Stimuli-responsive scaffolds

Ing. Martina Martínková, Ph.D.

Doctoral Thesis Summary

Tomas Bata University in Zlín

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Centre of Polymer Systems

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ABSTRACT

Tissue engineering (TE) is a multidisciplinary field that aims to preserve, restore, or enhance the physical and physiological properties of living tissues through combination of scaffolds and cells, engineering techniques, materials, and biochemical factors. Scaffolds for TE can be created from a variety of biomaterials, each with different properties suitable for specific applications. The biomaterial has to be selected with respect to the length of contact and the site of use in the biological system. Since several tissues in the body exhibit electrical activity, including brain tissues, cardiac muscle tissue, and skeletal muscles, a stimuli-responsive material is appropriate for TE of such tissues. Conducting polymers (CP) can be used to introduce stimuli-responsivity thanks to their electrical activity into a biomaterial. CP can be incorporated into composites or used to modify the surfaces of scaffolds made from other biomaterials, thereby providing specific bioactive properties. Especially combinations of CPs with natural polymers can be beneficial because they combine the electroactivity of CPs with the biocompatibility of biopolymers. In this work, different types of scaffolds suitable for modification with conducting polymers were prepared. Conducting polymers, specifically polyaniline and polypyrrole, were used to create conductive stimuli-responsive composites.

Key words: *Tissue Engineering, Cytocompatibility, Biomaterials, Conductive Polymers*

ABSTRAKT

Tkáňové inženýrství je multidisciplinární obor, jehož cílem je zachovat, obnovit nebo zlepšit fyzikální a fyziologické vlastnosti živých tkání pomocí kombinace scaffoldů a buněk, inženýrských technik, materiálů a biochemických faktorů. Scaffoldy pro tkáňové inženýrství lze vytvořit z různých biomateriálů, z nichž každý má jiné vlastnosti vhodné pro konkrétní aplikace. Biomateriál se vybírá s ohledem na délku kontaktu a místo použití v biologickém systému. Vzhledem k tomu, že několik tkání v těle vykazuje elektrickou aktivitu, včetně mozkové tkáně, srdeční svaloviny a kosterních svalů, je pro tkáňové inženýrství těchto tkání vhodný vodivě stimuli-responsivní materiál. K zavedení stimuli-responsivity (elektrické vodivosti) do biomateriálu lze použít například vodivé polymery. Ty mohou být začleněny do kompozitů nebo použity k modifikaci povrchu scaffoldů vyrobených z jiných biomateriálů, čímž získají specifické bioaktivní vlastnosti. Výhodné mohou být zejména kombinace vodivých polymerů s přírodními polymery, které kombinují elektroaktivitu vodivého polymeru s biokompatibilitou biopolymerů. V rámci této práci byly připraveny různé typy scaffoldů, vhodné pro modifikaci vodivými polymery. Pro vytvoření vodivých stimuli-responsivních kompozitů byly použity vodivé polmery, konkrétně polyanilin a polypyrrol.

Klíčová slova: *tkáňové inženýrství, cytokompatibilita, biomateriály, vodivé polymery*

TABLE OF CONTENT

1. INTRODUCTION

Tissue engineering (TE) has been explored in the last three decades. TE evolved from biomaterials development and its essence is the combination of biomaterials (e.g. in form of scaffolds), cells, biologically active molecules and physical signals into functional tissue-like structures. The main challenge is to modify the biomaterials used for this purpose so that their composition and/or structure mimic the native and physiological conditions for specific tissue cells. Advances in the knowledge and availability of stem cells, the emergence of new biomaterials as potential templates for tissue growth, improvements in bioreactor design, and a better understanding of healing processes are all contributing to the increasingly rapid development of this field.

This work deals with development of scaffolds, artificial implant structures, that support and control the growth of cells of the desired tissue. Supporting tissue growth is possible by selecting a suitable scaffold material, therefore the material is selected with respect to the site of application in the host tissue. For example, when designing scaffolds for hard TE (bone, cartilage and teeth), it is necessary to select a material that will have similar properties. Therefore, for bone TE, ceramics or polymeric materials such as polyetheretherketone (PEEK) can be considered. In the case of soft tissue, materials such as cellulose nanofibres can be used.

Various techniques can be used to obtain scaffolds with unique physical, chemical, mechanical, and biological properties. One of the possibilities of a simple and reproducible technological solution for scaffold preparation could be a manually hot-pressed technique. Another option for scaffold preparation is Powder Injection Molding technology (PIM). This technology could be beneficial in personalized medicine because it allows production with high precision. It is possible to obtain implants in various shapes with a defined pore size and overall porosity. All of these factors can affect implant acceptance. Another added value that will affect the instructive properties of cells is, for example, the electrical conductivity of biomaterials. This property is particularly important for electroactive tissues, because the electric field plays an important role in many biological processes. For this purpose, it is advisable to choose materials with combined electrical conductivity, appropriate candidates are conductive polymers.

Because most biological reactions take place at the interface between the biological system and the implant surface, the biointerface is a critical place. This is the reason why the surface properties of biomaterial are one of the main factors that affect its applicability *in vivo*. The chemical and physical characteristics of the biomaterial surface can affect many of cellular functions.

To reach the desired cell reaction, the surfaces could be functionalized. These are one of the main factors for the applicability of scaffolds in a real system.

2. TISSUE ENGINEERING

Tissue engineering (TE) is a multidisciplinary field that combines the targeted use of cells and biomaterials to maintain, restore or improve the function of living tissue (Langer and Vacanti, 1993). The term "tissue engineering" was defined by National Science Foundation in 1987. Nevertheless, TE approaches have been used since the seventies of the twentieth century (Ratner, 2013).

In particular, TE uses a combination of four key elements. 1) A suitable cell line, 2) the right environment such as a scaffold, 3) biomolecules (for example signalling molecules, growth factors, proteins) that keep cells productive, and finally, 4) external stimuli such as mechanical and electrical that affect cell behaviour. Figure 1 shows four key elements of TE.

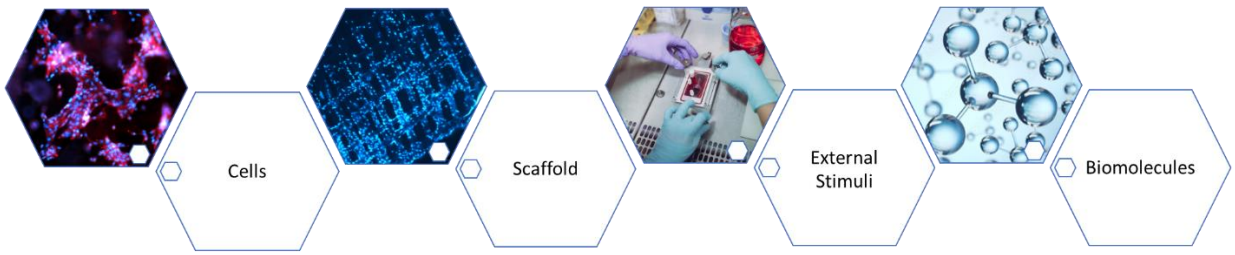

Fig. 1 Tissue engineering key elements

3. BIOMATERIALS

Materials used in contact with biological systems are referred as biomaterials. There are many definitions of the term biomaterial. One of the possible definitions is from the European Society for Biomaterials Consensus Conference II, where the term was defined as follows: "A biomaterial is a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body" (Leali and Merolli, 2009). Since then, the term has been re-defined many times, but the definitions have always met in one point: biomaterial is a material that interacts with the human body.

