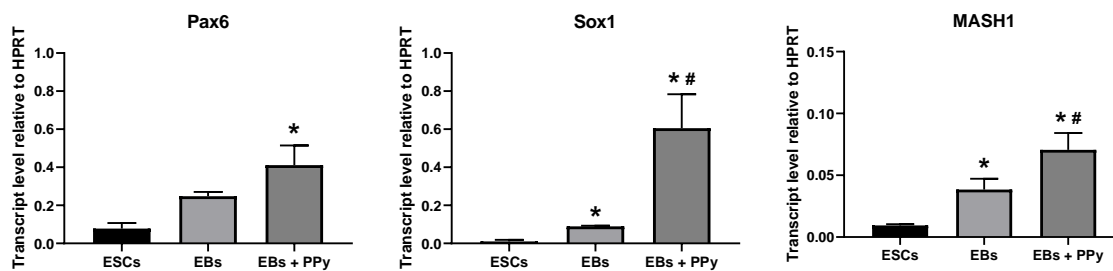
To date, it has not been possible to find studies in the literature dealing with the effects on cell behavior of PPy and its low molecular weight products released into the environment. The work by *Skopalová K.; Radaszkiewicz K.A.; Kašpárková V.; Stejskal J.; Bober P.; Junkar I.; Mozetič M.; Capáková Z.; Lehocký M.; Kašparová M.; Pacherník J.; Humpolíček P. Modulation of Differentiation of Embryonic Stem Cells by Polypyrrole: The Impact on Neurogenesis. International Journal of Molecular Sciences. 2021; 22(2):501* focused on the effect of polypyrrole extracts on the neurogenesis of mouse ESCs. No neuro-supportive effect was observed with PPy base; thus, the presented results demonstrate only the effect of PPy salt extracts. On the basis of previous tests of the cytotoxicity of PPy extract on mouse ESCs, EBs were formed by the hanging drop method in the presence of 1, 5 and 25 % concentrations of the extract for 5 days. Subsequently, EBs were cultured on adherent plastic without the presence of serum in culture medium for 7 to 11 days. As shown in Figure 14 A), EBs treated with 5 % PPy extract formed the most abundant neurites (Figure 14 B).





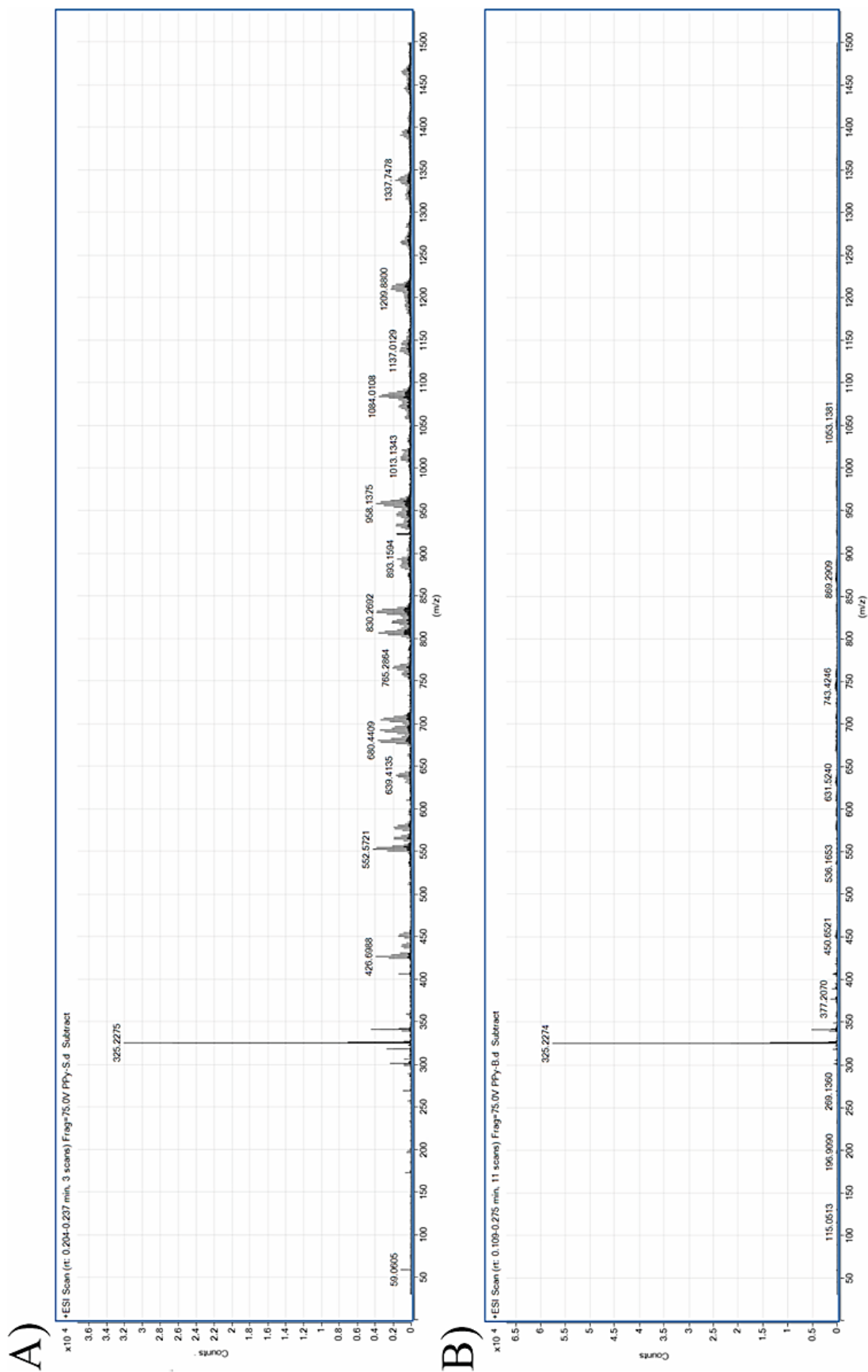
**Figure 10:** A) formation of EBs with neurite expansion after treatment with PPy extracts. B) microscopic visualization of expanded EB with neurites.

