



Tomas Bata University in Zlín

Faculty of Technology

Doctoral Thesis

Modification of polymers to the targeted cell response

Modifikace polymerních materiálů za účelem cílené buněčné reakce

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ABSTRACT

Polymers play a crucial role in the preparation of biomaterials for tissue engineering and regenerative medicine. The biocompatibility and ability to be combined with other materials to produce desirable 3D structure are critical properties for their real application. The thesis is focused on the modification of polymers in its native form, composites or copolymers with mentioned properties to achieve desirable interaction with the eukaryotic cells in terms of the cell adhesion, growth, proliferation, differentiation and death. The interaction of materials with the cells was studied in the cell laboratory using advanced *in vitro* techniques.

Key words: *biomaterials, tissue engineering, bioreactor*

ABSTRAKT

Polymery hrají klíčovou roli v oblasti vývoje biomateriálů využitelných v oblasti tkáňového inženýrství a regenerativní medicíny. Předpokladem pro jejich aplikaci je jejich biokompatibilita a kombinovatelnost s dalšími materiály vytvářejícími vhodnou 3D strukturu s požadovanými objemovými vlastnostmi. Předmětem práce je modifikace polymerů ať již samostatných či v kopolymerech a kompozitech za účelem cíleného ovlivnění buněčné vazby, růstu, proliferace, diferenciaci a smrti. Interakce materiálu s buňkami byla studována pomocí pokročilých *in vitro* metod v rámci laboratoře buněčných kultur.

Klíčová slova: *biomateriály, tkáňové inženýrství, bioreaktor*

1. INTRODUCTION

Today, polymers represent irreplaceable material in many fields of human activities. Various types of polymers already found their firm place in many areas of industry including building, electronics and textile industry as well as fabrication of products for daily use.

Besides above mentioned, polymers also have become very attractive for biomedical applications - either as materials utilized as sensors, medical devices or scaffolds in tissue engineering. In fact, they have become the most widely used material in these applications. To be specific, silicone hydrogel ocular lenses, polypropylene disposable syringes or blood bags made from polyvinyl chloride could be mentioned. Moreover, a lot of additional polymeric materials seem to be convenient for biomedical application - e.g. polyvinyl alcohol (Li, Jiang et al. 2015), polylactic acid (Ramot, Haim-Zada et al. 2016), polycaprolactone (Calandrelli, Calarco et al. 2008), polyglycolic acid (Gao, Niklason et al. 1998, DiCarlo, Hu et al. 2009), polypropylene fumarate (Wang, Lu et al. 2006) or polyhydroxyalkanoate (Li, Zhang et al. 2008).

In the first instance, the interest was focused on natural polymers, because of their good material-tissue compatibility. However, the use of natural polymers such as collagen or fibrin can bring also several disadvantages – e.g. limitation of availability, purity of animal-derived polymers, the risk of transfection of disease or inducing immune response, batch-to-batch consistency and mainly insufficient of mechanical properties for some applications and unsatisfactory degradation rates (Yao, Tao et al. 2011). The synthetic polymers lack some of these disadvantages and, moreover, exhibit additional advantages. One of the main advantages is their production flexibility (Liu and Ma 2004). Various low-cost products with controlled properties including mechanical properties (strength), biodegradation rate, chemical composition or (micro) structure can easily be prepared (Liu and Ma 2004). Synthetic polymers are also much more reproducible and show in general longer shelf-life in comparison with the natural ones (Ozdil and Aydin 2014). Furthermore, some synthetic polymers can offer some advanced properties such as intrinsic conductivity (polyaniline, poly(3,4-ethylenedioxythiophene), or polypyrrole). Because of mentioned properties, there has been noticed the increasing interest of scientists to prepare novel polymer-based materials suitable for biomedical applications¹ in recent years.

¹ *Despite (or perhaps because of) the great popularity of polymers in bio-application, the terms “biological application” or “biomedical devices” are not always properly used in literature. For the purpose of this thesis the “medical device” is defined according to ISO 10 993 -1 as any device, apparatus, instrument, material or any other product (including any software necessary for its utilize), which is determined to utilize within the human body, exclusive or predominantly for the purpose of a) diagnosis, prevention, monitoring, therapy or alleviation of the illness; b) diagnosis, monitoring, therapy or alleviation of injury or disability; c) investigation, compensation or modification of anatomical structure or physiological process; and d) control of conception. It also has no primary pharmaceutical, immunological*

2. BIOMATERIALS

During the event of the first Consensus Conference of the European Society for Biomaterials in 1976, a term “biomaterial” was presented as “a nonviable material used in a medical device, intended to interact with biological systems” (O'Brien 2011). However, since then the definition has been adjusted several times and several variants exist. Now, the most acceptable definition describes biomaterial as “any synthetic or natural substance (or combination of substances), which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual” (Bergmann, Stumpf et al. 2013). This definition seems to be not complete because it does not include the materials from which the e.g. medical disposable supplies or surgical instruments are made. This shift, however, is deliberate and corresponds to the shift of the biomaterials' field of study. Its issue of interest has been focused rather on tissue regeneration currently (O'Brien 2011). In this context, it should be stated the term “biomaterial” as an “abbreviation” of “biomedical material” - material for tissue engineering and regenerative medicine.

There are three main groups of biomaterial – the metals, ceramics and polymers. Moreover, the groups are further subdivided (Williams 2009). The metals include pure metallic materials and alloys, the ceramics subsume glasses, glass-ceramic and carbons. The last group, polymers, comprises thermosets, thermoplastics and elastomers. From another point of view, the polymers should be divided into both synthetic (human-made) polymers and biopolymers (naturally occurred in nature). Of course, biopolymers were the first choice to be utilized as biomaterials – e.g. sutures made from horse hair, linen or cellulose (Love 2017).

To be accepted as the biomaterial, the material has to satisfy a lot of individual criteria. In general, it should have suitable physical and chemical properties (including e.g. mechanical, thermal, electrical or magnetic characteristics, solubility or leaching), required biodegradability (biodegradable or biostable according to intend to use), biocompatibility and sterilizability (Viney 2013). Of course, the manufacturability is also important - it should be cost-effective and scale-up from making in a research laboratory to small batch production (O'Brien 2011). Moreover, to go more in depth, the surface properties are also very important and, in case of three-dimensional (3D) biomaterial scaffolds, there are also important bulk properties (Ratner 2013, Viney 2013).

or metabolic effect, however, its function should be supported with these effects. Medical devices are different from the drugs and their biological assessment requires a different approach.

3. POLYMERIC BIOMATERIALS

The term polymer originated from the connection of two Greek words poly (many) and meros (part) (Chanda 2013). Polymers naturally occurred in living systems e.g. deoxyribonucleic acid - gene coding polymer, cellulose - an important structural unit of the plant, collagen - the main component of the matrix surrounding cells in a living organism. As already mentioned, natural (polymeric) materials were used in surgery thousand years ago (Love 2017).

Nowadays, various polymeric materials should be artificially synthesized with tunable properties. Synthetic polymers should be divided into two main categories - non-biodegradable and biodegradable (Migonney 2014). Biodegradation is occurred hydrolytically or enzymatically under *in vivo* conditions (Migonney 2014). Based on their nature, biodegradable polymers have their application potential as temporary devices - e.g. transient implants, tissue engineering scaffolds and drug delivery system (Nair and Laurencin 2007).

Focusing on synthetic biodegradable polymers, aliphatic polyesters create one of the most discussed groups. The biodegradation of these polymers is based on the hydrolysis of the ester groups presented in the polymer backbone (Albertsson, Hakkarainen et al. 2008). This class of polymers should be easily obtained e.g. *via* ring opening and condensation polymerization based on the used monomer (Nair and Laurencin 2007). Polylactide, polyglycolide or poly(ϵ -caprolactone) and their copolymers are commonly utilized in medical devices (Lopes, Jardini et al. 2012) and interest of scientists still continue. As an example, the study of Ju and co-workers should be mentioned. They synthesized a self-assembled structure based on poly(ϵ -caprolactone) modified with glutathione and carnosine with promising potential in medicine (Ju, Zhang et al. 2017).

Also, non-biodegradable polymers have their no substitutable position in biomedical application, especially in devices, where long term stability is required (Migonney 2014). As an example, the poly(methyl methacrylate) based bone cement or intraocular lens (Teo, Mishra et al. 2016), polytetrafluoroethylene based heart valves (Jaganathan, Supriyanto et al. 2014) or polyamide or polypropylene sutures (Maitz 2015) should be mentioned.

4. BIOCOMPATIBILITY

As biomaterial is in direct contact with the living body, the biocompatibility is the very first criterion, which must be completely fulfilled. However, despite of its simple description as compatibility of material with the living system, the term of biocompatibility is really wide and is composed of complex of “non-properties” including non-toxic (including cytotoxicity, genotoxicity, reproductive, systematic, subchronic and chronic toxicity), non-immunogenic (not induce the immune response), non-thrombogenic (not result in thrombosis or coagulation of the blood), non-carcinogenic (not cause cancer) or non-inflammatory (not induce inflammation) (Elshahawy 2011, Davim 2014, Basu

2016). The criteria, which must any material fulfilled, depend on exposure time (temporary x permanent) and place of interaction (nature of body contact) (FDA 2000).

The overall process of biological testing of any material is complex and long term. It is composed of several individual steps. The testing usually starts with the risk assessment – the collecting relevant data on the materials, using the literature, clinical or animal study experiences, considering the proposed clinical use² of the device, manufacturing process and also the sterilization process (FDA 2000). Furthermore, the material characterization is typically conducted in advance of biological testing, because e.g. surface properties and material geometry could also affect biocompatibility (e.g. thrombogenicity) (dos Santos, Brandalise et al. 2017).

After this preparative step, the *in vitro*³ screening is performed. The utilizing of cell lines offers a lot of advantage for biocompatibility evaluation. First of all, the tests are generally cheaper and faster compared to *in vivo* tests, the lower amount of tested material is required and the lower amount of toxic waste is produced (Kandárová and Letašiová 2011). Moreover, the possibility of utilizing human cell lines or transgenic cells carrying the human genes makes *in vitro* methods very attractive (Kandárová and Letašiová 2011, Niu and Wang 2015). Furthermore, stem cells are considered as a prominent screening tool due to their ability to differentiate *in vitro* into various cell types (Tandon and Jyoti 2012). Embryonic stem cell test, which utilizes mouse embryonic stem cells to evaluate the embryo and related developmental toxicity, should be mentioned as an example (Conde-Vancells, Vazquez-Chantada et al. 2018). Moreover, stem cells should be also used for *in vitro* assessment of reproductive, organ and functional toxicity (Liu, Yin et al. 2017). Lately, with the discovery of somatic cell reprogramming into a stem-like state, originated induced pluripotent stem cells have found their application in developmental testing (Tandon and Jyoti 2012).

However, the biggest disadvantage of *in vitro* is the lack of system effect (Kandárová and Letašiová 2011). Therefore, the last step of a biological assessment is confirmation using *in vivo* testing and finally clinical trials. Nowadays, a great effort to reduce animal testing exists. As a consequence, the increase in a number of validated alternative methods has been recorded, e.g. tests utilizing *in vitro* reconstructed human 3D tissue models (as an example see Figure 1). These models have a lot of advantages. As they are constructed from normal

²The term “proposed clinical use” includes especially the anatomical location, duration exposure and intended use population. It must be also considered whether the device is proposed to be in direct or indirect contact with tissue and exposure should be one-time, constant or intermittent. The last mentioned could have a cumulative effect (FDA (2000). Use of International Standard ISO 10993-1, "Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process", FDA Maryland.

³ An *in vitro* screening is one that is done in glass, plastic vessels in the laboratory or elsewhere outside a living organism.

(non-transformed) human cells and structurally and functionally correspond to native tissues (OECD 2016), the obtained results are more reliable in comparison to *in vitro* testing with abundantly utilized mouse cells for preliminary testing. Epidermal, ocular, oral mucosa, tracheal/bronchial, alveolar, small intestinal and vaginal models have been already created and are commercially available, for example by company Mattek (Sheasgreen, Klausner et al. 2009). As skin irritation and corrosion are abundantly tested, of all mentioned, the epidermal model is one of the most commonly utilized.

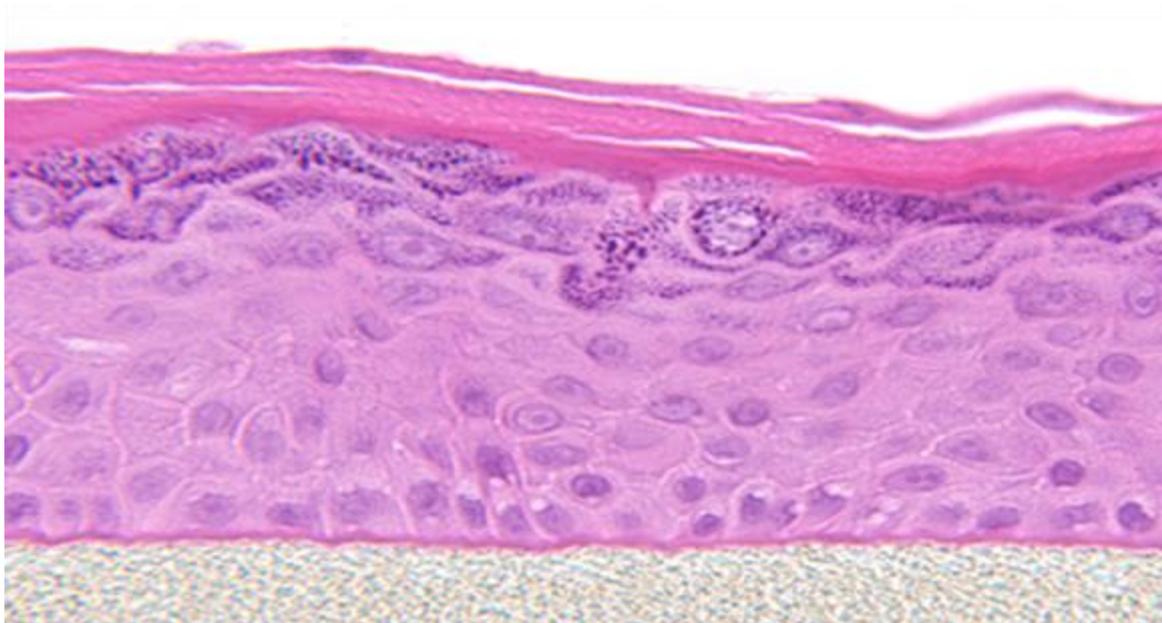


Figure 1. The Reconstructed Human Epidermis, EpiDerm from MatTek Corporation. EpiDerm exhibits human epidermal tissue structure and cellular morphology with greater uniformity and reproducibility. It's a 3D structure consisting of organized and proliferative basal cells, spinous and granular layers, and cornified epidermal layers are mitotically and metabolically active (Sheasgreen, Klausner et al. 2009).

Safety data should be obtained by proceeding the testing according to prescribed or recommended guidelines. Various guidelines addressed biocompatibility assessment of medical devices exist. Among other, the document created by International Organization for Standardization – ISO 10993 "Biological Evaluation of Medical Devices" and the guidance document released by U.S. Food and Drug Administration - blue book memorandum #G95-1, "Use of International Standard ISO 10993, 'Biological Evaluation of Medical Devices'—Part 1: Evaluation and Testing" should be mentioned primarily. Guideline ISO 10993 describes the accurate procedure, parameters and conditions established for biological evaluation. It consists of 20 separate parts including guidance on nanomaterials, tests for cytotoxicity, systemic toxicity, genotoxicity and others. All parts are summarized in Table 1.

Table 1. The individual parts of the series ISO 10993

| Title | Number/Year |
|--|----------------------|
| Evaluation and testing in the risk management | ISO 10993-1:2009 |
| Animal welfare requirement | ISO 10993-2:2006 |
| Tests for genotoxicity, carcinogenicity and reproductive toxicity | ISO 10993-3:2003 |
| Selection of tests for interaction with blood | ISO 10993-4 |
| Tests for <i>in vitro</i> toxicity | ISO 10993-5:2009 |
| Tests for local effects after implantation | ISO 10993-6:2007 |
| Ethylene oxide sterilization residuals | ISO 10993-7:2008 |
| Selection of reference materials | ISO 10993-8:2001 |
| Framework for identification and quantification of potential degradation products | ISO 10993-9:1999 |
| Tests for irritation and delayed-type hypersensitivity | ISO 10993-10:2010 |
| Tests for systemic toxicity | ISO 10993-11:2006 |
| Sample preparation and reference materials | ISO 10993-12:2012 |
| Identification and quantification of degradation products from polymeric medical devices | ISO 10993-13:1998 |
| Identification and quantification of degradation products from ceramics | ISO 10993-14:2001 |
| Identification and quantification of degradation products from metals and alloys | ISO 10993-15:2000 |
| Toxicokinetic study design for degradation products and leachables | ISO 10993-16:1997 |
| Establishment of allowable limits for leachable substances | ISO 10993-17:2002 |
| Chemical characterization of materials | ISO 10993-18:2005 |
| Physico-chemical, morphological and topographical characterization of materials | ISO/TS 10993-19:2006 |
| Principles and methods for immunotoxicology testing of medical devices | ISO/TS 10993-20:2006 |

The required test depends on the nature of body contact and contact duration. The concrete set-up of tests is defined in Figure 2.

| Medical device categorization by | | | Biological effect | | | | | | | | | | | | | | | | | | | |
|----------------------------------|----------------------------------|----------|-------------------|---------------------|-------------------------------|------------------------|----------------|---------------|---|-------------------------|--------------------------------|------------------------------|----------------|--------------|-------------------|------------------|-----------------|--------------------------------------|---|--|--|--|
| Nature of Body Contact | Contact Duration | Category | Contact | A – limited (<24 h) | B – prolonged (>24 h to 30 d) | C – permanent (> 30 d) | Cytotoxicity | Sensitization | Irritation or Intracutaneous Reactivity | Acute Systemic Toxicity | Material-Mediated Pyrogenicity | Subacute/Subchronic Toxicity | Genotoxicity | Implantation | Hemocompatibility | Chronic Toxicity | Carcinogenicity | Reproductive/Developmental Toxicity# | | | | |
| | | | | | | | Surface device | Intact skin | | A | X | X | X | | | | | | | | | |
| B | X | X | X | | | | | | | | | | | | | | | | | | | |
| C | X | X | X | | | | | | | | | | | | | | | | | | | |
| Mucosal membrane | | A | X | X | X | | | | | | | | | | | | | | | | | |
| | | B | X | X | X | O | | O | O | X | X | O | | | | | | | | | | |
| | | C | X | X | X | O | | O | X | X | O | | | | | | | | | | | |
| Breached or compromised surface | | A | X | X | X | O | | O | | | | | | | | | | | | | | |
| | | B | X | X | X | O | | O | O | | | | | | | | | | | | | |
| | | C | X | X | X | O | | O | X | X | O | | | | | | | | | | | |
| External communicating device | Blood path, indirect | | A | X | X | X | X | O | | | | | | | | X | | | | | | |
| | | | B | X | X | X | X | O | O | | | | | | | X | | | | | | |
| | | | C | X | X | O | X | O | X | X | O | X | O | X | O | X | O | O | | | | |
| | Tissue [†] /bone/dentin | | A | X | X | X | O | O | | | | | | | | | | | | | | |
| | | | B | X | X | X | X | O | X | X | X | | | | | | | | | | | |
| | | | C | X | X | X | X | O | X | X | X | | | | | | | O | O | | | |
| | Circulating blood | | A | X | X | X | X | O | | | | | O [‡] | | | X | | | | | | |
| | | | B | X | X | X | X | O | X | X | X | X | | | | | | | | | | |
| | | | C | X | X | X | X | O | X | X | X | X | | | | | | O | O | | | |
| Implant device | Tissue [†] /bone | | A | X | X | X | O | O | | | | | | | | | | | | | | |
| | | | B | X | X | X | X | O | X | X | X | | | | | | | | | | | |
| | | | C | X | X | X | X | O | X | X | X | | | | | | | O | O | | | |
| | Blood | | A | X | X | X | X | O | | | | | O | X | X | | | | | | | |
| | | | B | X | X | X | X | O | X | X | X | X | | | | | | | | | | |
| | | | C | X | X | X | X | O | X | X | X | X | | | | | | O | O | | | |

X = ISO 10993-1:2009 recommended endpoints for consideration*

O = Additional FDA recommended endpoints for consideration*

Note * All X's and O's should be addressed in the biological safety evaluation, either through the use of existing data, additional endpoint-specific testing, or a rationale for why the endpoint does not require additional assessment.

Note [†] Tissue includes tissue fluids and subcutaneous spaces

Note [^] For all devices used in extracorporeal circuits

Note [#] Reproductive and developmental toxicity should be addressed for novel materials, materials with a known reproductive or developmental toxicity, devices with relevant target populations (e.g., pregnant women), and/or devices where there is the probability for local presence of device materials in the reproductive organs.

Note [@] Degradation information should be provided for any devices, device components, or materials remaining in contact with tissue that are intended to degrade.

Figure 2. Biocompatibility Evaluation Endpoints (FDA 2000).

Beside the mentioned *in vitro* methods, also *in silico* approach conquer its firm place within the biocompatibility evaluation process. These approach uses computational methods to analyse, simulate, visualize or predict toxicity, more precisely toxicity endpoints (e.g. acute toxicity, carcinogenicity, organ toxicity or skin irritation) and related properties of the tested substance (such as

biodegradation or bioaccumulation) (Myatt, Ahlberg et al. 2018). The considerable development of *in silico* methods has been noticed in connection with the REACH (registration, evaluation, authorization and restriction of chemicals) regulation and was allowed by the development in the field of computational techniques. As any mentioned testing methods, *in silico* approach has also its advantages and disadvantages. Frequently mentioned benefits include lower price and its potential to reduce animal testing. Stanton and Kruszewski present the quantification of this advantage for the first time (Stanton and Kruszewski 2016). The authors summarize that 100 000 – 150 000 test animals and the expenditures of 50 000 000 – 70 000 000 US were avoided in the evaluation of 261 substances within the American Cleaning Institute's (Stanton and Kruszewski 2016). Another advantages of *in silico* methods in comparison with *in vivo/in vitro* approaches include higher throughput, less time consuming, and low compound synthesis requirements (Valerio 2009). The transparency of the program or quality, and transparency of some of the training set experimental data could be considered as the main limitations of this approach (Weaver and Gleeson 2008, Valerio 2009). In general, two main classes exist – comprehensive (global) and specific ones (e.g. interaction with specific receptors) (Raunio 2011). More specific aspect divides *in silico* models according to the level of biological organization target – molecular, cellular, tissue and organs or organism.

The three major *in silico* approaches are mostly discussed in the literature 1) statistical-based (quantitative structure-activity relationship, QSAR), 2) expert rule-based (or expert/structural alerts) and 3) read-across (Raunio 2011, Myatt, Ahlberg et al. 2018). Each approach has its advantages and limitations, which are thoroughly summarized in an advanced review of Raies and Bajic (Raies and Bajic 2016).

QSAR use statistical tools to correlate a chemical structure of the molecule (descriptors) with its response activity (Roy, Ambure et al. 2017). QSAR is intended to be the most discussed *in silico* method, and it has been also used for biological assessment of polymer material or their precursors. For example, Osimitz and co-workers screened 7 monomers of various polymers (ethylene glycol, diethylene glycol, polytetramethylene glycol, isophthalic acid, monosodium-5-sulfoisophthalic acid, 1,4-cyclohexanedicarboxylic acid, and dimethyl cyclohexanedicarboxylate) for their potential androgenic and estrogenic potential (Osimitz, Welsh et al. 2015). No androgenic and estrogenic potential was revealed using QSAR and obtained results were also confirmed by *in vitro* tests (Osimitz, Welsh et al. 2015). Moreover, several advanced types of QSAR have been developed from one/two dimensional *via* 3D to multidimensional QSAR (Verma, Khedkar et al. 2010). With the evolution of nanotechnology, the member of the QSAR family (nano-QSAR) have been also required (Gajewicz, Schaeublin et al. 2015).

5. TISSUE ENGINEERING

The aim of tissue engineering is to regenerate living tissues damaged by disease, injury or trauma (Dhandayuthapani, Yoshida et al. 2011). Conventional treatment of damaged tissues comprises the transplantation of the tissue from one side to another whether in the same patient (an autograft) or from another (allograft). However, even the lifesaving, the described approach brings not neglected side effects. The most famous one in the people's awareness is the shortage of donors or the rejection of the transplant by the patient's immune systems and also the possibility of the disease transmission from the donor (in case of allografts) (Amini, Laurencin et al. 2012). Moreover, the autograft transplantation is not without the problems as well. The painfulness, high cost connected with the harvesting of autograft or the possibility of transmission of infection should cause problems in case of allograft transplantation (O'Brien 2011). Scheme showing types of grafts and their relationship is depicted in Figure 3.

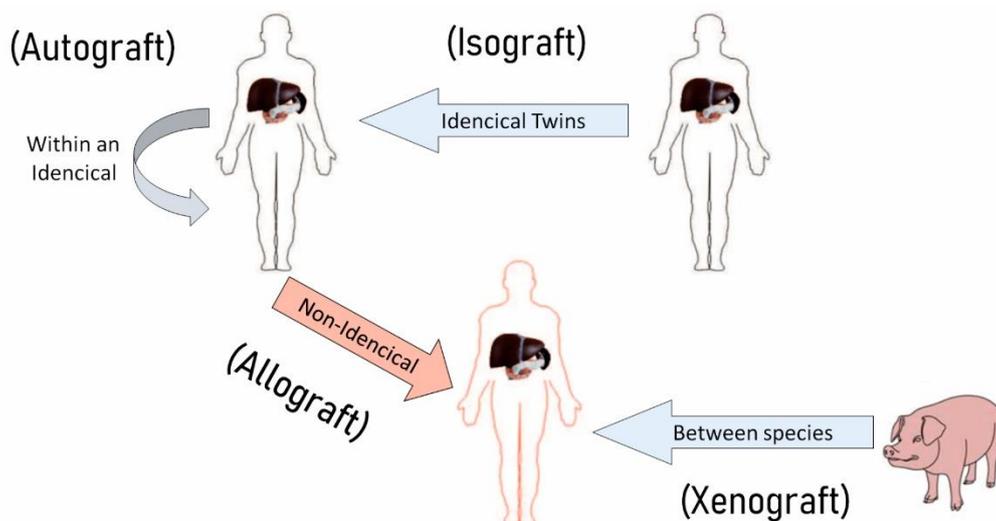


Figure 3. Types of grafts utilizing in tissue engineering (Lu, Rao et al. 2012).

Therefore, tissue engineering has appeared as an attractive alternative to standard transplantations. Tissue engineering creates tissues that aim to improve the function of diseased or damaged tissues (Nichol and Khademhosseini 2009). Perhaps because of this definition, it is misnamed the term tissue engineering and regenerative medicine⁴ in some circumstances. In fact, the tissue engineering is

⁴ Regenerative medicine is the branch of medicine that develops methods to regrow, repair or replace damaged or diseased cells, organs or tissues. Regenerative medicine includes the generation and use of therapeutic stem cells, tissue engineering and the production of artificial organs Sampogna, G., S. Y. Guraya and A. Forgione (2015). "Regenerative medicine: Historical roots and potential strategies in modern medicine." *J Microsc Ultrastruct* 3(3): 101-107.

only the part of the extensive field of the regenerative medicine and combines the biomaterial scaffolds, cells from the patients' body and growth factors to restore, maintain or improve tissue function (Howard, Buttery et al. 2008). Therefore, this multidisciplinary field connects the chemistry, material science, molecular and systems biology and mechanical engineering. Although the idea originated earlier, the term tissue engineering was officially introduced at a National Science Foundation in 1988 (O'Brien 2011).

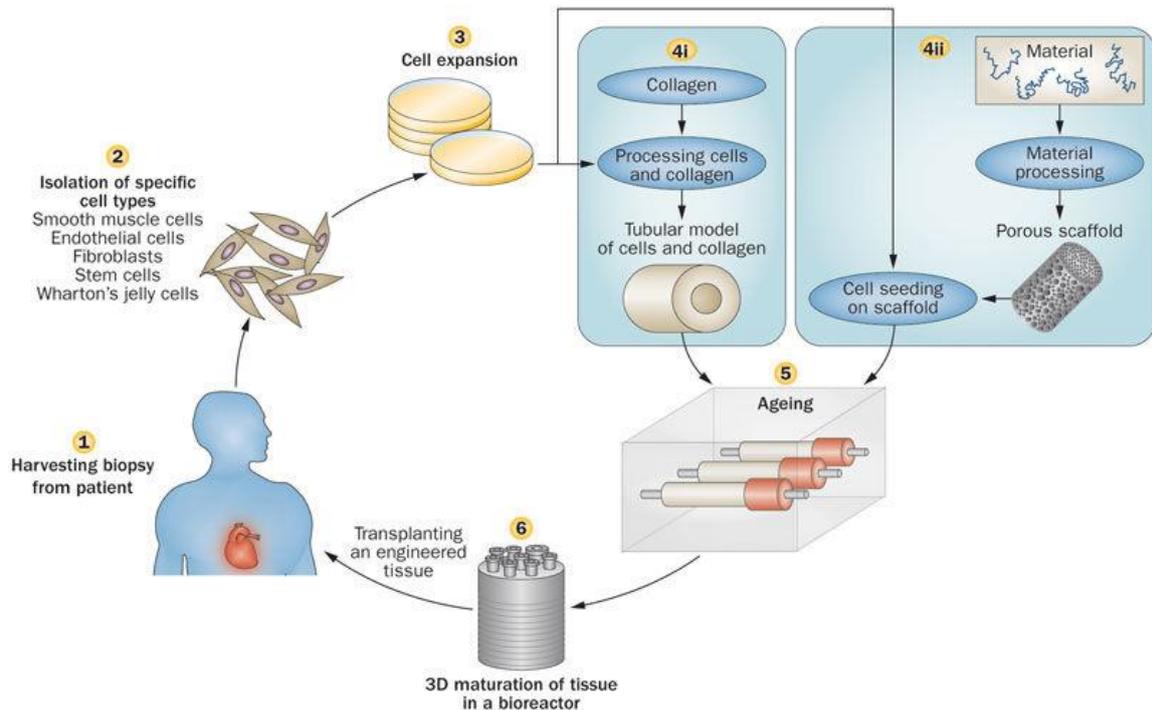


Figure 4. Scaffold-guided vascular tissue engineering (Seifu, Purnama et al. 2013).

One of the possible processes of tissue engineering is depicted in Figure 4. The process starts with the isolation of tissue-specific cells from the patient's biopsy. The isolated cells are cultivated and expanded *via in vitro* techniques to obtain their sufficient amount. The cells are seeded along with the growth factors into biomaterial scaffold, which acts as a template for regenerated tissue formation. In some cases, the seeded cells are subjected to mechanical stimuli. Biomaterial scaffold together with the growth factors and/or other biophysical stimuli create the appropriate environment for cell adhesion, proliferation and new tissue growth. The combination of scaffold, cells and stimuli are called tissue engineering triad (O'Brien 2011). Subsequently, the two ways are possible. In the case of the first one, the scaffold with seeded cells is cultured *in vitro* to enable the tissue formation, which is then implanted back into the patient's body. To obtain the 3D tissue formation, the using traditional culture methods is insufficient and therefore the bioreactors are utilized. The second approach is based on the direct implantation of the seeded scaffold into the injured site. So the tissue

regeneration is induced *in vivo* (Uchegbu and Schatzlein 2006). Concerning cell type, stem cells have a huge potential for tissue engineering. Firstly, because of their unique ability to self-renewal – cells can easily expand under define *in vitro* conditions and cells should be obtained in sufficient amount for subsequent seeding onto scaffolds (Zhang, Gupte et al. 2013). Secondly, under specific stimulation, they have capacity to differentiate into all desired cell types, such as neurogenic, osteogenic, chondrogenic or myogenic lineages (Zhang, Gupte et al. 2013). Nevertheless, the quantity of stem cells is limited in some cases and therefore, the induced pluripotent stem cells should be utilized as an alternative tool in tissue engineering (Bastami, Nazeman et al. 2017). Furthermore, contrary to embryonic stem cells, induced pluripotent one pose neither ethical conflict, nor immunological hazard (Amirabad, Massumi et al. 2017).

For the purpose of the doctoral thesis, the term “scaffold” describes the 3D biomaterial before cell settlement. On the contrary, the term “tissue engineered construct” refers to biomaterial already seeded with cells and subjected *in vitro* cultivation prior to implantation (O'Brien 2011).



Figure 5. Polyaniline cryogel mimicking the properties of native tissue. Source: Cell biology laboratories, Centre of polymer systems, Tomas Bata University in Zlin.

Biomaterial scaffolds for tissue engineering can be described as highly porous biomaterials, which operate as templates for the regeneration of tissues or organs (Dhandayuthapani, Yoshida et al. 2011). As newly regenerated tissue, in the overwhelming majority, does not create itself without assistance any “buttress”, the biomaterial scaffolds manufacturing plays a crucial role in tissue engineering. In other words, scaffolds represent support for cell adhesion, proliferation and subsequently the new tissue formation.

Before the utilization of artificial scaffold, the decellularized extracellular matrix⁵ (ECM) of targeted tissue was used with success (Rijal 2017).

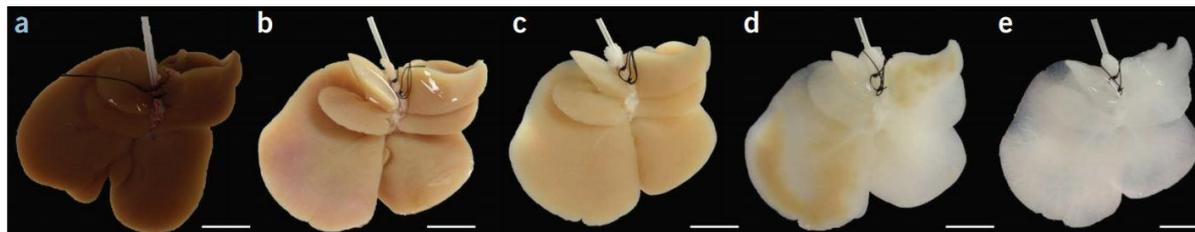


Figure 6. Representative images of ischemic rat livers during decellularization process at a) 0h; b) 18h; c) 48 h; d) 52h; e) 72h (Uygun, Soto-Gutierrez et al. 2010).

As each biomaterial, also scaffolds for tissue engineering must be biocompatible. Moreover, the scaffold should allow cell adhesion, proliferation, migration and ingrowth of cells through the bulk of the scaffold. Also, in the case of stem cells, the scaffold should promote their differentiation into the desired cell line. Implanted scaffolds also should not induce an immune reaction, because it leads to the inflammatory response, which might have a negative influence on the healing process and can lead to rejection of the implanted scaffold by the body (Chan and Leong 2008). The problematics of biocompatibility is described more detailed in Chapter 4. Biocompatibility.

Furthermore, as was mentioned in the general properties of biomaterials, the manufacturability is also important. To introduce the utilization of scaffold into clinical practice, the manufacturing should be cost-effective and scale-up from preparing several samples in the research laboratory to small batch production. Moreover, each scaffold for tissue engineering must be prepared under good manufacturing practice⁶ and its quality must be stable in time (O'Brien 2011). Compliance with good manufacturing practice standards is regularly inspected. The storage and transport conditions must also be defined and the principles of good manufacturing practise must be applied there.

⁵ Extracellular matrix should be described as non-living material secreted by cells that fills spaces between the cells in a tissue, protecting them and helping to hold them together. It is the aggregate of proteins secreted by cells containing collagen, elastin, proteoglycans and glycosaminoglycans (Winters, N. and J. H. Kelley (2017). *The Metabolic Approach to Cancer: Integrating Deep Nutrition, the Ketogenic Diet, and Nontoxic Bio-Individualized Therapies*, Chelsea Green Publishing.).

⁶ A good manufacturing practice is a system for ensuring that products are consistently produced and controlled according to quality standards. It is designed to minimize the risks involved in any pharmaceutical production that cannot be eliminated through testing the final product (Karnieli, O. (2016). *Bioreactors and Downstream Processing for Stem Cell Manufacturing*. *Stem Cell Manufacturing*. C. L. d. Silva, L. G. Chase and M. M. Diogo. Boston, Elsevier: 141-160.).

6. CELLS UTILIZED IN TISSUE ENGINEERING

At its beginning, tissue engineering utilized terminally differentiated cells (Gelinsky, Bernhardt et al. 2015). Although mature and differentiated primary cells are readily available (Jhala and Vasita 2015), their expansion in culture is deficient and therefore their application in tissue engineering is limited (Sundelacruz and Kaplan 2009). Therefore, stem cells seem to be more suitable for this purpose. Stem cells should be defined as unspecialized precursor cells with the ability to self-renew and to differentiate into diverse tissue- or organ-specific cells under appropriate stimuli (Wang and Chen 2013).

In the living system, stem cells reside in particular microenvironment called stem cell niche. This niche should be characterized as “specific microenvironment in the tissue, where stem cells can live for a quiescent stage and can differentiate or self-renew in a controlled manner (Jhala and Vasita 2015). The fundamental elements of the stem cell niche are an ECM, growth factors, support (niche) cells and stem cells (Willerth 2017). This structure is usually connected with blood vessels ensuring the delivery of nutrients and factors (Willerth 2017). Stem cells are in a tight connection with niche cells *via* gap or adherens junctions (Jhala and Vasita 2015). The connection of the daughter cell with the niche cells indicates the fate of the daughter cell after division (Jhala and Vasita 2015). The various stem cells processes (e.g. proliferation, migration, differentiation and apoptosis) should be also operated by altering properties of ECM. It comprises biochemical (chemical composition of ECM or presence of functional peptide sequence) (Gesteira, Sun et al. 2017, Donnelly, Salmeron-Sanchez et al. 2018), mechanical (Hilderbrand, Ovadia et al. 2016) as well as geometrical characteristics (porosity or topography) (Gattazzo, Urciuolo et al. 2014). Moreover, the hypoxic condition has an impact on the differentiation status of stem cells (Bino, Kucera et al. 2016).