3.1 Biological properties of biomaterials

The material in contact with the organism (directly or indirectly) can affected cellular functions. In order for a biomaterial to be used, it must meet a number of criteria. The most important is biocompatibility. The first commonly used definition of term biocompatibility comes from David F. Williams in 1987 "the ability of a material to perform with an appropriate host response in a specific situation" (Williams, D. F. and European Society for Biomaterials, 1987). Overall

the material must elicit an appropriate biological response for the application in the body (O'Brien, 2011). Thus the material must be non-cytotoxic, non-carcinogenic and cannot cause immunological rejection, must not cause an inflammatory reaction and should contribute to a harmonious biological function. (Gad and Gad-McDonald, 2015).

The material must also possess suitable mechanical properties with regard to the application. In particular, these properties are important for orthopaedic applications. However, good vascularization is also essential for the production of bone and cartilage scaffolds. Therefore, attention must also be paid to porosity, pore distribution, exposed area and overall scaffold architecture (Carletti et al., 2011).

3.2 Material properties of biomaterials

Material properties are most often divided into two categories: surface properties and bulk properties.

3.2.1 Surface properties

Most biological reactions take place at the interface between the biological system and the surface of the biomaterial (biointerface) (Castner and Ratner, 2002). The surface properties of biomaterial are one of the main factors that affect its applicability in a real system. In TE, the chemical and physical characteristics of the biomaterial surface can affect cellular functions, such as proliferation, or migration, phenotype, differentiation and so on (Parisi et al., 2020).

The cellular response is manifested primarily in cell morphology, adhesion, migration, proliferation and differentiation. The determining factor in the level of cell adhesion to the surface is topography, surface chemistry (for example functional groups) and surface energy. The surface energy of the biomaterial affects the cell adhesion. This is related to the polar and dispersive components of surface free energy. It is believed that in order for the cells to adhere to the surface successfully, the total surface energy of the material is crucial. In fact, surface energy is closely related to surface wettability. A certain balance between hydrophobicity and hydrophilicity is desirable. Nevertheless, protein absorption is easier on the hydrophobic surface (Ferrari et al., 2019; Wang et al., 2004).

The first reaction after contact of the biomaterial with the host system (body fluid) is the adhesion of proteins to the surface, which affects cell adhesion. Protein adsorption is faster than cell migration to a foreign surface, so the initial adsorbed protein layer is thought to be a critical factor in cell adhesion rate (Murphy et al., 2016). It is a complex process mainly influenced by various protein-surface

physicochemical/intermolecular interactions such as Van der Waals, hydrophobic and electrostatic forces. The adsorption characteristics of a protein (e.g. the amount of protein adsorbed, the type of protein) are also influenced by the surface properties, such as its topography, roughness, surface energy and charge (Ma, 2014). Vroman and Adams observed competitive protein exchange on surfaces, wherein proteins that had already adsorbed onto a surface from a protein mixture solution were displaced by subsequently arriving proteins (Fig. 2) (Vroman and Adams, 1969a; Vroman and Adams, 1969b).

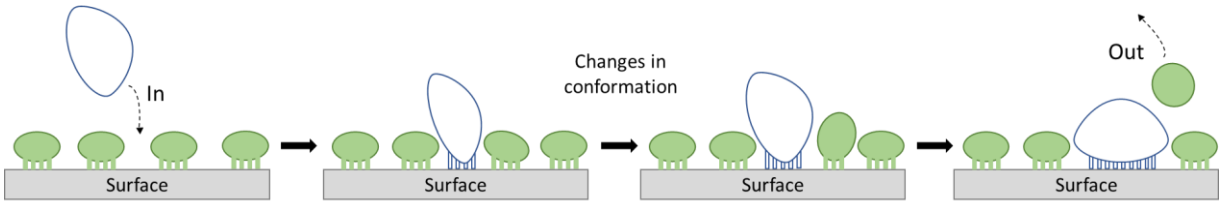

Fig. 2 Schematic representation of the competitive adsorption of proteins known as the Vroman effect

3.2.2 Bulk properties

In all cases the mechanical properties of a biomaterial must be as similar as possible to the characteristics of the tissue they will replace. Mechanical properties often analysed for most materials are its modulus of elasticity (Young modulus), tensile yield stress, fatigue strength, and toughness (Case et al., 1994). All these properties are assessed with respect to the mass density of the materials.

Many of the materials used for bioapplications are non-porous. This can be justified by compliance with the required conditions for mechanical properties and as corrosion protection. It is caused by the fact, that the corrosion rate is faster near defects and accessible pores of a structure (Vrana et al., 2020). Also, the mechanical properties of porous scaffolds depend on the pore diameter and the overall porosity. When larger pores and overall pore interconnection correlate with lower mechanical strength due to higher void volume. Nevertheless, porosity, pore sizes, and pore shapes are important for some applications such as bone tissue reconstruction. The pore structure is one of the key factors in the development of scaffolds.

4. STIMULI-RESPONSIVE BIOMATERIALS

A special group of biomaterials, that can change their properties with the change of external stimuli (such light, pH, temperature, magnetic fields, or electricity), are the so-called stimuli-responsive biomaterials (Gelmi and Schutt, 2021). A specific example of such materials is thermo-responsive polymer – poly(N-isopropyl acrylamide) (PNIPAAm). With increasing temperature the conformation changes and the surface of the material changes its hydrophilic/

hydrophobic properties (Nagase et al., 2018). This property can be exploited in a number of biomedical applications, such as drug delivery systems, “smart” cell culture setups, sensors, and separation technologies (Yang et al., 2020). For the drug delivery system pH-sensitive biomaterials can also be used. A system of targeted drug release based on pH change is a particular advantage because many diseased tissues are surrounded by an acidic microenvironment (Zhuo et al., 2020).

4.1 Electrical Stimulation

The meaning of an electric field in TE is mainly because of many excitable tissues/organs, such as the brain, heart, and skeletal muscle. Every human sense uses ionic currents and electric fields in its transduction mechanism. Hearing, sight, touch, taste, and smell all of these senses have receptor systems enabling generate electrical signals to the brain (Pullar, 2011). The electrical phenomena that occur in living organism is called bioelectricity. Bioelectricity arises from the transmission of electrical signals *via* ion channels (ionic conductivity) and pumps located on or within the plasma membrane. The plasma membrane possesses the capacity to generate and sustain distinct charges on its opposing sides. This capability stems from disparities in ion concentrations between the cytosol and the external environment of the cell (Grimnes and Martinsen, 2015; Otero et al., 2012).