In the light of previous findings, the expressions of early neurogenic transcripts in 5-day-old EBs growing in the presence of 5 % PPy extract were evaluated. The PAX6, SOX1 and MASH1 transcripts were selected as representatives of important factors in neurogenesis. For comparison, the expressions of these transcripts were also observed in undifferentiated ESCs and in EBs formed in the absence of extract of PPy. As expected, the expressions of all transcripts were the lowest in undifferentiated ESCs; in the case of EBs formed without extract treatment, the increase in expression was higher due to spontaneous differentiation. The highest expression was recorded for EBs formed in the presence of 5% PPy extract (Figure 15). The effect of PPy extract on neurogenesis was also confirmed by Western blot assay. Neuron-specific proteins such as N-cadherin, N-CAM,  $\beta$ -III-tubulin, and doublecortin were evaluated on day 16 of differentiation. Similar results were obtained as for the expressions of neuro transcripts. A significant increase in the expressions of all tested neuroproteins in the cells was recorded in samples treated with 5 % PPy extract, the values higher by 50 % or more.



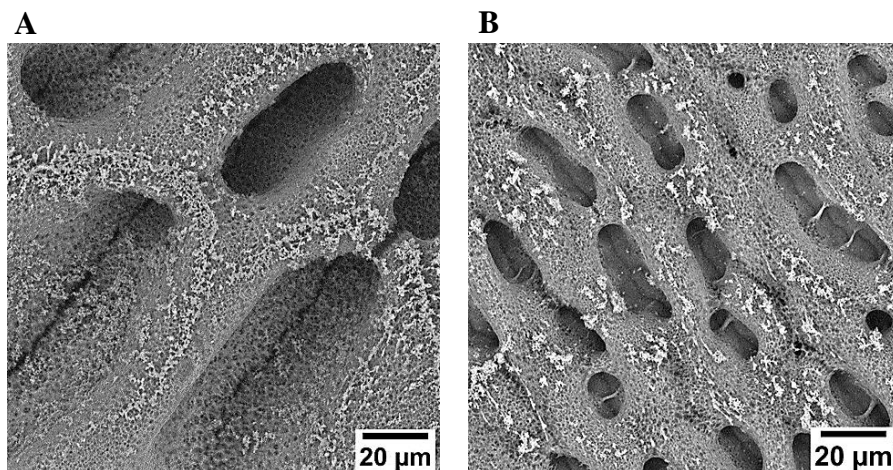
**Figure 11:** The expression of early neurogenic transcripts (*Pax6*, *Sox1*, and *MASH1*) in non-differentiating ESC and in 5-day-old EBs growing in the absence (EBs) or in the presence of 5% PPy extract (EBs + PPy)