Stem cells can be divided based on their differentiation potential as totipotent (able to differentiate into all cell types) (Godara, Nordon et al. 2008), pluripotent (capable of differentiating into any tissues except placental cells) (Bacakova, Zarubova et al. 2018), multipotent (more limited than pluripotent, have the capacity to develop into a variety of specialised cells) (Mirzaei, Sahebkar et al. 2018), oligopotent (able to differentiate into limited cell lineages) (Burgess 2016) and unipotent (can create only one cell type) (Godara, Nordon et al. 2008). The totipotency is exhibited only by embryonic stem cells derived from a morula, early stage of embryonic development (Bacakova, Zarubova et al. 2018).

Stem cells can be also distinguished according to their source on embryonic, foetal, adult and induced (Bacakova, Zarubova et al. 2018). Initially, the attention was focused on embryonic stem cell owing to their pluripotency. Embryonic stem cells are derived from the inner cell mass of the 5 to 6 day-old blastocyst (Godara, Nordon et al. 2008). However, the use of embryonic stem cells aroused ethical debates (Barenys and Fritsche 2018). Moreover, the embryonic stem cells require a feeder layer (commonly mouse fibroblasts) for their successful isolation and

expansion (Godara, Nordon et al. 2008). Therefore, an alternative cell source was obtained – adult stem cells and induced pluripotent stem cells.

Adult stem cells can be described as undifferentiated cells resided in mature organs and tissues (Sell 2013). The range of potency of adult stem cells varies from pluripotency (Jaramillo-Ferrada, Wolvetang et al. 2012), through multi- (Bhartiya, Boheler et al. 2013) and oligopotency (Bacakova, Zarubova et al. 2018) up to unipotency (Bacakova, Zarubova et al. 2018). Adult stem cells occur within the various tissues including e.g. bone marrow, skin, placenta subcutaneous adipose tissue or dental pulp (Danišovič, Polák et al. 2013). However, not all sources are suitable for tissue engineering due to required complex and invasive procedures for extraction or presence of a small number of desired adult stem cells (Hodgkinson, Yuan et al. 2009). The promising population of adult stem cells comprise bone marrow-, adipose-, and hair follicle-derived stem cells (Hodgkinson, Yuan et al. 2009). Moreover, stem cell populations derived from one location should be heterogeneous – e.g. hematopoietic and also mesenchymal stem cells should be derived from the bone marrow (Hodgkinson, Yuan et al. 2009). Among all adult stem cells, mesenchymal ones are of great interest. They are located in various tissues including adipose tissue, skin, skeletal muscle or lung (Dvorakova, Hrubá et al. 2008). They are able to differentiate into various cell types and have potential e.g. in bone (Toosi, Behravan et al. 2018), cartilage (Li, Truong et al. 2018), myocardium tissue engineering (Roura, Galvez-Monton et al. 2017). Moreover, they are really promising in dermal tissue regeneration based on their capability to stimulate scar-less wound healing (Jackson, Nesti et al. 2012, Li, Wang et al. 2015).

The milestone in the field of tissue engineering was passed in 2006, when Takahashi and Yamanaka reprogrammed somatic, already differentiated, cells to an embryonic-like state by introducing transcription factors *Ocr3/4*, *Sox2*, *c-Myc* and *Klf4* and referred them to induced pluripotent cells (Centeno, Cimarosti et al. 2018). Several techniques utilizing to reprogramming of differentiated cells exist. Currently, viral transfection utilized by Takahashi and Yamanaka is being abandoned today and novel delivery systems have been already described. These systems comprised non-viral vectors (e.g. human artificial chromosome vector (Hiratsuka, Uno et al. 2011), or nanoparticle carriers (Lee, Kim et al. 2011), or small chemical molecules (primarily inhibitors of specific signalling pathways such as valproic acid or 5'-azacytidine) (Rony, Baten et al. 2015). Moreover, it has been already described that pluripotent stem cells express a characteristic set of noncoding RNA (e.g. micro RNA), which is involved in the regulation of gene expression influencing unique stem cell cycle (Sherstyuk, Medvedev et al. 2018). This noncoding RNA is also known to influence pluripotency (Gonzalez, Boue et al. 2011) and, therefore, it should represent a useful tool of cell reprogramming process (Leonardo, Schultheisz et al. 2012).

7. TECHNIQUES OF CELL CULTIVATION

Since introducing *in vitro* cell cultivation technique, the standard (two dimensional (2D)) cell cultivation has been utilized. It represents technique, in which cells grow in a monolayer on a flat bottom of tissue culture, mostly polystyrene, plastic flask or plate (see Figure 7). However, these 2D methods do not reflect the natural physiology of the cell. Therefore, a novel 3D cell culture has been developed to approach more natural microenvironment of cells in a living system (Haycock 2011). The 3D cell culture creates an artificial environment in which biological cells are permitted to grow or interact with its surroundings in all three dimensions (Antoni, Burckel et al. 2015). Several various methods have been developed for this purpose and have enabled the improvement in cell culture research. These methods could be formally divided into scaffold-free systems (culturing spheroids using the hanging drop technique or spheroid microplates) or scaffold-based systems.



Figure 7. The cultivation flasks with mouse embryonic fibroblasts NIH/3T3 during cultivation in the incubator. Source: Cell biology laboratories, Centre of polymer systems, Tomas Bata University in Zlin.

Traditional and still routinely utilized “2D” monolayer cell culturing methods are insufficient for tissue engineering demands (Edmondson, Broglie et al. 2014). The main disadvantage of standard 2D cultivation is that the cells grow in a monolayer on a flat substrate. However, this is unnatural for the cells in the living organism, in which almost all cells are surrounded and in contact in all three dimensions with other cells and/or with ECM produced by neighbouring cells (Edmondson, Broglie et al. 2014). Thus, the cells culturing in 2D manner exhibits

significant structural differences in comparison to *in vivo* conditions. In general, the cells are flattened and strained (Knight and Przyborski 2015). This is a general problem of science as the holistic approach is not possible to be used and reductionism must be applied. Thus, although it is known that cultivation of cells on 2D surfaces are far from the *in vivo* conditions, the scientist must use this approach for decades because of lack of scaffolds (3D) and cultivation techniques (bioreactors).

The huge differences between the 2D and 3D cultivation can be seemed from the different morphology of cells (see Figure 8). The differences between both cultivation methods are aptly described in a review by Knight and Przyborski (Knight and Przyborski 2015). In conventional 2D cultivation, cells create monolayer and are flatter. In contrast, cells adopt a natural structure in all dimensions in 3D cultivation. Moreover, cell-cell interactions in standard 2D cultivation methods are limited to the periphery of cells (only in axes x and y), contrary to 3D cultivation techniques, where cell-cell interactions can be conducted in all directions (also axe z). All mentioned facts can be observed in Figure 8B, in which confocal images of a single fibroblast cell are depicted. The cell under 2D cultivation condition are unnaturally flat (see visualization from the side).

It should seem like a non-essential factor, but this shift in natural morphology influences the spatial organization of the cell surface receptors participated in cell-cell interactions. Therefore, it can also affect the outside-inside signal transduction pathways and this impact can finally lead to modified gene expression and altered cell physiology (Edmondson, Broglie et al. 2014). Thus, the construction of regenerate tissue and organs in 2D manners is impossible.

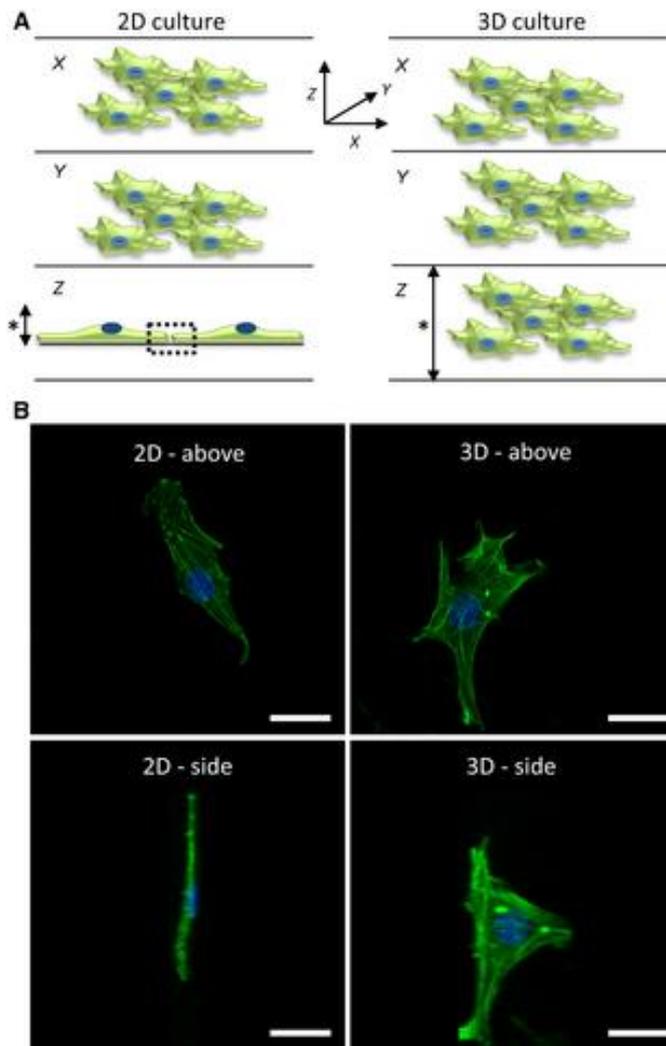


Figure 8. The differences between the cell morphology in “2D” and 3D cultivation conditions (Knight and Przyborski 2015).

Based on improved techniques, several pilot studies have focused on the description of cellular behaviour divergence in 2D versus 3D models from different angles. Hakkinen *et al.* in their study compared fibroblasts’ behaviour in four types ECM under 2D and 3D conditions. Fibroblasts observed in 3D matrices were more spindle-shaped, had fewer lateral protrusions and reduced actin stress fibres than on 2D ones (Hakkinen, Harunaga *et al.* 2011). The influence of 3D on cell differentiation has been also investigated. Petrakova *et al.* studied the effect of cultivation conditions on the differentiation of endodermal cells. Under 2D cultivation, the cells remained in an undifferentiated state, contrary to 3D cultivation, during which cells underwent differentiation in the absence of any additional stimulation by cytokines and growth factors (Petrakova, Ashapkin *et al.* 2012). The different differentiation level of MC3T3-E1 cell line between 2D and 3D cultivation conditions was observed also by Li and co-workers. In their study, they cultivated the cells under 2D and 3D conditions using the same type of material in form of thin film and scaffolds, respectively. The cells cultured within 3D scaffolds showed multiple morphologies, depending on e.g. the initial

seeding concentration (Li, He et al. 2017). Finally, Bellis *et al.* described the activation of a distinct set of transcription factors in the case of 2D model compared to 3D one (Bellis, Bernabe et al. 2013). Moreover, it should not be omitted, that certain cell cultures are sensitive to mechanical (Santos, Bakker et al. 2011) or electrical stimulation (Balint, Cassidy et al. 2013). It has been already described, that electrical stimulation is required to evoke high-level differentiation of induced pluripotent stem cells into cardiomyocytes (Amirabad, Massumi et al. 2017). The medium flow should also influence cellular behaviour (Meinel, Karageorgiou et al. 2004). For example, the medium flow perfusion is an essential factor in the engineering of the bioartificial liver (Pekor, Gerlach et al. 2015, Starokozhko, Hemmingsen et al. 2018).

Therefore, scientists and material engineers have made a huge effort to create the device providing the cells the appropriate environment, which is closer to natural (*in vivo*) one. One of the key components of 3D cell culturing is a bioreactor.

7.1. Bioreactors

A bioreactor is a general term applied to a closed culture environment that enables control of environmental or operation variables that affect biological processes (Chaudhuri 2005). In biotechnology, the bioreactors (in this branch also called fermenters) are used for prokaryotic cells cultivation and subsequent to the production of enzymes or other proteins.

For the demands of tissue engineering, the bioreactors are utilized to give controlled and reproducible cell cultivation conditions (Chaudhuri 2005). They enable the dynamic cell cultivation, which assumes the cells the homogenous concentration of nutrients, oxygen and other required elements as well as the toxic by-products removal and pH level preservation. This is allowed due to continuous culture media mixing or perfusion. Contrary to dynamic cultivation in a bioreactor, during static cultivation (cultivation in cultivation flasks or plates, nutrient supply and waste removal are driven only by diffusion) the concentration gradient of all indispensable components in culture medium could appear during the time period between culture medium exchanges. This un-natural environment (the sufficient nutrient support and toxic by-product removal are provided by capillary bed⁷ inside each living system) leads to an alteration in cellular behaviour.

Three dimensional cell culturing in the bioreactor (in fact, as tissue regeneration is a dynamic process ongoing during the time, it could be defined as an issue of a four-dimensional challenge) mimic the *in vivo* cellular behaviour and therefore purvey more physiologically relevant outcomes (Edmondson, Broglie et al. 2014).

⁷ Capillary bed is a network of capillaries in a living system (Barral, J.-P. and A. Croibier (2011). *General organization of the cardiovascular system. Visceral Vascular Manipulations.* Oxford, Churchill Livingstone: 3-26.).

From this point of view, the bioreactors should be considered as an intermediate step between the traditional static cell cultivation and natural *in vivo* cell existence.

Moreover, besides the appropriate amount of the spectrum of biochemical components contained in the culture medium, certain cells require also different physical stimuli based on the function of complete tissue, e.g. shear stress, mechanical or electrical stimulation. As a good example, the cells of cartilage should be mentioned. The cartilage in a natural state is exposed to mechanical compression (Tran, Cooley et al. 2011). The tissue is repeatedly compressed and cells are exposed to fluid shear stress developed by pressurization of the internal fluid. By virtue of this knowledge, the suitable bioreactor with mechanical loading was constructed (Tandon, Marolt et al. 2013). The modern bioreactors can mediate all of the mentioned stimulation.

Several general types of bioreactor exist for the purpose of tissue engineering application. There are three basic bioreactor types including spinner flasks, perfusion bioreactor and low gravity bioreactor (Chaudhuri 2005).

7.2. Spinner flasks

The most simple and still abundantly utilized bioreactor type is spinner vessel. This type should be described as a vessel (often simple cylindrical glass flask) filled with the culture media, which is inoculated with the cells. The culture medium is continuously mixed by the stirring element placed at the bottom of the vessel (Chaudhuri 2005). Besides it has been still widely used, this bioreactor arrangement has two very important disadvantages (Massai, Isu et al. 2016). The results of cell culturing should be affected by the cells collisions with the stirring element and also with the scaffolds that are also suspended in the culture medium. Another factor is shear stress caused by the stirring. For this reason, the upper limit to the intensity of mixing is specified (Chaudhuri 2005). Several mathematical approaches utilizing commercial software, e.g. FLUENT, are used to simulate the flow field under given operation conditions (Sucosky, Osorio et al. 2004). The wavy profile of the vessel is commonly used for reduction of the turbulence effect occurred in the cylindrical flask (Hutmacher and Singh 2008). Overall, the agitation speed must be optimised for specific cell type to obtained required cellular behaviour. For example, the expansion of induced pluripotent stem cells and maintain its differentiation capability depends on the really narrow range of spin rates (Gupta, Ismadi et al. 2016).

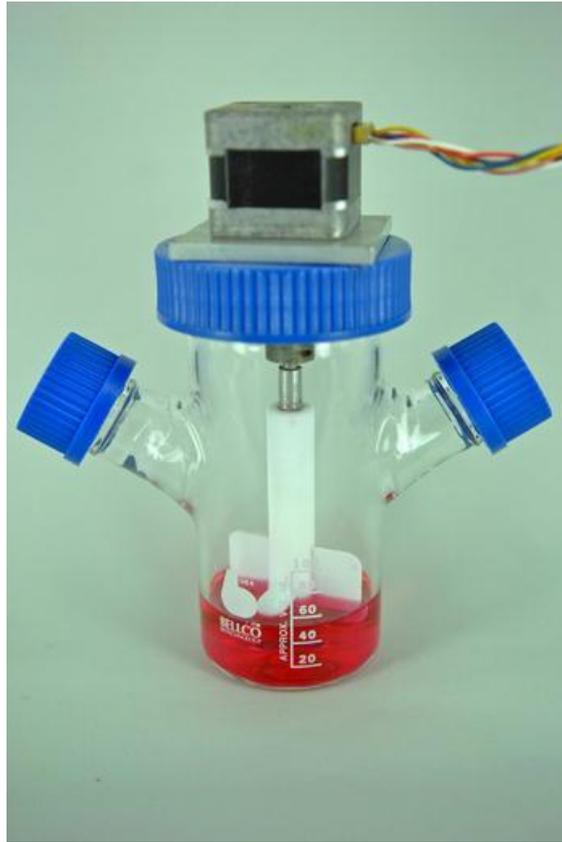


Figure 9. Spinner flask (Gupta, Ismadi et al. 2016).

7.3. Perfusion bioreactors

The substantial advantage of the second model, bioreactor with the cultivation medium perfusion, is minimal concentration gradient and efficient nutrient support. The mixing of cultivation medium is substituted by continuous medium perfusion through a scaffold seeded with cells (Chaudhuri 2005). The scaffold is firmly fixed inside the bioreactor chamber to restrict its movement inside the bioreactor chamber and to secure the correct function of the bioreactor. Rohar and co-workers studied the effect of the direct medium flowing through a porous 3D scaffold with pre-cultured cells. For this purpose, they utilized the typical perfused bioreactor consisted of a scaffold holder, an oxygenator, a medium reservoir and a peristaltic pump, and described the 3D neovascularization promotion under flow conditions (Zohar, Blinder et al. 2018).

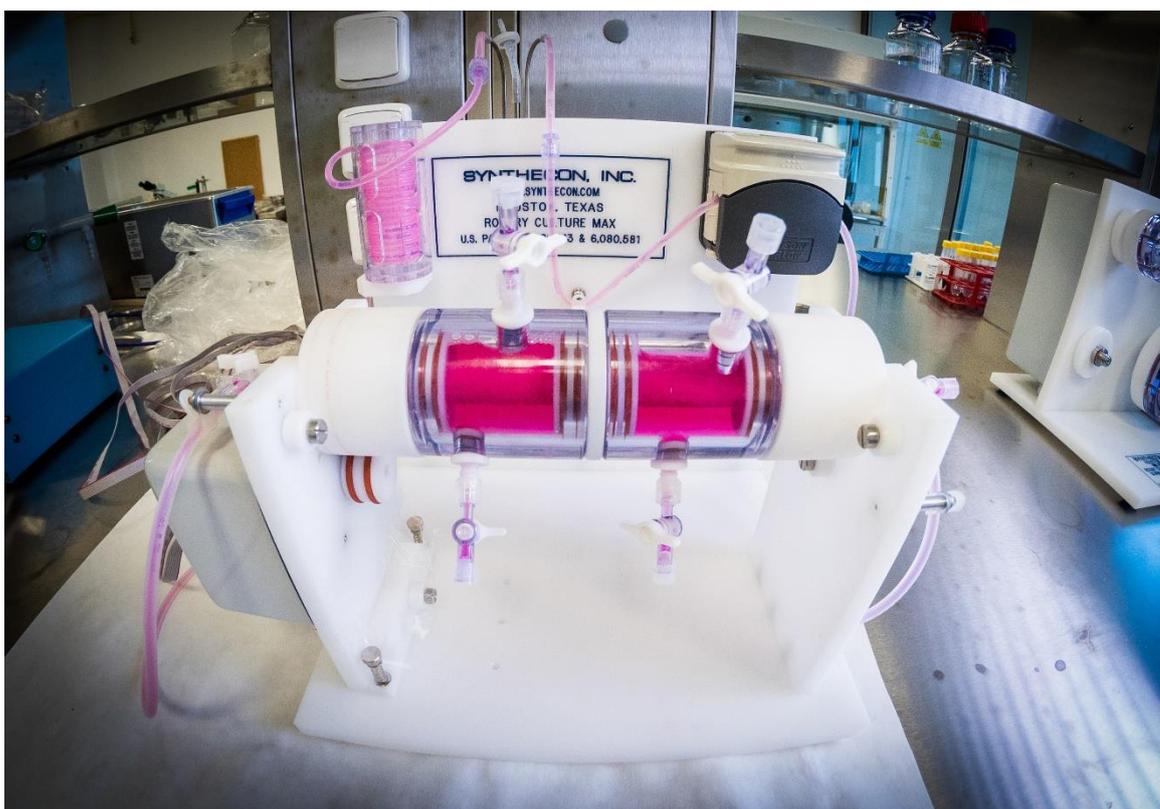


Figure 10. Continuous flow perfusion system from Synthecon Incorporated. Source: Cell biology laboratories, Centre of polymer systems, Tomas Bata University in Zlin

7.4. Low gravity bioreactors

As low gravity bioreactor is used in the practical part of the thesis, this section will be described in more detail compared to others. The rapid development in space engineering has also caused the growing interest of natural scientists in research accomplished in the space environment. This research has also been supported with a national agency, e.g. National Aeronautics and Space Administration (NASA) supported the research based on the study of zero gravity on cell morphology (Chaudhuri 2005).

Moreover, the morphology is not the only aspect influenced by microgravity⁸. A lot of research papers focused on the effect of microgravity on the behaviour of various cell type have been published. The modulation of gene expression and consequent protein synthesis, signalling pathways or structured organization has been previously described (Chaudhuri 2005). Furthermore, changes in the

⁸ Contrary to physics, in regenerative medicine the term “microgravity” (or “micro-g environment” or “1 g”) is frequently used as a synonym of “weightlessness” and “zero-g”, which indicates that the g-forces are not actually zero but just “very small” (Herranz, R., R. Anken, J. Boonstra, M. Braun, P. C. M. Christianen, M. de Geest, J. Hauslage, R. Hilbig, R. J. A. Hill, M. Lebert, F. J. Medina, N. Vagt, O. Ullrich, J. van Loon and R. Hemmersbach (2013). "Ground-Based Facilities for Simulation of Microgravity: Organism-Specific Recommendations for Their Use, and Recommended Terminology." *Astrobiology* 13(1): 1-17.).

regeneration ability and response for tissue engineering have been also investigated. For example, cell migration is central in tissue repair and regeneration (Borm, Requardt et al. 2005). Ranieri and co-workers described increased keratinocyte migration in a microgravity environment. The test was based on Scratch assay⁹ simulated wound healing and the subsequent fluorescent analysis revealed that the induced migration was connected with the cytoskeletal reorganization – the lamellipodia¹⁰ and ruffles were frequently observed in the peripheral cell (Ranieri, Proietti et al. 2017).

Another goal of tissue engineering is to acquire a sufficient number of cells. It should be difficult in some cases, therefore the attention of researches is focused on differentiation potential of stem cells. In this context, several various studies have been performed. From all, the research guided by Xue and co-workers should be emphasised. They described the regulation of the differentiation fate of mesenchymal stem cells (MSCs) with the duration of simulated microgravity. A shorter period of microgravity promoted MSCs to differentiate into endothelial, neuronal and adipogenic cells (Xue, Li et al. 2017). On the other hand, a longer period of simulated microgravity (10 days) promoted MSCs to differentiate into osteoblasts (Xue, Li et al. 2017). However, focused on osteogenesis, the miscellaneous results were published and some authors allege the inhibition effect of the microgravity environment on osteogenic differentiation (Chen, Luo et al. 2015). Furthermore, Cazzaniga and co-workers concluded that simulated microgravity alone reprograms bone mesenchymal stem cells towards an osteogenic phenotype, which results in complete differentiation only after exposure to a specific stimulus (Cazzaniga, Maier et al. 2016).

It must be also added that simulated microgravity (ground-based) is not identical to real (outer space) one and each type of microgravity simulating bioreactor has own individual capacities and limitations (Herranz, Anken et al. 2013). This aspect, as well as using of different cell culturing protocols or method to differentiation induction, should impute to the inconsistency of obtained results dealing with the osteogenic differentiation (Cazzaniga, Maier et al. 2016).

In addition to scaffold-based tissue regeneration, it should be mentioned, that also scaffold-free tissue formation in microgravity environment seems to be a promising novel method in regenerative medicine and tissue engineering (Aleshcheva, Bauer et al. 2016).

⁹ The Scratch assay is an *in vitro* method to measure cell migration. It is based on the forming of „scratch“ in a cell monolayer and the quantification of cell migration rate (Liang, C. C., A. Y. Park and J. L. Guan (2007). "In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*." *Nature Protocols* 2(2): 329-333.

¹⁰ Lamellipodium should be described as a broad, flat protrusion at the leading edge of moving cells. Beside the cell motility, it is involved in mechano-sensing process. (Krause, M. and A. Gautreau (2014). "Steering cell migration: lamellipodium dynamics and the regulation of directional persistence." *Nature Reviews Molecular Cell Biology* 15(9): 577-590.

Despite the above-mentioned fact that the microgravity simulators are not able to precisely imitate the real outer space microgravity environment, they are abundantly utilized in research. The reason is simple – spaceflights are rare and expensive. Therefore several devices were constructed to simulate microgravity on Earth. The four basic types of simulators have been already developed – 2D or 3D clinostats, random position machine, rotating wall vessel and diamagnetic levitation. The clinostat was originally developed to the research of plant roots grow predominantly in direct to the centre of the Earth (Russomano, Cardoso et al. 2005).

The two basic types of rotating wall vessel bioreactor exist. The slow turning lateral vessel (STLV) and high aspect rotating vessel (HARV). These type of bioreactors were developed in cooperation with NASA and their entrenched producer is Sythecon (Texas, U.S.) (Salerno-Goncalves, Fasano et al. 2016).

The STLV bioreactors have shown to be suitable for cultivation of mouse embryonic stem cells, enable their aggregation into embryonic bodies and subsequent differentiation of these embryonic bodies into cardiomyocytes (Rungarunlert, Klincumhom et al. 2013). The embryonic bodies' formation and its quality are the key steps in cardiogenesis. Based on quality and quantity evaluation of embryonic bodies, the STLV bioreactors were proved to allow their forming in the homogeneous sized, improving further proliferation and finally cardiac differentiation of embryonic stem cells in comparison to conventional techniques (hanging drop, static suspension cell culture) (Rungarunlert, Klincumhom et al. 2013). Analogous research was performed by Gerecht-Nir and co-workers using human embryonic cells with similar results. The cultivation in STLV led to its increased viability and proliferation, enabled satisfactory control over the embryonic bodies agglomeration and the natural progression of differentiation (Gerecht-Nir, Cohen et al. 2004).

Moreover, Aubry and co-workers described the improved STLV bioreactors. The technological upgrade was based on the perfusion and dialysis chamber adaptation. The perfusion secured the continuous delivery of culture medium and allow optimized oxygenation, pH and nutrient support as well as waste removal. The dialysis chamber decrease the quantity of often expensive media supplements (e.g. growth factors) inside the bioreactor chamber. Perfused and dialyzed STLV bioreactors promoted the formation of human embryonic bodies that were differentiated more rapidly. (Aubry, Tournois et al. 2008)

The research is also focused on an effective monitoring system for continuous control of cell assembling and tissue formation. Manley and co-workers described a fluorescent-based real-time analysis of the process of diverse cell type assembling into spheroids (Manley and Lelkes 2006). This system should represent the beginning of a complex feedback regulatory system allowing automatic control required rotational speed to maintain nascent tissue in continual free fall (Manley and Lelkes 2006).

In addition to fluorescent based systems, the fluorescent free online monitoring system for *in situ* computerized monitoring of 3D *in vitro* cell aggregation has been already published (Sawyer, Worrall et al. 2008).

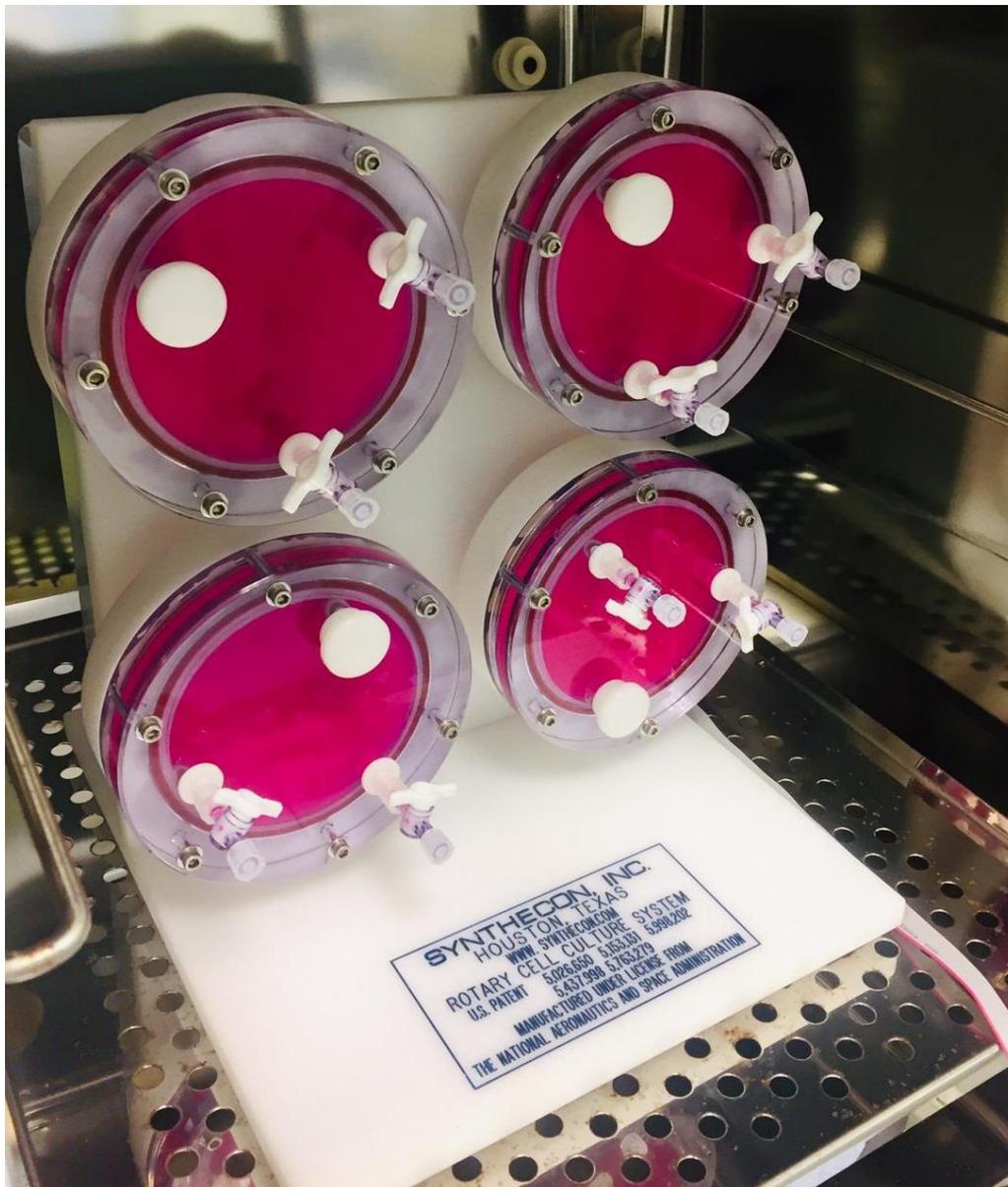


Figure 11. The rotary cell culture system (RCCS-4) from Synthecon Incorporated. Source: Cell biology laboratories, Centre of polymer systems, Tomas Bata University in Zlin.

Each tissue could be described as a complex material showing different intricate properties and requiring specific conditions. Therefore, the specific types of bioreactors have been constructed to fulfil all the requirements of individual tissue. These specific bioreactors are based on the natural physical conditions of concrete tissue and could be the combination of above mentioned general types with the additional properties including mechanical or electrical stimulation (Plunkett and O'Brien 2011).

7.5. Advanced bioreactors

In the natural environment, the cells respond not only to biological factors (e.g. growth factors or signalling molecules), but also to physical stimuli. Cells are in permanent contact with their surrounding ECM and receive various forces in dynamic environment – e.g. endothelial cells in a blood vessel sensing fluid shear stress of blood, compression of chondrocytes in knee cartilage or stretching of muscle during contraction (Wang and Chen 2013).

Therefore, the utilization of advanced bioreactors enabling mechanical or electrical stimuli is highly demanded to mimic the natural cells' environment as much as possible. For this purpose, multi-modal bioreactors are usually assembled.

The systems for mechanical stimulation of cells could be subdivided into 3 main categories based on their primary loading modality – comprehensive loading systems (using hydrostatic pressure or direct platen contact), longitudinal stretch system and fluid shear system (Brown 2000).

Focusing on the development of the artificial cartilage, the compression is the key stimulus dictated generation of functional tissue. Wimmer and co-workers were inspired by the physiological articular motion and designed a device that generates joint-like movement (Wimmer, Grad et al. 2004). The “pin-on-ball” concept is composed of rotating “pin” (cell-seeded scaffold), which is pressed onto conforming ball (Wimmer, Grad et al. 2004). This type of bioreactor is depicted in Figure 12, and it has been subsequently utilized in a number of studies focused on mechanical cell stimulation (e.g. study of Neumann and co-workers (Neumann, Gardner et al. 2015) or work of Gardner and co-workers (Gardner, Musumeci et al. 2017)).

The novel and different approach had Sun and co-workers, who designed rolling-compression loading bioreactor, which fully mimics the complex mechanical stimuli of natural articular cartilage (Sun, Lv et al. 2010). On the other hand, vascular cells placed in their physiological environment are sensitive to fluid shear stress (caused by blood flow), substrate stretching (due to pulsatile pressure) and hydrostatic pressure of blood (Davis, Zambrano et al. 2015). Lin and Mequanint used commercially available a dual pump bioreactor designed for maturing functional blood vessel (Lin and Mequanint 2017). The key component of this device is pulsatile pump providing luminal pulsatile medium flow and generating physiological-like stimuli (Lin and Mequanint 2017). Focusing on the biomechanical milieu of native arteries, which exhibit both circumferential and axial stress, improved device (providing biaxial stretching) is required (Huang, Lee et al. 2015). Therefore, Huang and co-workers have developed a novel biaxial bioreactor system enabling except circumferential strain (pulsatile pump) also axial one (Figure 13). Axial stress is driven by a linear motor through bellows (Huang, Lee et al. 2015).



Figure 12. Pin-on-ball bioreactor (left) with display detail (right): (1 and 2) step engines providing reciprocating motion for scaffold/tissue pin (P) and/or ceramic ball (B); (3) linear actuator providing compression; (4) specimen holder with nutrition chamber;(5–7) fluid channels providing various pathways of nutrition; (8) contamination glass cover (Wimmer, Grad et al. 2004).

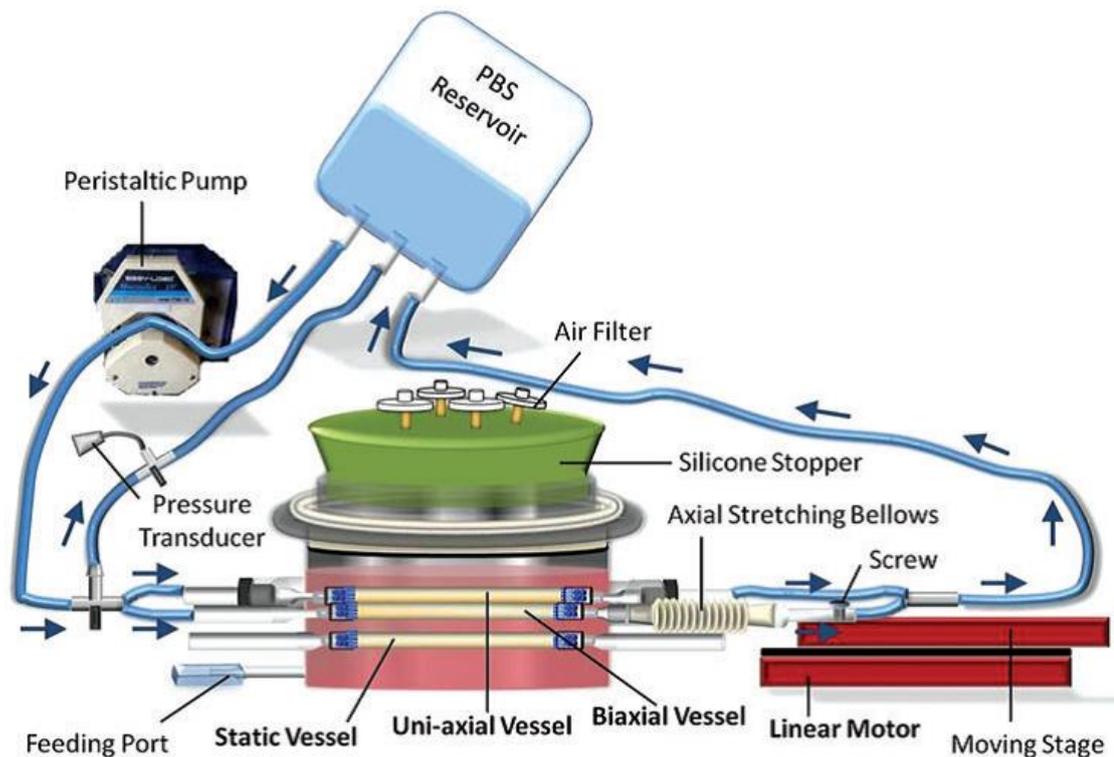


Figure 13. Biaxial bioreactor system. Three tissue-engineered vessels were cultured under different mechanical stimuli within the same biochemical milieu. The biaxial bioreactor is connected to a flow system and linear motor to achieve cyclic biaxial stretching (circumferential and axial stretching). Axial stretch is achieved when the linear motor axially stretches the movable connector of the bioreactor through a bellows. Circumferential stretch is achieved through pulsatile phosphate-buffered saline (PBS) flow that is maintained by a peristaltic pump. Arrows indicate the direction of flow. (Huang, Lee et al. 2015).