A lot of studies revealed the presence of electrical fields influence a variety of biological processes such as migration, proliferation, differentiation, orientation (Fig. 3), cytoskeletal organization, apoptosis and necrosis (Thrivikraman et al., 2018). The reason is, that external electrical stimulation can help with modulation of cellular responses and this could lead to enhance tissue regeneration (Lee, 2013).

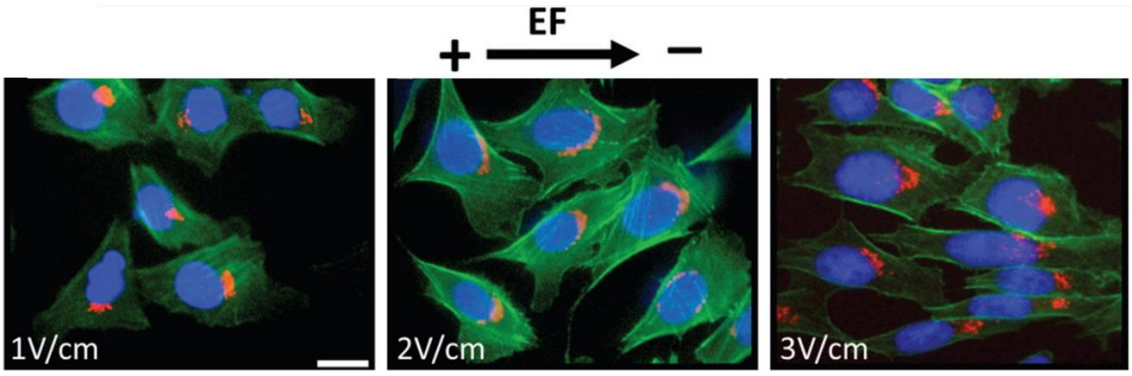

Fig. 3 Orientation of cells due to electric field, edited from (Pu et al., 2015)

The electrical signal can be transferred *via* conductive biomaterials through two possible routes. The first is a series connection of conductive biomaterial and an electrical circuit. It follows the fact, that the electrical signal is transmitted

by the biomaterial. This induces a local electrical field and its charge is not transferred from the biomaterial to the environing electrolyte. In the second case, the conductive biomaterial is used only as an electrode, which allows the transfer of charge by the surrounding electrolyte from the opposite electrode (Gelmi and Schutt, 2021). Whole mechanisms of electrical stimulation of cell are uncertain. However, it is believed that the external electric field is able to change the distribution of the membrane receptors and intracellular levels of cell regulators (Lee, 2013).

5. TYPES OF BIOMATERIALS

Biomaterials are usually divided into three basic categories: metals, ceramics and polymers. Each of these groups is suitable for different applications and has its advantages and disadvantages. Only the materials used in the practical part of the disertation will be described below.

5.1 Ceramic

In general, ceramics are divided into groups based on the different reactions of ceramic materials to the biological environment (Ben-Nissan et al., 2019). The first group is bioinert ceramics and it is characterized mainly by the fact that the materials do not show any interaction with the surrounding tissue after implantation. This group includes for example alumina and zirconia. They have good biocompatibility, corrosion and wear resistance, mechanical strength and they are relatively biologically inactive (Boniecki et al., 2020; Huang and Best, 2014).

Another group consists of bioactive ceramics, this category involves glass-ceramics and calcium phosphate ceramics (e.g. tricalcium phosphate, hydroxyapatite, …). The materials in this group must elicit specific biological activity. They interact with surrounding living tissues after implantation and form a bond between hard and soft tissues (Rahaman et al., 2011).

Overall, ceramic materials are characteristic for their strength and good biocompatibility. Due to their structural similarity to native bone, they are suitable for bone TE (O'Brien, 2011). Ceramics are also used in orthopaedics, load-bearing applications, dentistry, and spinal surgery (Vaiani et al., 2023). The properties of ceramics and its microstructure depend mainly on the technology of production. One way to prepare ceramics for biomedical applications is powder injection molding (PIM) technology. This manufacturing technology could be beneficial in personalized medicine because it allows production with high precision (various shapes and defined pore size). All of these factors can affect implant acceptance. More about PIM technology is in the practical part of disertation.

5.2 Polymers

In general, polymers provide scaffolds with considerable processing flexibility, good biocompatibility or even biodegradability. It is the most common group from which scaffolds can be made. Polymer properties that determine the applicability of a polymer as a biomaterial include molecular weight, polymer structure, crystallinity, thermal and electrical properties. Polymers are divided into two groups, natural polymers and synthetic polymers, and each of these groups has its own advantages and disadvantages for use. Often used natural polymers are chitosan (Sukpaita et al., 2021), collagen (Jiang et al., 2018), alginate (Venkatesan et al., 2015), hyaluronic acid (Mohammadi et al., 2018), cellulose (Torgbo and Sukyai, 2020) etc. Synthetic polymers include polyetheretherketone (PEEK) (Mavrogenis et al., 2014), polyurethane (Cooke et al., 2020), polylactide (Gregor et al., 2017) and many others.

5.2.1 Natural polymers

Natural biomaterials include materials with the protein or polysaccharide origin. Thus, these are natural polymers that take advantage of better cytocompatibility than for example synthetic polymers. Natural biopolymers such as collagen, fibrinogen, or hyaluronan, can provide biochemical stimuli to promote cell adhesion or differentiation. The main disadvantage of natural biopolymers isolated from natural sources is their less defined composition and also their susceptibility to biological contamination (Milne et al., 2003). Because their composition can be variable and thus the reproducibility of products becomes problematic.

5.2.1.1 Alginate

Alginate is a linear natural polymer obtained mainly from brown seaweed (Phaeophyta) or from soil bacteria. It is a block copolymer consisting of α-L-guluronic acid (G) and β-D-mannuronic acid (M) (Berthiaume and Yarmush, 2003). Its formula can be seen in figure 4. Alginates are constituted of tree types of blocks. The first option is alternating sequence of M and G blocks and thus form the most flexible part of the chain. Then there are the blocks of the GG itself and third type are MM blocks with polymerization degree greater than or equal to twenty ($DP \geq 20$) (Rinaudo, 2008). Alginate is able to make a reversible hydrogel by the reaction of metal cations with functional carboxyl groups. Crosslinking of the alginate takes place ionically using bivalent cations (such as Ca^{2+}, Mg^{2+}, Ba^{2+}) in an aqueous solution (Blitterswijk and Thomsen, 2008).