Pyrrole-containing compounds have the ability to inhibit the activity of the GSK3 signaling protein<sup>125, 126</sup>. Other kinases such as Akt (or PKB) or ERK can phosphorylate and thus also modify the activity of GSK3<sup>127, 128</sup>. Akt kinase is involved in processes such as proliferation, differentiation, and protein synthesis. When the signaling pathway is disrupted, it leads to neurodegenerative diseases<sup>129</sup>. The ability of PPy extract to inhibit Akt signal protein and ERK kinases was also determined. Modulation of these signaling pathways by 10 % PPy extract was noted. On the basis of all the results obtained, it can be stated that PPy extracts contain low molecular weight products that play a role in the neurogenesis of ESCs. The presence of low molecular weight substances was determined by electrospray mass spectrometry. Figure 16 shows the results for both PPy salt (A) and PPy base (B). In both graphs, we see a dominant molecular ion at 325 m/z. This weight is assigned to the pentamer of PPy. Since the existence of pentamer is present in both salt and base, but base had no effect on neurogenesis, it could be said that pentamer is not a key low molecular weight product. Higher weights then correspond to oligomers containing, for example, oxygen and by-products. Thus, neurogenesis is induced by species with higher m/z than 325. However, the identification of these species was beyond the scope of this study and will need to be further investigated in the future. This research reveals new and remarkable properties of PPy which have never been observed before. Scientists should keep in mind that the effect of native PPy on neuronal differentiation must also be considered when working with this polymer.



**Figure 12:** Positive-ion ESI-MS spectrum of polypyrrole extract; (A) polypyrrole salt, (B) polypyrrole base

3) *Testing the influence of material topography on cell behaviour.* Culturing cells on the smooth surface of tissue plastic, which is commonly used technique, does not mimic the 3D environment in which cells usually occur *in vivo*. By modifying the topography of the material surfaces, it is possible to create a so-called pseudo-3D models that are advantageous for relatively stable and repeatable experiments. The ready-to-submit manuscript **Skopalová K.; Radaszkiewicz K. A.; Kadlečková M.; Capáková Z.; Pacherník J.; Minařík A.; Kašpárková V.; Daďová E.; Humpolíček P.** *Hierarchically structured surfaces amplifying fluorescence signals: cytocompatibility with human induced pluripotent stem cells*, the publication of which is expected in the coming weeks, is devoted to hierarchically structured PS surfaces. Two types of surfaces were tested, one with a macro/micro structure and the other with a meso/micro structure (Figure 17).