Bioreactors enabling electrical stimulation

The term bioelectricity may be described as ion flows, voltage gradients or/and electric fields produced or occurring endogenously within living systems and mediating informative information (Levin 2012). The beginnings of the investigation focused on electrical signalling within animals are connected with the research of Luigi Galvani and his experiments with contracting frog's leg muscles from the 18th century (Cajavilca, Varon et al. 2009). However, electric sensitivity is not only the privilege of nervous systems and muscles. It has been subsequently reported that bioelectric signals can influence e.g. gene expression, cell migration and proliferation, stem cells differentiation, and are included in embryogenesis or wound healing (Isaacson and Bloebaum 2010, Levin 2012, Levin 2014, Mathews and Levin 2018). The generation of bioelectrical signals is based on the activity of ion channels or pumps (Levin 2014). Furthermore, apart from the cell membrane, also nuclear envelope and membrane of other intracellular organelles (e.g. mitochondria) are involved in this process (Jang, Byun et al. 2015). The transduction of electrical signalling is frequently based on

voltage-gated receptors – sodium and/or calcium channels (Catterall, Wisedchaisri et al. 2017). The methods of *in vitro* electrical stimulation could be subdivided according to electric signal delivery into direct coupling (electrodes in direct contact with the cell culture) and an indirect one (Balint, Cassidy et al. 2013). Indirect stimulation comprises capacitive coupling (the homogenous electromagnetic field is created between metal or carbon layers above and below the cell culture), inductive coupling (electromagnetic field is produced by coils placed around the cell culture), or combination of both (Balint, Cassidy et al. 2013). Concerning the design of bioreactor, Barash and co-workers described a novel system combining electrical and medium flow stimulation (Barash, Dvir et al. 2010). They improved basic perfusion bioreactor by simple inserting of two carbon rod electrodes (Barash, Dvir et al. 2010). Pavesi and co-workers also created the bioreactor enable delivering of controlled electrical stimulations (Pavesi, Soncini et al. 2014). The cells were seeded on the glass slide and inserted into culture chambers with two embedded electrodes (steel rods) (Pavesi, Soncini et al. 2014). These chambers were placed into chassis with embedded connectors allowing chamber electrodes connection with the electrical stimulator (Pavesi, Soncini et al. 2014). Mobiny and co-workers utilized for their study the standard 6-well cell culture plate with L-shaped platinum electrodes incorporated into the lid of the plate (Mobini, Leppik et al. 2016) (see Figure 14).

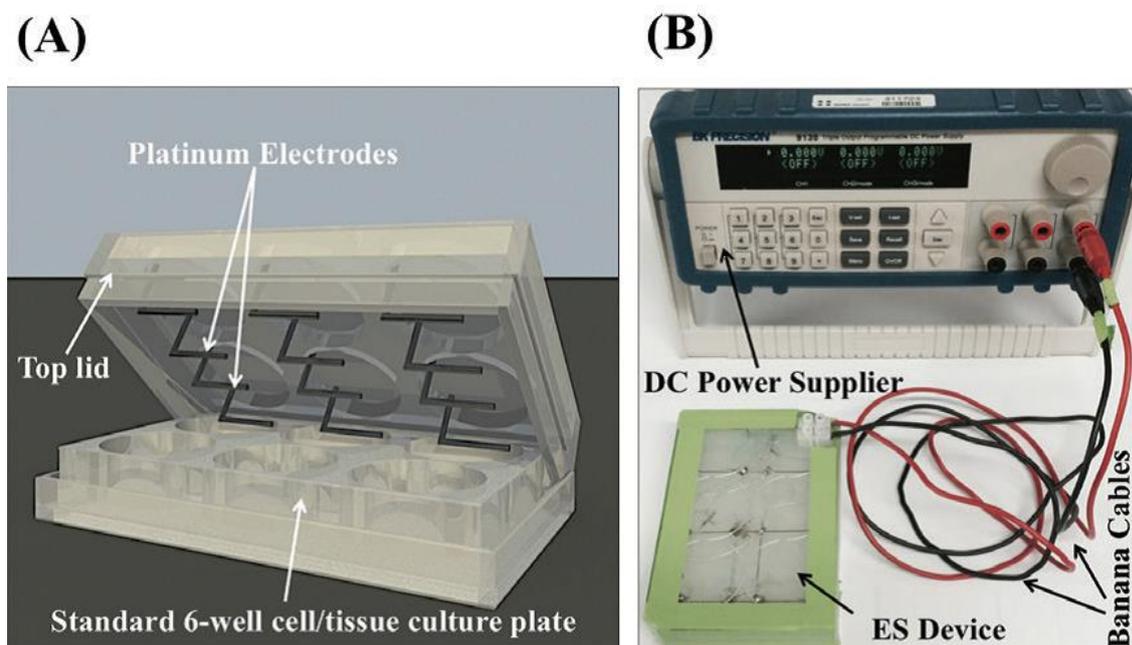


Figure 14. Electrical stimulation (ES) chamber. (A) ES device design of the chamber with L-shaped platinum electrodes incorporated into the lid of a standard 6-well plate. (B) ES device connected to the power supply with cables equipped with banana clips, facilitating transfer to an incubator while remaining connected (Mobini, Leppik et al. 2016).

Donnelly and co-workers also used cell culture plastic to design a bioreactor for *in vitro* electrostimulation of cells in monolayer and also in 3D engineered muscles (Donnelly, Khodabukus et al. 2010). In this case, the lid contained U-shaped stainless steel electrodes (Donnelly, Khodabukus et al. 2010).

The magnetic field could be mentioned among other physical factors, which could influence cellular behaviour. Fahy and co-workers investigated the effect of shear stress and magnetic field on cells, both separated and simultaneously (Fahy, Alini et al. 2018). The shear stress caused alignment and elongation of cells along the flow direction, but on the other hand a decrease in a number of cells (Fahy, Alini et al. 2018). The moderate magnetic field enhanced cells proliferation without changing cells shape (Fahy, Alini et al. 2018). However, the simultaneous stimuli of both factors changed cytoskeleton without decreasing number of cells, therefore the developed system allowed the creation of optimized culture condition of calf pulmonary artery endothelial cells (Fahy, Alini et al. 2018).

8. MATERIAL PROPERTIES AFFECTING CELLULAR BEHAVIOUR

It has been already described that material properties significantly influence cellular behaviour.

8.1. Two-dimensional view (2D)

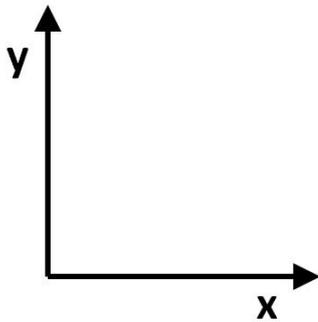


Figure 15. Two-dimensional space – relating to two dimensions, usually describable in terms of breadth or length and height (lying on a plane, having an area but not enclosing any volume)(Anderson 2006).

In the case of 2D point of view, the surface properties in the sense of both chemical and physical characteristics represent the key factors of interactions between cells and material.

8.1.1. Physical characteristics

Of the whole group of physical characteristics, the surface energy and surface topography should be highlighted. The surface energy determines the wettability and significantly influence not only cellular behaviour, but also influence the protein absorption (Saini, Singh et al. 2015). Although synergic effect both surface energy and topography is expected to be a crucial factor for cell adhesion and proliferation (Borges, Benetoli et al. 2013), it has been described the greater importance of surface topography in several research papers.

The importance of surface topography is most likely related to the structure of the ECM, which surround cells in natural environment. The ECM is a component of all tissues and organs and its composition and topography are specific for each tissue/organ (Frantz, Stewart et al. 2010). In general, ECM is composed of interwoven fibrillary proteins (e.g. collagen, elastins, fibronectins or laminins) embedded in a network of proteoglycans (Skoog, Kumar et al. 2018). The ECM constitute a complex, hierarchically structured environment based on micro/nano-scaled pores, grooves, ridges and fibres (Skoog, Kumar et al. 2018).

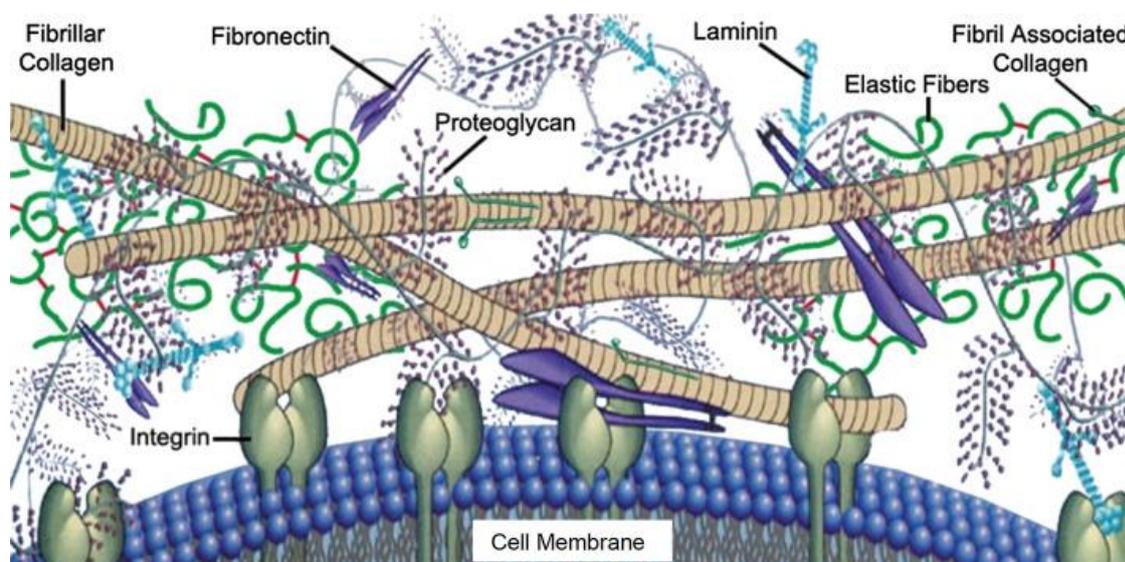


Figure 16. Scheme of extracellular matrix components and their composition (Aamodt and Grainger 2016).

As substructures of ECM have a size in micrometre/nanometre range, it is clear that the effort of researchers was focused on the preparation of micro- and nanostructured material (Grespan, Martewicz et al. 2016, Skoog, Kumar et al. 2018). Surface texturing can be achieved using lithography techniques (Acikgoz, Hempenius et al. 2011) self-assembly approaches (Gunkel-Grabole, Palivan et al. 2017), thermal processing (Lizundia, Saenz-Perez et al. 2017), chemical (Chun, Khang et al. 2009) or laser treatment (Nedela, Slepicka et al. 2017). Electrospinning or inkjet printing could be also used (Skoog, Kumar et al. 2018).

The texture of the surface comprises roughness (a complex of irregular elements with variable sizes) or creating of regularly repeated features (pits, pillars/protrusions, grooves etc. as can be seen in Figure 17).

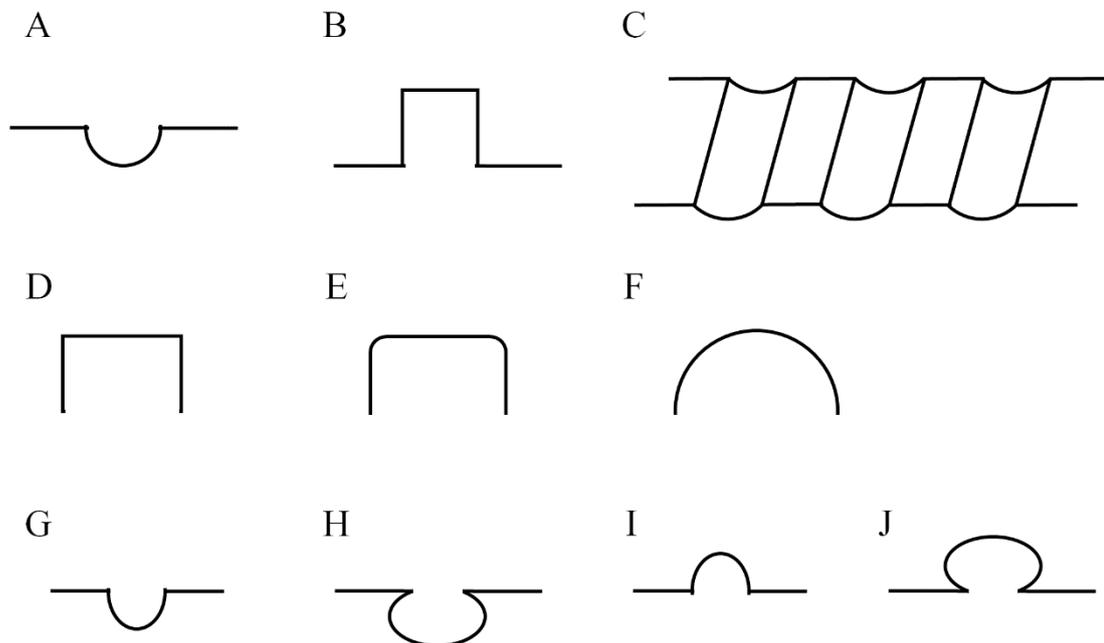


Figure 17. Scheme of basic surface texture features: A) pits; B) pillars/protrusions; C) grooves. Individual features can moreover vary in their “sharpness” (D-F) and convexity (G-H)/ concavity (I-J) (Gui, Xu et al. 2018).

The curvature of a single feature (convex, concave), as well as its “sharpness” (sharped, curved or curved edges) also influence the cell response (Harvey, Hill et al. 2013). It has been already described that surface topography influence proliferation (Taskin, Xia et al. 2017), migration (Kim and Kim 2018), differentiation (Chen, Han et al. 2018), influence adhesion strength (Hampe, Li et al. 2018) or altered gene expression (Gasiorowski, Liliensiek et al. 2010).

However, the results describing relationships between material topography are ambiguous and vary with the cell types, substrate material, and surface feature character (Skoog, Kumar et al. 2018). Dalby and co-workers studied cellular behaviour on island topography (Dalby, Marshall et al. 2002). Human mononuclear blood cells and platelets showed no difference in comparison to flat control (Dalby, Marshall et al. 2002). Contrary to blood response, the fibroblasts and endothelial cells changed their morphology (Dalby, Marshall et al. 2002). Fibroblasts became rounded and their cytoskeleton was poorly developed (Dalby, Marshall et al. 2002). On the other hand, the endothelial cells were more spread with morphology similar to *in vivo* (Dalby, Marshall et al. 2002).

Moreover, cell source also affects the cellular behaviour on structured surfaces. Rebollar and co-workers described the effect of the corrugated surface on the orientation of rat skeletal myoblasts and human myoblasts (Rebollar, Frischauf et al. 2008). Human myoblasts aligned on foils, contrary to rat myoblasts, which

preferably showed more random orientation on majority of tested surfaces (Rebollar, Frischauf et al. 2008).

Finally, the surface topography also affects protein absorption on the material surface and generated protein layer modulates the interaction between cells and surface (Lackner and Waldhauser 2010). In the context of nanostructure topography and its influence on cellular behaviour, the studies on topo chip could be mentioned. This topic is more detailed described in one of the following chapters.

8.1.2. Surface chemistry

The term surface chemistry is mainly connected with the chemical composition, especially the presence of functional groups on the surface of the material. Considering the cell interaction with the biomaterial surface on molecular level, cells interact with the artificial surface *via* the cell adhesion molecules (integrin, cadherin or selectin receptors) enabling cell-cell and cell-ECM connection (Petruzzelli, Takami et al. 1999). The extracellular domain of cell adhesion molecule mediates the bonding with the ligand presented on the surface of another cell, ECM or artificial substrate, and therefore mediate the cell adhesion. In fact, the extracellular domain bonds to specific functional groups, more precisely to specific amino acid sequence (Srichai and Zent 2010). The most famous sequence is artificial RGD (a tripeptide composed of Arginine, Glycine and Aspartic acid). This sequence is presented for example in fibronectin, protein occurred abundantly in the ECM, which plays a crucial role in mediating cell attachment, cell migration during embryonic development, and it is also crucial for self-renewal of satellite stem cells. (Jhala and Vasita 2015) Based on the significance of RGD sequence, it is commercially available and commonly utilized for material modification and improvement of cell attachment (and of course, for improvement of biocompatibility in general) (Hersel, Dahmen et al. 2003). Moreover, it is used in both aliphatic and cyclic form. Beside the RGD, several other adhesive motifs have been described. As an example, YIGSR (Tyrosine - Isoleucine - Glycine - Serine - Arginine) detected in laminin (Wang, Tang et al. 2015) or LDV (Leucine - Aspartic acid - Valine) could be mentioned (Humphries, Byron et al. 2006). Besides cell binding domains, also growth factors could be immobilized onto the surface of the material and purposefully influence the cellular behaviour (Tallawi, Rosellini et al. 2015).

Multifarious methods of chemical modification of polymer surface exist. Plasma treatment, UV/ozone treatment, chemical grafting or their combinations can be used for introducing of new functional groups, in general (Rashidi, Yang et al. 2014). Apart from individual functional group, the chemical composition of the surface could be modified with bioactive substances (frequently with growth factors, ECM components or above mentioned adhesive motifs) by physical adsorption or covalent bonding (Tallawi, Rosellini et al. 2015). The plasma treatment introduces different chemical groups (varying with the used gases and

operating parameters (Sengupta and Prasad 2018)) onto the material surface and influences surface properties such as wettability and surface energy (Jacobs, Declercq et al. 2013). Moreover, this treatment also influences surface roughness (Jacobs, Declercq et al. 2013). All mentioned factors directly influence cellular behaviour, as was demonstrated by Wang and co-workers (Wang, Favi et al. 2016). They used cold atmospheric plasma to modify the 3D printed polylactic acid (Wang, Favi et al. 2016). The altered roughness, hydrophilicity and chemical composition of the surface of material promoted osteoblast and mesenchymal stem cells adhesion and proliferation (Wang, Favi et al. 2016). Described results were supported with the study of Jeon and co-workers. They utilized improved oxygen plasma generator by using templates with various holes sizes (Jeon, Lee et al. 2014). Using this technology, the surface properties (also chemical composition, roughness and hydrophilicity) of poly (ϵ -caprolactone) based scaffold were modified (Jeon, Lee et al. 2014). The positive impact of the mentioned modification on human osteosarcoma cells adhesion, proliferation and differentiation was described (Jeon, Lee et al. 2014). Plasma treatment is also often used to activate the material surface for its further functionalisation. For example, Novotná and co-workers activated polyethylene surface with argon plasma and activated surface subsequently grafted with fibronectin or bovine serum albumin (Novotna, Bacakova et al. 2013). The improvement of vascular smooth muscle cells adhesion and proliferation was observed on functionalized materials (Novotna, Bacakova et al. 2013).

8.2. Three-dimensional view

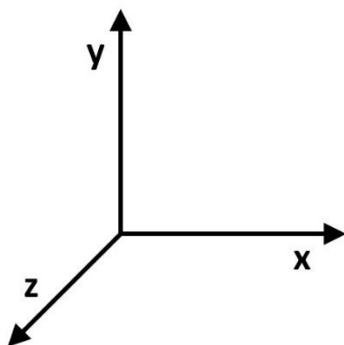


Figure 18. Three-dimensional view – relating to three dimensions (depth, width and height), having volume (Anderson 2006).

As scaffolds for tissue engineering are three-dimensional, besides the above mentioned 2D properties, the bulk properties of scaffolds should also be taken into account. First of all, the mechanical characteristics are firstly discussed in scientific articles. Moreover, also thermal, optical, electrical and magnetic properties must be taken into account.

8.2.1. Mechanical properties

As mentioned above, the mechanical characterization of novel scaffold for tissue engineering is the most common characteristic element. In general, the scaffold should exhibit the same mechanical properties as the tissue or organ to be regenerated (O'Brien 2011). This is important both for the fulfilment of the function of a temporary implant and for formation the appropriate environment for cellular behaviour. The scaffold should be also fixed enough to allow safe manipulation during the *in vitro* pre-cultivation and following surgical procedure. To be more specific, the very first criterion is the modulus of elasticity (Viney 2013). The modulus of elasticity (Young's modulus) can be considered as one of the most important mechanical characteristics of any scaffold and solid material generally. It is defined as the gradient (tensile stress/extensional strain) of the linear (initial) part of a stress-strain diagram (Koltzenburg, Maskos et al. 2017). It describes the stiffness of scaffolds. The elastic modulus of natural tissue vary from very stiff (rat Achilles' tendon ~ 310 MPa) to very soft ones – (e.g. human fat ~ 17 Pa or mammary glands ~ 160 Pa) (Levental, Georges et al. 2007). The importance of this material characteristic is supported by several studies focusing on changing the behaviour of cells cultured on substrates with varying stiffness/elasticity. Engler and co-workers described the strong influence of the material's stiffness on the differentiation of stem cells (Figure 19). The soft gels mimicking brain elasticity induced neurogenesis of mesenchymal stem cells, stiffer matrices with elasticity similar to striated muscle one (8 – 17 kPa) were myogenic and comparatively rigid matrices (25 – 40 kPa) were osteogenic (Engler, Sen et al. 2006).

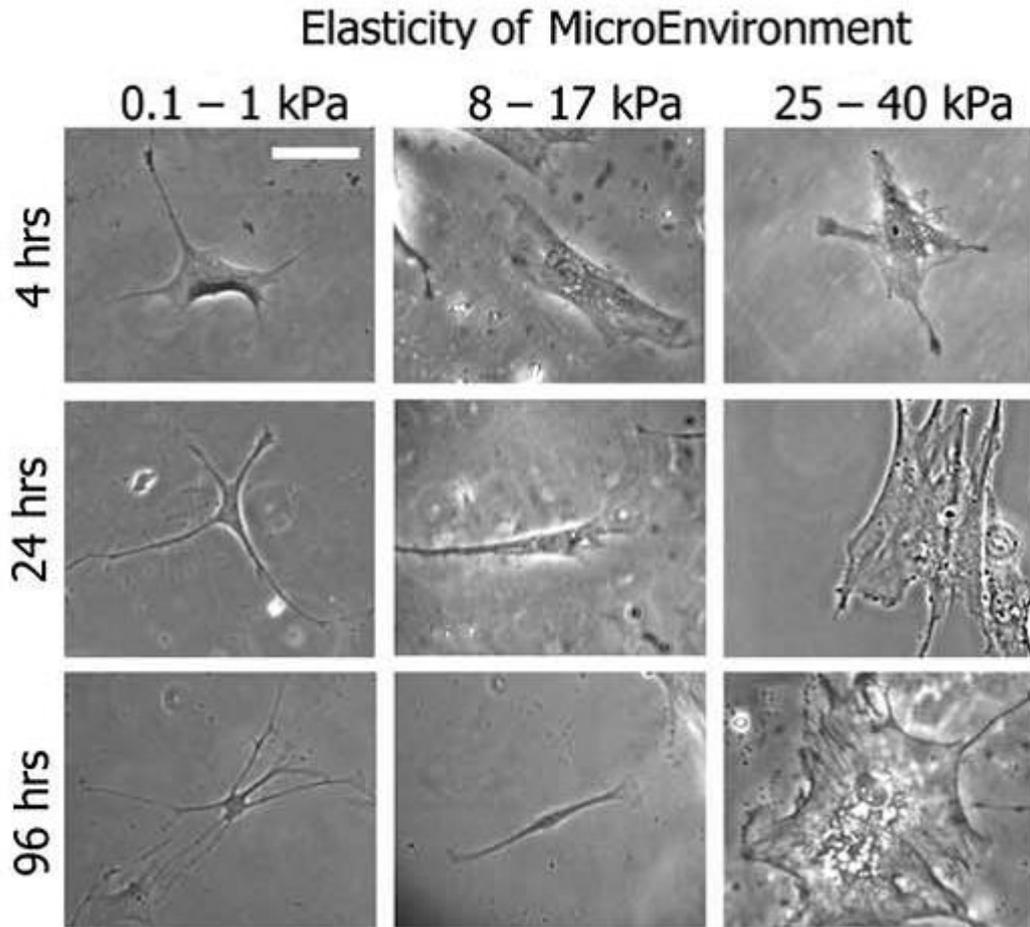


Figure 19. Naive MSCs of a standard expression phenotype are initially small and round but develop increasingly branched, spindle, or polygonal shapes when grown on matrices respectively in the range typical of E_{brain} (0.1–1 kPa), E_{muscle} (8–17 kPa), or stiff crosslinked-collagen matrices (25–40 kPa). Scale bar is 20 μ m (Engler, Sen et al. 2006).

These findings correlate with the study of Leipzig and co-workers, in which the stiffness of substrate below 1 kPa was demonstrated as optimal stiffness for neurogenesis of neural stem/ progenitor cells (Leipzig and Shoichet 2009). The stiffness of material influence not only the differentiation of stem cells but also other elements of cell physiology. Evans and co-workers showed that increasing substrate stiffness from 0.041 MPa to 2.7 MPa promotes cells spreading and proliferation of embryonic stem cells (Evans, Minelli et al. 2009).

Moreover, many tissues exhibit fibre alignments, such as tendons and ligaments, blood vessels, skin or skeletal muscles. This fibre alignment leads to anisotropic mechanical properties of tissue (properties vary depending on the testing directions) optimal for its specific physiological function. For example, Lynch and co-workers revealed that tensile properties of the tendon are about two orders higher along fibre direction compared to perpendicular one (Lynch,

Johannessen et al. 2003). Moreover, Takaza and co-workers studied the stress-stretch behaviour of skeletal muscle of porcine (Takaza, Moerman et al. 2013). The results showed that the tissue in cross-fibre direction is stiffer (77 kPa stress at a stretch of 1.1) and in the longitudinal direction is less stiff (10 kPa stress at a stretch of 1.1) (Takaza, Moerman et al. 2013). Certainly, the scientists follow that requirements. For example, Li *et al.* created electrospun anisotropic nanofibrous scaffold based on poly (ϵ -caprolactone) by incorporating of moving element, on which the producing fibres were deposited and aligned (Li, Mauck et al. 2007). Wakuda and co-workers revealed the optimised methodology for the production of fibrous, mechanically anisotropic collagen hydrogel scaffold (Wakuda, Nishimoto et al. 2018). Collagen fibre in scaffold maintained the characteristic triple helical structure and should exhibit the physiological activity as natural collagen (Wakuda, Nishimoto et al. 2018). Moreover, it should be added that prepared scaffold also induced cell extension of cultivated human umbilical vein endothelial cells along the fibre direction (Wakuda, Nishimoto et al. 2018). The last example represents the study of Hsu *et al.* (Hsu, Lee et al. 2018). They prepared mechanically anisotropic poly(glycerol sebacate) membrane with incorporated alignment poly(vinyl alcohol) fibres (Hsu, Lee et al. 2018).

Focusing on the mechanism how matrix stiffness influence the cellular behaviour, Swift and co-workers used proteomic analysis to reveal that tissue elasticity directly influenced protein laminin-A level (Swift, Ivanovska et al. 2013). Laminins are filamentous proteins located close to cell nuclei contributing nuclear stiffness and stability (Swift, Ivanovska et al. 2013). They are believed to modulate the process of transcription (Swift, Ivanovska et al. 2013).

8.2.2. Scaffold architecture

Nowadays, tissue engineering tends to follow natural structures (tissue histology) to generate biomimetic scaffold (Li, Ding et al. 2015). The scaffold architecture is also a key factor, which must be taken into account. The most important feature of each scaffold to be fulfilled is an appropriate porosity. Porosity is described as the percentage of the void volume of the sample over its bulk volume (Kuila, McCarty et al. 2014). However, porosity significantly influences the mechanical properties of the scaffold (Olubamiji, Izadifar et al. 2016). Moreover, the porosity is not important in itself, the pores must be interconnected to properly perform their function (Loh and Choong 2013). This is important for several reasons. At first, the interconnected pores enable the cell to migrate within the scaffold. Therefore, the pore interconnection is a crucial characteristic for appropriate cell ingrowth and penetration within the whole bulk of the material. The second aspect is connected with the compensation of vascularization. The interconnected pores allow efficient nutrients support and, on the other hand, the waste products removal. The degradation products of the scaffold can also directly abandon the system. (O'Brien 2011)

Another important factor is the pore size, more precisely the mean pore size. The general rule is that the pores should be large enough to enable cell penetration and creation of the ECM. According to size, the pores should be divided into nano-sized (< 100 nm), micro-sized (100 nm – 100 µm) and macro-sized pores (100 µm – millimetres) (Bruzauskaite, Bironaite et al. 2016). The recommended pore sizes of scaffolds depend on tissue, which is proposed to be regenerated.

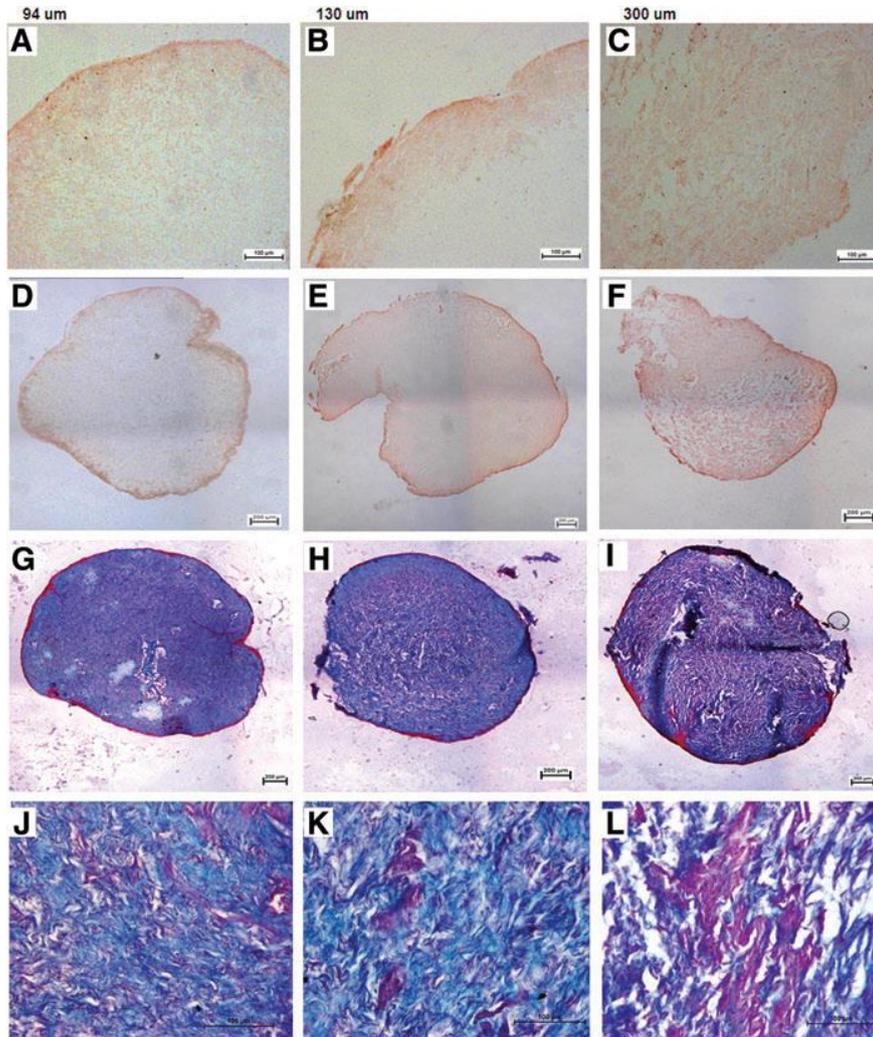


Figure 20. Histological sections of scaffolds with a mean pore size of 94 µm (A, D, G, J), 130 µm (B, E, H, K), and 300 µm (C, F, I, L) after 28 days of culture. The sections were immunohistochemically stained for collagen type I (A–C) and collagen type II (D–F) and safranin-O with a fast green counterstain and imaged at x4 magnification (G–I) and x10 magnification (J–L). Collagen type I and II are represented by the brown staining in (A–F), respectively, whereas the deep pink stain in (G–L) represents synthesized sGAG. There is evidence of collagen type I and II and sulfated glycosaminoglycans in all three scaffold groups after 28 days of culture. The scaffolds with the largest mean pore size (300 µm) supported the greater synthesis of sGAG and collagen type II in the centre of the scaffold in comparison to the scaffolds with smaller mean pore sizes (94 and 130 µm) (Matsiko, Gleeson et al. 2015).

For example, Matsiko and co-workers revealed that scaffold with the mean pore size about 300 μm stimulated chondrogenic gene expression and cartilage-like matrix deposition of mesenchymal stem cells in comparison to scaffolds with the mean pore size of 94 and 130 μm (Matsiko, Gleeson et al. 2015). Histological sections of scaffolds are depicted in Figure 20.

Moreover, the significance of pore size could vary more or less with the used scaffold, the condition of cell cultivation and also with the type of cells. For example, the behaviour of differentiated chondrocytes isolated from Wistar rats on gelatine scaffolds, with various pore sizes ranging from 50 to 500 μm , was studied by Lien *et al.* (Lien, Ko et al. 2009). The positive correlation between pore sizes, cell growth and expression of the gene marks for aggrecan (cartilage-specific proteoglycan), collagen I, II and X was described (Lien, Ko et al. 2009). The chondrocytes preferred the scaffolds with pore size ranging from 250 μm to 500 μm (Lien, Ko et al. 2009). Zhang *et al.* also examined the behaviour of chondrocytes, however, isolated from the calf (Zhang, Lu et al. 2014). In the presented study, the collagen porous scaffolds with pore size ranging from 150 to 500 μm were utilized and these scaffolds with pre-cultivated cells were *in vivo* implanted into athymic nude mice (Zhang, Lu et al. 2014). Interestingly, the pore size did not influence either capability of adhesion, nor proliferation rate, however, the effect on gene expression and production of the cartilaginous matrix were observed (Zhang, Lu et al. 2014). Scaffold with pore size ranging from 150 to 250 μm promoted the best cartilage regeneration (Zhang, Lu et al. 2014).

Moreover, it should be mentioned that several tissues exhibit a gradient porous structure (e.g. skin, in which structure the pore sizes increase with the distance away from the surface of the skin) (Loh and Choong 2013). The biomimetic scaffolds with gradient structure have been already prepared (Wang, Xu et al. 2016).

The pore size correlates also with the specific surface area¹¹. The specific surface area is decreasing with the increasing pore size (Murphy and O'Brien 2010). Murphy *et al.* revealed the importance of the specific surface area in the context of adhesion and proliferation of osteoblast on the scaffold with different pore sizes (Murphy, Haugh et al. 2010). Cells proliferated preferably on scaffolds with the largest pore size (a mean pore size of 320 μm). However, in the case of cell adhesion and early stage of proliferation (48h), the scaffolds with smaller pores (a mean pore size of 120 μm , exhibiting more extensive specific surface area) were preferable for cells (Murphy, Haugh et al. 2010). Scaffolds are profitable to exhibit a high specific surface area for tissue engineering. In this point of view, the high specific surface area permits the high incorporated ligands density and improve the cell-material interaction. The cells have therefore enough opportunity to interact with the interstitial surface area and successfully create

¹¹ The specific surface area of a porous material is defined as the interstitial surface area of the voids and pores either per unit mass or per unit bulk volume of the porous material (Schetz, J. A. and A. E. Fuhs (1999). *Fundamentals of Fluid Mechanics*. Jefferson City, Wiley.

desired tissue or organ. (O'Brien 2011) Described in a simple way, the more specific surface area the more opportunity for cells to interact.

Finally, the shape of pores is also important (e.g. the long pores are beneficial in the engineering of tissue of peripheral axons) (Bruzauskaite, Bironaite et al. 2016).

8.3. Material properties in “four-dimensional” view

Besides already mentioned characteristics, the last, but not least factor is time. So, there are considered 3D aspects changing in time. In this section, it is important to note that the implanted scaffolds should not be considered to be permanent implants. They serve as temporary support to enable regenerate tissue formation.

Therefore, the scaffolds should degrade in the rate allowing the cells to produce their own ECM and to gradually create the required functional tissue (O'Brien 2011). During the scaffold's degradation, the new empty “pores” are formed. These empty places are subsequently ideally colonized with proliferated cells. Too slow degradation limited cell proliferation and migration and could lead to a deficiency of nutrient and oxygen support (Loh and Choong 2013). On the other hand, fast degradation can negatively affect the mechanical and structural integrity of scaffold and developing tissue (Loh and Choong 2013).

Besides the disruption of the initial tissue formation, the rapid degradability can also influence the microenvironmental conditions in place of regeneration, for example the pH increase/decrease (Tandon, Cimetta et al. 2013). The degradation should also be in conformity with the healing rate, which unfortunately varies with the age. This variation is the most obvious in the case of scaffolds for orthopaedic application.

The degradation is not the only factor influencing the time-dependent interaction between the cells (tissues) and scaffolds. Moreover, it is necessary to mention that cells are not in the rigid microenvironment in living systems. Besides the changing in concentration of dissolved nutrients and signalling molecules, the properties of the ECM itself are also changing. The changes in mechanical properties may occur. So, the microenvironment changes can influence the cells fate. This aspect is very important especially in the case of stem cells, where microenvironments provide a dynamic biophysical and biochemical instrument, which influence stem cells proliferation, migration and mainly differentiation (Hilderbrand, Ovadia et al. 2016). Therefore, researchers currently focus their efforts in developing of strategies that include active compound delivery systems in order to add a fourth dimension to the design of 3D scaffolds (Hanauer, Latreille et al. 2015).

9. POLYMERIC BIOMATERIALS WITH ADVANCED PROPERTIES

In following chapter, only two chosen advanced properties of many possibilities will be mentioned - the electrical conductivity and nanostructured surfaces. These two properties are in the centre of attention of the presented dissertation thesis.

9.1. The electrical conductivity of polymers

As electrical signalling plays a non-substitutable role in the living system and several cell types require electrical stimulation for its proper function, the conductive polymers (CP) have the indispensable site in tissue engineering. In general, an electric conductor could be described as a material with free electrons allowing the transport of electrical current (Mercier, Zambelli et al. 2012). Voltage (V) of electrical current of intensity (I) moving through a conductor of resistance (R) is based on the Ohm's law (Mercier, Zambelli et al. 2012).

$$V = RI$$

Based on geometrical parameters of the conductor (for example the cross-section S and the length L), electrical resistivity ρ could be calculated (Mercier, Zambelli et al. 2012).

$$\rho = \frac{RS}{L} = \frac{1}{\delta}$$

Resistivity (with the unit of ohm·cm) is, therefore, inverse of electrical conductivity (unit S/cm) (Taherian and Kausar 2018).