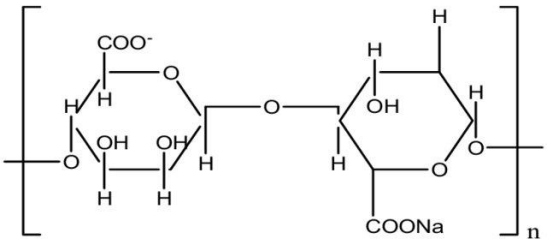

Fig. 4 Chemical structure of sodium alginate (Tamilisai et al., 2021)

Alginate is used for biomedical applications mainly due to its nontoxicity, good biocompatibility, biodegradability and easy gelation process (Sabu et al., 2018). Also, it facilitate wound healing (Davis and McLister, 2016). It is clear that it is used for wound dressing (Varaprasad et al., 2020) and skin repairing. Alginate is one of the options for the production of soft tissue scaffolds (Yuan et al., 2017) and also for supporting connective tissue as cartilage (Klein et al., 2009). This polysaccharide is also used as a drug delivery system and as a cell encapsulation material (Hariyadi and Islam, 2020).

5.2.1.2 Cellulose

Cellulose is a linear homopolymer which consist of covalently bond units of D-anhydroglucopyranose established in chair conformation. The units are linked by β-1,4-glycosidic bonds and form cellobiose (see fig. 5). This biopolymer is found in plants and some other organism such as tunicates and bacteria (Sabu et al., 2018). Sources of cellulose from higher vascular plants include cotton, sisal, jute and the rigid cell walls of wood. Lower non-vascular plants containing cellulose are algae, lichen, and fungi (Sacui et al., 2014). Cellulose is possible to isolated in different forms such as cellulose nanocrystals (CNC) and cellulose nanofibrils (CNF) and both can be obtained by chemical and mechanical disintegration or enzymatic digestion methods (Sabu et al., 2018).

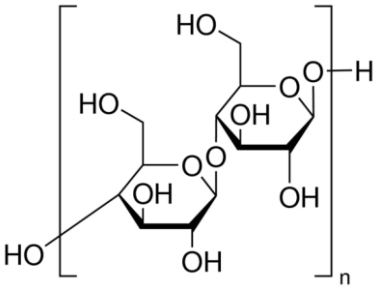

Fig. 5 Chemical structure of cellulose ("Cellulose C6288, Sigma-Aldrich,")

Cellulose is used for TE due to its tunable mechanical properties, high biocompatibility, ability to release drugs and retain moisture (Hickey and Pelling, 2019). In general, cellulose-based biomaterials are used for artificial skin (Vatankhah et al., 2014) and wound dressing (Liu et al., 2012; Rees et al., 2015). As already mentioned, cellulose has adjustable mechanical properties suitable

even for rigid and mechanically demanding environments such as bones. It goes without saying that another use for cellulose is bone tissue (Zhang et al., 2015; Zhou et al., 2013). This natural polymer also finds use for neural applications (Yang et al., 2018) and blood vessels (Fink et al., 2011).

Derivates of cellulose

In order to achieve higher utilization of cellulose for bioapplications, various chemical treatments and functionalizations have been introduced and many cellulose derivatives are also used. The properties of the derivatives depend on the degree of substitution and on the functionalization pattern (Seddiqi et al., 2021). Cellulose ethers, for example, are known to have a high-water retention and thermo-gelling ability. Their properties make them suitable for use in wound healing, e.g. carboxymethyl cellulose is used for wound dressing (Capanema et al., 2018). Other possible applications are in TE (Schütz et al., 2017) and drug delivery. Cellulose is insoluble in water and most solvents. However, cellulose esters are soluble in common solvents. They have the same biological uses as cellulose ethers (Mwesigwa and Basit, 2016; Schunck et al., 2005). The oxidation of cellulose by periodate salts leads to cellulose dialdehyde (DAC). Applications of DAC could be in TE (Li et al., 2009) and drug deliver carrier (Dash and Ragauskas, 2012).

5.2.1.3 Chitosan

Chitosan is a derivative of chitin, the second most common natural polysaccharide found mainly in crustacean shells, exoskeletons of insects and the cell wall of fungi. Chemically, chitin is formed by glucosamine molecules linked by a β (1→4) glycosidic bond (Shukla et al., 2013). It is thus a copolymer of D-glucosamine and N-acetyl-D-glucosamine units (Fig. 6). The solubility of chitosan is pH-dependent, but is also affected by several factors such as the degree of deacetylation and ion concentration.

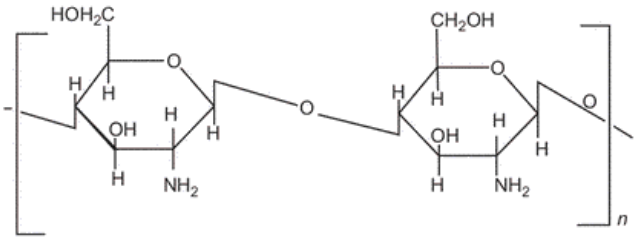

Fig. 6 Chemical structure of chitosan (Sabu et al., 2018)

Chitosan has been widely investigated for bioapplications due to its biodegradability (Vila et al., 2002), biocompatibility (Chellat et al., 2000) and wound healing properties (Adekogbe and Ghanem, 2005). Also, its structural similarity to glycosaminoglycans offers active sites for the binding of other

molecules (e.g. growth factors) and increases cell adhesion, and induces beneficial responses within biological systems (Kumbar et al., 2014). Chitosan also has antibacterial and haemostatic properties. Moreover, chitosan positively affects formation of osteoblast. Therefore, chitosan is considered a suitable material for the regeneration of various tissues such as bone (Azaman et al., 2022; Moreira et al., 2019) or cartilage (Shen et al., 2021, 2015). Another possibility is its use in blood vessel (Chupa et al., 2000) and corneal regeneration (Rafat et al., 2008). Chitosan can be used in several form such as gels, films, membranes and fibers.

5.2.1.4 Hyaluronan

Hyaluronan (hyaluronic acid, HA) is a unique polysaccharide with great promise for TE. It occurs in epithelial, neural and connective tissues (Sabu et al., 2018). Hyaluronan belongs in to the family of glucosaminoglycans and it is present in extracellular matrix (ECM) tissues and therefore shows high biocompatibility. It is also a polysaccharide that promotes cell proliferation and migration (Kumbar et al., 2014). Hyaluronan can be extracted from animal sources, but it is also produced in large quantities by *Streptococcus zooepidemicus* and *Streptococcus equiwit*, with good yield and high purity. In bacterial extraction, HA is isolated in the form of the sodium salt (Rinaudo, 2008).

HA is a copolymer composed of repeating monomeric units of β-(1,4)-D-glucuronic acid and β-(1,3)-N-acetyl-D-glucosamine (Fig. 7) (Sabu et al., 2018). Depending on the application, HA is used in different concentrations and molecular weights (Rinaudo, 2008).