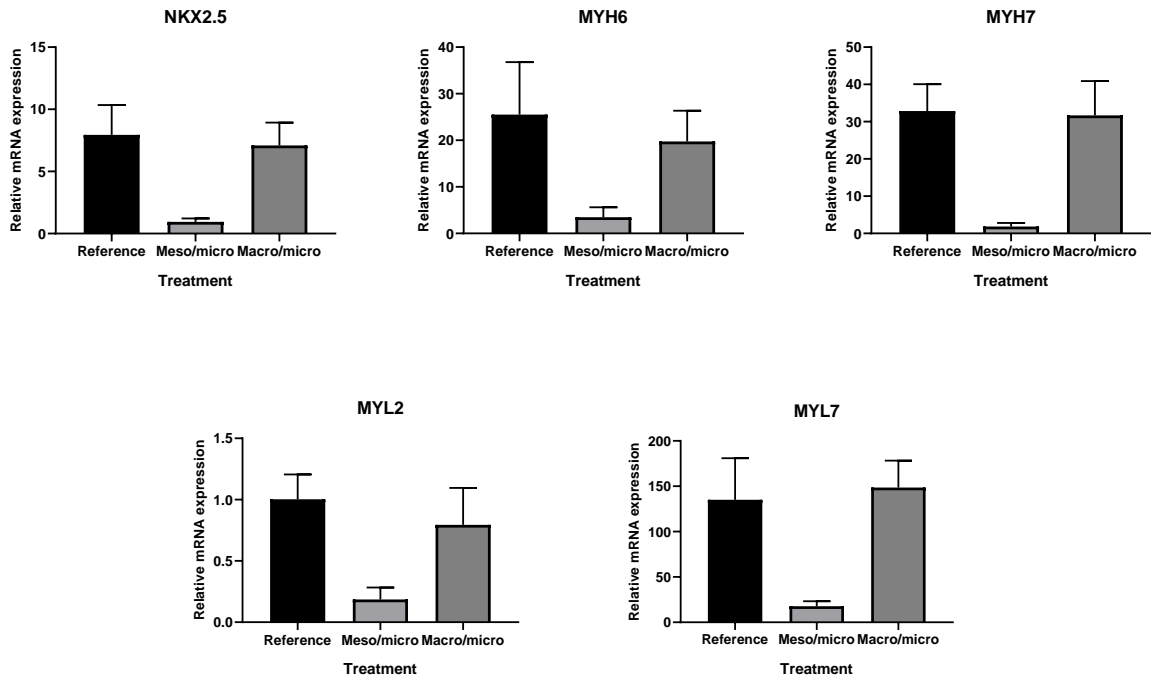
**Figure 13:** SEM images of macro/micro structured substrate A), and meso/micro structured substrate

Wrzecionko et al.<sup>130</sup> studied the effect of similarly structured surfaces (although without a hierarchical structure) on the proliferation of NIH/3T3 mouse embryonic fibroblasts. In addition, these authors modified the wettability of the surfaces by plasma treatment. It was found that the surface structure itself did not affect the morphology of fibroblasts and played a secondary role here, in contrast to the plasma treatment. Nevertheless, mouse fibroblasts are already differentiated cells that do not respond as sensitively to external stimuli as undifferentiated cells. In our work, the influence of PS-structured substrates on the behavior of ESCs was studied. The results are summarized in a submitted manuscript *Kadlečková, M. .; Skopalová K.; Ptošková, B.; Wrzecionko, E.; Daďová, E.; Kocourková, K.; Mracek, A.; Musilová, L.; Smolka, P.; Humpolíček, P.; Minařík, A.* *Hierarchically*

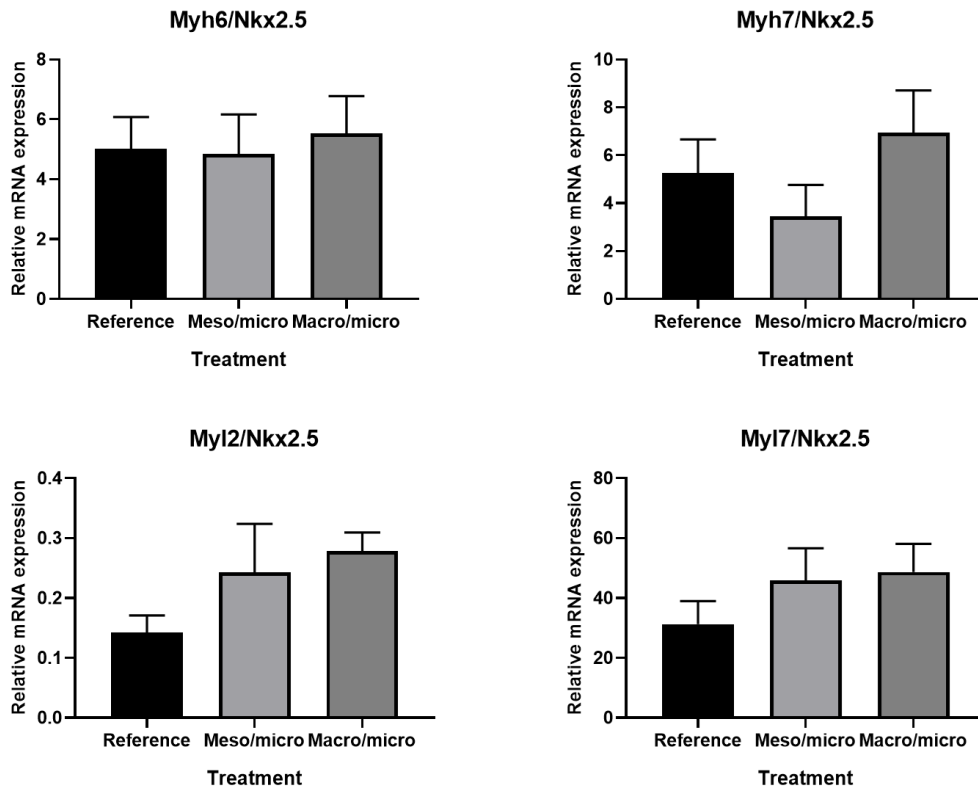
*structured surfaces prepared by phase separation: Induction of stem cells differentiation, Langmuir 2021.* Briefly, hierarchically structured surfaces were found to affect the shape of ESCs. Cells that had more space for differentiation and growth took on a polygonal shape, while cells growing on a surface with smaller pores took on a spherical shape similar to the undifferentiated state. However, the manipulation of ESCs could face ethical issues. The ready-to-submit manuscript tested hierarchically structured surfaces using human iPSCs (hiPSCs). The properties of iPSCs and the advantages of using them are described in chapter 3.2.2 Induced pluripotent stem cells. One of their main advantages is that the patient's own cells can be used in experiments and thus also for therapy and treatment<sup>131</sup>. First, it was determined whether undifferentiated forms of hiPSCs cultivated on a sample were capable of growing and proliferating. The cells were able to proliferate on both types of surfaces (hierarchical meso/micro and macro/micro).