Contrary to the electrical conductivity of metals, which is based on the random movements of the electrons, the intrinsic conductivity of conduction polymers is grounded in their conjugated backbone consisted of alternating single and double bonds (Cui, Yang et al. 2016). This unique structure enables the electrons to move along the chain (Cui, Yang et al. 2016). The conductivity could be modulated by phenomena called doping (generating charge carriers). Dopants utilized for this purpose could vary from small ions (chlorine ions) to large (polymers) (Kaur, Adhikari et al. 2015). Used dopant agent (especially in case of large ones) could influence also the surface properties (e.g. roughness) and subsequently cellular behaviour (Collier, Camp et al. 2000).

Additionally, to electric conductivity, CP exhibit also ionic one (Stejskal, Trchová et al. 2015). The ionic conductivity is related to ionic molecular bonding and with the mobility of free anions or cations (Taherian and Kausar 2018). The atoms are ionized and coulomb forces are responsible for keeping ions together (Grimnes and Martinsen 2014). Based on this force, ions are bounded and solid material shows no ionic conductivity (Grimnes and Martinsen 2014). However, in water, the dissociated ions demonstrate the ionic conductivity (Grimnes and Martinsen 2014).

9.2. Conductive polymers

As the most famous representatives, the polypyrrole (PPy), polyaniline (PANI) and poly(3,4-ethylenedioxythiophene) (PEDOT) could be mentioned. The utilization of conducting polymers as biosensors or drug delivery devices have been already described (Inzelt 2017, Sankoh, Vagin et al. 2017). Nowadays, the interest of scientists have been directed to tissue engineering (Kaur, Adhikari et al. 2015). In this context, it is important to produce 3D electrically conductive scaffolds. There are generally three possible ways how the electrically conductive scaffolds can be prepared (see Figure 21). Focusing on the first-mentioned group, the electrospinning method is abundantly utilized with success. The nano/microfiber form of the scaffold is attractive because it mimics the structure of ECM or tissue structure. Chen and co-workers developed aligned electrospun nanofibers composed of PANI and poly(ϵ -caprolactone) (Chen, Sun et al. 2013). The fibre alignment directed mouse myoblast orientation and together with the incorporation of conductive element promoted myotube formation (Chen, Sun et al. 2013). Concerning the coating of material with a CP, polylactide nanofibers covered with PANI promoted differentiation of bone marrow-derived mesenchymal stem cells into osteoblasts (Chen, Yu et al. 2018). Moreover, PEDOT nanoparticles deposited on chitosan/gelatin porous scaffold surface promoted adhesion and proliferation of neuron-like rat pheochromocytoma cells and (based on gene expression assessment) enhanced cellular neurite growth (Wang, Sun et al. 2017). Moreover, improvement of mechanical properties and thermal stability were observed (Wang, Sun et al. 2017). Finally, the preparation of scaffolds based on CP is an attractive topic for researchers. Guex *et al.* produced porous conductive scaffold based on PEDOT and polystyrene sulfonate (PSS) by freeze-drying of PEDOT:PSS dispersion (Guex, Puetzer et al. 2017). Scaffold promoted differentiation of mouse pre-osteogenic precursor cells into osteoblasts (based on increasing osteogenic markers and osteocalcin deposition) (Guex, Puetzer et al. 2017).

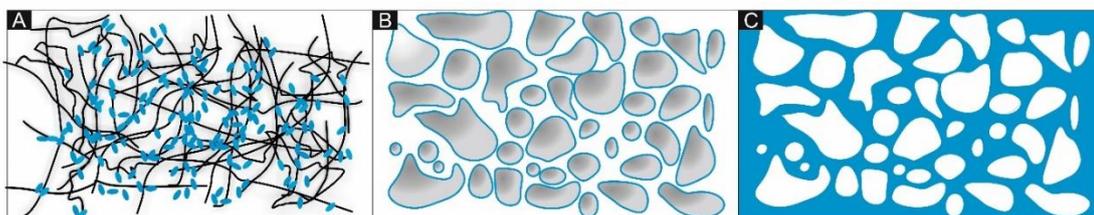


Figure 21. Three-dimensional materials based on CP; A) electrospun fibres containing conducting colloids, B) matrices of biodegradable polymer covered with a thin conducting film, C) conducting polymer scaffold (CP within the respective 3D structure is marked in blue). Source: non published schema from Cell biology laboratories, Centre of polymer systems, Tomas Bata University in Zlin.

As an example of advance scaffold approaching the native structure of the tissue, the work of Wang and co-workers could be mentioned (Wang, Wu et al. 2015). They designed scaffold mimicking skeletal muscle tissue consisting of series of core-shell columns and sheets (Wang, Wu et al. 2015). The aligned nanofiber core consisted of blended poly(ϵ -caprolactone), silk fibroin, and PANI. Cores mimic myofibers' structure in skeletal muscles (Wang, Wu et al. 2015). The poly(ethylene glycol)-co-poly(glycerol sebacate) hydrogel create the shell mimicking the extracellular connective tissue in natural tissue (Wang, Wu et al. 2015). This unique artificial structure induced 3D myoblasts alignment and formation of elongated myotubes (Wang, Wu et al. 2015). Preparation scheme of scaffolds is depicted in Figure 22.

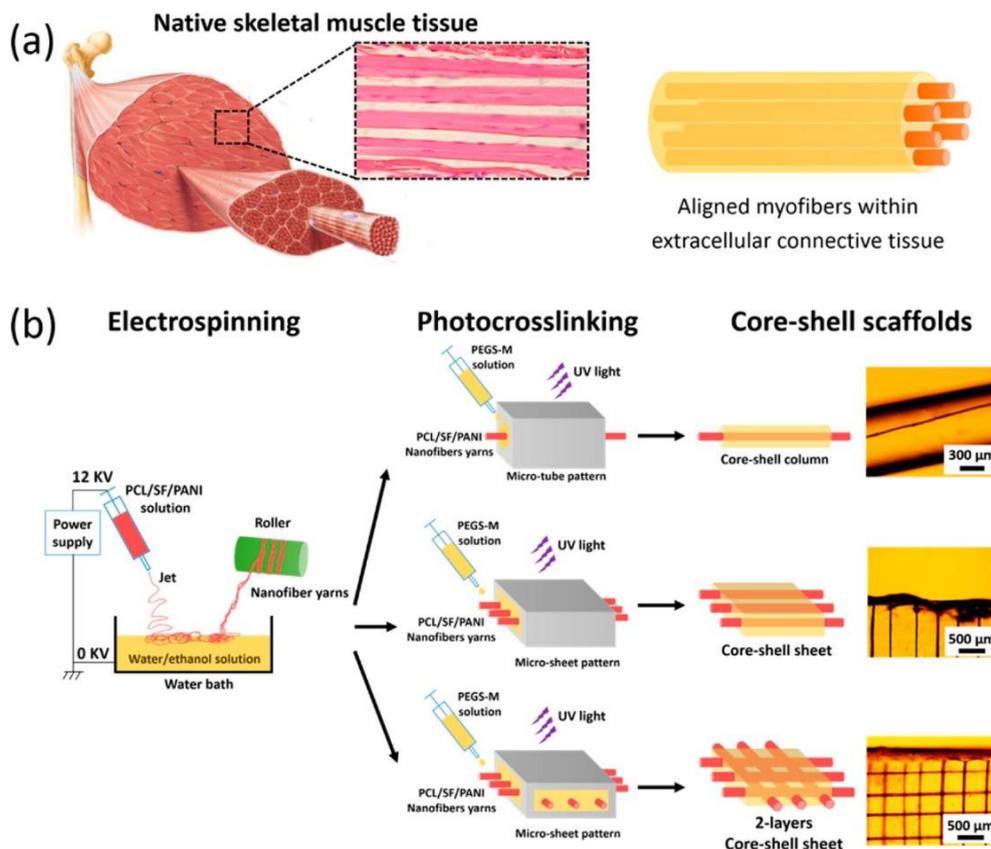


Figure 22. a) The structure of skeletal muscle tissue, consisting of aligned myofibers formed through myoblast fusion together into multinucleated myotubes surrounded within the extracellular connective tissue b) Preparation scheme of core-shell column and sheet scaffolds that mimic the native skeletal muscle tissue by the combination of aligned nanofiber yarns via electrospinning and hydrogel shell via photocurable microfabrication of poly(ethylene glycol)-co-poly(glycerol sebacate) (PEGs-M) solution (Wang, Wu et al. 2015).

9.3. Nanostructured polymer surfaces

There is emerging evidence that nano-topography can be a very important factor influencing the cell (tissue) interaction with biomaterials. One of the initial studies that opens this field of science, was published in Nature in 2011 (Baker 2011). In this and the following works, the cell line dependent effect of nanotopography was described.

Manson *et al.* revealed the NIH/3T3 behaviour on surfaces modified with polymer nanopillars fabricated by 3D direct laser writing (Buch-Månson, Spangenberg *et al.* 2017). Strong cells' interaction with nanopillars was observed (Buch-Månson, Spangenberg *et al.* 2017). On surfaces with higher nanopillars densities, the cell cytoskeleton was remodelled; actin filaments tend to enwind nanopillars resulted in altered morphology (Buch-Månson, Spangenberg *et al.* 2017). Yu and co-workers in their study investigated the combined effect of both nanotopography and mechanical stimulation (Yu, Prodanov *et al.* 2013). Periodontal ligament stem cells demonstrated an extended, spindle-like shape with alignment parallel with the nanogrooved surface texture (Yu, Prodanov *et al.* 2013). However, this alignment changed with mechanical stimulation (Yu, Prodanov *et al.* 2013). The cells were arranged perpendicularly to the direction of the mechanical stretching force and retained their shape (Yu, Prodanov *et al.* 2013). Moreover, the increased amount of DNA was observed after 3 days of cell culturing on a stretched nanostructured substrate (Yu, Prodanov *et al.* 2013). Altered gene expression was also noted in this case – the expression of *Runx2* gene (a typical bone marker) increased (Yu, Prodanov *et al.* 2013).

Nevertheless, the cellular response to topography is really complex and various factors of surface geometry could altered cellular behaviour. Therefore, the advanced approach in an effort to reveal the relationship between cell behaviour and surface topography is necessary. Unadkat *et al.* (Unadkat, Hulsman *et al.* 2011) prepared chips of poly(lactic acid) with 2,176 different topographies and, using high-content imaging, revealed heretofore unknown surface topographies induced human mesenchymal stromal cells proliferation or osteogenic differentiation. They described an effective tool for deciphering the “Braille code” of cell-topography interactions. A scheme of proposed high-throughput screening of biomaterial is presented in Figure 23A including a created library of surface topographies (TopoChip). Preparation of TopoChip is subsequently depicted in Figure 23B. Its design is based on the three basic “primitives” (circles, triangles and thin rectangles). The topographic units are assembled from these “building blocks”. The block of repeated features is called TopoUnit. The resulting TopoChip (Figure 23C) consists of two distinct TopoUnits, each in two repetitions.

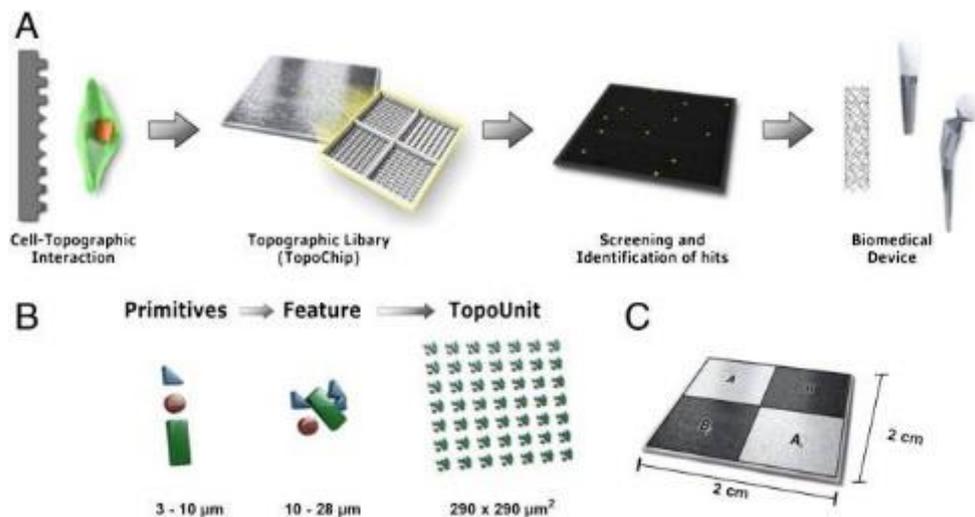


Figure 23. TopoChip design (Unadkat, Hulsman et al. 2011).

Beside influenced stem cell proliferation and differentiation, it was also evaluated the different effect on cell morphology (see fluorescent micrographs in Figure 24 A-D or scanning electron microscopy images in Figure 24 E-H, in which the spread or aligned cells can be seen), or texture of cell membrane (see scanning electron microscopy images in Figure 24 I-J).

Lately, the effect of topography on pluripotency of human-induced pluripotent stem cells was determined using the same design of micro-TopoChips (Reimer, Vasilevich et al. 2016). Moreover, the model predicting relationships between the material topography design and cell morphology was constituted (Hulsman, Hulshof et al. 2015). Recently, the nano-TopoChip has been developed to study the effect of nano-sized topography on cell behaviour (Hulshof, Zhao et al. 2017).

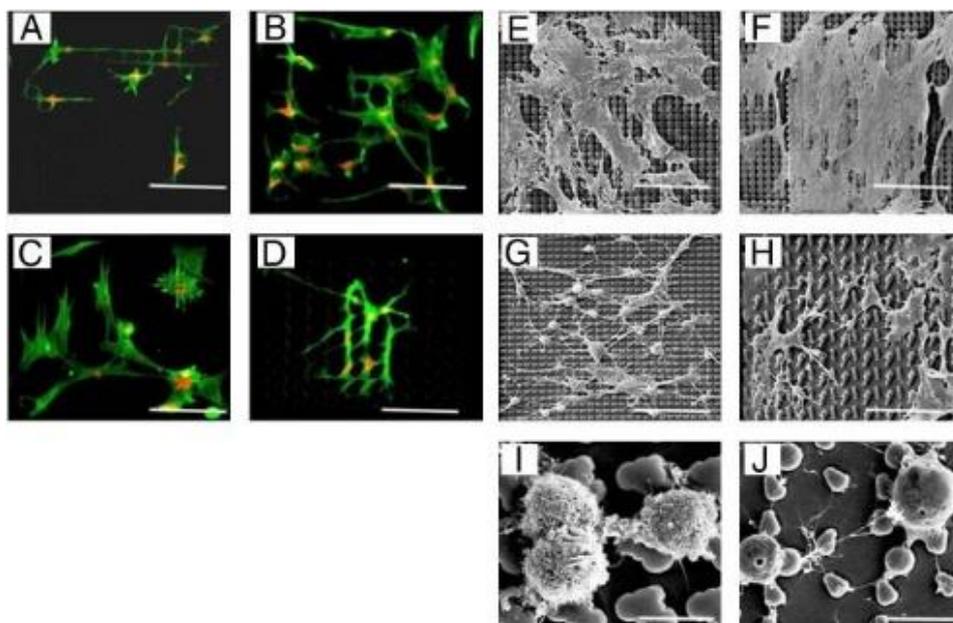


Figure 24. Diverse cell morphologies and differences in the texture of cell membrane (Unadkat, Hulsman et al. 2011).

10. AIMS OF DOCTORAL THESIS

The aim of the presented thesis is the study of the interaction of eukaryotic cells and polymer materials. This aim has been defined in accordance with formerly achieved results of research conducted in laboratories at our institution and with the aid of information gathered from literature review summarized in previous sections. The aim of this thesis may be achieved by pursuing following goals. According to the current state of knowledge, the aims of work were defined:

- 1) Determination of interaction of eukaryotic cells with conductive polymers.
- 2) Study of cell response to structured polymer surfaces with focusing on cell adhesion, proliferation and cytoskeletal alternations.
- 3) Implementation of methodology for testing the ability of eukaryotic cells to incorporate within 3D polymeric scaffolds with the utilization of bioreactors. Testing of cell ability to expand within 3D porous polymeric scaffolds.

11. EXPERIMENTAL PART

The main aim of the presented doctoral thesis was to widespread the current knowledge about the interaction of materials, especially the polymeric-based materials, and eukaryotic cells. To reach this goal, it was necessary to come familiar with various experimental approaches used in material and polymer science, and also to work in the laboratory of cell biology. The important part of presented study was oriented to the introduction of new methodological approaches of cell cultivation within the bioreactors to become a common instrument for determination of material/cell interaction. Therefore, the interest will be focused not only on the results of the work, but also on the introduction of crucial problems solved referring to work with bioreactors.

11.1. Materials and sample preparation

Various polymeric materials were used during the study. Due to the fact that the author of thesis was also a member of a team solving the project of Czech Grant Agency no. 17-05095S, it has been intensively worked with conducting polymers, mainly with PANI. PANI can be prepared by various methods, but the IUPAC protocol were mostly used for preparation of PANI powders or films, which will be referred here as “standard”. This standard PANI was then modified by a variety of methods, which will be described below. In some cases, the IUPAC protocol was modified for preparation of a material, which was expected to have some advantageous properties. The modification of PANI films with acids was one of the alternations.

11.1.1. PANI films prepared according to IUPAC and modified with acids

The PANI films were formed *in situ* directly on the tissue culture plates (TPP, Trasadingen, Switzerland). The polyaniline salt (PANI-S) was prepared by chemical oxidation of aniline hydrochloride with ammonium persulfate in aqueous solution according to IUPAC technical report (Stejskal and Sapurina 2005). An appropriate amount of aniline hydrochloride (2.59 g, Neratovice, Czech Republic) and ammonium persulfate (5.71 g, Sigma-Aldrich, St. Louis, MO, USA) was separately dissolved in 50 mL of water. Then, both solutions were mixed, briefly stirred and poured into culture plates. The polymerization reaction lasted 1 h at room temperature. Then, the solution was poured out and deposited a green film of PANI-S was rinsed with 0.2 M hydrochloric acid followed by methanol. The films were left to dry in air overnight.

To prepare the polyaniline base (PANI-B), the films were immersed in 1 M ammonium hydroxide for 12 h.

In order to prepare films doped with sulfamic acid (PANI-SULF) and phosphotungstic acid (PANI-PT), the PANI-B films were re-protonated with either 1M sulfamic acid (Sigma-Aldrich, St. Louis, MO, USA) or 50 wt %

aqueous solution of phosphotungstic acid (Sigma-Aldrich, St. Louis, MO, USA). The solutions of acids were poured onto the surface of the PANI-B film and the reaction was left to proceed for 24 h. Afterwards, the residual solutions were poured out and the films were rinsed with methanol and left to dry in air.

The second type of modified film was prepared using direct polymerization with poly(2-acrylamido-2-methyl-1-propanesulfonic) acid (PAMPSA) present in the reaction mixture of aniline hydrochloride and ammonium persulfate. Modified procedures published by Bayer *et al.* (Bayer, Trenchard *et al.* 2010), Stejskal *et al.* (Stejskal, Sapurina *et al.* 1999), and Yoo *et al.* (Yoo, Cross *et al.* 2007) were employed. Firstly, an aqueous solution of PAMPSA was prepared, its target concentration corresponding to 0.028 mol of its monomer (acrylamido-2-methyl-propanesulfonic acid). Aniline hydrochloride (0.028 mol) was then added to the PAMPSA solution and stirred at room temperature for 1 h. The mole ratio of aniline hydrochloride to PAMPSA was adjusted to 1:1 (PANI-PAMPSA-1:1) and 2:1 (PANI-PAMPSA-2:1). The oxidant, ammonium persulfate (0.025 mol), was dissolved separately in ultrapure water and added to the reaction mixture at the mole ratio for aniline hydrochloride to an oxidizing agent of 1:0.9. Polymerization was completed within 60 min. The films were rinsed with water and methanol to remove any adherent PAMPSA and left to dry in air.

The PANI films prepared by above-described methods were used for the study of adhesion, proliferation and migration of NIH/3T3 cells, published in article entitled “Adhesion, Proliferation and Migration of NIH/3T3 Cells on Modified Polyaniline Surfaces”.

11.1.2. PANI films prepared in colloidal dispersion mode

Similarly to the above mentioned modified PANI films, films prepared in colloidal dispersion mode were also formed *in situ* directly on the tissue culture plates (TPP, Trasadingen, Switzerland). The methodology of synthesis PANI films prepared in colloidal dispersion mode is based on the procedure described in IUPAC Technical Report with modifications (Stejskal and Sapurina 2005). To produce PANI films, aniline hydrochloride (2.59 g) was dissolved in 50 mL of an aqueous solution of the respective stabilizer (40 g.L⁻¹), namely poly(N-vinylpyrrolidone) (PVP), sodium dodecyl sulfate (SDS), Tween 20 (T20), or Pluronic F108 (F108) (all Sigma-Aldrich). Then, an aqueous solution (50 mL) containing 5.71 g of ammonium persulfate was added, and the reaction mixture was stirred and poured over the substrate. The oxidation of aniline was left to proceed for 1 h. PANI films formed on supports were then rinsed with 0.2 mol.L⁻¹ HCl and left to dry in air. Similar films were also prepared with aniline hydrochloride and stabilizer dissolved in 1 mol.L⁻¹ hydrochloric acid instead of water that is under higher acidity of the reaction medium. The films prepared in the absence of stabilizers were referred to as standard films.

Cell compatibility of PANI films prepared in described colloidal dispersion mode was investigated and published in article “Cell-compatible conducting polyaniline films prepared in colloidal dispersion mode”.

11.1.3. Green synthesis of PANI powder

During the Erasmus traineeship in Italy, set of PANI powder samples was prepared at the chemical department of University degli Studi di Milano. The adopted methodology protocols was already described in the literature (Falletta, Costa et al. 2014).

PANI prepared by oxidation of aniline with potassium persulfate in the presence of hydrochloric acid was set as a reference powder (PANI_A). Aniline (2 mL, Sigma Aldrich, USA) was stirred with 36 mL of 6 M HCl at low temperature (ice bath). Then, an aqueous solution (8.8 g, in 40 mL of water) of potassium persulfate (Sigma Aldrich, USA) was added dropwise to the solution keeping the mixture still at ice bath. Green product was filtered after 6 hours, washed several times with water and acetone and dried in an oven at 40°C.

One of the green chemistry methods for synthesis employs $\text{H}_2\text{O}_2/\text{Fe}^{3+}$ system as oxidant and N-(4-aminophenyl)aniline (aniline dimer, AD) as initial reactant (PANI_AD). N-(4-aminophenyl)aniline (1g, Sigma Aldrich, USA) was dissolved in 36 mL of 6 M HCl. After the complete dissolution, 2 mL of an aqueous solution of 31% H_2O_2 was added followed by adding of 0.75 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Sigma Aldrich, USA). The reaction was kept 24 hours under room temperature. The obtained powder was filtered, washed several times with water and acetone and dried in an oven at 40°C.

The third sample, PANI_EtOH, was prepared by oxidation of AD with H_2O_2 in the presence of ethanol in the reaction mixture. AD (1 g) was dissolved in 100 mL of ethanol. Then, the HCl (2.27 mL) and H_2O_2 (2.35 mL) were added. The reaction was kept 24 hours under room temperature. Obtained PANI_EtOH powder was separated by filtration, washed firstly several times with water, then with acetone, and dried in an oven at 40°C.

Additionally to the first set of samples prepared in presence HCl (PANI_A_HCl, PANI_AD_HCl, PANI_EtOH_HCl), the second set was synthesized using H_2SO_4 (PANI_A_ H_2SO_4 , PANI_AD_ H_2SO_4 , PANI_EtOH_ H_2SO_4).

All samples were finally doped with salicylic acid (Sigma Aldrich, USA) according to protocol already described in the literature (Sironi, Marinotto et al. 2015). Firstly, samples were separately deprotonated in 300 mL of 0.1M NH_4OH aqueous solution. After 4 hours, the powders were filtered, washed several times with water until the mother liquor became neutral, and dried in an oven at 40°C. Sample reprotonation with salicylic acid was performed by putting each sample in contact with an aqueous solution of this acid, maintaining an aniline/acid molar ratio of 2:1 (theoretical protonation degree of polyaniline salt). All samples were filtered, washed with water and dried in an oven at 40°C.

Besides previously described and studied forms (powders, films, colloids), the preparation of 3D porous scaffold exhibits a novel approach expanding the application potential of PANI into tissue engineering.

11.1.4. PANI cryogels

PANI cryogels were prepared by polymerization of aniline in frozen poly(vinyl alcohol) solutions in cooperation with the Institute of Macromolecular Chemistry of the Academy of Sciences of the Czech Republic. The detailed methodology is summarized in (Humpolicek, Radaszkiewicz et al. 2018). Prepared PANI cryogels were utilized for the study of their cytotoxicity. The results are published in article entitled “Polyaniline cryogels: Biocompatibility of novel conduction macroporous material” (Humpolicek, Radaszkiewicz et al. 2018).

Additionally, lyophilised PANI cryogel was prepared by freeze-drying of native cryogels for 24 h in a CoolSafe Pro (Scanvac, Denmark).

11.1.5. PpPDA powders

Besides PANI, the interest was also focused on poly(*p*-phenylenediamine) (PpPDA) - although categorised into non-conducting polymers, it exhibits structure and properties closely related to PANI (Stejskal 2015). Similarly to PANI, PpPDA could be synthesized in various forms depending on the reaction conditions.

PpPDA powders were prepared in cooperation with the Institute of Macromolecular Chemistry of the Academy of Science of the Czech Republic by chemical oxidation of *p*-phenylenediamine in acidic media (Kuceková, Rejmontová et al. 2017). The ammonium persulfate was used as an oxidant in two concentrations (0.25 and 0.5M) (Kuceková, Rejmontová et al. 2017). The concentration of oxidant is expected to influence the structure of chain (Stejskal 2015).

The cytotoxicity of prepared PpPDA powder was studied and discussed in article “Cytotoxicity od poly(*p*-phenylenediamine)”.

Furthermore, for the purpose of study cell-material surface interaction, the nanostructure polystyrene structures were prepared at the Department of physics and materials engineering, Tomas Bata University in Zlín. Prepared samples were utilized in study cell-material surface interaction. Results of this study are presented in article “Variations of Polymer Porous Surface Structures *via* the Time-Sequenced Dosing of Mixed Solvents”.

11.1.6. Scaffolds based on PVA and fractionated Kraft lignin

Finally, beside conductive polymers, also scaffold originate from biodegradable, renewable resources are highly demanded in tissue engineering. Concerning the presented thesis, the work was focused on lignin as representative of the low-cost, biodegradable natural biopolymer.

The set of scaffolds based on PVA and fractionated Kraft lignin (KL^f) were prepared in cooperation with Graz University of Technology and Brno University of Technology. The concentration of fractionated Kraft lignin varied from 1 to 20 wt%. Briefly, a fraction of Kraft lignin was dispersed in PVA solution and the pH value was adjusted to 2.00 using 2.0 M HCl. Subsequently, Glutaraldehyde was added to the solution and stirred for 30 min. Then, the solution was densified by repletion of six freeze-thawing cycles. The detailed preparation procedure is described in (Rejmontová, Kovalcik et al. 2019, in press) Prepared hydrogels have been designated as PVA_X-KL^f, where X indicates the concentration of lignin (wt%).

Scaffolds based on PVA and KL^f were used for investigation of their biological properties in terms of cell toxicity and the ability of cells to ingrowth and proliferate through the 3D structure of scaffolds. Obtained results are summarized in article “The use of Fractionated Kraft Lignin to improve the mechanical and biological properties of PVA-based scaffolds” (Rejmontová, Kovalcik et al. 2019, in press).

11.2. Characterization of material properties

Various material characteristics are able to alter cellular response in cell-material interaction, e.g. surface chemistry, topography, and in case of 3D scaffold elastic modulus, porosity or pore size. For thin films and solid surfaces, the surface energy is a fundamental characteristic. It can be determined indirectly assessing wetting properties of liquids with known surface tensions (Kohli and Mittal 2013). The wetting properties are characterized by contact angle¹² measurements. The contact angle could be determined directly (measuring the tangent angle at the three-phase contact point on a sessile drop profile) or indirectly (e.g. by Wilhelmy balance method) (Yuan and Lee 2013). For the purpose of the presented doctoral thesis, the conventional sessile drop method was utilized for contact angle determination by telescope-goniometer.

¹² A contact angle is an angle at which a liquid-vapour interface meets the solid surface (Yuan, Y. and T. R. Lee (2013). *Contact Angle and Wetting Properties. Surface Science Techniques*. G. Bracco and B. Holst. Berlin, Heidelberg, Springer Berlin Heidelberg: 3-34.

11.2.1. Surface energy evaluation

The surface energy is one of the fundamental surface properties and, as affects the ability of the cells to adhere, it strongly correlates with biocompatibility and determines the suitability of the material for particular biomedicine application. To determine the surface energy of materials tested during the doctoral study, the Surface Energy Evaluation System (See System, Advex Instruments, Czech Republic) was utilized. For evaluation of contact angle, deionized water, ethylene glycol, and diiodomethane (Sigma Aldrich; USA) were used as testing liquids. The droplet volume was set to 2 μL and ten separate readings were averaged to obtain one representative value. Using this data, the surface energy of the substrate was determined by the “acid-base” method.

11.2.2. Measurement of electrical conductivity

As the main portion of studied materials represents conductive polymer, PANI, the conductivity of prepared samples was also determined. The conductivity of thin films could be determined by measuring the resistance and dimensions of specimens.

To determine the conductivity of flat samples, the four-point van der Pauw method was used. A programmable electrometer with an SMU Keithley 237 current source and a Keithley 2010 Digital Multimeter with a 2000 SCAN 10-channel scanner card (USA) were employed. Measurements were carried out at laboratory temperature.

11.2.3. Scanning electron microscopy

Furthermore, focusing on 3D polymer scaffolds, the internal structure can be investigated by microscopic techniques, for example by confocal or scanning electron microscope. Within the presented thesis, scanning electron microscopy of sectioned scaffolds was used.

Scanning electron microscopy (SEM) was utilized to reveal the internal structure of PVA and Kraft lignin-based scaffolds in cooperation with the Department of Physics and Materials Engineering, Tomas Bata University in Zlin. The scaffolds were sectioned and measurements were carried out on samples without prior metallization using model Phenom Pro (Phenom-World BV) with a special sample holder that allows the reduction of charges on nonconductive materials. The samples were studied at the acceleration voltage of 10 kV in the backscattered electron.

11.2.4. High-performance liquid chromatography

The presence of toxic residual impurities in polymeric materials has an undesirable impact on the biological properties of the material. Chromatography represents a suitable method for determination of an amount of releasing impurities. For the purpose of the presented thesis, the high-performance liquid chromatography (HPLC) was utilized.

For HPLC analysis, samples were extracted in accordance with ISO 10993-12 in the ratio of 0.1 g of cryogel per 1 mL of ultrapure water. Prior to extraction, the dry cryogel was saturated to equilibrium with ultrapure water. Concentrations of residual impurities were determined using a modular HPLC system consisting of a Waters 600E pump, a VD 040 vacuum degasser (Watrex, Czech Republic), and a UV200 ultraviolet detector (Watrex, Czech Republic). A reversed-phase C18 column X-select (300 mm × 7.8 mm; Waters) was employed. The analysis was performed in isocratic mode with an acetonitrile/acetate pH 4 buffer at a ratio of 60/40 (v/v) as the mobile phase. A flow rate of 0.8 mL per min and 20 µL injection volume were employed. Analytes were monitored at 235 nm. Data acquisition and analysis were performed using a Clarity Chromatography Station.

11.3. Biological properties

The evaluation of cells' response represents the crucial part of the doctoral thesis. The work was focused on the interaction of eukaryotic cells with polymeric material both under *in vitro* and also under simulated *in vivo* cultivation conditions. Moreover, the evaluation of the biological properties of novel polymeric materials is also included within the presented doctoral thesis.

In vitro testing methods are abundantly utilized and well established in this field of study. Various standards defining the biological evaluation could be used, for example, ISO standard 10993 Biological evaluation of medical devices. Moreover, various successfully utilized protocols can be found in already published articles. Contrary to well characterized *in vitro* tests, methods using bioreactors (simulated *in vivo* conditions) are not sufficiently described and unified in the scientific literature. The process of developing a methodology for testing in the bioreactor will be briefly described in Results and discussion section.

The methodology of tests used within the doctoral study and utilized cell lines are summarized below. The set comprises some of common *in vitro* methods (determination of cytotoxicity, cell adhesion, proliferation and migration) and novel evaluation of the ability of the cells to ingrowth within 3D material. Additionally, the impact of the material was studied not only on the cell level, but 3D reconstructed human tissue model was also used.

Prior to biological *in-vitro* testing, materials were disinfected. PANI films (both modified with acids and prepared in colloidal dispersion mode) were disinfected by 30 min exposure to a UV-radiation source operating at a wavelength of 258

nm emitted from a low-pressure mercury lamp. PpPDA powders were disinfected by dry heat at 120°C for 40 min. Finally, scaffolds based on PVA and fractionated Kraft lignin were disinfected by 70% ethanol.

11.3.1. Cell lines

The mouse embryonic fibroblast cell line (ATCC CRL-1658 NIH/3T3, USA) was utilized for the purpose of the presented thesis. The mouse embryonic fibroblasts are recommended in the above mentioned ISO standard 10993 and, moreover, the NIH/3T3 cell line is one of the most frequently used lines in material/cell interaction research. Since continual cell lines could have a slightly different reaction, also primary mouse embryonic fibroblasts (a kind gift of Jiří Pacherník from the Institute of Experimental Biology, Faculty of Science, Masaryk University) were used in certain tests to reveal the interaction of primary cells.

11.3.2. Cytotoxicity

The cytotoxic effect is a fundamental characteristic of biological testing of any material proposed to be in contact with the biological system.

Cytotoxicity in direct contact could be evaluated as a preliminary test. For the purpose of this thesis, cells were seeded in the presence of samples at a concentration of 10^5 cells per mL. Cell proliferation around the tested samples was evaluated after three days using an inverted Olympus phase contrast microscope (Olympus IX81, Japan). Cells seeded on tissue plastic (TPP; Switzerland) were used as a reference.

Cytotoxicity testing of extracts of tested samples was performed according to ISO 10 993-5. Samples were extracted according to ISO 10993-12 in the ratio of 0.2 g of hydrogel sample or 0.1 g of powder per 1 mL of cultivation medium. Extraction was performed in chemically inert closed containers using aseptic techniques at 37 ± 1 °C under continuous stirring for 24 ± 1 h. The parent extracts (100%) were then diluted in a culture medium to obtain a series of dilutions with required concentrations. All extracts were used within 24 h. Cells were pre-cultivated for 24 h and the culture medium was subsequently replaced with individual sample extracts. As a reference, cultivation in a pure medium was used. All tests were conducted in quadruplicates and Dixon's Q test was used to remove outlying values.

Various endpoints are measured in cytotoxicity determinations. They could be grouped into three basic categories – morphological changes, cell growth and proliferation, and specific aspects of cellular metabolism. Several methods enabling the quantitative evaluation of the cytotoxic effect exist. One of them is the colorimetric MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay (Invitrogen Corporation, USA). The absorption was measured at 570 nm with an Infinite M200 Pro NanoQuant (Tecan, Switzerland).

Dixon's Q test was used to remove outlying values. The results are presented as a percentage of cells present in the respective extract relative to cells cultivated in a pure medium (100%).

However, in some circumstances, MTT assay could not be used, e.g. when the MTT interaction with tested extract is observed. In those cases, flow cytometry should be used. Viable cells were counted after one day of cell cultivation (37 ± 0.1 °C) in the presence of sample extracts using a BD FACS Canto flow cytometer (BD Biosciences, Canada) employing SYTO 61 red fluorescent nucleic acid stain (Life Technologies, USA) to assess cytotoxic effects. First, the diluted extracts were sucked up, and the layers of cells were washed with phosphate buffered saline (PBS, BioSera, France). The adhered cells were released using trypsin and stained with SYTO at a final concentration of 30 nM. After 30 minutes, the viable cells were analysed in the dark.

To distinguish healthy, apoptotic, and necrotic cells after cultivation, staining with annexin V/propidium iodide (BD Biosciences, Canada) was carried out and the cells were analyzed with a BD FACS Canto flow cytometer (BD Biosciences, Canada) following the above-mentioned protocol.

The morphology of the cells (qualitative evaluation) was also observed using an inverted Olympus phase contrast microscope (Olympus IX81, Japan).

11.3.3. Cell adhesion

The cell adhesion is one of the main factors permitting the material biocompatibility, as it antedates all another cell behaviour. To perform the cell adhesion assay, the cell suspension was seeded at a concentration of $1 \cdot 10^7$ cells per mL both on surface of tested material and reference culture dishes (TPP; Switzerland). After one hour, the cells were gently rinsed and micrographs were taken.

Considering the cell adhesion to sliced scaffolds, the cells were seeded at a concentration of 10^6 cells per mL. Nuclei counterstaining using Hoechst was utilized for evaluation of this test. Cells were fixed before staining for 15 min with 4% formaldehyde (Penta, Czech Republic) and rinsed by phosphate-buffered saline (PBS, Invitrogen, USA). The required amounts of PBS and $5 \mu\text{g mL}^{-1}$ of Hoechst 33258 were added and left to incubate for 30 min in the dark. The cell nuclei were observed with an inverted Olympus phase-contrast microscope Olympus IX81, Japan).

11.3.4. Cell proliferation

Cell adhesion is the first criterion, but it is not the only one. The cell must be able to both to adhere and proliferate on the surface of the biomaterial. To evaluate the cell proliferation, the cell suspension at a concentration of $1 \cdot 10^5$ cells per mL was seeded on the tested material and micrographs were taken every 24 hours. Cells seeded on tissue plastic (TPP; Switzerland) were used as a reference.