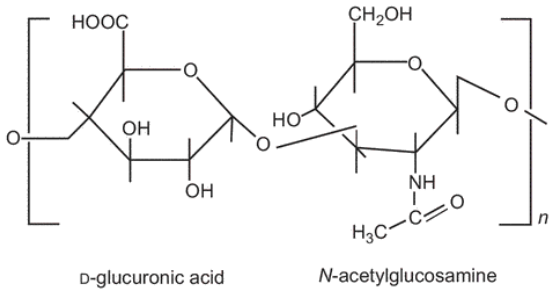

Fig. 7 Repeating monomers of hyaluronic acid (HA) (Sabu et al., 2018)

In regenerative medicine, HA finds use in the form of fibers, films and hydrogels (Ma, 2014). Due to the wide distribution of HA in the human body and its importance in certain body functions, it can be used in various field of medicine such as vascular (Zhu et al., 2014), cartilage (Matsiko et al., 2012), bone (Townsend et al., 2018), and skin TE (Monteiro et al., 2015) and cancer therapy as drug delivery (Trombino et al., 2019).

5.2.2 Conductive polymers

Another group of potentially suitable materials for TE from the category of polymers are electroactive biomaterials such as conductive polymers (CPs). CPs are synthetic organic polymers possessing conductivity up to 200 S/cm. Aromatic rings with conjugated π-orbitals and delocalized electrons occur in their formula, which allows electrical activity (Lee, 2013). The CPs are capable to transform the ionic conductivity to electronic conduction (Lindfors and Ivaska, 2002). CPs can be synthesized using various methods. The most common methods include electrochemical polymerization and chemical polymerization. In their pristine state, CPs have low electrical conductivity, but these can be enhanced by the use of dopants (K and Rout, 2021).

Overall their properties almost correspond to those of inorganic semiconductors. With relatively easy synthesis, CPs provide the desired electrical and optical properties. Thanks to that they can be used in a wide range of bioapplications, such as neural interfaces (Green et al., 2008), drug delivery systems (Chapman et al., 2020) or biosensors (Aydemir et al., 2016; Mawad et al., 2012). Overall, CPs are widely studied for regenerative medicine applications.

5.2.2.1 Polypyrrole

PPy (Fig. 8) is conjugated polymer which can be easy to synthesize. It could be prepared by electrochemical or chemical oxidation of pyrrole. Reaction takes place at room temperature, and a variety of solvents can be used for preparation (Balint et al., 2014). Several different oxidants can be used in chemical oxidative polymerization, such as iron (III) chloride or ammonium persulfate. However, various oxidants and their concentrations can affect thermal stability, conductivity and morphology (Yussuf et al., 2018). The resulting intrinsic properties of PPy are determined by the polymerization conditions. Overall, PPy powder or films has excellent mechanical and electrical properties and good *in vitro* biocompatibility. Thanks to its excellent properties that can respond to stimuli, PPy is a smart biomaterial that is allowed to dynamically control properties using an electric field application. Due to its stimuli-responsive characteristics, it is one of the most widely used CPs for biomedical applications (Khan et al., 2019). Its bioapplications are also aided by the fact that the pyrrole ring structure can be found in the aminoacids proline and hydroxyproline.

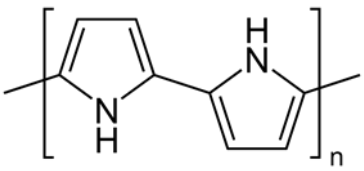

Fig. 8 Polypyrrole

There are studies that investigate PPy as biosensors (Pandey et al., 2018; Van Hao et al., 2018), scaffolds for TE (Naghavi Alhosseini et al., 2019), and drug delivery systems (Puiggalí-Jou et al., 2019; Shah et al., 2018).

5.2.2.2 Polyaniline

Polyaniline (PANI) could be easily synthesized by various methods, commonly used are chemical or electrochemical oxidation (Pina and Falletta, 2022; Stejskal and Sapurina, 2005). The conductivity of PANI depends on the degree of its oxidation. Three redox forms are known: 1) a completely reduced leucoemeraldine base (Fig. 9A), 2) a fully oxidized pernigraniline base (Fig. 9B) and 3) an emeraldine base (Fig. 9C) (Qazi et al., 2014). The most conductive of these three forms is PANI emeraldine. This form is not inherently conductive, but the electrically conductive form can be transformed by doping to the emeraldine salt (Chiang and MacDiarmid, 1986). The advantage is good environmental stability and possibility of making PANI as a powder, thin film, hydrogel/cryogel or colloidal suspension (Humpolíček et al., 2018; Kašpárková et al., 2017; Kucekova et al., 2014). The morphology of polyaniline depends on the degree of oxidation and also on the reaction conditions. The growing interest in polyaniline for bioapplications is mainly due to its biocompatibility, adjustable conductivity, processability, and antibacterial efficacy (Kucekova et al., 2013; Qazi et al., 2014; Roshanbinfar et al., 2020). However, the main disadvantage of PANI is, that it exhibits pH-dependent conductivity. It loses its conductivity upon contact with physiologic pH (Lindfors and Ivaska, 2002). The potential of PANI applications lies in biosensors (Zheng et al., 2020), in drug delivery systems (Li et al., 2018)(Li et al., 2018) and TE (Massoumi et al., 2020; Roshanbinfar et al., 2020).

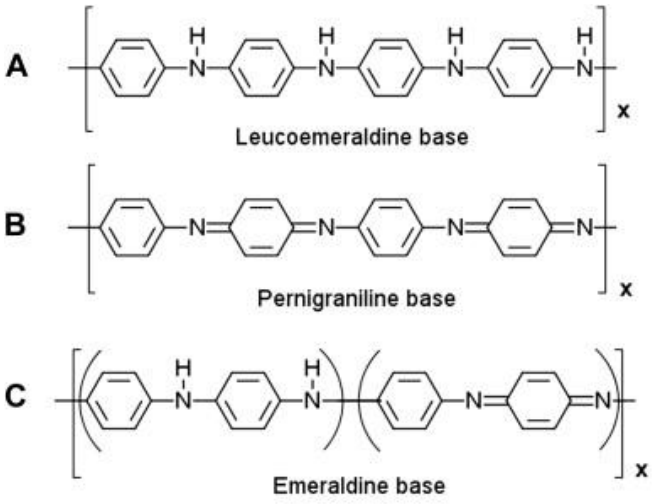

Fig. 9 Polyaniline forms, edited from (Qazi et al., 2014)

6. AIMS OF DOCTORAL THESIS

The aim is to prepare and modify the biomaterials in the form of a scaffold and to determine the interactions between the prepared scaffolds and the cells. Overall, the aim is to prepare stimuli-responsive material enabling targeted interaction with the organism and cytocompatibility testing of prepared scaffolds. Another goal is to observe cellular behaviour on scaffolds using static and dynamic cultivation.