hiPSCs can give rise to different cell lines. However, the methodology used in the manuscript led to cardiomyogenesis. In the body, cardiomyocytes have only a limited proliferative capacity, and if the heart is damaged, for example by a myocardial infarction, adult cardiomyocytes are unable to completely regenerate the tissue<sup>132</sup>. hiPSCs could serve as a rich source of cardiomyocytes and thus participate in the treatment of damaged hearts. However, iPSC-derived cardiomyocytes (iPSC-CM) are often phenotypically immature compared to adult cardiomyocytes<sup>133</sup>. By appropriate selection of the material and structure for cultivation, the maturation could be improved. For example, in the work of Xu et al., a three-dimensional structure similar to onion epithelium which promoted cardiomyocyte maturation was prepared<sup>134</sup>.

Several representatives of cardiomyogenesis-related genes, namely Nkx2.5, MYH6, MYH7, MYL2 and MYL7, were thus selected to determine the presence of a cardiomyocyte population on hierarchically structured surfaces. As can be seen in Figure 18, the expression of cardio genes was significantly higher on the macro/micro structure. Nevertheless, if we relate the ratio of the MYH6, MYH7, MYL2 and MYL7 genes to Nkx2.5, which could be taken as a reference in the case of the cardiomyocyte population, we see that neither surface inhibited their maturation (Figure 19). The results show that none of the surface types inhibited hiPSC-CM maturation. Different topography played a role only in the capture of hiPSC-CM and not in maturation. Cardiomyocytes adhered better to the macrostructure.