Moreover, the MTT assay mentioned above can be utilized for quantification of cell proliferation.

11.3.5. Cell migration

To simulate wound healing, the modified Scratch assay was performed according to Liang *et al.* (Liang, Park et al. 2007). The scratch was created in a confluent cell monolayer and set of micrographs was periodically captured with an Olympus inverted fluorescent microscope (Olympus, IX 51; Japan) equipped with a digital colour camera (Leica DFC480; Germany). Cells migration on tissue plastic (TPP; Switzerland) was utilized as a reference.

11.3.6. Cell morphology

The impact on cell cytoskeleton was evaluated using an ActinRed 555 (Thermo Fisher Scientific, United States). The proliferated cells were firstly fixed using 4% formaldehyde (Penta, Czech Republic) for 15 min, washed by a PBS buffer, and subsequently poured with a 0.5% Triton X-100 (Sigma-Aldrich, United States) for 5 min until permeabilization. The required amount of PBS and ActinRed 555 (one drop per 1 mL of PBS, Thermo Fisher Scientific, USA) were added and left to incubate for 30 min in the dark. The morphology of the cells was observed with an inverted Olympus phase-contrast microscope (Olympus IX81, Japan)

11.3.7. Testing the ability of the cells to ingrowth within the biomaterial

In comparison to the above mentioned commonly used tests, a methodology for cell expansion within 3D scaffolds using bioreactors was not established in Cell biology laboratories at Centre of polymer systems at the beginning of the study. The introduction of the procedure of cell cultivation within 3D scaffolds was, therefore, the crucial aim of presented thesis as well as the establishment of the methodology for evaluation of this test.

To cultivate cell within 3D polymeric scaffolds, there are two types of bioreactors available in Cell biology laboratories at the Centre of polymer systems – Rotary Cell Culture System and Continuous Flow Perfusion system. Both types simulate microgravity and were manufactured by Synthecon Incorporated. The Rotary Cell Culture System has a disc-shaped culture vessel with the oxygenator membrane at the rearward side of the chamber. In contrast, the Continuous Flow Perfusion System has a two-part cylindrical shaped vessel with microporous perfusion core placed in the centre of the culture chamber.

Cell cultivation in a bioreactor

The optimised protocol used within the doctoral study is following. The cell suspension at a concentration of 1×10^6 cells per mL was gently injected by syringe into each scaffold and cultivation medium was added. The cells were let to adhere in an incubator for 24 hours. After this pre-cultivation period, the seeded scaffolds were inserted separately into the bioreactor vessel, cultivation medium was added and the forward rotation was adjusted to 15.5 RPM. Cultivation in the bioreactor lasted 14 days with the medium changing after seven days.

Evaluation of cell incorporation within the scaffold

First of all, cells within scaffolds were fixed with 4% paraformaldehyde, subsequently washed with PBS, permeabilized with 0.5% Triton X-100 and again washed with PBS three times.

Secondly, the scaffolds were embedded and subsequently sectioned. Focused on methodology, the scaffolds were covered with embedding medium for frozen tissue specimens to ensure optimal cutting temperature (O.T.C. compound, Sakura, Japan) and kept on dry ice to completely freeze and store at $-80\text{ }^{\circ}\text{C}$. For sectioning of the scaffolds, hand microtome (Euromex Microtome MT 5503, Holland) was used (section thickness ranging from 0.5 to 1 mm).

Finally, the embedding medium was removed by PBS and the fixed and permeabilized cells were stained using ActinRedTM 555. Finally, the tested samples were sliced, and the cell morphology was observed using fluorescent microscopy (Olympus IX81, Japan).

11.3.8. Skin irritation test

During the doctoral study, the author of thesis attended the laboratory training *in vitro* testing using the 3D reconstructed human tissue models in MatTek Corporation. Several tests were performed within this training – e.g. eye irritation, skin irritation and skin corrosion test.

One of the mentioned methods, skin irritation test, was also utilized for the purpose of presented thesis. The term skin irritation refers to the induction of reversible damage of skin (erythema, dryness skin, itching). The skin irritation potential was tested *in vitro* after application onto a 3D reconstructed human tissue model (RHT) EpiDerm (MatTek, Slovakia). The exposure time was adjusted to 1 h. After exposure, RHT was carefully rinsed and incubated in fresh medium for 42 h. After the incubation period, skin irritation was evaluated using the MTT assay. The test was performed according to OECD Guideline for the Testing of Chemicals, No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method (OECD 2015) and tested samples were classified as irritants or non-irritants.

11.4. Results and discussion

The presented doctoral thesis deals with the relationship between polymer-based materials and eukaryotic cells or artificial tissue. The understanding of the mentioned relationship is crucial for development of new materials with anticipated biological properties and with application potential in rapidly developing field of biomedicine. Despite the huge interest in biomaterials in last decades, the knowledge of material-cell interactions on bio-interfaces has not been completely accomplished.

Obtained results will be presented in the same order corresponding to defined goals of this thesis. Firstly, the results observed by testing under standard cultivation conditions (2D) will be presented and discussed. In this step, the goals “Cell interaction with conductive polymers” and “Cell interaction with micro-structured polymers” are included. In the next part of the work, the last goal focusing on “Cell interaction with polymeric scaffolds” will be presented. This step include both implementation of methodology as well as testing of cell ability to expand within 3D porous polymeric scaffolds.

Some text in the discussion section could follow in a certain extent the text in articles, in which the author of the thesis is an author or co-author. However, the text is supplemented with additional, unpublished results.

11.4.1. Cell interaction with conductive polymers

Based on cells' sensitivity towards electrical signals, conductive polymers have acquired a firm place in a group of biomaterials. Previously, the emphasis has been paid especially to polypyrrole. However, the growing interest in PANI has been noticed recently. Nowadays, the application potential of PANI tends to expand from microelectronics and corrosion protection to biomedicine, where PANI could be used e.g. as a biosensor. More recently, the interest of scientists has been paid on PANI-based scaffolds for tissue engineering, especially in cardiac and neural tissue regeneration.

Polyaniline (PANI)

PANI could be prepared in various form ranging from powder, colloidal suspension to thin films. Several methods can be used for preparation of those forms of PANI. The interest was mainly focused on IUPAC protocol described by Stejskal and Saporina (Stejskal and Saporina 2005) which is used for production of “standard” PANI. Within the work on the thesis, the altered PANI films were prepared based on the IUPAC methodology. Concretely, either films modified with acids or films prepared in colloidal suspension were synthesized. Additionally, PANI powder was also prepared using green synthesis. Finally, PANI-based scaffold was tested as a novel 3D material.

a) PANI films modified with acids

The utilization of PANI in biosensing and tissue engineering depends on the acquirement of efficient bio-interface properties. In this context, biological properties of pristine PANI have been previously described. For example, the study focused on embryonic stem cell differentiation on pristine PANI films were conducted by Humpolíček and co-workers (Humpolíček, Radaszkiewicz et al. 2015). The results indicated no harmful effect on cardiomyogenesis or neurogenesis of tested PANI films. Furthermore, no embryotoxic effect was found (Humpolíček, Radaszkiewicz et al. 2015). Moreover, adhesion and proliferation of PC-12 cells on PANI films doped with perchloride, hydrochloride, malic and citric acid were studied (Wang, Ji et al. 2008). In addition to interaction with cells, the interaction of this material with blood must be also taken into account concerning the biomedical application. The anticoagulation effect of PANI reprotonated with poly(2-acrylamido-2-methyl-1-propanesulfonic) acid (PAMPSA) *via* the interaction of film with coagulation factors X, V and II has already been described. This films also significantly reduced a platelet adhesion in comparison to standard PANI films or uncoated reference surface. (Humpolíček, Kuceková et al. 2015). The mentioned biological impact of tested films was ascribed to the presence of PAMSA anion on the surface of PANI film (Humpolíček, Kuceková et al. 2015). Apart from PAMPSA, other acids may also be utilized, e.g., sulfamic and phosphotungstic acid.

Within the presented thesis, an investigation was made into the adhesion, proliferation and migration of mouse embryonic fibroblasts on pristine PANI films (PANI-S, PANI-B) and films doped with sulfamic (PANI-SULF) and phosphotungstic acids (PANI-PT). In addition, PANI films supplemented with PAMPSA at various ratios were tested (PANI-PAMPSA-1:1, PANI-PAMPSA-2:1).

Prior to biological testing, the surface energy of tested films was evaluated. Moreover, the surface energy of cell monolayer was also measured, because cell surface properties (e.g. cell surface hydrophobicity or surface charge) could affect the ability of cells to adhere to a solid surface (discussed particularly in the context of bacteria) (An and Friedman 1998, Popovici, White et al. 2014). Therefore, the total surface energy (γ^{tot}) was obtained and the absolute value of the difference between the surface energy of the cells and the sample was calculated ($\gamma^{\text{dif}} = |\gamma^{\text{tot}}$ of the sample - γ^{tot} of cells|). All obtained results, perceived the changes in surface properties of modified samples, are summarized in Table 2.

Table 2. Surface energy evaluation of different polyaniline surfaces.

| Sample | Surface Energy Components (mN.m ⁻¹) | | | |
|-----------------|---|----------------------|----------------------|-----------------------|
| | γ^{tot} | γ^{LW} | γ^{AB} | γ^{dif} |
| PANI-S | 52.54 * | 46.05 * | 6.49 * | 3.33 |
| PANI-B | 50.88 * | 46.54* | 4.35 * | 1.67 |
| PANI-SULF | 52.13 | 44.97 | 7.17 | 2.92 |
| PANI-PT | 51.89 | 47.39 | 4.50 | 2.68 |
| PANI-PAMPSA-1:1 | 41.85 | 40.98 | 0.87 | 7.36 |
| PANI-PAMPSA-2:1 | 56.35 | 43.91 | 12.45 | 7.14 |
| Cells | 49.21 | 23.21 | 26.00 | - |

* The values presented in (Humpolíček, Radaszkiewicz et al. 2015).

An interesting phenomena was observed for PANI samples containing PAMPSA in the reaction mixture. The value of the total surface energy of PANI-PAMPSA-1:1 obtained in this study (41.85 mN.m⁻¹) significantly corresponded to the value of total surface energy of PANI doped with PAMPSA (39.96 mN.m⁻¹) published previously in study of Humpolíček and co-workers (Humpolíček, Radaszkiewicz et al. 2015). This may indicate that the characteristics of PAMPSA predominated and significantly impacted the surface properties of PANI-PAMPSA-1:1 sample. Surface energy changed dramatically when a reduced amount of PAMPSA was used during PANI synthesis (the ratio of aniline hydrochloride to PAMPSA for synthesis was 2:1). In this case, the measured value of γ^{tot} of PANI-PAMPSA-2:1 approximated to γ^{tot} of pristine PANI-S and PANI-B. Thus, it can be concluded that the surface properties of PANI-PAMPSA-2:1 are primarily governed by PANI, and only to a lesser extent by PAMPSA. Moreover, as can be seen, doping PANI with both sulfamic acid and phosphotungstic acid did not influence the surface properties in terms of γ^{tot} in comparison with PANI-S and PANI-B. Furthermore, the obtained γ^{tot} resembled that for the cell monolayer and so should indicate that suitable biological properties exist.

To reveal the interaction of cells with surface of synthesized films, the adhesion, proliferation and migration of the mouse embryonic fibroblast NIH/3T3 cell line were investigated. Concerning the results, cell behaviour corresponds to the surface energy of individual samples. The micrographs in Figure 25 clearly show that the NIH/3T3 cells were able to adhere to all the tested surfaces in a similar way as the reference. However, remarkable differences in the subsequent cell proliferation and morphology existed (see Figure 26).

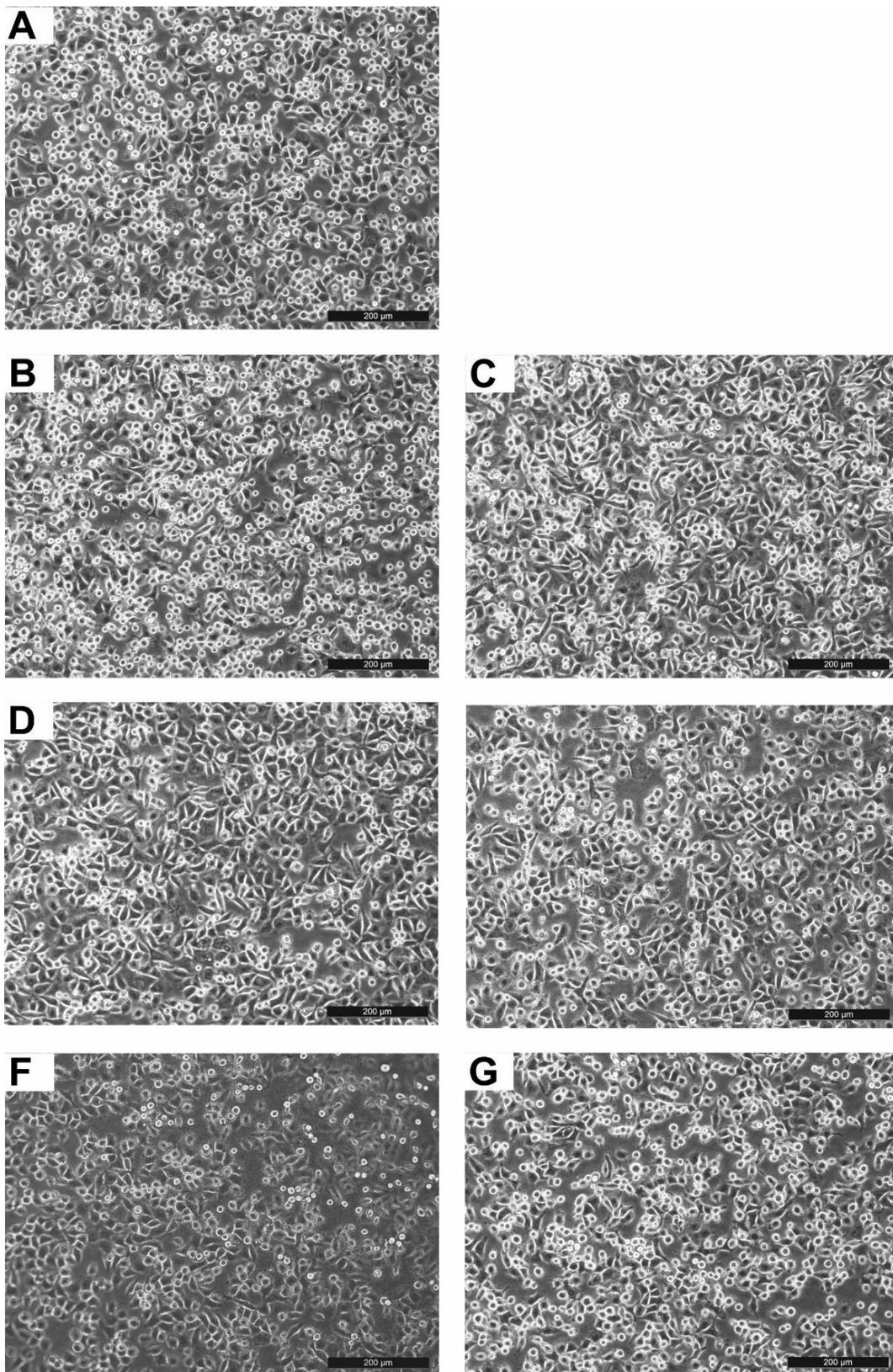


Figure 25. Adhesion: A) Reference; B) PANI-B; C) PANI-S; D) PANI-SULF; E) PANI-PT; F) PANI-PAMPSA-1:1; G) PANI-PAMPSA-2:1. Scale bars correspond to 200 μm .

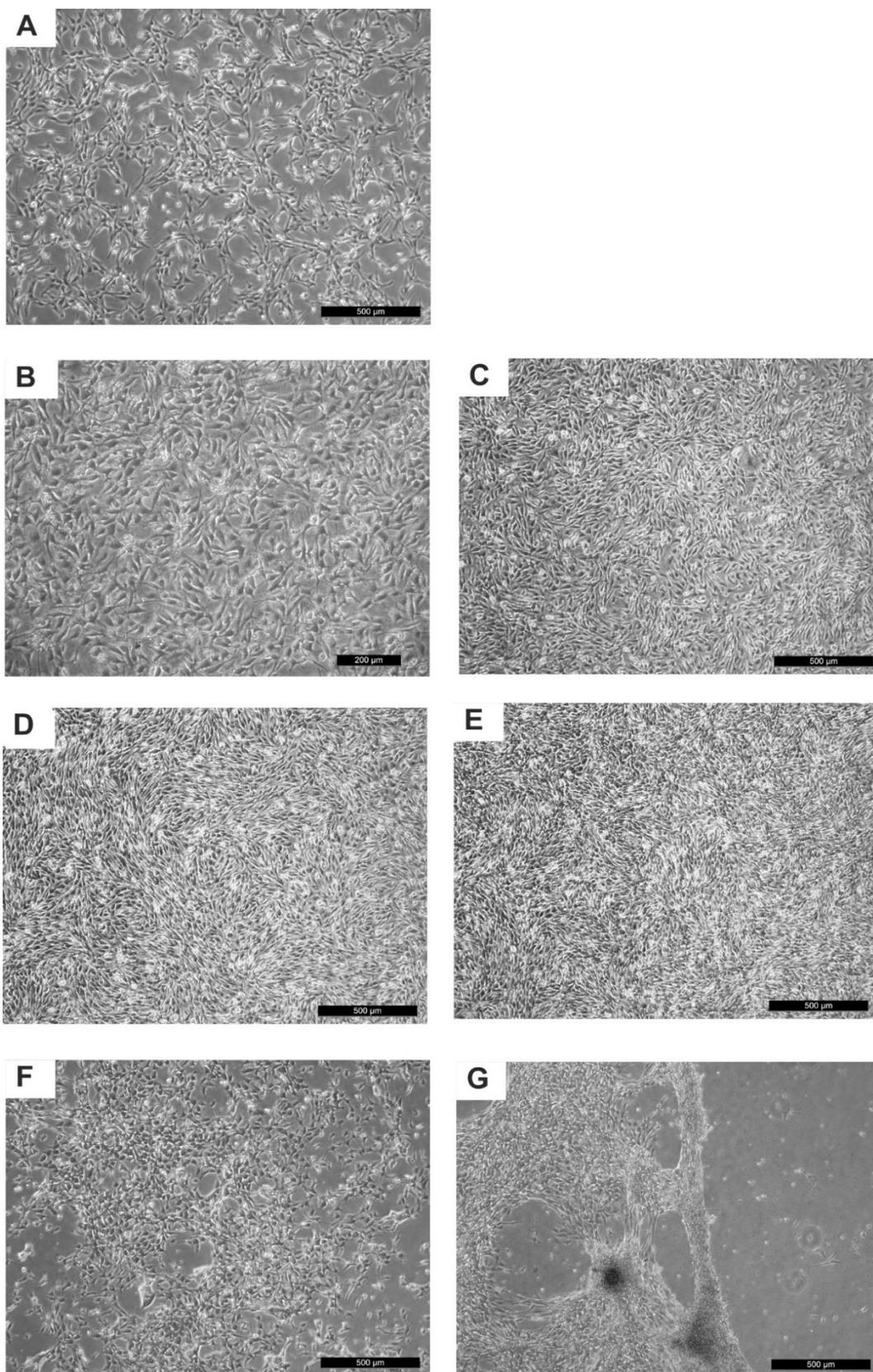


Figure 26. Proliferation: A) Reference; B) PANI-B; C) PANI-S; D) PANI-SULF; E) PANI-PT; F) PANI-PAMPSA-1:1; G) PANI-PAMPSA-2:1. Scale bars correspond to 500 μm .

It was clearly demonstrated that the cells reached semi-confluence on the reference sample after 24 h (Figure 26A). Behaviour comparable to that for reference was observed on the surfaces PANI-S, PANI-B, PANI-SULF, PANI-PT (see Figure 26B, C, D, E, respectively). In contrast, proliferation was significantly decreased on the PANI-PAMPSA-1:1 sample, and the cells initially reached a semi-confluent state after 144 h (see Figure 26F). Cell proliferation was improved on PANI-PAMPSA-2:1, which contained a lower amount of PAMSPA in comparison to PANI-PAMPSA-1:1 sample. This is also in a good agreement with the better cell proliferation observed on pristine PANI-S and PANI-B surfaces, where PAMPSA was absent. Nevertheless, the attachment of cells was weak and any cells adhered on PANI-PAMPSA-2:1 easily detached from its surface; even gentle handling during media exchange caused cell detachment (Figure 26G). Consequently, it can be concluded that introducing PAMPSA into the polymer bulk during synthesis notably affected NIH/3T3 cell proliferation in comparison with reference and pristine PANI. These results correspond to observed preferable behaviour of mouse embryonic stem cells on pristine PANI forms, compared to PANI sample deposited with PAMPSA (Humpolíček, Radaszkiewicz et al. 2015). Although PANI films modified with PAMSA had no negative influence on cardiomyogenesis or neurogenesis, the decreased adhesion of embryonic stem cells was observed (Humpolíček, Radaszkiewicz et al. 2015). Moreover, limited cell spreading was observed on these surfaces (Humpolíček, Radaszkiewicz et al. 2015).

The results of cell migration on the tested surfaces are presented in Figure 27.

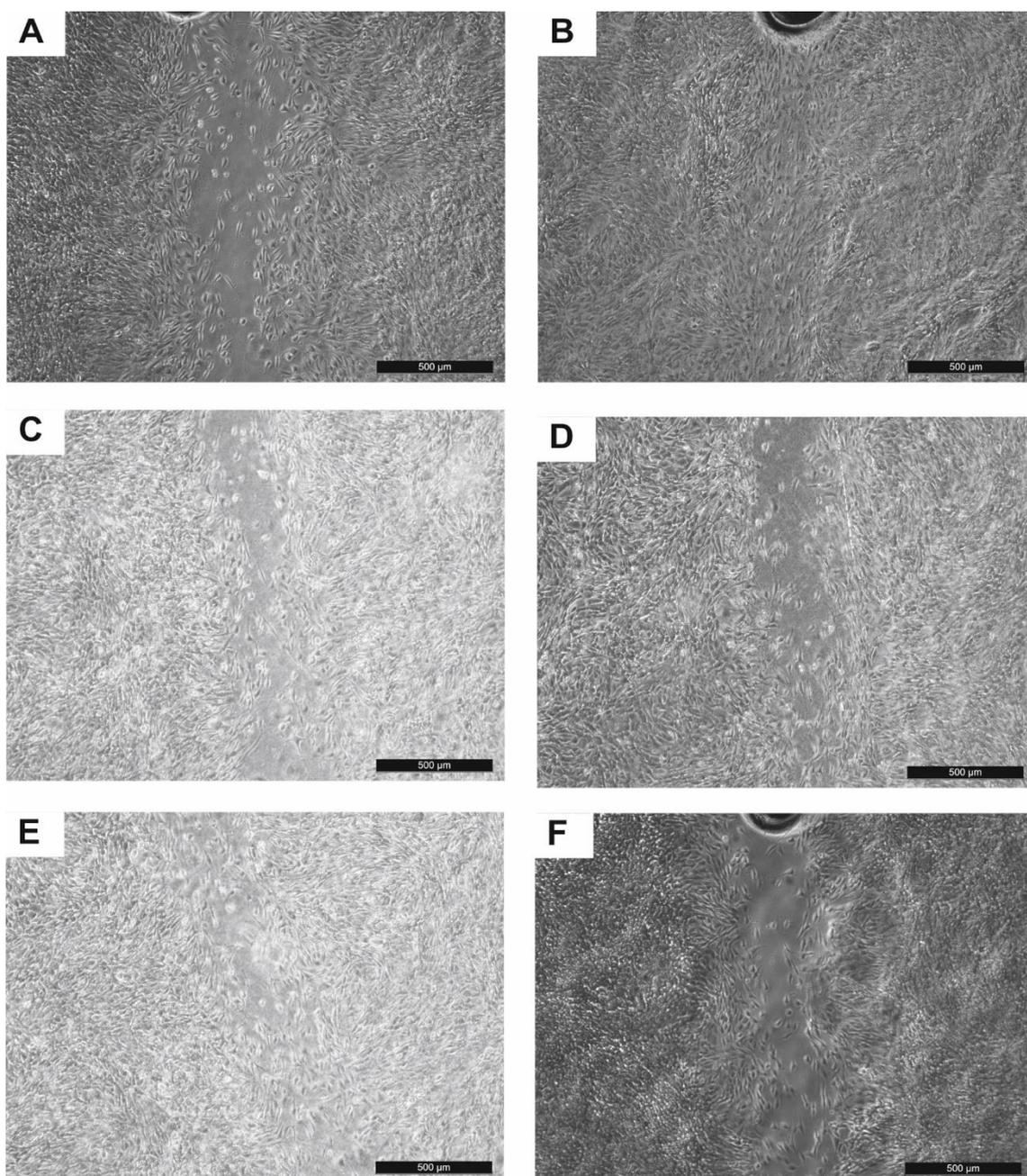


Figure 27. Migration 48 h: A) Reference; B) PANI-B; C) PANI-S; D) PANI-SULF; E) PANI-PT; F) PANI-PAMPSA-1:1. Scale bars correspond to 500 μm .

As can be seen, cells migrated on PANI-PAMPSA-1:1 surface (Figure 27F) significantly more slowly than on the reference. This finding fully corresponds to the limited cell proliferation observed on these surfaces and correlates with the results obtained for the surface energy. Nevertheless, the migration of cells on PANI-B, PANI-S, PANI-SULF and PANI-PT was comparable to cell behaviour on the reference sample (Figure 27A-E).

To conclude this first study, the mouse embryonic fibroblasts were able to adhere, proliferate and migrate on pristine PANI films and those doped with sulfamic or phosphotungstic acids. Therefore, these PANI forms could be suitable for utilization in biomedicine, e.g., in tissue engineering of electrically responsive

tissues such as nerve or muscle ones. Nevertheless, the incorporation of PAMPSA affected the surface properties of the PANI films, significantly influencing the cell proliferation and migration. Therefore, their potential for application in the biomedical sector is limited, but on the other hand, opens the door for utilization as a biosensor or bio-interface, where limited cell adhesion can be desirable. Moreover, cell behaviour corresponds to the surface energy of individual samples and the surface energy constitutes the crucial factor that influences cell/surface interaction. The determination of surface energy is essential to attaining appropriate surface modifications. Obtained results were presented in Article Adhesion, Proliferation and Migration of NIH/3T3 Cells on Modified Polyaniline Surfaces by Rejmontová *et al.*, published in International Journal of Molecular Science (Rejmontova, Capakova et al. 2016). In future, the interaction of those surfaces in context of Vroman effect could be also studied.

b) PANI films prepared in colloidal dispersion mode

Various stabilizers (surfactants or water-soluble polymers) could be used to control PANI cytocompatibility. In the presence of suitable stabilizer, PANI could create a colloidal dispersion, which represents a composite form including a water-soluble stabilizer (Stejskal, Trchová et al. 2015). Colloidal PANI was successfully synthesized using different types of surfactants (Alves, de Melo et al. 2012). Some stabilizers were even tested for their biological properties (Kucekova, Humpolicek et al. 2014). Focusing on material properties, films proceeded during dispersion polymerization are in general thinner and smoother compared to those proceeded from the reaction mixture without stabilizer (Stejskal and Sapurina 2004). The PANI films synthesized in colloidal dispersion mode could, therefore, represent interesting conducting material with application potential in tissue engineering or as biosensors.

During the doctoral study, PANI films were synthesized (with participation of Nela Maráková, doctoral student) in presence of four stabilizers, namely poly-N-vinylpyrrolidone (PANI-PVP-H₂O), sodium dodecyl sulfate (PANI-SDS-H₂O), Tween 20 (PANI-T20-H₂O) and Pluronic F108 (PANI-F108-H₂O). The polymerization was also carried out in 1M hydrochloric acid instead of water considering higher acidity of the reaction medium and altered properties of synthesized films. Thereby, the second set of PANI films was obtained and marked as PANI-PVP-HCl, PANI-SDS-HCl, PANI-T20-HCl, PANI-F108-HCl. Since prepared PANI films represent novel materials without any described biological properties, the standard tests of cell adhesion, proliferation and migration on PANI films were performed (likewise in case of the first mentioned study). Moreover, the biological evaluation was extended by the study of tissue interaction with selective samples. Concretely, the interaction with the reconstructed human epidermal model was examined.

Cell adhesion to the tested PANI films after 1h is visualised in Figure 28. Cell behaviour was similar to reference material.

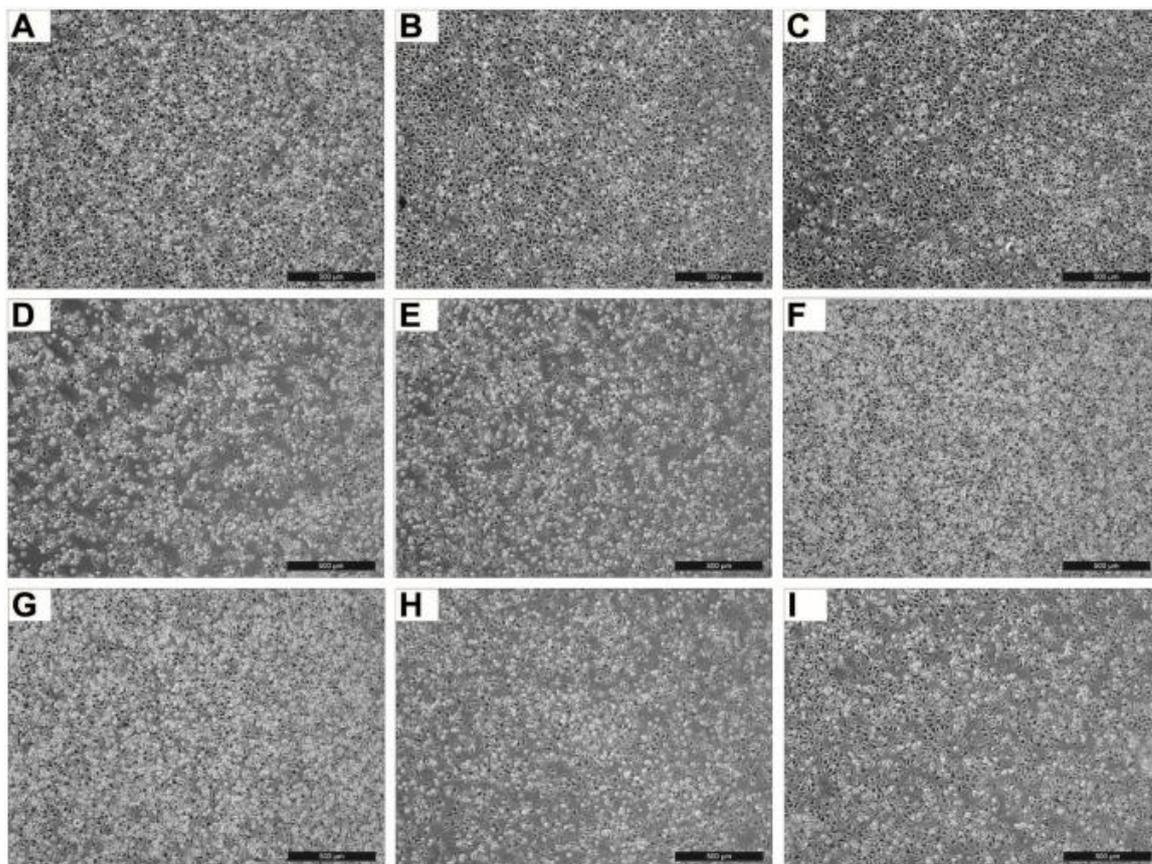


Figure 28. Adhesion of NIH/3T3 cells on the surfaces of PANI films prepared in the presence of different stabilizers evaluated after 1 h incubation. A) reference; B) PANI-SDS-H₂O; C) PANI-SDS-HCl; D) PANI-PVP-H₂O; E) PANI-PVP-HCl; F) PANI-F108-H₂O; G) PANI-F108-HCl; H) PANI-T20-H₂O; I) PANI-T20-HCl. Scale bars correspond to 500 μ m.

However, significant differences were observed in the subsequent behaviour of cells. Cells were able to grow and proliferate only on PANI-SDS-H₂O and PANI-SDS-HCl films (Figure 29B and C, respectively), but not on the remaining surfaces. On PANI-PVP-H₂O and PANI-PVP-HCl films, the cell growth was rare and only few cell clusters were presented (Figure 29D, E). The situation was even “worse” on PANI films containing F108 or T20 surfactants, where the cells were not able to proliferate at all (data is not shown).

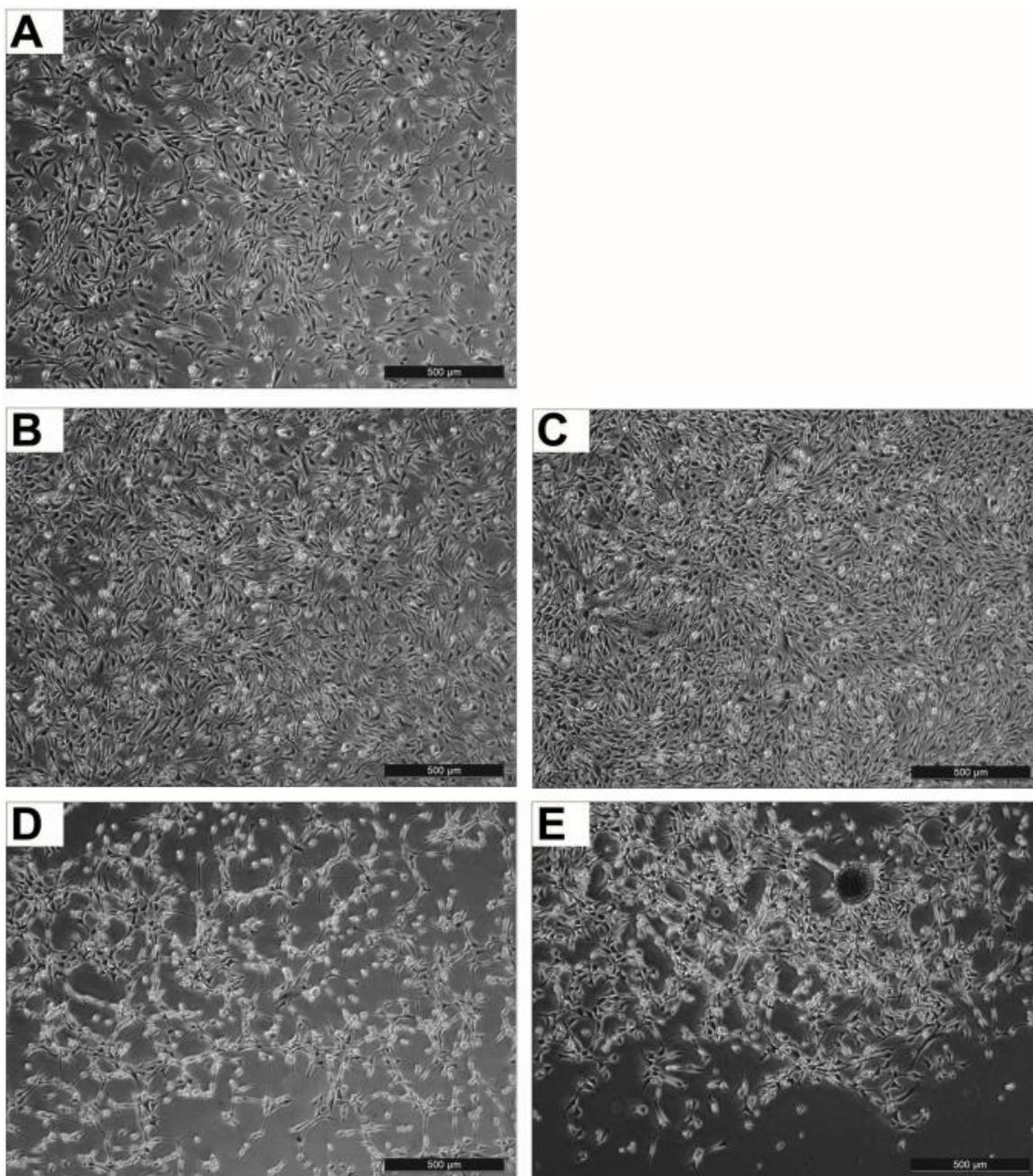


Figure 29. The proliferation of NIH/3T3 cells on surfaces of PANI films prepared in the presence of different stabilizers evaluated after 72 h incubation. A) reference; B) PANI-SDS-H₂O; C) PANI-SDS-HCl; D) PANI-PVP-H₂O; E) PANI-PVP-HCl. Scale bars correspond to 500 μm.

As cells were able to proliferate well only on PANI-SDS-H₂O and PANI-SDS-HCl, scratch assays were performed only on these surfaces. The corresponding micrographs (Figure 30) demonstrate that cells migrated into the scratches on these films in a comparable manner to cells on the reference. Therefore, it can be concluded that both PANI films containing SDS offer good cell compatibility.