7. EXPERIMENTAL PART

The experimental part of the disertation was focused on the preparation and surface modification of scaffolds. Furthermore, the interactions between material and cells were investigated. Overall, routine tests for cytotoxicity and biocompatibility were performed in the cell laboratory. The MTT assay was used for cell quantification, evaluation was performed by spectrophotometry. NIH/3T3 (ECACC 93061524, England) mouse fibroblast lines were mostly used during the experiments. However, in some studies, the embryonic stem cell ES R1 line (Nagy et al., 1993) or cell line of mouse osteoblastic precursors (MC3T3-E1) obtained from the European Collection of Cell Culture (c.n. 99072810) were used. Furthermore, the effect of dynamic cultivation and electrical stimulation on cell cultures was investigated

A substantial part of this thesis deals with testing CPs as they are suitable for the preparation of stimuli-responsive scaffolds. From the theoretical part of the thesis, it can be understood that there are many material properties that affect cell behaviour. CPs were investigated for their conductivity because, as mentioned in the electrical stimulation subchapter, electrical signals affect cell fate. Of the CPs, PANI and PPy were used for the work mainly because of their relatively high conductivity and ease of synthesis. Interesting results were obtained when testing PANI films and coatings of ceramic substrate. In addition, these films and coatings were enriched with biopolymers that also affect cell behaviour. During the research, ceramic samples produced by powder injection technology (PIM) were investigated. Ceramic material has been studied primarily for its potential in bone TE. This was followed by the study of another material that could be used in TE of hard tissues. PEEK was studied for its elastic modulus similar to that of natural bone. Therefore, the cytocompatibility and bioactivity of PEEK grafts modified with farringtonite were investigated.

Another part of research focused on stimuli-responsive material suitable, for example, for soft TE. The study was aimed at preparation of PPy in combination with DAC, and combination of PPy with cotton modified with DAC, dialdehyde alginate (DAAL), or dialdehyde hyaluronate (DAH). To the authors' best knowledge, in this research, a completely new approach to template-controlled polymerization was used. Due to ongoing patent proceedings, the preparation and results of this study will only be described briefly.

7.1 Cell lines

Here, the cell lines that were used for the biological evaluation of the materials will be briefly introduced.

7.1.1 Mouse embryonic fibroblasts

The mouse fibroblasts NIH/3T3 cell line (ECACC 93061524, England) was used to test the biological properties of the scaffolds. It is a line of adherent mouse embryonic fibroblasts. The culture medium for this cell line consists of DMEM (Dulbecco's Modified Eagle Medium, Biosera) containing 10% Calf Sera (Biosera) and 1% Penicillin/Streptomycin (Biosera). For cultivation, TPP tissue-polystyrene bottles and dishes were used. Cultivation was carried out under constant conditions in an incubator (Heracell 150i, Thermo Scientific) at 37 °C, with a CO_2 concentration of 5% and a constant relative humidity of 90%.

7.1.2 Stem cells

Stem cells could play an essential role in regenerative medicine. They are unspecialized cells that can be found in most multicellular organisms. Those cells have the ability to convert into another cell type based on their purpose, also they can renew themselves (Bishop et al., 2002).

"The embryonic stem cell ES R1 line (Nagy et al., 1993) *was propagated in an undifferentiated state by culturing on gelatinized tissue culture dishes in complete media. The gelatinization was performed using 0.1% porcine gelatin in water. Complete medium with the following composition was used for the cultivation: Dulbecco's Modified Eagle's Medium (DMEM), 15% fetal calf serum, 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, 100 mM non-essential amino acids solution (all from Thermo Fisher, Waltham, MA, USA), 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA) and 1000 U mL^{-1} of leukemia inhibitory factor (LIF) (Gibco, MA, USA)"*(Skopalová et al., 2021). Cultivation conditions in incubator are the same as for the NIH/3T3 line.

7.2 Samples preparation

The following part of the thesis briefly describes the methodology of preparation of the materials, which could have been prepared by myself or in collaboration with colleagues. Most of the tested materials were prepared in collaboration with other faculties or other universities. These methodologies are described in the articles.

7.2.1 Ceramic-based scaffold

Ceramic-based substrates (CBS) were prepared in collaboration with colleagues from department of production engineering Tomas Bata University by PIM technology. This method consists of four steps, where the first step is to create a homogeneous mixture that consists of powder material, polymer binder and space holder. *"The powder components of the PIM compound were aluminum oxide (Martinswerk – Huber Corporation, USA) (ρ = 3.98 g/cm^3, size range 0.1-3.0 μm) and a powder space holder (PSH), potassium chloride (KCl, Sigma*

Aldrich, Germany) (ρ = 1.98 g/cm^3, size range 125-500 μm). The powders were admixed into a partially water-soluble binder (Licomont EK 583, ρ = 1.08 g/cm^3, viscosity 1.5 mPa.s at 130 °C) in a batch mixer (Plasti-Corder, Brabender, Germany) with counter-rotating blades" (Martínková et al., 2022). The architecture of the scaffold and shape of pores is significantly influenced by the shape and size of PSH. Particles KCl of irregular shape were used.

The second step is the injection of the prepared mixture into the desired shape. Injection molding was performed on an injection molding machine (Allrounder 370S, Arburg, ARBURG GmbH + Co KG, Lössburg, Germany).

The third step is the removal of the binder by a suitable solvent or thermal decomposition and finally the sintering of the powder material to the final density. *"The water-soluble binder component and part of the PSH were removed by immersion in distilled water (60 °C) for 24h. The remaining binder (the backbone) was debound thermally (280 °C) at atmospheric pressure. Sintering was carried out in a PIM furnace (CLASIC CZ s.r.o., Revnice, Czech Republic) up to a maximum temperature of 1670 °C and for a holding time of 1h. The surfaces of CBS were inspected using SEM microscopy (VEGA, Tescan)"* (Martínková et al., 2022).

The surfaces of the CBS were functionalized by electrically-conductive polyaniline and polyaniline stabilized by biopolymer films prepared in a colloidal dispersion mode. Sodium alginate, sodium hyaluronate and chitosan were used, mainly due to their good biocompatibility. Subsequently, the cytocompatibility of the native ceramic substrate and bioactive coatings were investigated. Cytocompatibility was investigated under static and under dynamic conditions with electrical stimulation.

Surface functionalization of native substrate

The surface of a CBS prepared by powder injection molding was coated to become bioactive. For surface functionalization, four different coatings were designed. First of all, electroconductive PANI was used. However, several studies indicate the lack of cytocompatibility of PANI itself. Therefore, composite surfaces were further prepared. An innovative approach to the preparation, *in-situ* polymerization of aniline hydrochloride (AH; Sigma Aldrich, Germany) with oxidizing agent ammonium persulfate (APS; Sigma Aldrich, Germany) in the presence of stabilizers was used. This technique of colloidal dispersion mode of preparation was used for three biopolymer stabilizers – specifically, sodium hyaluronate (SH; Contipro a.s.), sodium alginate (SA; IPL, Czech Republic), and chitosan (CH; Sigma Aldrich). In this arrangement, biopolymers provide cytocompatibility and the conducting polymer contribute with electroactivity. A schematic representation of the reaction is shown in Fig. 10.