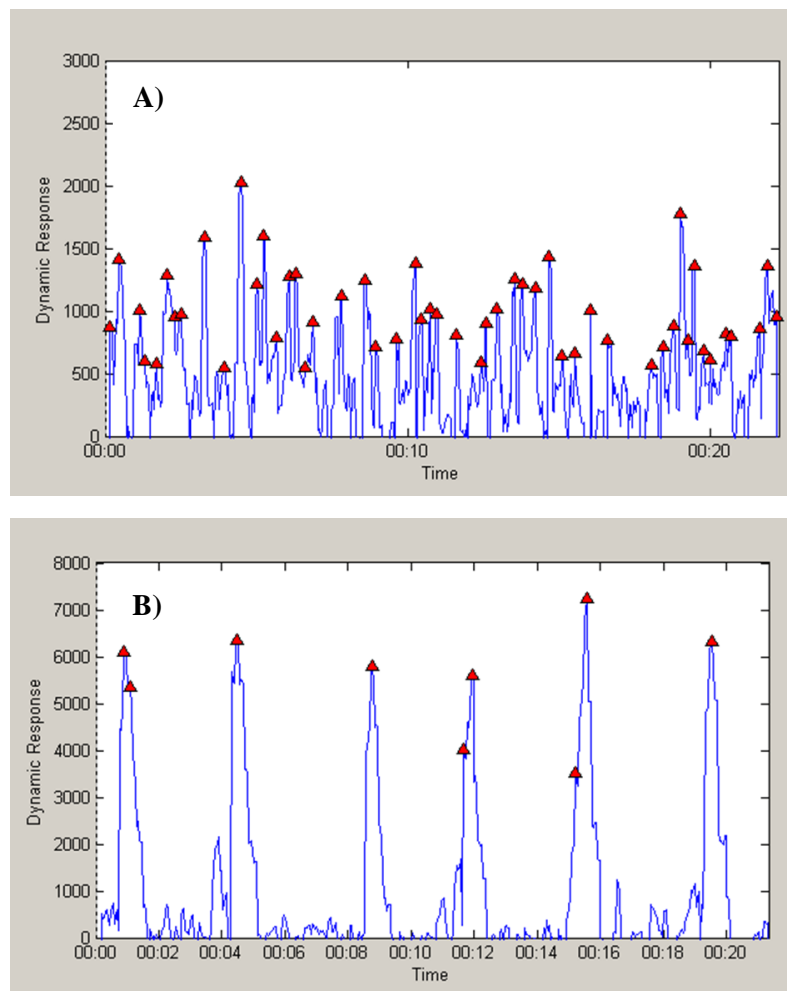


*Figure 14: The expression of markers of cardiogenesis*



*Figure 15: Ratio of gene expression (Myh6, Myh7, Myl2 and Myl7) to reference gene (Nkx2.5). Evidence of mature cardiomyocytes.*

The presence of mature cardiomyocytes was also determined by monitoring  $\text{Ca}^{2+}$  flow on the substrates. This highly specific intricate regulatory system develops gradually, with progressive maturation of specialized structures and increasing capacity of  $\text{Ca}^{2+}$  sources and sinks<sup>135</sup>. Remarkably, through this analysis the extraordinary properties of structured substrate was determined. Modification of tissue culture plastic leads to amplification of fluorescence signal (Figure 20). The modified substrates (based on common tissue culture plastic) can be thus well used for increasing sensitivity of  $\text{Ca}^{2+}$  transition analyse in contracting cardiomyocytes. It simply make possible to used common camera for taking of record or low concentration of  $\text{Ca}^{2+}$  sensitive fluorescent probes, and thus decrease of phototoxicity in this assay.



**Figure 16:** Representative records of  $\text{Ca}^{2+}$  intracellular wave in beating cardiomyocyte determined by Fura4 probe. Record in hiPSC-CM growing on native (A); macro/micro (B) substrates.

During her studies, the author also participated in testing the biological properties of polymeric materials intended for the production of scaffolds. Specifically, she took part in the work by *Shah R.; Stodulka P.; Skopalová K.; Saha P. Dual Crosslinked Collagen/Chitosan Film for Potential Biomedical Applications. Polymers. 2019, 11(12):2094*, where, inter alia, the adhesion and proliferation of NIH/3T3 mouse fibroblasts were tested on films prepared by a combination of collagen and chitosan. Furthermore, in the work by *Kovalčík A.; Sangroniz L.; Kalina M.; Skopalová K.; Humpolíček P.; Omastová M.; Mundigler N.; Müller A.J. Properties of scaffolds prepared by fused deposition modeling of poly(hydroxyalkanoates). International Journal of Biological Macromolecules. 2020, 161, 364-376*, the author participated in testing the effect of poly (hydroxyalkanoates) on cell viability and proliferation.



## 9. CONTRIBUTION TO SCIENCE

Although biomaterials have known to human activity since ancient times, and research into new biomaterials for the preparation of scaffolds has been at a high level in recent years, interactions between cells and materials are still not fully explored. Preparing a functional scaffold that will find its use in biomedicine is still a considerable challenge for researchers today. The presented work deals mainly with the impact of the biochemical and geometric properties of the material on the behavior of different cell lines. During her doctoral studies, the author was able to exploit the laboratory handling skills she acquired during her Master's degree studies at the Faculty of Technology and the Center for Polymer Systems. These skills were enriched by the possibility not only of culturing common lines of differentiated cells but also of initiating the culturing of stem cells at the Center of Polymer Systems. For example, in cooperation with the Faculty of Science of Masaryk University in Brno, the methodology for the cultivation of mouse ESCs was gradually introduced. The introduction of lines that are able to differentiate due to external stimuli has offered new possibilities for the study of cell-material interactions.