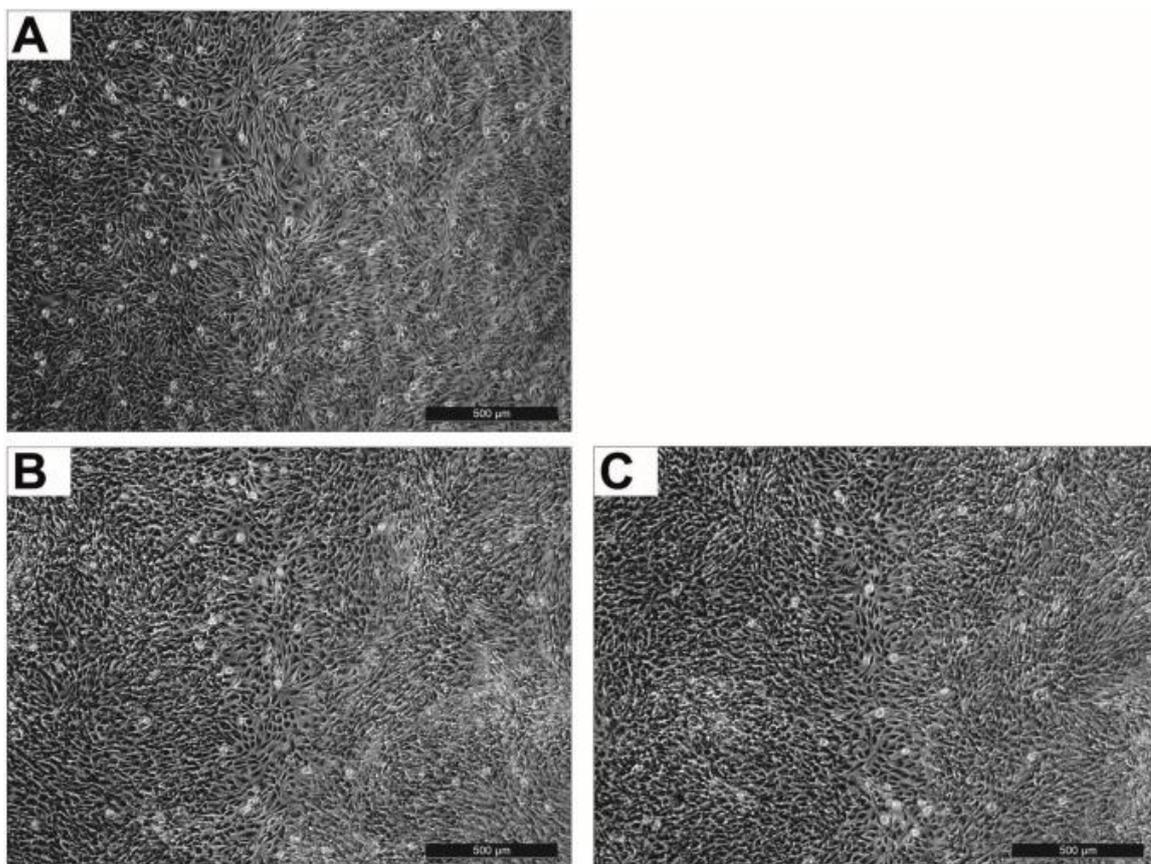


Figure 30. Scratch assay – cell migration of NIH/3T3 cells on surfaces of PANI films prepared in the presence of different stabilizers evaluated after 24 h incubation. A) reference; B) PANI-SDS-H₂O; C) PANI-SDS-HCl. PANI-PVP, PANI-F108 and PANI-T20 were not tested. Scale bars correspond to 500 μm.

Considering the above-described behaviour of cells, it can be summarized that cells were able to adhere on all surfaces but, apart from PANI-SDS-H₂O and PANI-SDS-HCl, were incapable to proliferate. This was likely due to a non-receptor mediated cell/surface binding, which allows cells to adhere but was not sufficient for the survival of anchorage-dependent cells (NIH/3T3), which could, therefore, undergo anoikis, apoptosis induced by lack of correct attachment of cells to the ECM or artificial surface (in this case). The surfaces of PANI-SDS-H₂O and PANI-SDS-HCl must allow for receptor-mediated adhesion which facilitates not only the adhesion of cells on these films but also their growth and proliferation. Explanation of the exceptional behaviour of PANI films containing SDS can be attributed to the fact that SDS contains sulfate groups, which were reported to stimulate cell adhesion, activate the spreading of cells, and influence cytoskeleton reorganization (Kowalczyńska and Nowak-Wyrzykowska 2003). The mentioned study revealed hypothesis that sulfonic groups presented on surface may influence the absorption and, more precisely, the arrangement of fibronectin (glycoprotein naturally occurring in ECM and also presented in serum-containing cell cultivation medium) (Kowalczyńska and Nowak-Wyrzykowska 2003). This

arrangement determines integrins involved in process of secondary cell adhesion (Kowalczyńska and Nowak-Wyrzykowska 2003).

Furthermore, the best performing film with respect to cell compatibility (PANI-SDS-HCl) was tested for the production of reversible damage to the skin using epidermal model. Utilization of this model shifted the testing from study single cell response to the response of a more complex system and brought improved information about biological properties of PANI-SDS-HCl sample. Based on the results obtained from skin irritation test, this film was classified as a non-irritating material, since the viability of cells in the epidermal model treated with the tested sample reached $(112 \pm 6) \%$. This value indicated even less irritating potential than that of the reference. The obtained result supported previous findings of Humpolíček *et al.* (Humpolíček, Kašpárková *et al.* 2012). They determined the skin irritation potential of standard PANI powders without stabilizers *in vivo* on a number of volunteers and they reported that tested material induced significantly less irritation than the positive control (Humpolíček, Kašpárková *et al.* 2012). None of the volunteers developed skin reaction either immediately or after the specified tested periods (up to 72 h) (Humpolíček, Kašpárková *et al.* 2012).

To conclude this section, the novel PANI films were synthesized and their biological properties were described. The presence of stabilizers during the synthesizes of PANI films significantly affected cell response within the meaning of cell adhesion, proliferation and migration. Among used stabilizers, incorporation of SDS had the best positive impact on the cell biocompatibility of prepared PANI films. Moreover, these films (PANI-SDS-H₂O and PANI-SDS-HCl) were non-irritating and showed no harmful effects on human skin. This significantly enhances the applicability of PANI in tissue engineering and, more generally, in biomedicine. In particular, its use in biosensors capable of conductometric monitoring of ongoing changes on the skin surface should be of particular interest. Described results were presented in Article Cell-compatible conducting polyaniline films prepared in colloidal dispersion mode by Kašpárková *et al.*, published in *Colloids and Surfaces B: Biointerfaces* (Kašpárková, Humpolíček *et al.* 2017).

c) Green synthesis of PANI

In general, green synthesis aims to make chemical reactions environmentally friendly. Considering PANI synthesis, new “green” approach could involve the utilization of environmentally friendly oxidants, e.g. molecular oxygen or hydrogen peroxide, instead of ordinarily used toxic persulfates or silver nitrate. Moreover, aniline, which is commonly used as starting reactant, is also regarded to be toxic. The primary toxicity of aniline is related to the accumulation of methemoglobin and associated erythrocytotoxicity (Pauluhn 2004). The environmentally unfriendly nature of aniline has been previously described. Chen and co-workers described a significant adverse effect of aniline on soil microbes

(Chen, Zhuang et al. 2014). Tao and co-workers demonstrated the harmful impact of aniline on the plant. The results of their study showed deleterious genotoxic effects of aniline to wheat root tip cells (Tao, Liu et al. 2017). Moreover, growth toxic effects of aniline to wheat seedlings were presented (Tao, Liu et al. 2017).

For the purpose of study focused on green synthesis of PANI presented in this thesis, two basic approaches were applied (both instructed during the Erasmus traineeship at the chemical department of University degli Studi di Milano). First, PANI synthesis was based on the oxidation of N-(4-aminodiphenyl)aniline (aniline dimer) with hydrogen peroxide in the presence of Fe^{3+} as a catalyst. More correctly, poly(4-aminodiphenylaniline) was synthesized in this case. This novel “green” approach allow to obtain water as co-product and avoid the formation of toxic pollutants, e.g. benzidine (Della Pina, Capáková et al. 2017).

The second synthesis way of PANI considers the presence of ethanol in the reaction mixture. The interest is still focused on alcohols presented in the reaction mixture during PANI preparation. Al-Ghamdi and co-worker reported that strong intermolecular hydrogen bonding exist between molecules of PANI and ethanol (Al-Ghamdi and Al-Saigh 2002). Lately, the addition of ethanol into reaction mixture was proved to make a reaction rate of electrochemical polymerization of PANI faster (Kan, Lv et al. 2004). The altered morphology of PANI prepared by electrochemical polymerization in the presence of methanol in the reaction mixture was investigated in study performed by Zhou and co-workers (Zhou, Wu et al. 2007). The presence of methanol affected the growth of PANI fibres, moreover, the morphology of formed fibres was observed to be more distinct (Zhou, Wu et al. 2007). Recently, Morávková and co-workers also described the influence of ethanol on the molecular and supramolecular structure of PANI synthesized by oxidative polymerization (Morávková, Trchová et al. 2013).

Overall, PANI powder samples were synthesized in three ways. Firstly, PANI prepared by oxidation of aniline with potassium persulphate (PANI_A) represents a reference powder (common, standard chemical oxidation method). Secondly, PANI_AD sample employs $\text{H}_2\text{O}_2/\text{Fe}^{3+}$ system as oxidant and aniline dimer as an initial reactant. Thirdly, PANI_EtOH sample was prepared by oxidation of aniline dimer with H_2O_2 in the presence of ethanol in the reaction mixture. All samples were also prepared in the presence of both hydrochloric acid (PANI_A_HCl, PANI_AD_HCl, PANI_EtOH_HCl) and sulfuric acid (PANI_A_H₂SO₄, PANI_AD_H₂SO₄ and PANI_EtOH_H₂SO₄). All prepared samples were finally doped by salicylic acid, intended to improve biological properties.

The aim of this study was focused on the description of the cytotoxic effect of prepared PANI powders. For this purpose, cytotoxicity testing was performed using mouse embryonic fibroblasts NIH/3T3 cell line. The test was conducted according to the EN ISO 10993-5 standard procedure to be comparable with previously published results about PANI cytotoxicity. The results are expressed as a relative number of viable cells cultivated in the presence of extracts for 24 h. The completed results are summarized and depicted in Figure 31. As can be seen,

the extracts exhibit a significant impact on cell viability. The direct proportion between the concentration of extract and cytotoxic effect is observed and the linear dependency of concentration of extract and cell viability is presented. Regarding the way of PANI synthesis (PANI_A vs PANI_AD vs PANI_EtOH), the lowest cytotoxic effect shows sample PANI_EtOH, prepared by oxidation of aniline dimer with H₂O₂ in the presence of ethanol. The impact of acid (HCl vs H₂SO₄) utilized during synthesis seems to be not so prominent. The micrographs of cell morphology support the results obtained in the cytotoxic test (as can be seen in Figure 32). The significantly lower amount of cells and stress morphology is related to the significant toxic effect of tested extracts. Obtained results could be also discussed in term of comparison with the cytotoxic effect of PANI powder prepared by IUPAC procedure presented in the study of Humpolíček and co-workers (Humpolíček, Kašpárková et al. 2012). All tested samples show slightly higher cytotoxic effect toward to NIH/3T3 cell line compared to the cytotoxicity of reprotonated PANI powder prepared by Humpolíček and co-workers towards to HaCaT and HepG2 cell lines (Humpolíček, Kašpárková et al. 2012). Similarly, all samples exhibit slightly higher cytotoxicity in comparison to PANI samples prepared by oxidation of aniline dimer with H₂O₂/Fe³⁺ system (Della Pina, Capáková et al. 2017). The toxicity could be addressed to decreased pH of extract (indicated by the colour of prepared extract, see Figure 33). The observed phenomenon could be caused by the insufficient purification of samples after final doping step by salicylic acid. The additional washing could decrease the toxic effect of all tested PANI powder samples.

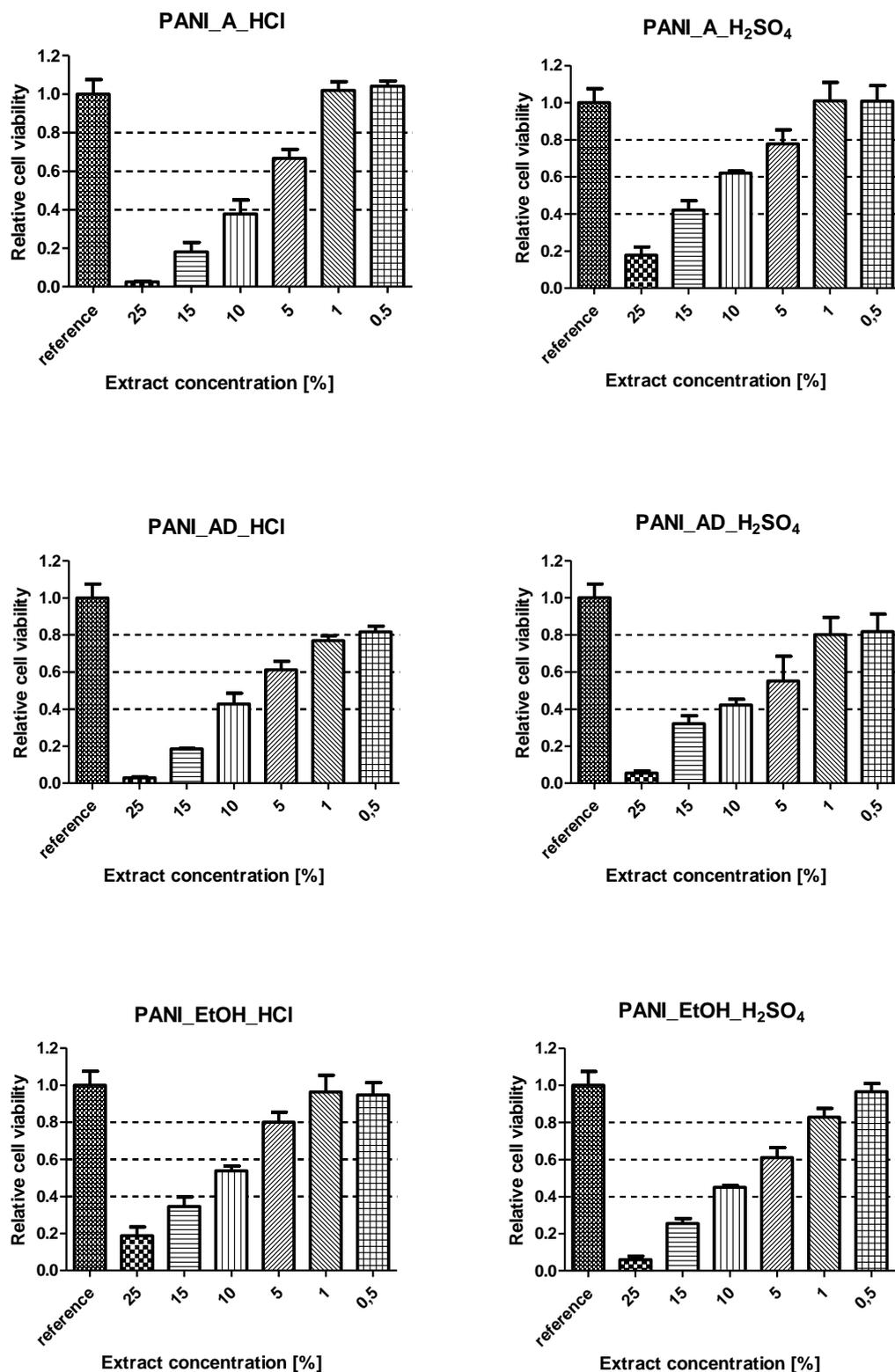


Figure 31. Cytotoxicity of extracts of tested PANI powders determined as a relative number of viable MEF cells cultivated in the presence of extracts for 24 h. The dashed lines highlight the limits of viability according to EN ISO 10993-5: viability >0.8 corresponds to no cytotoxicity, >0.6–0.8 mild cytotoxicity, >0.4–0.6 moderate toxicity and <0.4 severe cytotoxicity.

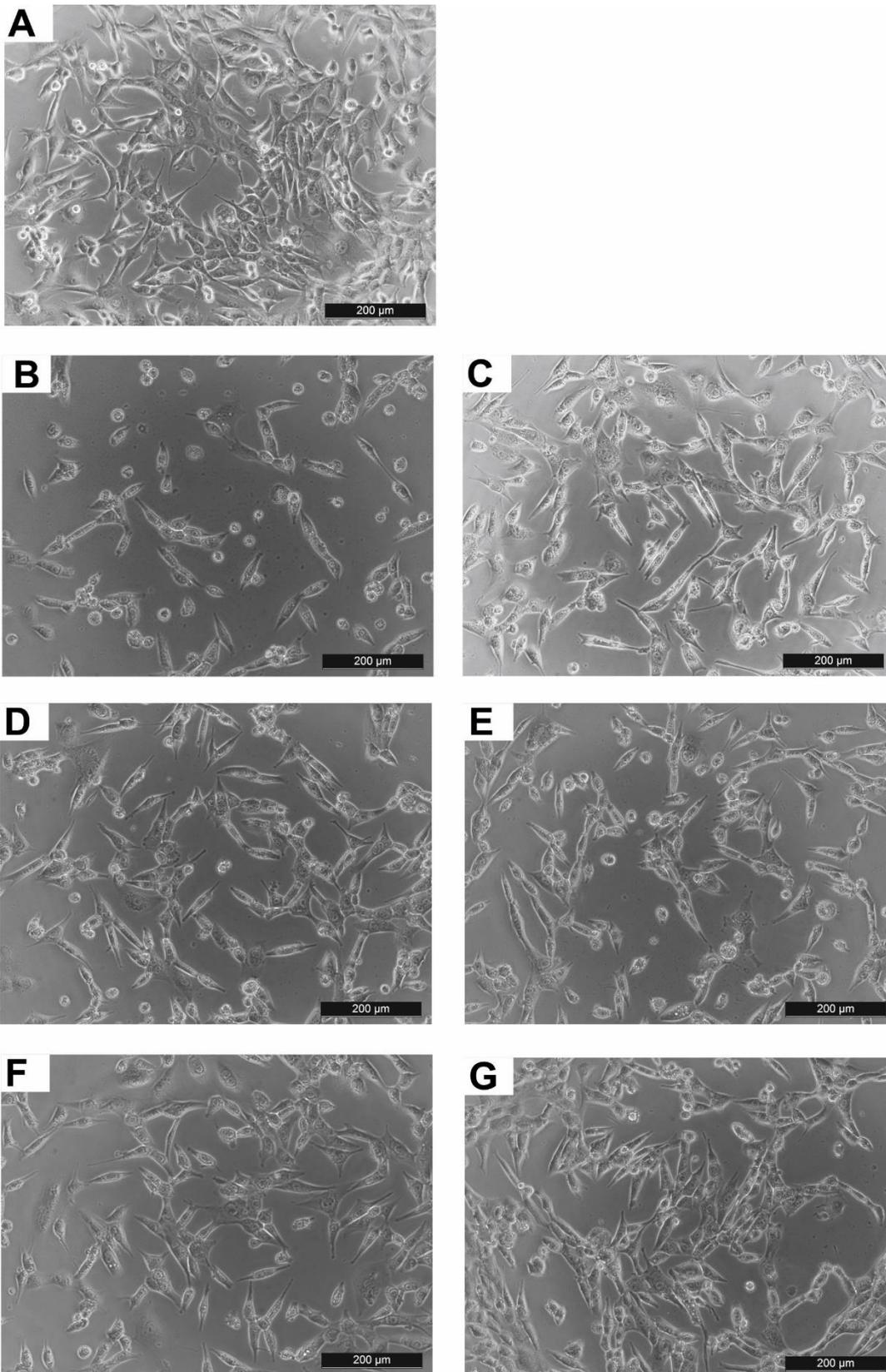


Figure 32. Morphology of NIH/3T3 fibroblasts in presence of 10% extract of PANI powders: A) reference; B) PANI_A_HCl; C) PANI_A_H₂SO₄; D) PANI_AD_HCl; E) PANI_AD_H₂SO₄; F) PANI_EtOH_HCl; G) PANI_EtOH_H₂SO₄. Scale bars correspond to 200 μm.



Figure 33. Change in the colour related to acidic pH.

d) PANI cryogel

As was already mentioned several times, PANI represents promising material for tissue engineering. However, to be utilized in tissue engineering, each material is assumed to constitute the 3D structures. Nevertheless, the main limitation of PANI is the lack of required bulk parameters. However, three possible manners exist for the preparation of 3D PANI-based scaffolds: 1) electrospinning of mixture of PANI with a thermoplastic polymer, 2) independently prepared scaffold coated by PANI, and 3) combination of PANI with supporting polymer (carrier medium). These carriers provide the mechanical properties of synthesized scaffolds.

For the purpose of this thesis, the PANI cryogel was prepared at the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic *via* polymerization of aniline in frozen poly(vinyl alcohol) solutions. The prepared scaffold combines intrinsic electrical conductivity and the material properties of hydrogels. This properties make it more than desirable for application in tissue engineering and regenerative medicine.

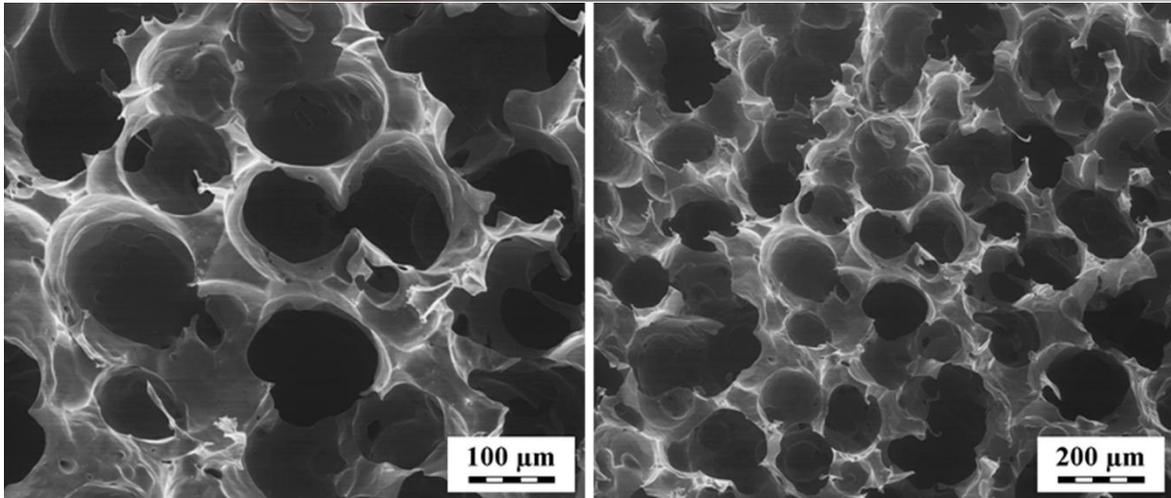
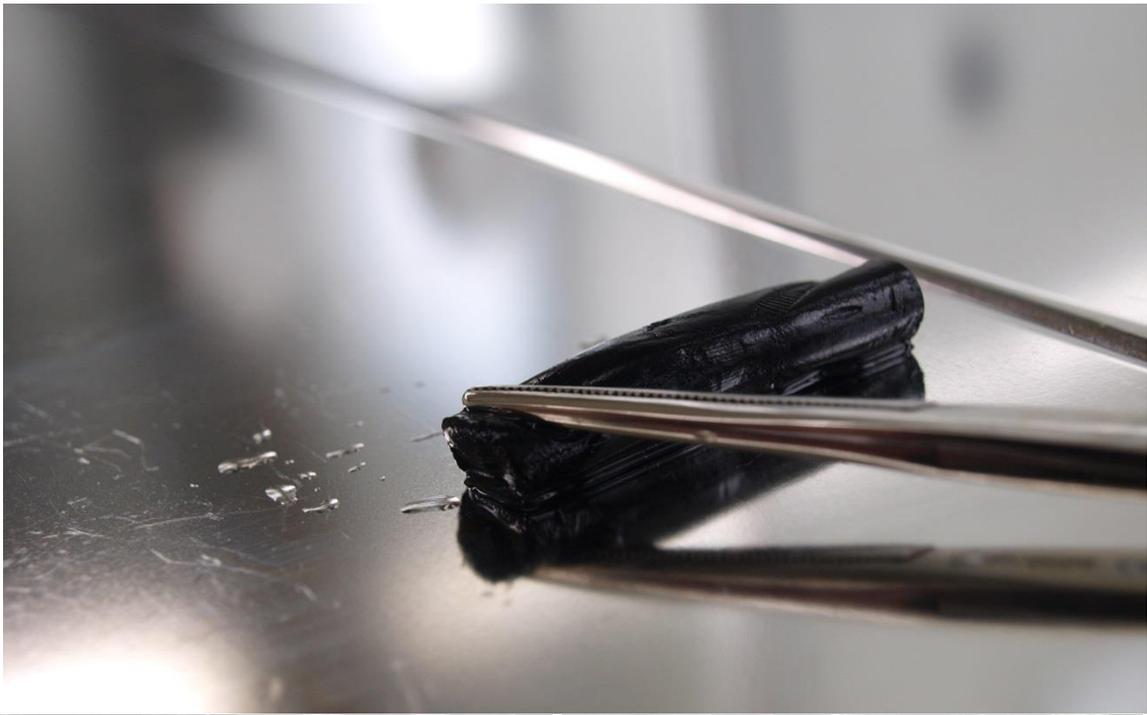


Figure 34. Above: PANI cryogel mimicking the properties of native tissue. Source: Cell biology laboratories, Centre of polymer systems, Tomas Bata University in Zlin. Bottom: Scanning electron micrographs of PANI cryogel (Humpolicek, Radaszkiewicz et al. 2018).

Cytotoxicity of extract was primarily studied as the basic parameter of biocompatibility. Two cell lines were utilized for this purpose - mouse fibroblasts NIH/3T3 and mouse embryonic fibroblasts (MEF). The cytotoxic effect was tested both on native and purified PANI cryogels (repeated extraction of 5 g of cryogel with 50 mL of ultrapure water). The results of cytotoxicity testing are transparently illustrated in Figures 35 and 36). Concerning the cytotoxic effect on both cell lines, the results obtained using NIH/3T3 and MEF are comparable. The NIH/3T3 cells represent a continuous cell line, which underwent the artificial process transformation. They are able to divide indefinitely owing to this artificial procedure. On the contrary, MEFs are primary cells, which are obtained from

tissue and should more closely mimic the native physiology of cells. As the sensitivity of cells differs with used cell lines (Wataha, Hanks et al. 1994), differences between primary and continuous cell lines are also expected. However, the inconsistency of results can be found in the literature (Hanks, Anderson et al. 1981, Berstein, Bemauer et al. 1992), most likely because of donor-to-donor variability of primary cells. (Roggen 2011)

Cell viability was also comparable for native and purified samples, but only up to their extract concentration of 75%. Mentioned extracts exhibited no cytotoxic effect (cell survival was higher than 80%, compared to the reference). The parent 100% extracts of purified cryogel showed mild cytotoxicity (cell survival ranging from 60 to 80 %) using both NIH/3T3 and MEF. The parent extracts of native caused mild cytotoxicity (cell viability ranging from 40 to 60 % of the viability of reference). This shift in the cytotoxic effect could be attributed to the presence of low molecular weight impurities and purification step led to a decrease in their amount. It has been previously described that standard PANI powder exhibits cytotoxic effect caused rather by the reaction of by-products and residues. Considering the preparation procedure, the residues of reactants (aniline hydrochloride and ammonium persulphate) and oxidation products of aniline are expected as the most principal impurities. Concerning the comparison of toxicity of native and purified cryogels, the impact of washing on cytotoxicity could be considered as negligible.

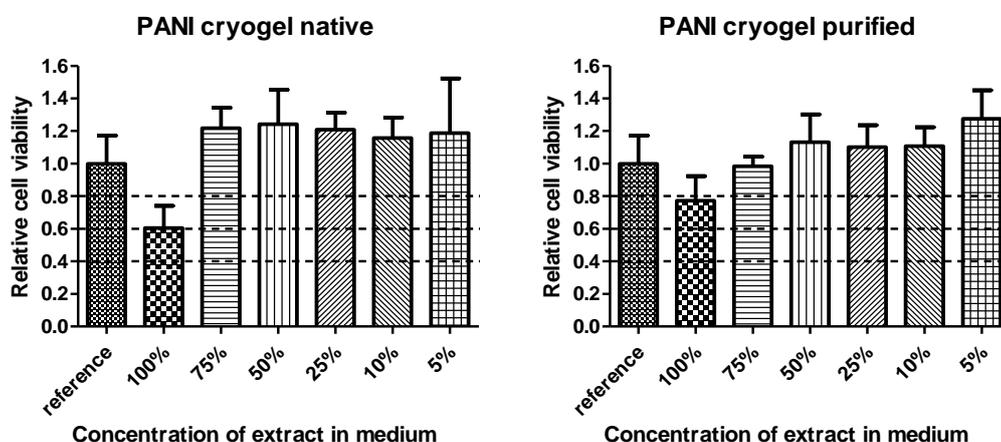


Figure 35. Cytotoxicity of extracts of native (A) or purified (B) PANI cryogel determined as a relative number of viable MEF cells cultivated in the presence of extracts for 24 h. The dashed lines highlight the limits of viability according to EN ISO 10993-5: viability >0.8 corresponds to no cytotoxicity, >0.6–0.8 mild cytotoxicity, >0.4–0.6 moderate toxicity and <0.4 severe cytotoxicity.

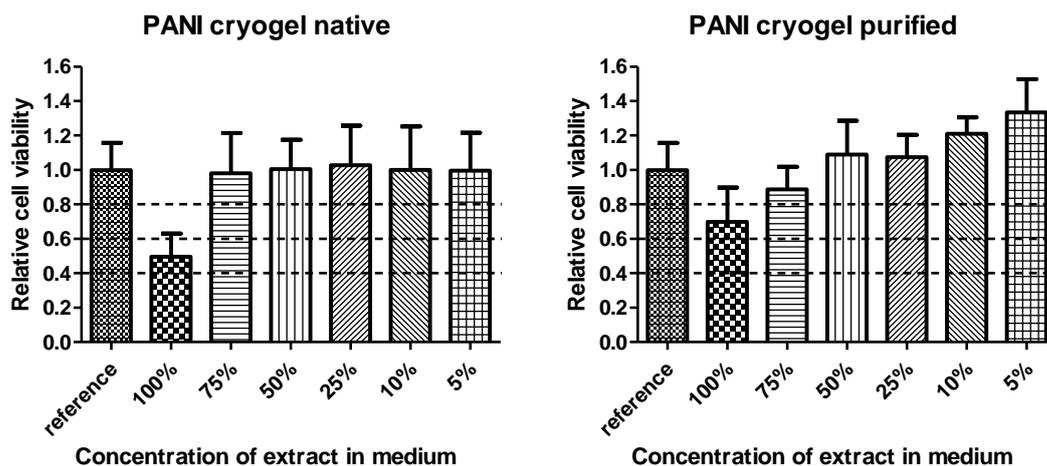


Figure 36. Cytotoxicity of extracts of native (A) or purified (B) PANI cryogel determined as a relative number of viable NIH/3T3 cells cultivated in the presence of extracts for 24 h. The dashed lines highlight the limits of viability according to EN ISO 10993-5: viability >0.8 corresponds to no cytotoxicity, >0.6–0.8 mild cytotoxicity, >0.4–0.6 moderate toxicity and <0.4 severe cytotoxicity.

Discussed results correlated with the observed morphology of cells (see Figures 37 and 38 for MEF and NIH/3T3, respectively). The morphology of cells remained unaffected up to 75 % of extract concentration. However, the both MEF and NIH/3T3 cells were unable to grow in presence of parent extract (100 %) in both native and purified cryogels. The correlation with the cytotoxicity quantification is noteworthy. It is the typical model of false-positive results of MTT assay occurring in the necrotic state of the cell. MTT assay is abundantly used colorimetric method utilized for cell viability/cytotoxicity assessment. The principle of this method is the reduction of water-soluble yellow tetrazolium salt by the mitochondria dehydrogenase system of metabolically active/live cells into water-insoluble blue/magenta formazan crystals (Karakas, Ari et al. 2017). However, it has been previously described that tetrazolium reduction could be observed in non-viable cells if the toxic effect of a tested sample does not interfere directly in the mitochondrial function and leave the mitochondria intact (Page, Bejaoui et al. 1988).

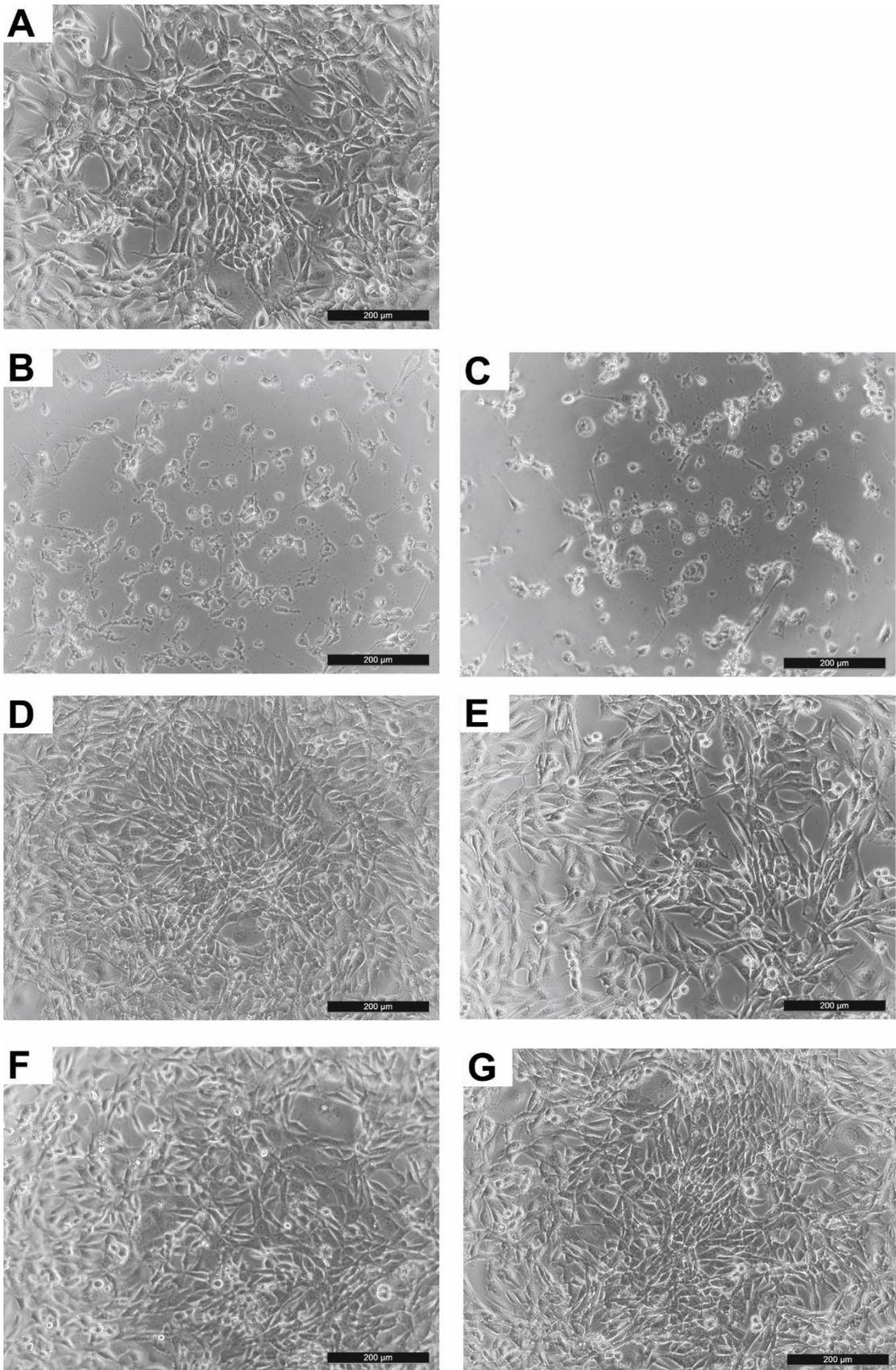


Figure 37. MEF cell proliferation in the presence of PANI cryogel extracts: a)reference; b) PANI cryogel native 100%; c) PANI cryogel purified 100%; d) PANI cryogel native 75%; e) PANI cryogel purified 75%; f) PANI cryogel native 50%; g) PANI cryogel purified 50%. Scale bars correspond to 200 μm.

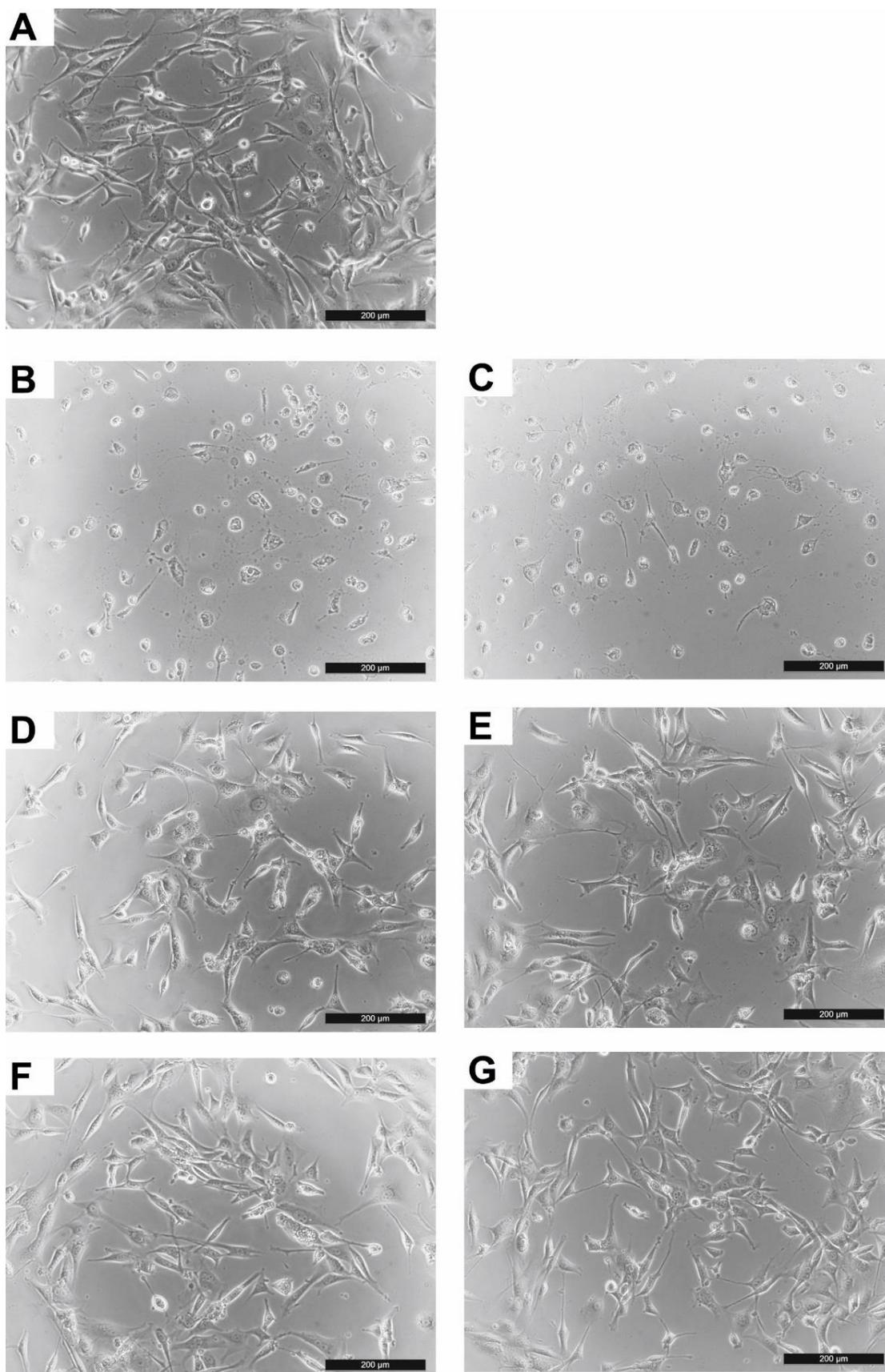


Figure 38. NIH 3T3 cell line proliferation in the presence of PANI cryogel extracts: a)reference; b) PANI cryogel native 100%; c) PANI cryogel purified 100%; d) PANI cryogel native 75%; e) PANI cryogel purified 75%; f) PANI cryogel native 50%; g) PANI cryogel purified 50%. Scale bars correspond to 200 μm .