The methodology for each surface is given in the publication "Powder injection molded ceramic scaffolds: the role of pores size and surface functionalization on the cytocompatibility" (Martínková et al., 2022).

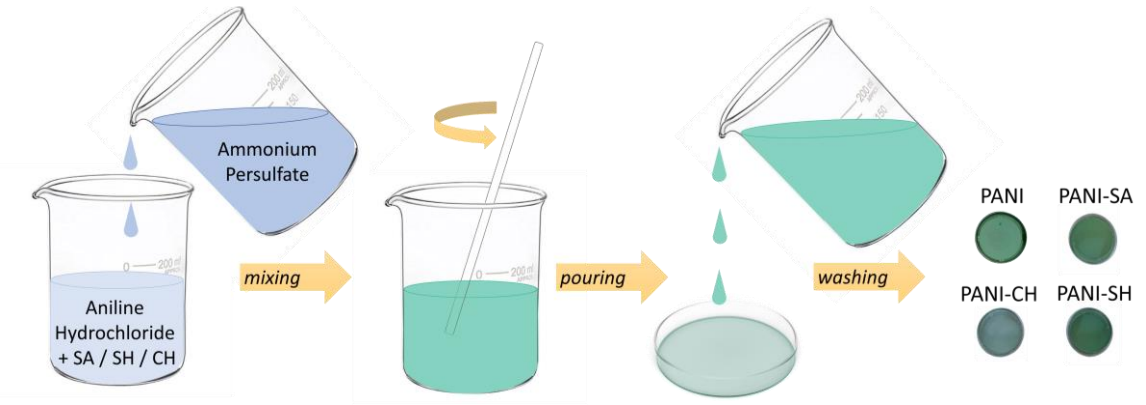

Fig. 10 Scheme for the preparation of PANI films in colloidal dispersion mode stabilized by SA, SH or CH

7.2.2 Preparation of dialdehyde cellulose nanofibrils with PPy (CNF-DAC PPy)

Solution in concentration of 0.5 wt % of Cellulose nanofibrils (CNF; 3 wt % in water, Cellulose lab, Canada) in ultra-pure water (UPW) was prepared. Then the solution was heated to 55 °C overnight under shaking. The solution was homogenized using a sonicator for 60 min.

Then the cellulose nanofibrils were oxidized from 10%. For oxidation was used periodate salt (sodium periodate $NaIO_4$; PENTA, Czech Republic). The reaction was carried out for 72 h in the dark with stirring at 30 °C. After this time the reaction was terminated by the addition of ethylene glycol (PENTA, Czech Republic). Subsequently, dialysis of cellulose nanofibrils dialdehyde (CNF-DAC) against UPW was performed for 72 hours. The solution of CNF-DAC was investigated for the presence of residual oxidizing agents (periodate) by iodometric titration. The presence of iodine was detected in the sample, so the sample was purified by centrifugation and homogenization on a mechanical homogenizer. Then dialysis against 0.05 M NaCl (Sigma Aldrich Co.) using a 14 kDa MWCO membrane, until iodometric titration and XRF measurements confirmed complete removal of all oxidizing agents. Subsequently mass analysis was done.

To the solution of CNF-DAC, a pyrrole (Py; Sigma-Aldrich Co.) in different concentrations was added. To a CNF-DAC suspension of 0.5 wt% in UPW was added an amount of pyrrole corresponding to $n_{py} : n_{CHO}$ molar ratios of 1:1, 2:1, 4:1, and 8:1. This was followed by incubation for 24 hours on a shaker. Before polymerization, the solution was homogenized by ultrasonication for 10 minutes. This was followed by the addition of an oxidizing agent to the solution (in mass ratio 1:4 Py : $FeCl_3$ Sigma-Aldrich Co.) under gentle shaking for 24h. After this

time, the samples were filtered and washed with 0.2 M hydrochloric acid (HCl; PENTA, Czech Republic), then with methanol (PENTA, Czech Republic) and finally with UPW. The samples were transferred into aqueous solution and lyophilized for SEM.

The different preparation procedure resulted in the formation of more rigid samples suitable for conductivity measurements. CNF-DAC dialysis was followed by filtration through a 0.4 μm pragopore filter. The pellet was dried between two vapor permeable membranes (Fig. 11) at 40 °C with a load of 1kg. The prepared CNF-DAC disks were consequently immersed in a solution of pyrrole (several different concentrations) and placed for 24h on an orbital shaker. Finally, iron (III) chloride was added four times the mass excess compared to pyrrole. After 24 h, the samples were washed with UPW and then placed in an ultrasonic bath in 0.2 M HCl for 5 min. This was followed by washing with UPW, methanol, and finally 5 min in the ultrasonic bath with UPW. CNF-DAC PPy disk were dried between two vapor permeable membranes at 40 °C with a load of 1kg.

Fig. 11 CNF-DAC disk on vapor permeable membrane after drying (left), CNF-DAC PPy after polymerization before washing (right)

7.3 Biological properties

7.3.1 Cytotoxicity

In the theoretical part in the chapter biological testing the permitted conditions for testing according to ISO standard 10993-5 and 12 are given.

Extract preparation

The preparation of the extracts was carried out in accordance with the ISO standard 10993-12. If the material properties of the sample allow, the sample should be crushed or cut into small pieces for preparation in order to increase the surface area of the extracted material. The extraction ratio of the material depends on the thickness, the preparation method and type of material. For example, in the case of the ceramic substrate prepared by the PIM method, the samples were crushed and extracted at a concentration of 0.2 g/mL of culture media. The extraction was carried out for 24 hours at 37 °C with stirring.

Subsequently, the extracts were sterilized by filtration on syringe filter with a pore size of 0.22 μm (Merck, Darmstadt, Germany). The parent extracts (100 vol.%) were then diluted in culture medium to achieve the desired final concentration. In a second study testing the cytotoxicity of PEEK grafts, samples were extracted in concentration of 3 $cm^2 \cdot mL^{-1}$ of culture medium. Prior to extraction, samples were sterilized with ethanol for 1 hour, thus eliminating the need for filtration of extracts. All extracts were used within 24 hours. *In vitro* testing of cytotoxicity was performed according to the ISO 10993-5.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromid)) (Invitrogen Corporation, USA) in the form of a yellow solubilized solution is added to the cells. MTT is reduced in living and metabolically active cells by mitochondrial dehydrogenases and reducing agents to purple coloured formazan crystals (Freimoser et al., 1999; Liu et al., 2002). Reduction of MTT to formazan is limited upon cytotoxic damage or destruction of the cell. A strong detergent is required to dissolve formazan. Dimethyl sulfoxide (DMSO) is considered the best solvent and is applied especially where large amounts of residual medium remain in the wells of the used microtiter plate (Twentyman and Luscombe, 1987). The absorbance is measured spectrophotometrically with an Infinite M200 Pro NanoQuant instrument (Tecan, Switzerland) at 570 nm. The degree of absorbance is directly proportional to the amount of formazan i.e. the number of living cells.