Due to the author's involvement in the project GAČR 17-05095S (Biomimetic materials based on conductive polymers), a large part of her work was devoted to the study of the biological properties of CPs. This research mainly concerned two representatives: PANI and PPy. The degree to which the properties of CPs are biocompatible is connected with the method of preparation. PANI is usually prepared by the oxidation of AnH and APS in an acidic environment. The toxicity of individual precursors is reported in the literature. During the author's research into the cytobiocompatibility of PANI, her findings were enriched with information on the synergistic effects of precursors. These results are important, especially when choosing the method of PANI synthesis. The hemocompatibility of the material is also important for practical applications. In the light of the known anticoagulant effects of heparin and PAMPSA, the author tried to prepare novel modified PANI films with anticoagulant properties. Unfortunately, the used low molecular weight substances (SDBS, taurine, ACES) did not meet the given expectations. However, even these results could be useful to researchers interested in a similar goal.

As with PANI, the synthesis of PPy is accompanied by the formation of low-molecular-weight by-products that could be extracted into the culture medium or directly into the body. However, during the study of these low molecular weight

products, new and important findings were revealed. The author's work shows that low molecular weight PPy products induce neurogenesis in mouse ESCs. So far, most published studies have focused on the induction of neurogenesis by PPy itself but in the presence of an electric field. Due to these properties, PPy could serve as a bioactive material for targeted cell differentiation in the future, perhaps without the need for electrical stimulation.

Another part of the author's doctoral research was devoted to the topographic properties of the material. Structured surfaces of macro and meso PS were studied in the context of hiPSC behavior. In this context, only limited information about the impact of surface properties and structures on hiPSC behavior is so far available. The cytocompatibility of these structured surfaces was determined on undifferentiated hiPSCs; it was found that both surfaces were suitable candidates for culturing hiPSCs. In addition, the material did not inhibit cardiomyocyte maturation. Moreover, the structured surface amplified the fluorescence signals when measuring Ca<sup>2+</sup>, by a normal camera for recording signals.

Further, the author was involved in the GAČR 19-16861S project (Interaction between biomaterials and stem cells in simulated *in vivo* conditions). Here, she focused on the introduction of the cultivation of common differentiated cell lines and pluripotent cell lines in a flow chamber in dynamic conditions. The results achieved so far are only preliminary and the author will continue to work in this area. In the future, the author would like to test the behavior of ESCs, MSCs, and hiPSCs on different topographies of materials by means of culturing in dynamic conditions.

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# LIST OF ABBREVIATIONS AND SYMBOLS

Alphabetically ordered

2D	Two-dimensional
ACES	N-(2-acetamido)-2-aminoethanesulfonic acid
An	Aniline
AnH	Aniline hydrochloride
APS	Ammonium persulfate
ASCs	Adult stem cells
ATP	Adenosine triphosphate
CPs	Conductive polymers
D4	Octamethylcyclotetrasiloxane
DBD	Dielectric Barrier Discharge
DMSO	Dimethylsulfoxide
EBs	Embryonic bodies
ECM	Extracellular matrix
ESCs	Embryonic stem cells
EO	Ethylene oxide
ICM	Inner cell mass
iPSCs	Pluripotent stem cells
LIF	Leukemia inhibitory factor
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF	Nerve growth factor
PAMPSA	Poly(2-acrylamido-2-methyl-1-propanesulfonic acid)

PANI	Polyaniline
PCL	Polycaprolactone
PLA	Poly(lactide)
PLGA	Poly(lactide- <i>co</i> -glycolide)
PMMA	Poly(methylmethacrylate)
PPy	Polypyrrole
PS	Polystyrene
PSCs	Pluripotent stem cells
PUR	Polyurethane
SDBS	Dodecylbenzenesulfonic acid sodium salt
Taurine	2-aminoethane-1-sulfonic acid



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