Based on the promising results obtained from cytotoxicity test, an ability of cells to ingrowth within the scaffold was also performed. However, the cells were not able to ingrowth inside the scaffold, likely due to insufficient interconnectivity between the pores (see scanning electron micrographs of PANI cryogel in Figure 34).

In addition, the biological properties of lyophilised PANI cryogel were also investigated. Firstly, the cytotoxic effect of its extract was tested using NIH/3T3 cell line. The values of relative viability of cells cultivated in the presence of the extract of lyophilised PANI cryogel is depicted in Figure 39. The lyophilised PANI cryogel exhibited significantly higher cytotoxic effect compared to both native and purified PANI cryogel. Its extract caused severe cytotoxicity in case of all tested concentrations (100% - 5%).

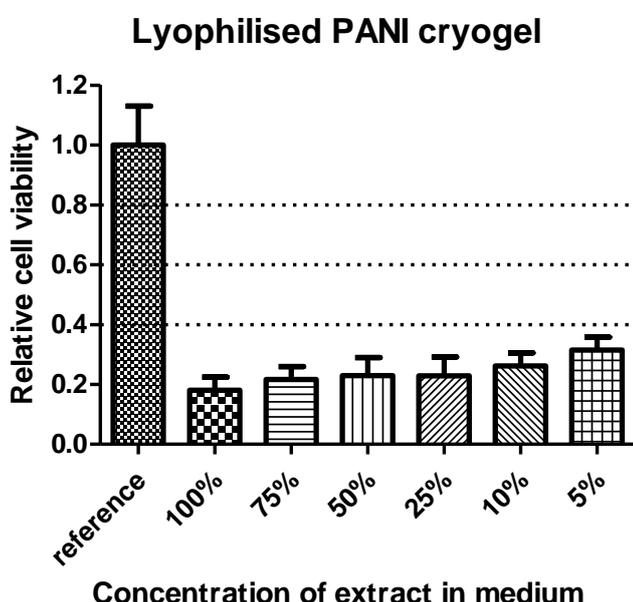


Figure 39. Cytotoxicity of extracts of lyophilised PANI cryogel determined as a relative number of viable NIH/3T3 cells cultivated in the presence of extracts for 24 h. The dashed lines highlight the limits of viability according to EN ISO 10993-5: viability >0.8 corresponds to no cytotoxicity, >0.6–0.8 mild cytotoxicity, >0.4–0.6 moderate toxicity and <0.4 severe cytotoxicity.

Morphology of NIH/3T3 cells cultivated in the presence of tested extract supported the obtained results, as can be seen in Figure 40). Contrary to reference that exhibited extended and elongated cell morphology typical for fibroblasts (Figure 40A), cells cultivated in the presence of the extract of tested material were unable to grow (Figure 40B-G). They were not able to expand, assumed a spherical shape and created filopodia.

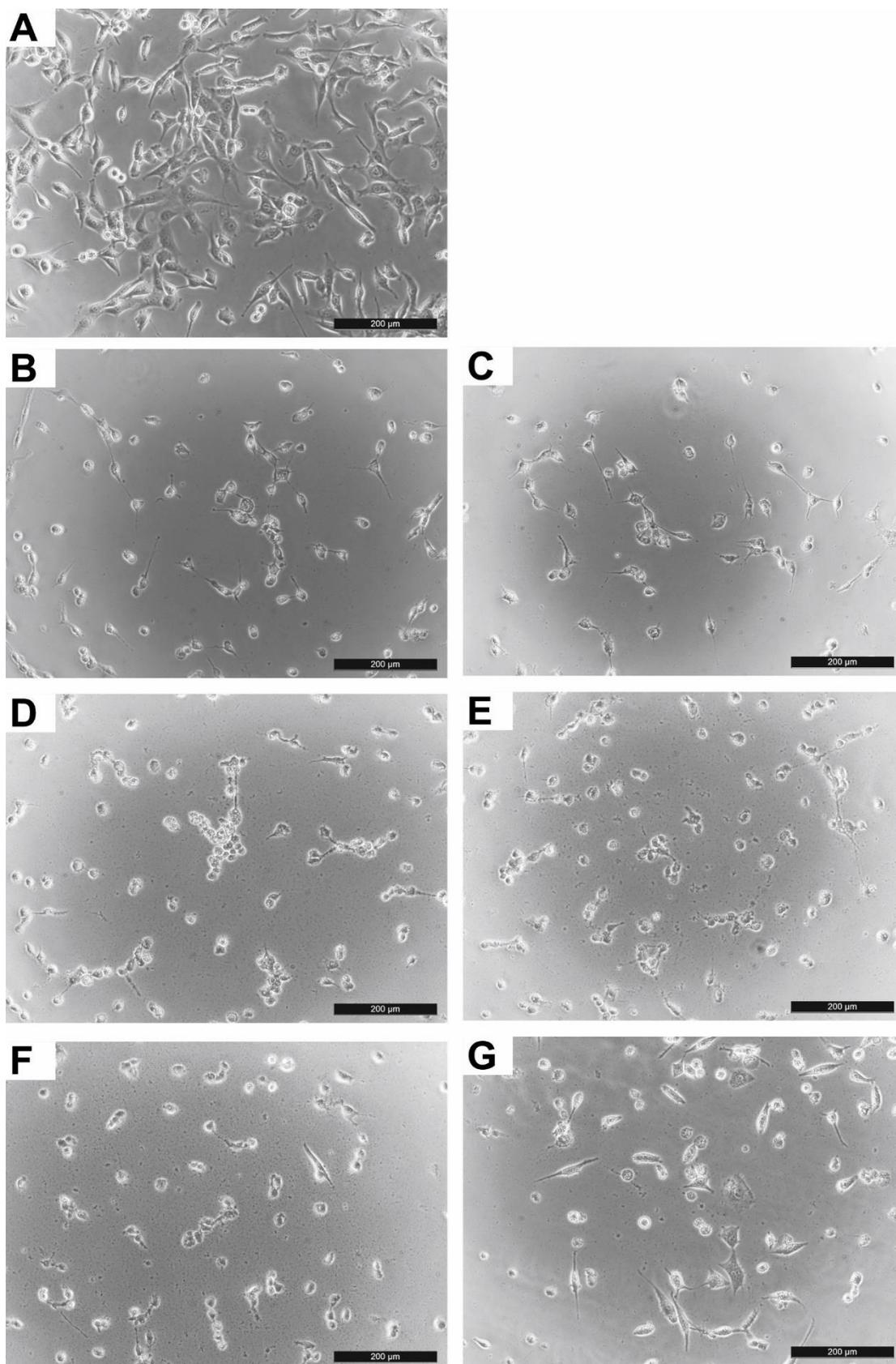


Figure 40. Morphology of NIH/3T3 cell line cultivated in the presence of extracts of lyophilised PANI cryogel: a) reference; b) 100% extract; c) 75% extract; d) 50% extract; e) 25% extract; f) 10% extract; g) 5% extract. Scale bars correspond to 200 μm .

The shift in toxicity could be attributed to the impurities released during the lyophilisation process. Therefore, the HPLC analysis of extract was performed in cooperation with Assoc. prof. Ing. Věra Kašpárková, CSc. The contents of impurities will be discussed with respect to the procedure used for their preparation, and precursors used for their synthesis, aniline hydrochloride and ammonium persulfate. The lyophilized sample contained $30.5 \pm 1.2 \mu\text{g.g}^{-1}$ and $1.2 \pm 0.1 \text{mg.g}^{-1}$ of aniline hydrochloride and ammonium persulfate, respectively. Oxidation by-products originating from synthesis, hydroquinone ($41.1 \pm 1.2 \mu\text{g.g}^{-1}$) and benzoquinone ($41.1 \pm 3.3 \mu\text{g.g}^{-1}$), were also identified in the extract. Interestingly, chromatographic analysis of native cryogel revealed the same types of impurities present as in the lyophilised sample; however, concentrations of individual residuals differed. To be concrete, aniline hydrochloride ($12.8 \pm 0.5 \mu\text{g.g}^{-1}$) and ammonium persulfate ($2.3 \pm 0.3 \text{mg.g}^{-1}$), hydroquinone ($8.4 \pm 0.3 \mu\text{g.g}^{-1}$) and benzoquinone ($35.6 \pm 2.9 \mu\text{g.g}^{-1}$) were detected (Humpolicek, Radaszkiewicz et al. 2018). As extraction rate was the same in both cases (lyophilised cryogel was saturated with deionized water prior both cytotoxicity testing and HPLC analysis), the differences between the amounts of impurities could be interpreted as being a result of different processes taking place in PANI cryogels upon its treatment. The process of lyophilisation, which is frequently used for the reduction of residual impurities through sublimation. The decrease of amounts of impurities can be occurred and is controlled by the sublimation rates of respective analytes. On the other hand, during lyophilisation of aqueous solutions, the ice crystals could be formed which can modify the structure of bulk material *via* the formation of “channels” or pores in the original gel structure. These channels can interconnect the originally closed pores to become more accessible to the extraction medium, which can result in an increase of the amounts of impurities in the extract.

Additionally to cytotoxicity testing, the ability of NIH/3T3 cells to adhere to lyophilised PANI cryogel was investigated. The results are shown in Figure 41. As can be seen, the cells were able to adhere to the tested sample, however, the amount of adhered cells was significantly lower in comparison with the reference (cell culture plastic, TPP, Switzerland). The results are in a good agreement with the detected cytotoxic effect of lyophilised PANI cryogel.

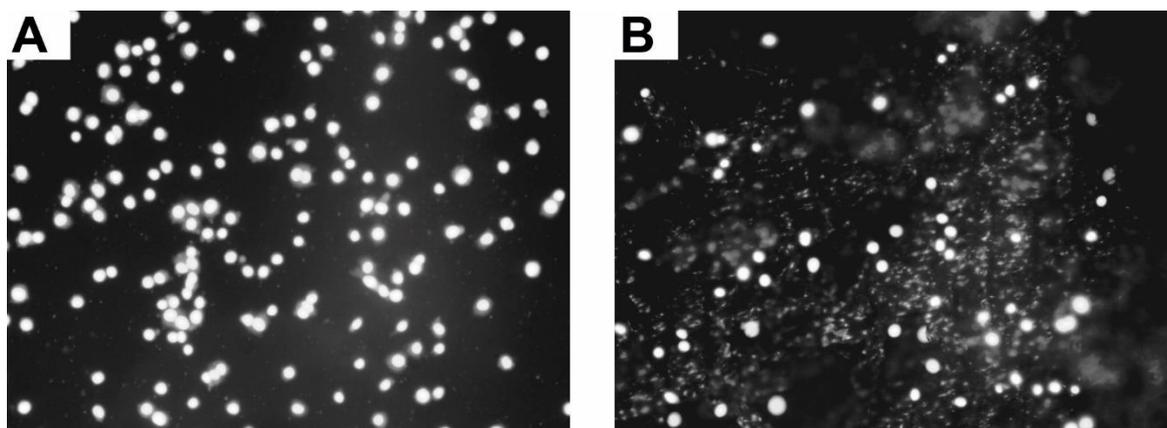


Figure 41. Cell adhesion (1h): a) reference; b) lyophilised PANI cryogel. Magnification $\times 100$.

To conclude, the novel PANI-based scaffold (with the mean value of Young modulus of 9.7 ± 0.5 kPa (Humpolíček, Radaszkiewicz et al. 2018)) mimics the properties of soft tissue and exhibits suitable biocompatibility. However, for the utilization in tissue engineering, the improvement of synthesis procedure is necessary to produce scaffold with pores, which enable cells to ingrowth within the bulk of the material. The results described above, except biological properties of lyophilised cryogel, were summarized in Article “Polyaniline cryogels: Biocompatibility of novel conducting macroporous material” published in Scientific Reports by Humpolíček *et al.* (Humpolicek, Radaszkiewicz et al. 2018).

e) Poly(*p*-phenylenediamine) (PpPDA)

Although the polyphenylenediamines, so-called “amino anilines” are considered as an electric non-conductor with very low conductivity varied between 10^{-10} and 10^{-9} S.cm⁻¹ (Stejskal 2015), they exhibit properties related to conductive polymers, especially PANI. They show PANI-like chain with pendant amino groups and exhibit a redox activity (salt-base transition) (Stejskal 2015). Therefore, besides their already established potential as corrosion protection or sensors, they could be attractive for application in biomedicine. However, the lack of information about the biological impact should be supplemented.

Within doctoral thesis, the fundamental cytotoxic effect of poly(*p*-phenylenediamine) was described. Two samples were synthesized at Institute of Macromolecular Chemistry, Academy of Science in Prague differing in the concentration of oxidant in reaction mixture – PpPDA-1 powder (0.25M ammonium persulfate) and PpPDA-2 powder (0.5M ammonium persulfate). The flow cytometer was used for the cytotoxic effect evaluation because of the observed interaction of tested extracts with MTT reagent. However, flow cytometry enabled quantification of viable cells only in case if 1% concentration of the extract was applied. The cells treated by extracts with concentrations higher than 1% showed strong fixation to the substrate, precluding the cell detachment

and analysis. Therefore, the cell morphology was observed microscopically in the cases of higher tested concentrations.

Concerning the results, Figure 42 depicts the flow cytometry results for PpPDA-1, where the number of viable, necrotic and apoptotic cells in the presence of 1% extract significantly differs in comparison with the reference. A significantly greater proportion of necrotic cells (Q2 sectors in flow cytometry plots) was detected in the case of cells cultivated in the presence of just 1% extract of PpPDA-1 sample. The similar results were observed in the case of PpPDA-2 sample (data not shown). The obtained results clearly showed the harmful impact of both samples on NIH/3T3 cell line.

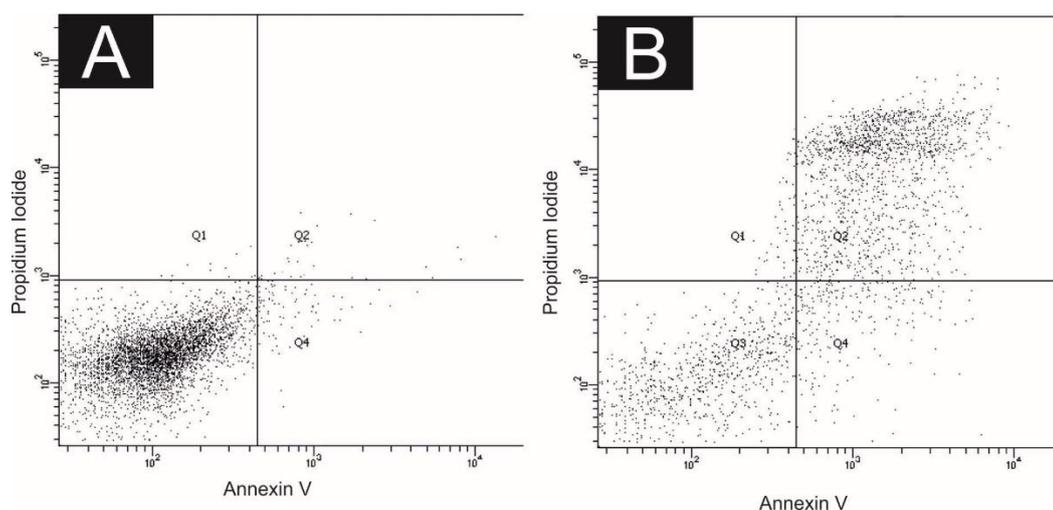


Figure 42. Number of viable (Q3), necrotic (Q2) and apoptotic (Q4) cells in reference (a) or in the presence of 1% extract of PpPDA-1 (b) determined by Annexin V/propidium iodide staining.

As was already mentioned, the microscopic observation of cell morphology was used for evaluation of the cytotoxic effect of extracts of concentration higher than 1%. The harmful effect of the extracts of both PpPDA-1 and PpPDA-2 on cell morphology are shown in Figures 43 and 44, respectively. The number of cells was reduced as well as their cytoskeleton.

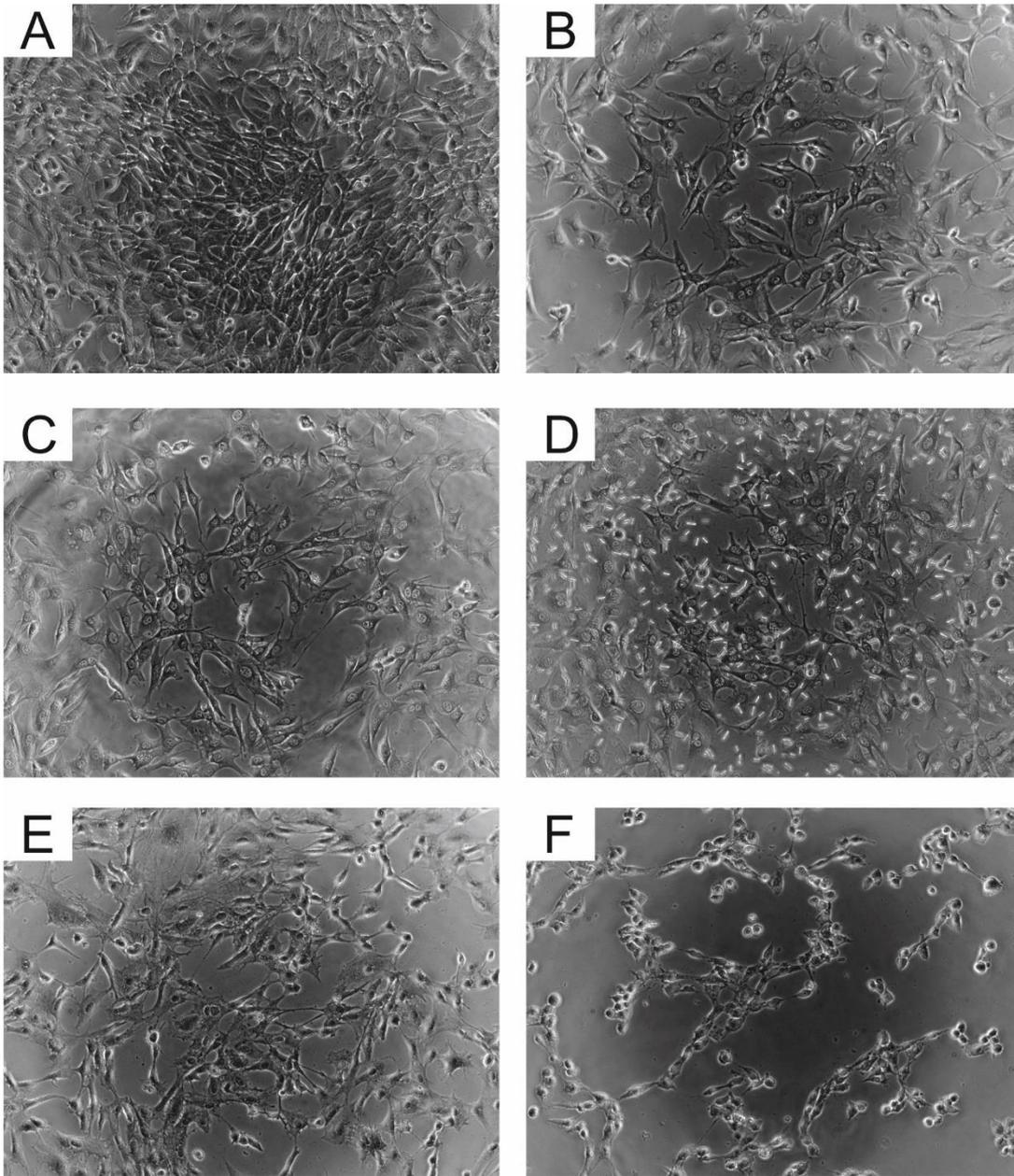


Figure 43. Micrographs depicting the viability and morphology of cells after treatment with extracts of PpPDA-1. (a) reference; (b) 100% extract; (c) 50% extract; (d) 25% extract; (e) 10% extract; (f) 1% extract. Magnification x40.

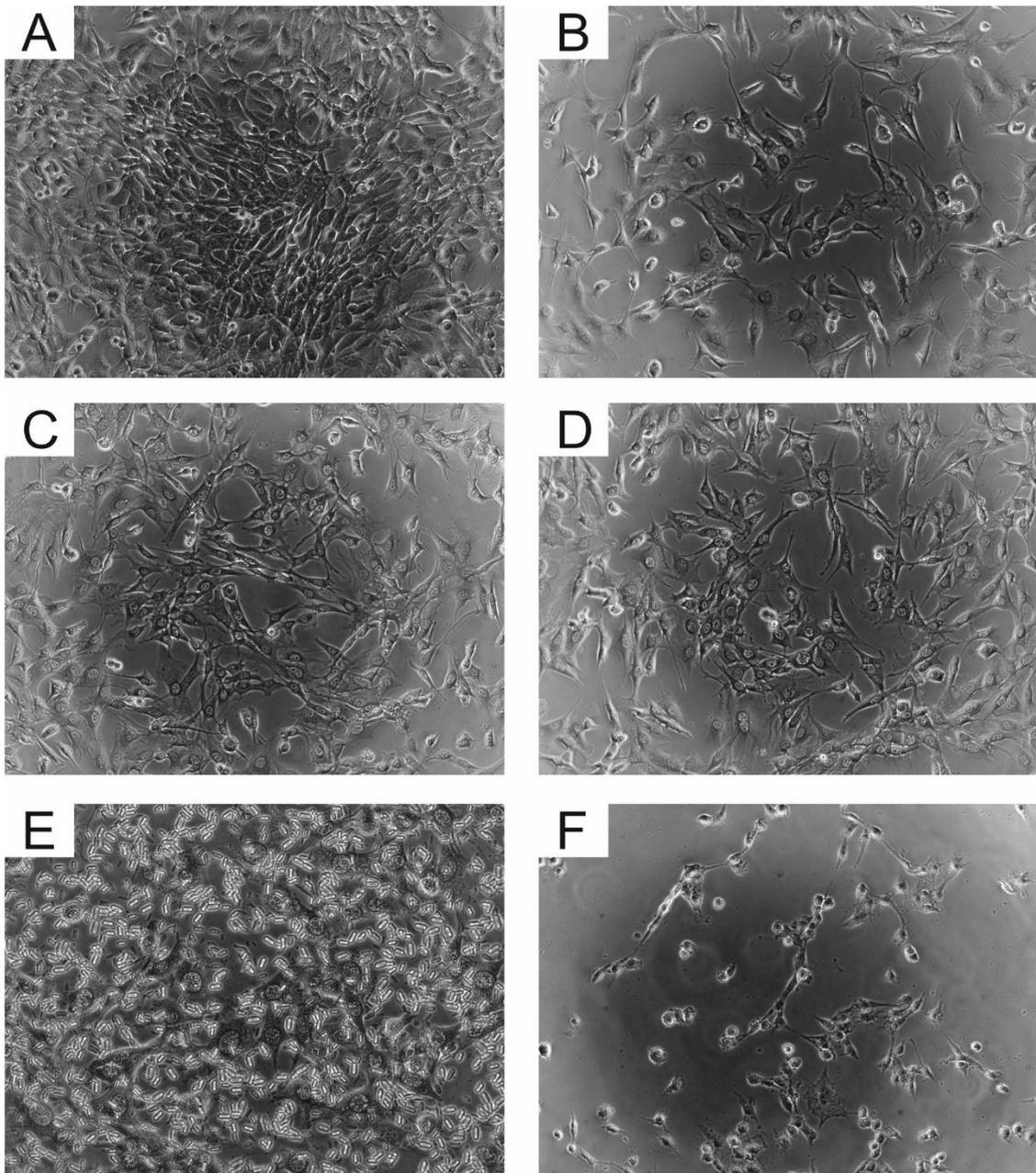


Figure 44. Micrographs depicting the viability and morphology of cells after treatment with extracts of PpPDA-2. (a) reference; (b) 100% extract; (c) 50% extract; (d) 25% extract; (e) 10% extract; (f) 1% extract. Magnification x40.

Finally, the harmful impact of extracts on cell nuclei is demonstrated in Figure 45. The destroyed nuclei and a significant amount of debris can be seen in this Figure.

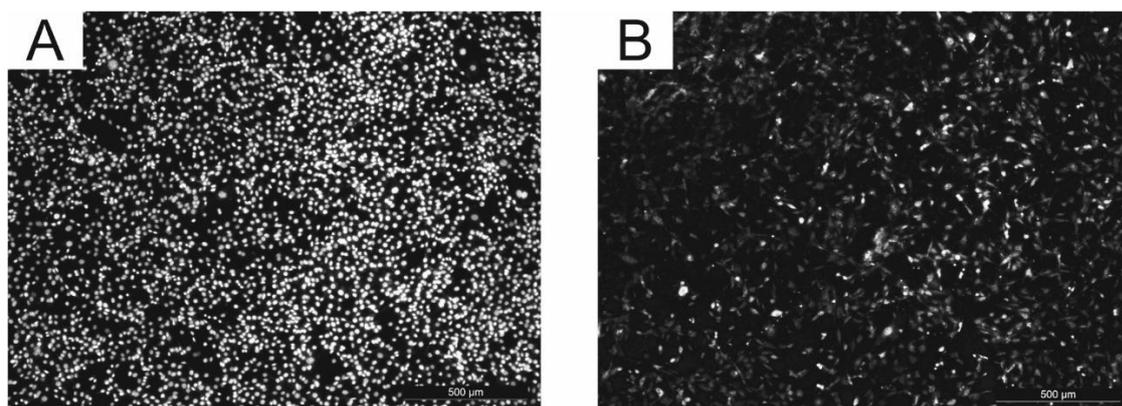


Figure 45. The harmful effect of the tested extract of PpPDA expressed by nuclei counterstaining by Hoechst 33258. The cell nuclei are viable in case of reference (a) while application of 50% extract of PpPDA-2 caused the nuclei destruction (b). Magnification $\times 40$.

Overall, both PpPDA samples exhibited severe toxicity and their application in biomedicine is conditioned by modification of preparation procedure and/or purification of resulting products, with the aim of significantly decreasing their cytotoxicity. Described results were presented in Article Cytotoxicity of poly(p-phenylenediamine) published in Chemical papers by (Kuceková, Rejmontová et al. 2017).

11.4.2. Cell interaction with micro-structured surfaces

Surface properties are a crucial factor determining the interaction between cells and (polymer) material. Besides surface chemistry, the surface topography and surface energy could be highlighted as the most discussed factors. Structured polystyrene surfaces were utilized for the study of cell interaction with topographic features. Polystyrene (PS) Petri dishes (TPP Tissue culture dishes, BioTech, Czech Republic), generally utilized for cell culturing, were modified with micropores (M-PS) *via* the time-sequenced dispensing of good and poor solvent mixtures on the rotating surface of treated substrate. The micro-structured surfaces were prepared at the Department of Physics and Materials Engineering, Tomas Bata University in Zlín (Wrzecionko, Minařík et al. 2017). The micro-structured polystyrene-based surfaces were subsequently modified with plasma treatment (M-PS-20W, M-PS-50W, M-PS-100W). The parent PS Petri dishes were also treated with plasma (PS-50W, PS-100W) (Wrzecionko, Minařík et al. 2017).

Pore size/depth/distribution as well as surface energy, significantly influence the cells in a various manner including their adhesion (Wan, Wang et al. 2005), proliferation (McGlohorn, Holder et al. 2004), morphology (Arai, Tanaka et al. 2008) gene expression (DeRosa, Hong et al. 2014) etc. Concerning the complexity of this topic, the interest of scientists still persists.

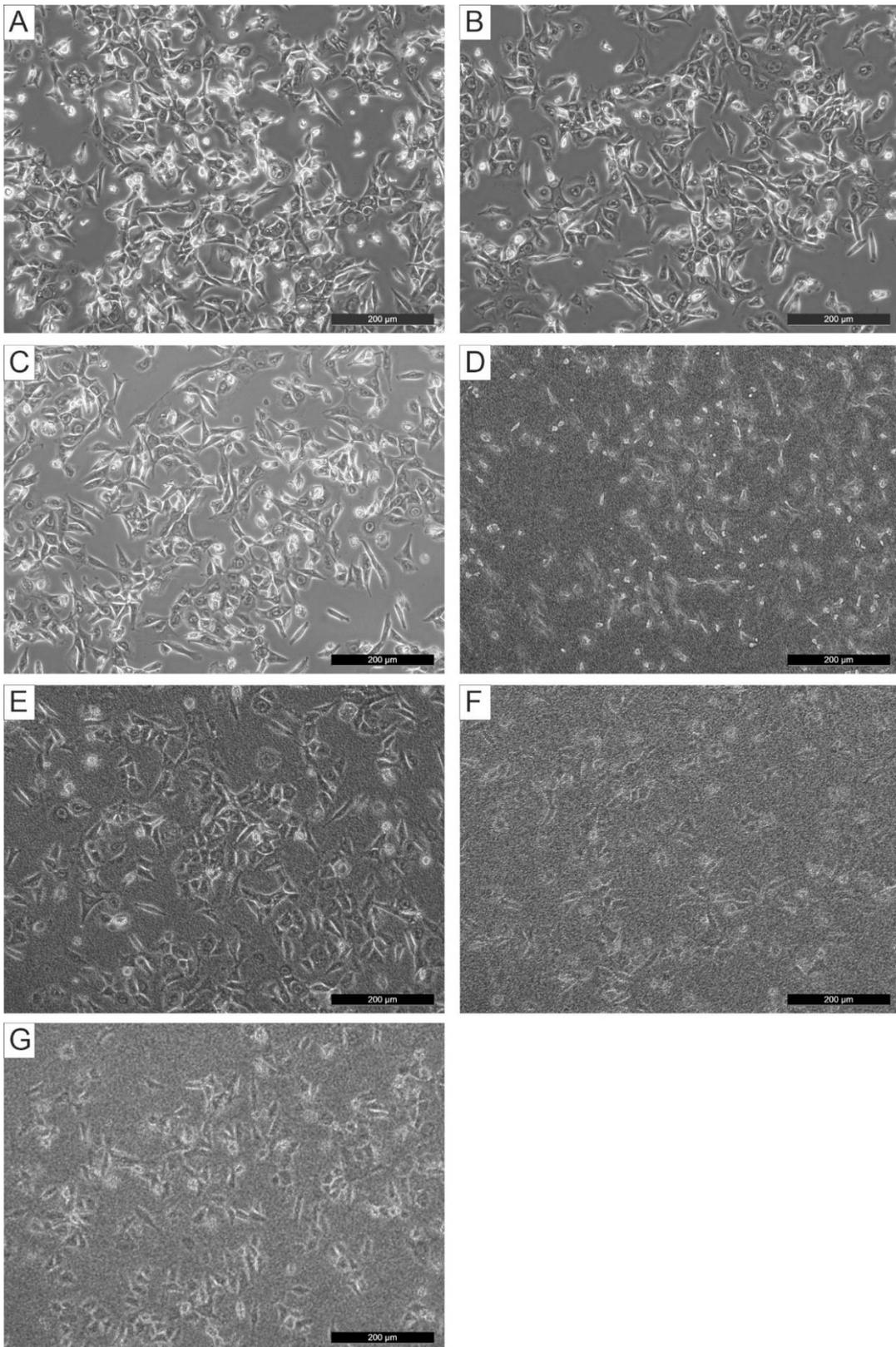


Figure 46. Adhesion of NIH/3T3 determined 3 h after seeding on (A) reference PS, (B) PS-50W, (C) PS-100W, (D) M-PS, (E) M-PS-20W, (F) M-PS-50W, and (G) M-PS-100W. Scale bars correspond to 200 μm.

The scheme of biological testing was the following:

- 1) Cell adhesion test evaluated 1 and 3 h after cell seeding
- 2) Cell proliferation test (48 h)
- 3) Evaluation of impact on cell cytoskeletons

Figure 46 displays adhered mouse embryonic fibroblasts on referential (PS) and micro-structured (M-PS) surfaces with and without plasma pre-treatment (3h after cell seeding). As can be seen, no impact on cell adhesion was observed.

Concerning the cell proliferation, the number of viable cells (Figure 47) was lower on M-PS, M-PS-20W, and M-PS-50W compared to that on the reference (PS).

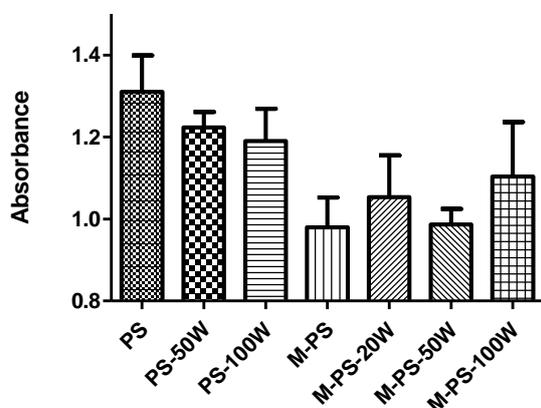


Figure 47. Amount of viable cells determined by MTT assay and expressed as average absorbance and its standard deviation.

However, the cell viability is only one parameter of the cellular behaviour. The cytoskeleton structure is another important cue that can indicate if the cell is under a physiological condition. It is known that the cytoskeleton structure and orientation are changed on flat surfaces compared to the *in vivo* state (e.g., the number of stress fibres is significantly increased in the case of fibroblasts). Considering the practical application of any biomaterial, the impact on cytoskeleton is at the centre of attention. The impact of topography on the cytoskeleton formation was detected by actin staining. From the micrographs presented in Figure 48, it can be concluded that the cytoskeletons of cells cultivated on samples PS-50W and M-PS-20W were similar to those in the reference PS.

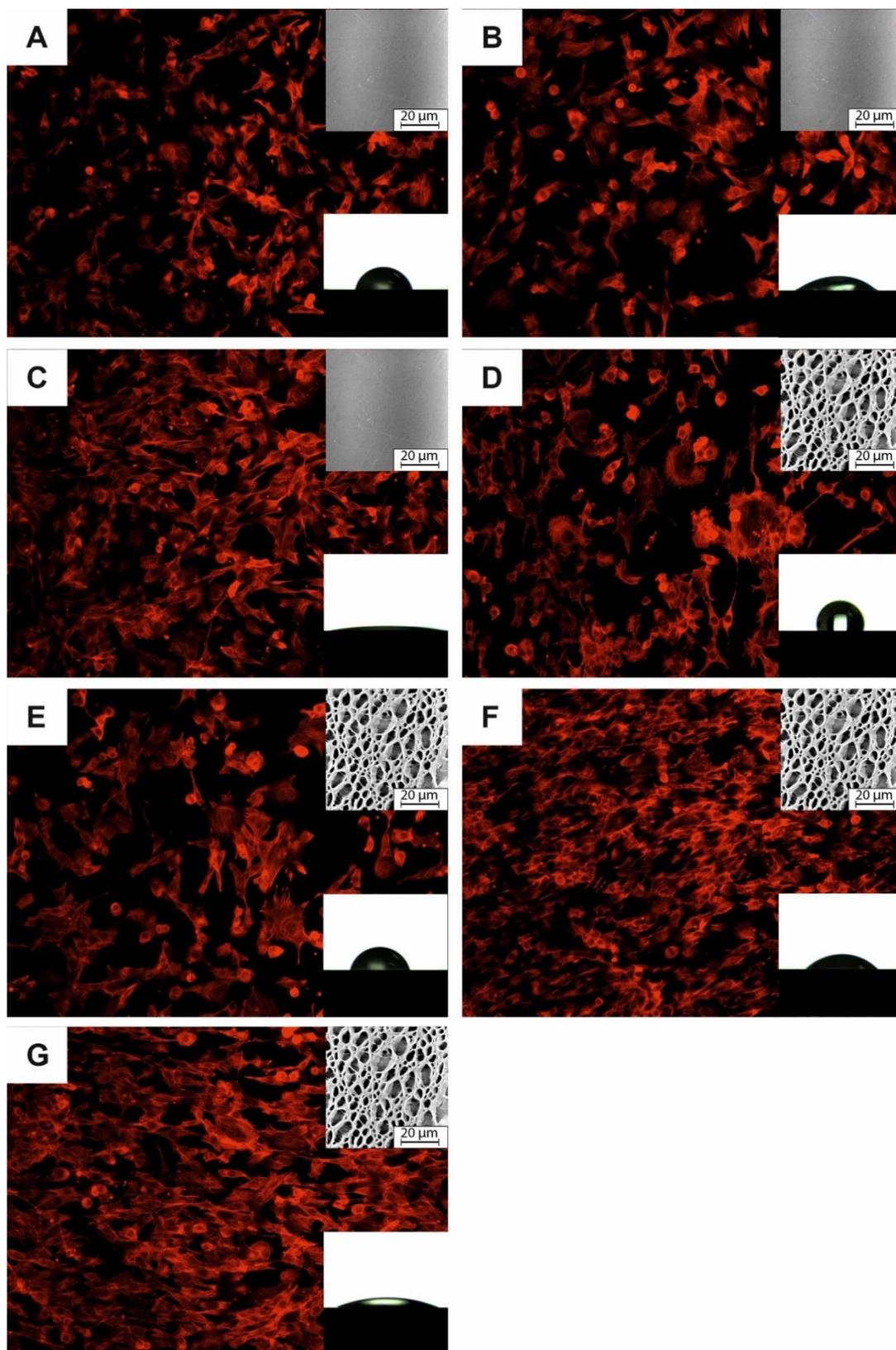


Figure 48. The cytoskeleton of NIH/3T3 visualized by ActinRed on (A) reference PS, (B) PS-50W, (C) PS-100W, (D) M-PS, (E) M-PS-20W, (F) M-PS-50W, and (G) M-PS-100W. Magnification $\times 100$. Top-right corner: SEM image of the used surface (scale bars correspond to $20 \mu\text{m}$); bottom-right corner: water drop on the studied surface as a measure of surface hydrophilicity (Wrzecionko, Minařík et al. 2017).

Despite the dramatic change of topography (samples marked as M-PS), the cytoskeletal structure did not change significantly with moderate hydrophilicity increase (plasma treatment 20 W). At more hydrophilic surfaces (plasma treatment 100 W for PS and 50/100 W for M-PS), the cell's cytoskeleton was altered noticeably at both substrates: the referential PS and modified M-PS. This suggests that the surface energy plays a primary role, and the surface relief plays a secondary role, probably because the structured and (at the same time) hydrophilic surfaces better mimic the native 3D structure of biological tissue.

In the case of surfaces M-PS and M-PS-20W, the cells are more compact, spherical, and create filopodia. On the M-PS-50W and M-PS-100W surfaces, the cells seem to be preferentially oriented, and their morphology is closer to the normal fibroblast morphology on planar surfaces. Therefore, it can be concluded that, based on both the cell viability and cell morphology experiments, the M-PS-100W represents the most promising surface for biological applications.

To conclude this section, presented study enable the deeper insight into the issue of cell interaction with nanostructured polymer surfaces, which is still not sufficiently described due to its complexity. Obtained results were presented in Article Variations of Polymer Porous Surface Structures *via* the Time-Sequenced Dosing of Mixed Solvents of Wrzecionko *et al.*, published in ACS Applied Materials and Interfaces (Wrzecionko, Minařík *et al.* 2017).

11.4.3. Cell interaction with the 3D polymeric material (scaffold)

The last section of the experimental part represents the study of cell interaction with 3D materials (scaffolds) utilizing bioreactors simulating *in vivo* conditions.

Establishing of procedure for cultivation of cell in bioreactor

Contrary to above-mentioned tests, no standardised protocol for cell cultivation in bioreactor existed in Cell biology laboratories at Centre of Polymer Systems. Moreover, no uniform method has been discovered in published articles. Therefore, prior to testing itself, the reliable pre-cultivation procedure was necessary to be established. Primarily, the optimal cell seeding concentration had to be determined. The commercially available porcine collagen-based scaffold Optimaix-3DTM (Matricel, Germany) was utilized for this purpose. Based on literature review, the seeding cell concentration varies in a broad range from 10^4 to 10^7 cells per mL of cultivation medium (Bueno, Laevsky *et al.* 2007). Based on several initial tests using the mentioned range, the efficient cell seeding concentration injected within the bulk of tested material was adjusted to 1×10^6 cells per mL. Moreover, the period of pre-cultivation had to be also defined to ensure the cell adhesion and to allow cells began to proliferate inside the structure of the tested samples prior to cultivation in bioreactor. No or too short pre-cultivation period can cause cells being incapable to attach to the scaffolds, and

lead to anoikis¹³ of cells independently on material biological properties. Based on the results of additional tests, the sufficient pre-cultivation period was adjusted to 24 hours under static cultivation conditions in incubator. Finally, the period of cultivation in bioreactor and rotational speed had to be determined. Results of performed initial studies indicated the minimum cultivation period of 14 days and confirmed the efficient rotational speed of 15.5 RPM. Finally, the methodology of evaluation of cell ingrowth had to be established, especially for scaffold embedding and sectioning. Several methods can be used for scaffold embedding, e.g. paraplast, gelatine and cryo embedding. Based on the initial tests utilizing the methodology of paraplast and cryo-embedding, the second mentioned procedure was chosen as more effective. The method was quick, simple and embedding medium was easy to remove after sectioning.

The scaffolds based on poly(vinyl alcohol) and Kraft lignin were used for study of cell interaction under simulated *in vivo* conditions. In recent years, there has been considerable interest in preparing scaffolds for tissue engineering from renewable sources. Kraft lignin, a waste product of a paper manufacturing, constitutes a promising material.

Scaffold-based on poly(vinyl alcohol) and Kraft lignin

Poly(vinyl alcohol) (PVA) has already been utilized in tissue replacement (Baker, Walsh et al. 2012, Hayes and Kennedy 2016). Therefore, it was supposed to be a suitable matrix for Kraft lignin-based scaffolds. However, few researchers have addressed the issue of limited mechanical properties of PVA (Karimi and Navidbakhsh 2014). This limitation was presumed to be improved by Kraft lignin.

In contrast to PVA, the biological properties of Kraft lignin (KL) have not yet been satisfactorily described. However, this renewable industrial biopolymer could exhibit several properties desirable for tissue engineering, for example stiffening effect (Naseem, Tabasum et al. 2016) and antioxidant efficiency (Kaur and Uppal 2015). Both properties could be advantageous especially for PVA-based devices. An antibacterial activity is another favourable feature by which KL can contribute to the final product (Dong, Dong et al. 2011). However, its utilization in tissue engineering is challenging because of its polydispersity. Therefore, a fractionation of KL is highly recommended before its utilization in any application. Fractionation can be for example performed using selective precipitation based on pH, or organic solvent extraction. The fractionation can improve its polydispersity index and lead to the derivation of specific lignin samples (Jiang, Savithri et al. 2017).

A set of scaffolds based on PVA and fractionated Kraft lignin were prepared in cooperation with the Graz University of Technology (see Figure 49). The concentration of fractionated Kraft lignin (KL^f) varied from 1 to 20 wt%.

¹³ *Anoikis is a detachment-induced apoptosis (Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter (2008). Molecular Biology of the Cell. New York, USA, Garland Science, Taylor & Francis Group.*

Hydrogels were designated as PVA_X-KL^f, where X indicates the concentration of lignin (1, 5, 10, 15 and 20 wt%).

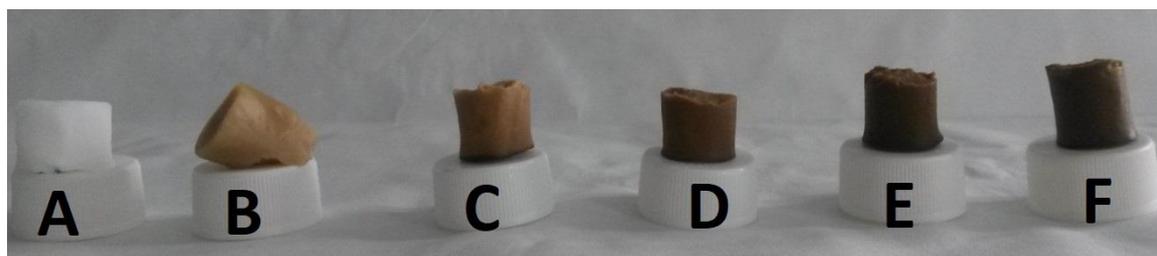


Figure 49. Scaffold-based on PVA and KL^f: A) PVA; B) PVA_1- KL^f; C) PVA_5- KL^f; D) PVA_10- KL^f ; E) PVA_15- KL^f ; F) PVA_20- KL^f.

The arrangement of biological testing included cytotoxicity testing (both in direct contact and cytotoxicity of extracts) and the evaluation of cell ability to ingrowth within the bulk of tested samples.

As the biological properties of KL^f have not been tested yet, to the best knowledge of the author, the testing of cytotoxicity in direct contact (preliminary method determining scaffold cytotoxicity) was employed first. It was found that cells were able to proliferate in direct contact with all samples. Moreover, no morphological changes were observed in comparison with reference. Therefore, the obtained results indicated no cytotoxic effect in direct contact. The cell proliferation in direct contact with tested samples is shown in micrographs in Figure 50.

For quantitative and more sensitive cytotoxicity assessments, extracts of the studied scaffolds were tested using flow cytometry and SYTO staining. First, the cytocompatibility of pure PVA material (matrix of the tested scaffolds), whose biocompatibility has already been described in previous studies (Kumar and Han 2017), was confirmed. The results for 50, 75 and 100% extracts are presented in Figure 51. The lower extract concentrations exhibited no cytotoxic effect. Concerning the scaffolds containing KL^f, a significant dependence between the cytotoxic effect and the amount of KL^f in the tested samples was observed (Figure 51). Moreover, cell morphology supported the obtained results confirming a direct correlation between the cytotoxic effect and the amount of KL^f in the sample (see Figure 52).

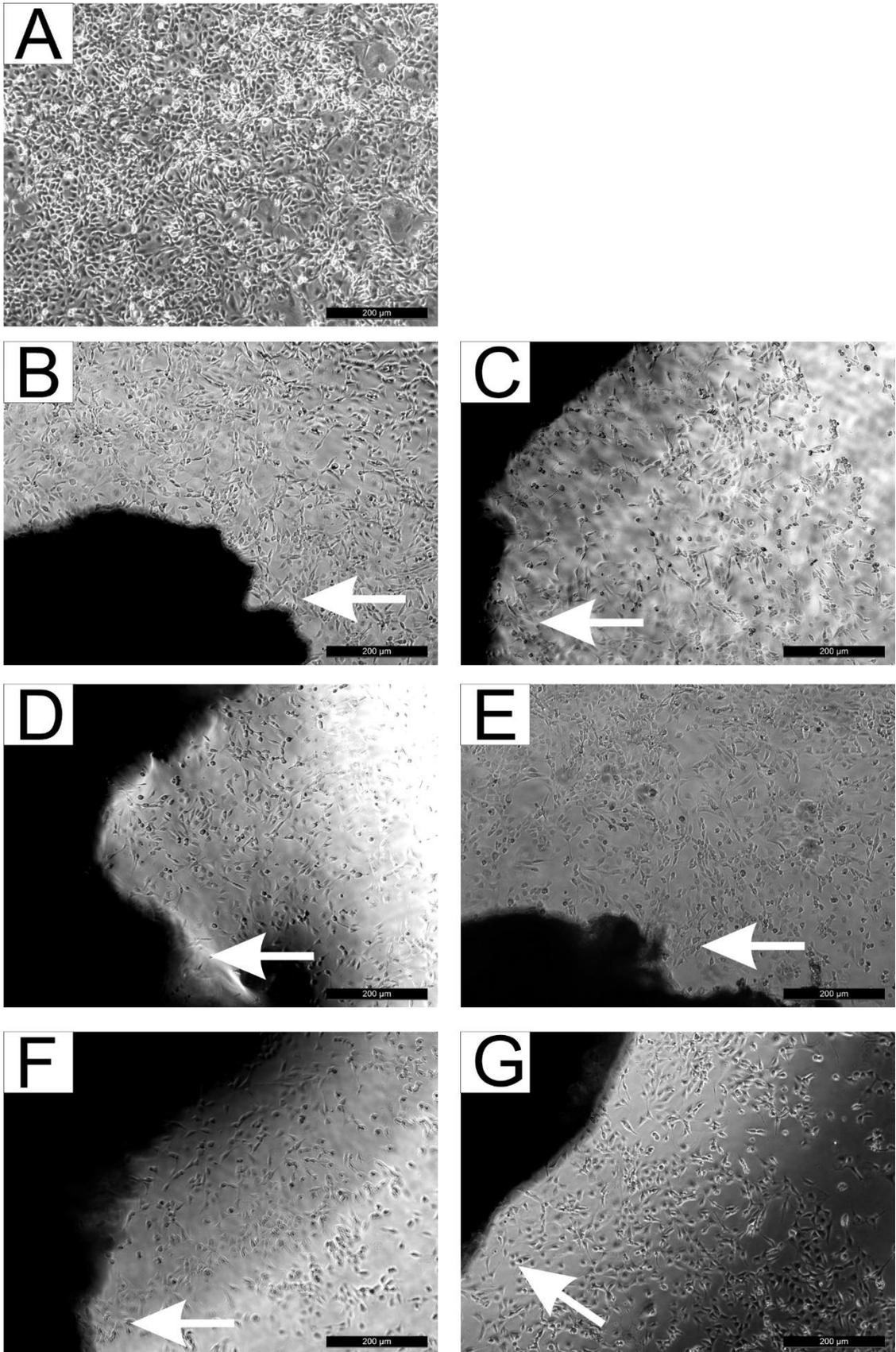


Figure 50. The mass of NIH/3T3 cultivated in direct contact with scaffolds based on PVA and KL^f. A) reference; B) PVA; C) PVA_1- KL^f; D) PVA_5- KL^f; E) PVA_10- KL^f; F) PVA_15- KL^f; G) PVA_20- KL^f (dark regions marked with white arrow). Scale bars correspond to 200 μm.

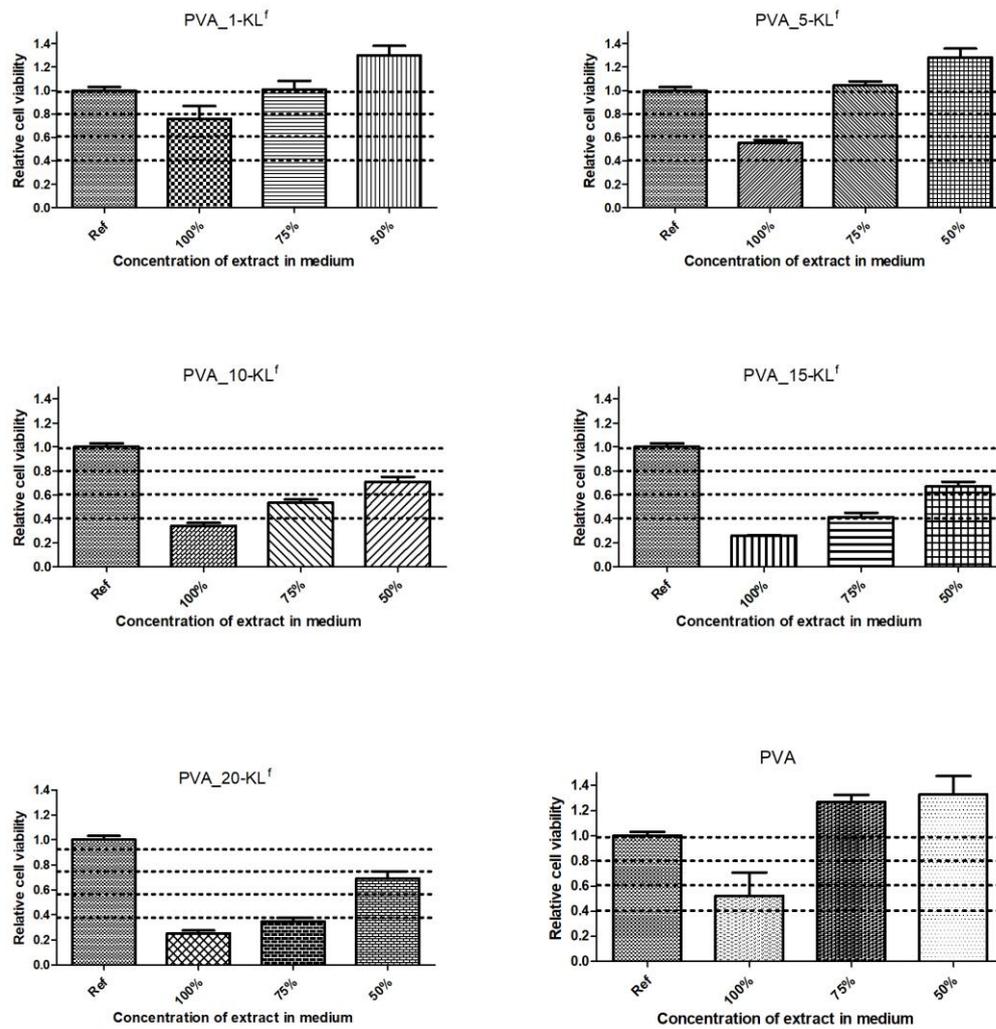


Figure 51. Cytotoxicity of scaffold extracts of various concentrations presented as a relative number of viable cells \pm the standard deviation compared to reference according to ISO 10993-5 standard. The dashed lines highlight the critical viabilities to be assessed according to requirements of EN ISO 10993-5, where viability > 0.8 means no cytotoxicity; 0.6–0.8, mild cytotoxicity; 0.4–0.6, moderate toxicity; and < 0.4 , severe cytotoxicity.

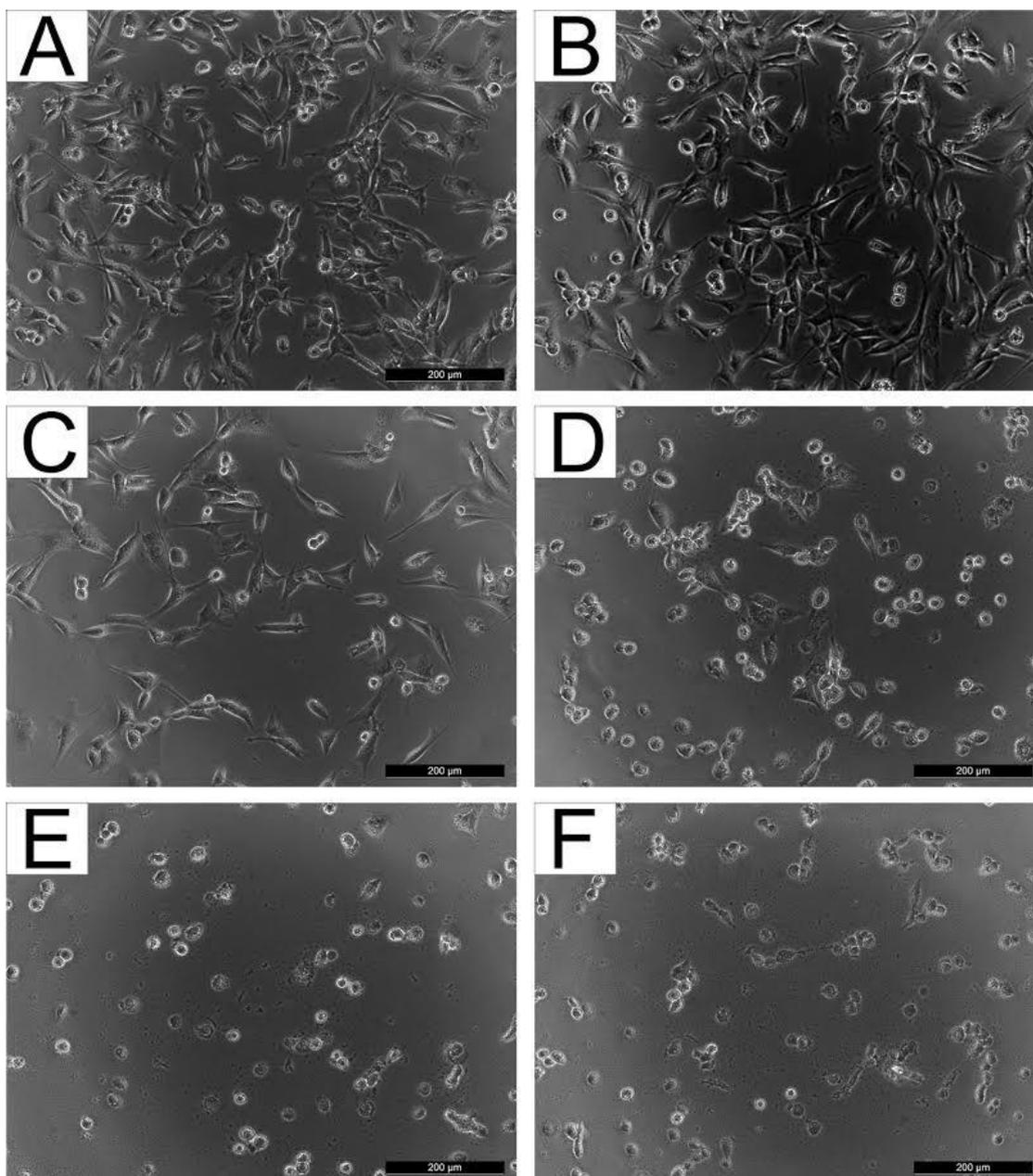


Figure 52. NIH/3T3 cultivated in the presence of 75% extracts of (A) PVA, (B) PVA_1-KL, (C) PVA_5-KL, (D) PVA_10-KL, (E) PVA_15-KL, and (F) PVA_20-KL. Scale bars correspond to 200 μm .

However, the comparison of the obtained results with the literature is problematic because of a lack of articles dealing with the cytotoxicity and biocompatibility of KL in general and especially its fractionated form. The obtained results are discussed in the context of more general studies of lignin. In spite of the fact that lignin-based copolymers or material with incorporated lignin are perceived as nontoxic (Kai, Low et al. 2015, Musilová, Mráček et al. 2018), it must be noted that lignin could have an adverse impact on cell viability. The data presented in this thesis is in a good agreement with the study performed by Kai *et al.* (Kai, Ren et al. 2016). They described the limitations of the application of alkali lignin resulting from its cytotoxicity at high concentrations. A concentration

of 6 % by mass was evaluated as the optimal amount of alkali lignin in PLLA/PLA-lignin nanofibers (Kai, Ren et al. 2016). Moreover, it was found that the cytotoxic effect of alkali lignin increased with time of exposure (Ugartondo, Mitjans et al. 2008). Based on the results obtained in our study, the highest possible concentration of KL^f that exhibits no or very low cytotoxicity was assessed to be around 5 wt%.

The absence of cytotoxicity is only one critical factor important for the successful application of materials in tissue engineering. The ability of cells to ingrowth and survive within the internal structure of scaffolds is another crucial parameter. Thus, NIH/3T3 cells were seeded inside the tested samples and cultivated in a Rotary Cell Culture systemsTM RCCS-4 for evaluation of the ability of cells to grow into the porous scaffolds. It was found that the cells were able to grow into all the tested scaffolds (as can be seen in Figure 53, in which only PVA, PVA_1-KL^f, PVA_5-KL^f, PVA_15-KL^f and PVA_20-KL^f are depicted as examples). However, a significant correlation was found between the ability of cells to grow into scaffolds and the amount of KL^f contained in samples. Greater cell growth into samples with lower amounts of KL^f was observed similarly to what was observed during the testing of cytotoxicity of extracts. The samples PVA_1- KL^f and PVA_5- KL^f are advantageous for mouse fibroblasts; therefore, the suggested amount of KL^f for tissue engineering scaffolds seems to be between 1 and 5 wt%. However, according to the comparison of stained cell cytoskeletons inside scaffolds, cell growth was homogeneous only in the case of PVA_1- KL^f (Figure 53B). In contrast, the cells inside the PVA_5- KL^f (Figure 53C) scaffold created clusters. The cell growth is an important parameter because a tissue-engineering scaffold should enable cells to grow homogeneously into its structure and gradually form a continuous tissue. The creation of separate clusters is undesirable. From this point of view, a concentration of KL^f of about 1 % seems to be optimal for use in tissue engineering.

On the basis of advanced biological testing, it must be noted that only scaffolds containing 99 wt% PVA and one wt% KL^f can be proposed as advanced materials suitable for use in tissue engineering.

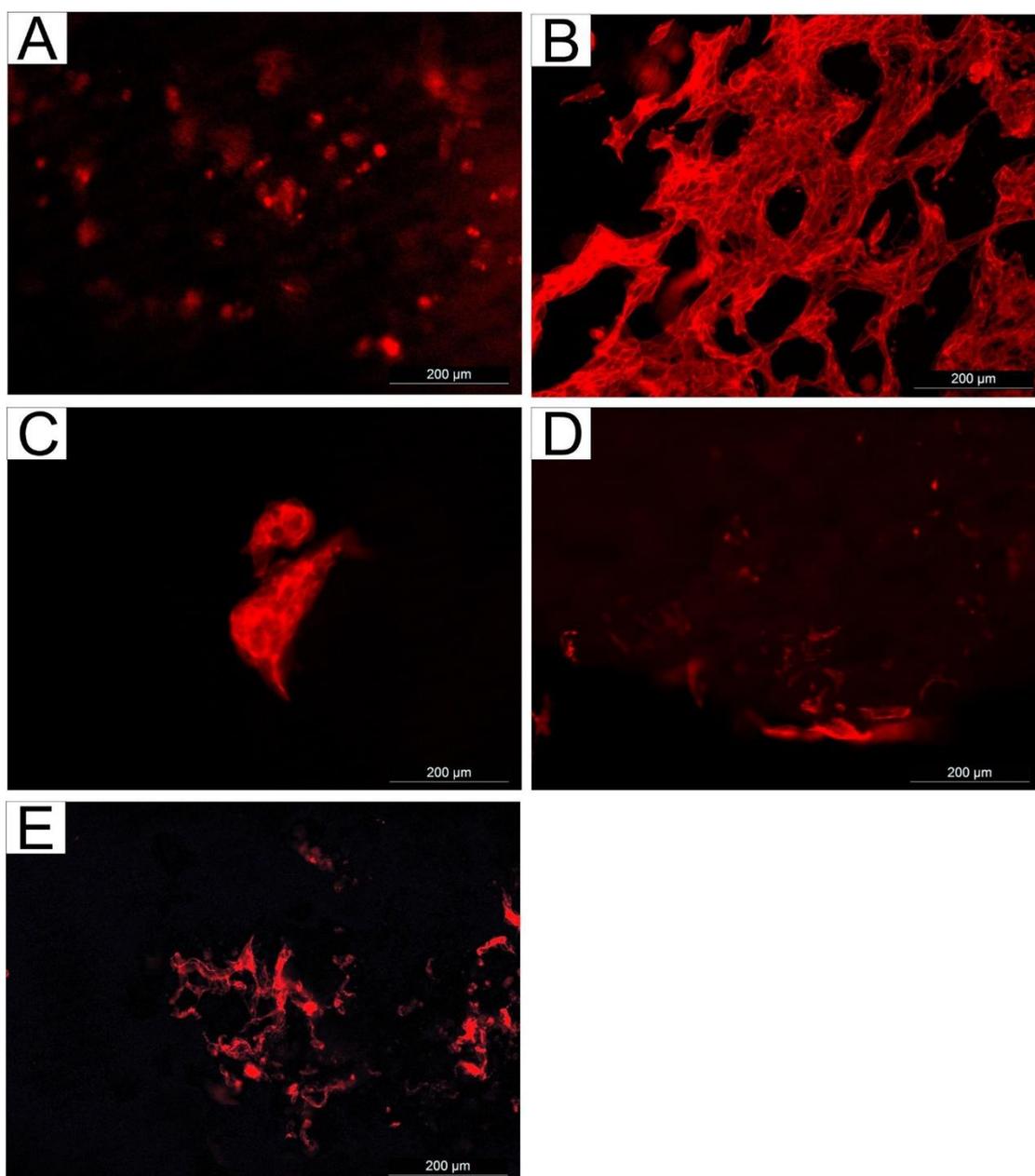


Figure 53. NIH/3T3 fibroblasts stained with AnctinRed[™] 555 grown within A) PVA, B) PVA_1- KL^f, C) PVA_5- KL^f, D) PVA_15- KL^f, E) PVA_20- KL^f. Scale bars correspond to 200 µm.

To conclude this part, the biological properties of novel PVA and Kraft lignin-based scaffolds was described using both standardized *in vitro* methods and bioreactors simulating *in vivo* culturing conditions. The cell-3D material interaction under simulated *in vivo* conditions was evaluated at Cell biology laboratories of the Centre of polymer systems for the first time. The results were summarized in Manuscript entitled “The use of fractionated Kraft lignin to improve the mechanical and biological properties of PVA-based scaffolds” accepted in RSC Advances in May, 2019 (Rejmontová, Kovalcik et al. 2019, in press).

CONCLUDING SUMMARY

The understanding of the principles of cell-material interaction is essential for developing of functional biomaterials with desired biological properties. Toxicity (in polymers mainly connected to the presence of residual impurities), surface and bulk properties (consider 3D scaffolds) could be mentioned as crucial aspects influencing the biological properties of any material. Based on its complexity and despite the perpetual effort of scientists, this topic has been still poorly understood.

The thesis is focused on cell interaction with a polymeric material, particularly with conductive ones. The topic of the interaction of eukaryotic cells with artificial materials is discussed in relatively broad scope extended from already well-established 2D *in vitro* to 3D simulated *in vivo* cell cultivation conditions. *In vitro* cell cultivation conditions enable controlled, quantifiable experimental characterization, however, it lacks the complexity occurs *in vivo*. The bioreactors simulated *in vivo* conditions could be considered as an intermediate step between *in vitro* and *in vivo* conditions combining the advantages of both approaches.

Experiments performed during the doctoral study cover all above mentioned basic aspects related to the biological properties of polymeric material. Obtained results could be divided according to defined aims of doctoral thesis into three groups: 1) cell interaction with conductive polymers; 2) cell interaction with micro-structured surfaces; 3) the implementation of methodology and subsequent testing of cell interaction with the polymeric scaffolds.

The first part, cell interaction with conductive polymers (especially with PANI), comprised the majority of doctoral study. PANI in several forms (including film, powder or novel 3D scaffold) was studied. In summary, PANI films with modified surface properties were prepared and the principal cell response to this surface of individual films was investigated. Firstly, the surface energy of the sample, especially its correlation with a surface energy of cells, was indicated to be the key factor influencing the cytocompatibility of PANI films modified with acids. Secondly, the using of colloidal dispersion during PANI synthesis can lead to improving the interaction of cells with the surface of the material. Focusing on the powder form of material, the residual impurities (such as non-bounded monomers, oligomers, used chemicals or additives) was decided to be the major source of the toxic effect of polymeric material. Therefore, the selection of appropriate purification step (to avoid the unwanted releasing toxic impurities under physiological conditions) is necessary for successful utilization of polymeric material in biomedicine. Moreover, a preliminary study focused on the biocompatibility of novel soft 3D porous PANI-based cryogel was presented and the impact of its lyophilisation on cell behaviour was described. The PANI cryogel represents promising material for tissue engineering showing appropriate biological properties.

Concerning the second aim, the cell response to micro-structured PS surfaces exhibited various surface hydrophilicity was evaluated. The results suggested the principal effect of surface energy on cell behaviour, primarily on cell morphology, more than surface topography alone.

Finally, the methodology for testing the ability of eukaryotic cells to incorporate within 3D polymeric scaffolds was introduced in Cell biology laboratories at the Centre of polymer systems. The appropriate seeding concentration of the cells, parameters of cell cultivation as well as the procedure suitable for the evaluation of cell ingrowth was defined. This novel method was utilized for evaluation of cell ingrowth within several polymeric scaffolds during the doctoral study. The cell interaction with 3D polymeric scaffolds based on PVA and fractionated KL under simulated *in vivo* conditions was also described in the presented thesis. The appropriate amount of KL within scaffolds in the sense of lack of toxicity and homogeneous cell ingrowth was set up.

CONTRIBUTION TO SCIENCE

The presented topic of cell interaction with artificial material remains still not fully explored. The presented thesis deals not only with the cell interaction under standard 2D cultivation conditions, but also extend the knowledge about cell-material interaction under conditions close to the cells natural environment (under simulated *in vivo* conditions). The methodology for testing of cell ingrowth within 3D porous scaffold was established. Therefore the range of routine biological tests employed in Cell biology laboratory at Centre of polymer systems has been expanded. The cell interaction within porous 3D polymeric scaffolds can be studied using cell culture vessel, both rotary and continuous flow perfusion systems.

During the study, the biological properties of several novel materials were described and their possible application in biomedicine were discussed. To be concrete, PANI films modified with acids or prepared in colloidal mode, green synthesized PANI powders, PANI cryogel and scaffold based on PVA and KL^f were studied. Of them, the PANI cryogel could be mentioned as one of the most interesting materials mimicking the mechanical properties of native tissue, with promising application potential in tissue engineering.

The acquired knowledge can be used in the process of development of novel material with properties desired for utilization in biomedicine and tissue engineering. Nowadays, the nontoxicity itself is not sufficient property of any biomaterial. High demand is paid to stimuli-responsive material, which is able to specifically modulate cellular behaviour and trigger a targeted cell response (e.g. differentiation). Therefore, the understanding of the interaction between cells and materials at the interface remains a crucial precondition for the successful application of any material in modern medicine.

Moreover, the results described in this thesis were presented at several national and international conferences and were published in impacted international journals.

FUTURE PROSPECTIVE

Based on the cell sensitivity to diverse stimuli previously described in the theoretical part and continuing researcher effort to become cell culturing closer to physiological conditions, the presented study could be further extended in several areas. Firstly, the cell interaction with polymeric materials under dynamic cell cultivation condition could be studied (survey the impact of perfusion rate on cell behaviour could be mentioned as an example). Additionally, as the predominant part of the presented thesis was focused on the conductive polymer, the cell response to electrical and/or mechanical stimuli could be considered as an interesting topic for further investigations. Finally, the attention could also be paid to development of porous scaffolds mimicking tissue properties prepared by the combination of conductive polymers with natural biopolymers.

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LIST OF ABBREVIATIONS

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| | |
|--------------------------------------|--|
| 2D | Two-dimensional material/ cell culturing technique |
| 3D | Three-dimensional material/ cell culturing technique |
| AD | Aniline dimer or N-(4-Aminophenyl)aniline |
| CP | Conductive polymers |
| DNA | Deoxyribonucleic acid |
| <i>e.g.</i> | <i>Exempli gratia</i> |
| ECM | Extracellular matrix |
| ES | Electrical stimulation |
| <i>etc.</i> | <i>Et cetera</i> |
| EtOH | Ethanol |
| F108 | Pluronic F108 |
| FDA | Food and Drug Administration |
| FeCl ₃ .6H ₂ O | Iron(III) chloride hexahydrate |
| FLUENT | Computational Fluid Dynamics code |
| H ₂ O ₂ | Hydrogen peroxide |
| H ₂ SO ₄ | Sulfuric acid |
| HARV | High aspect rotating vessel |
| HCl | Hydrochloric acid |
| HPLC | High-performance liquid chromatography |

| | |
|------------------------|--|
| ISO | International Organization for Standardization |
| ISO/TS | ISO Technical Specifications |
| IUPAC | International Union of Pure and Applied Chemistry |
| KL and KL ^f | Kraft lignin and fractionated Kraft lignin, respectively |
| LDV | Leucine - Aspartic acid - Valine |
| MEF | Primary mouse embryonic fibroblast |
| micro-g | Microgravity |
| M-PS | Micro-structured Polystyrene |
| MSCs | Mesenchymal stem cells |
| MTT | 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide |
| NASA | National Aeronautics and Space Administration |
| NH ₄ OH | Ammonium hydroxide |
| OECD | The Organisation for Economic Co-operation and Development |
| PAMPSA | Poly(2-acrylamido-2-methyl-1-propanesulfonic) acid |
| PANI | Polyaniline |
| PANI-A | Polyaniline prepared by oxidation with potassium persulphate |
| PANI-B | Polyaniline base |
| PANI-PT | Polyaniline doped with phosphotungstic acid |
| PANI-S | Polyaniline salt |
| PANI-SULF | Polyaniline doped with sulfamic acid |
| PBS | Phosphate-buffered saline |
| PEDOT | Poly(3,4-ethylenedioxythiophene) |
| PEDOT:PSS | Poly(3,4-ethylenedioxythiophene) polystyrene sulfonate |
| PEGs-M | Poly(ethylene glycol)-co-poly(glycerol sebacate) |
| pH | Potential of hydrogen |
| PpPDA | Poly(p-phenylenediamine) |
| PPy | Polypyrrole |
| PS | Polystyrene |
| PSS | Polystyrene sulfonate |
| PVA | Poly(vinyl alcohol) |
| PVP | Poly(N-vinylpyrrolidone) |
| QSAR | Quantitative Structure-Activity Relationship |
| RCCS-4 | The rotary cell culturing system |
| REACH | Registration, Evaluation, Authorisation and Restriction of Chemical Substances |
| RGD | Tripeptide composed of Arginine, Glycine and Aspartic acid |
| RHT | The Reconstructed Human Tissue |
| RNA | Ribonucleic acid |

| | |
|---------------|---|
| RPM | Revolutions per minute |
| SDS | Sodium dodecyl sulfate |
| SEE System | Surface Energy Evaluation System |
| SEM | Scanning electron microscopy |
| sGAG | Sulfated glycosaminoglycan |
| STLV | Slow turning lateral vessel |
| SYTO 61 | Red fluorescent nucleic acid stain |
| T20 | Tween 20 |
| TPP | Tissue culture plastic |
| TRITON – X100 | Polyethylene glycol tert-octylphenyl ether |
| U.S. | The United States |
| UV | Ultraviolet |
| YIGSR | Tyrosine – Isoleucine – Glycine – Serine - Arginine |

LIST OF SYMBOLS

Alphabetically ordered

| | |
|----------------|---|
| E | Elasticity |
| I | Current |
| L | Length |
| M | Molarity |
| Q | Quartile |
| R | Resistance |
| S | Cross-section |
| V | Voltage |
| v/v | volume/volume ratio |
| W | Watt |
| wt% | Percentage by mass |
| γ^{AB} | Polar part of surface energy of material |
| γ^{DIF} | Difference between the surface energy of the cells and the sample |
| γ^{LW} | Apolar part of surface energy of material |
| γ^{TOT} | Total surface energy of material |
| δ | Electrical conductivity |
| ρ | Electrical resistivity |

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GAČR 13-08944S Conductive Polymers and Their Interaction with Cells (2015)

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