ATP assay

ATP assay is a method that can be used to evaluate cell viability. Adenosine triphosphate (ATP) serves as the main chemical energy carrier for living cells. When membrane integrity is disrupted, the cell loses its ability to synthesize ATP (Riss et al., 2004). Cellular ATP is one of the most sensitive indicators in measuring cell viability (Strehler and McElroy, 1957). This method is based on a reaction when the substrate D-luciferin is converted by enzyme luciferase to oxyluciferin. This conversion is driven by ATP in the presence of oxygen, magnesium ions and luciferase accompanied by visible light emissions (Lee et al., 2012). The resulting oxyluciferin produces a chemiluminescent signal whose intensity is directly proportional to the ATP concentration. The Cellular ATP Kit HTS (Invitrogen Corporation, USA) was used for ATP assessment in my co-authored research. Luminescence was measured on an Infinite Lumi luminometer (Tecan, Switzerland).

7.3.2 Cytocompatibility determination

The cytocompatibility of materials can be studied by determining cell adhesion, growth and proliferation. In cell adhesion assays, cells are seeded onto sterile samples at a concentration of 10^6 cells per 1 mL of culture medium. After 1 hour, unadhered cells were rinsed and the cell nuclei of adherent cells were visualized through nuclei counterstaining by Hoechst 33258 (Invitrogen, USA).

For the determination of cell proliferation, cells were seeded at a concentration of 10^5 cells per mL on the sample surface. Cultivation was carried out under standard incubation conditions for 48 h (can be extended if necessary). Subsequently, cells were fixed and stained by Hoechst 33258 and actin filaments were visualized through staining by ActinRed™ 555 (Thermo Fisher Scientific, USA).

Depending on previous results, the growth and ingrowth of cells under dynamic conditions with electrical stimulation can be further investigated. Cells were seeded on a scaffold at an initial concentration of 10^5 per mL and pre-cultivated for 72 h. The cell-seeded sample was then transferred to a bioreactor for 72 h. Electrical stimuli run 6 hours a day "*each successive hour-long period alternating between electrical stimulation and no stimulation. The medium flow was 54 RPM. The pulse had a rectangular waveform with a width of 3000 ms, and the voltage was set at 0.1 V."*(Martínková et al., 2022).

A number of tests with unsuccessful setups were performed before the correct electrical stimulation settings and testing times were achieved in the bioreactor.

8. SUMMARY OF RESULTS

The main focus of my doctoral study was to deepen the knowledge in the field of stimuli-responsive scaffolds. There are various materials available for creating a stimuli-responsive scaffold. However, many materials do not naturally respond to external stimuli but still possess appropriate characteristics for use in TE. Therefore, my research has also focused on modifying materials to enhance their bioactivity. The choice of material for the creation of scaffolds is made with regard to the intended use. For scaffolds to regenerate or restore soft tissues, materials from the polymer group are more likely to be used. For hard tissues such as bone, biomaterials need to have the required high degree of hardness and for this reason, ceramics are used. In the beginning, my research dealt with the preparation of ceramic-based biomaterials and functionalization of surfaces of sintered porous scaffolds by electrically-conducting polyaniline or polyaniline/biopolymer films prepared in a colloidal dispersion mode. CBS prepared by PIM technology has potential usage as bone scaffolds. However, there are also polymers such as PEEK that have suitable properties for use in bone TE. The research then moved on to another material that would be also suitable for bone TE and that was PEEK. The addition of farringtonite particles ($Mg_3(PO_4)_2$,) was introduced into the PEEK matrix to obtain physical-chemical and mechanical properties suitable for bone-related applications. In contrast, biomaterials suitable for soft TE should possess enhanced flexibility and mechanical properties that align with the specific functions of the tissues. For instance, load-bearing tissues like cartilage and tendons exhibit greater rigidity compared to nonload-bearing tissues like the brain. The utilization of conductive polymers holds promise in the development of electroactive soft tissues, including cardiac muscles, nerves, and skin. With this in mind, a comprehensive investigation was conducted on the combination of polypyrrole and cellulose as a potential approach. The theoretical portion of the thesis highlighted the various requirements that biomaterials must meet in order to be utilized in TE. Consequently, this thesis focused on the experimental evaluation of the prepared materials and their interactions with cells.

8.1 Scaffolds for hard TE

Hard tissue is calcified tissue in the human body, such as bones and teeth (tooth enamel, dentin and cementum). This mineralized tissue is mainly characterized by a firm intercellular matrix. The need for hard tissue surgery increases with the increasing lifespan of the population as the incidence of fractures increases. This is partially related to increased bone fragility. However, bone fractures and defects are a significant problem worldwide at any age. Bones are characterized by high compressive strength (cortical bone approximately 125 MPa along its longitudinal axis) (Kundu et al., 2014), of course in immature bones, where the mineralization process is still ongoing, this value is lower. In any

case, for bone TE, it is necessary to select a material that has high strength but is also flexible. Therefore, most of the materials that are brittle are not suitable, as the risk of failure increases (Zioupos et al., 2020). Biomaterials used for bone TE include metals, ceramics, natural or synthetic polymers and composites. On this basis, the experimental section was therefore divided into two parts according to the materials that were investigated in my research. One part will be dedicated to ceramic material and the other for polymer material.

1) *Ceramic scaffolds for bone TE applications* are ideal as ceramic combine good biocompatibility, osteoconductivity and corrosion resistance. However, hard and brittle ceramics limits its clinical use for TE. One of the representatives of ceramics is aluminium oxide. Alumina (Al_2O_3) is biocompatible, poses high hardness and good abrasion and corrosion resistance (Rahmati and Mozafari, 2019). The architecture of the scaffold is influenced by its manufacturing. The ability to produce individually design products with defined scaffold architecture is possible by additive technologies or here used PIM technology. It enables the production of personalized medical devices for hard TE. The pore size can be effectively controlled by the particle size of the space holder. Ceramic parts from alumina prepared by this technique was studied by Thomas-Vielma et al., 2008. However, this publication does not study the interaction of cells with material. The alumina parts prepared in this work are not porous and do not exhibit the defined porosity that is desirable for bone scaffolds.

Nevertheless, it is not only the bulk properties (porosity, thermal conductivity or elasticity) that are important for scaffold, but also the surface characteristics are critical. Since alumina is bioinert and does not interact with the surrounding bone tissue, it is possible to modified a surface with a biocompatible coating that will promote cell adhesion and proliferation. The study by Bertazzo et al., 2009 deals with bioactivation of alumina surface with low molecular weight dicarboxylic acid. The biocompatibility of this surface modification was proved by adhesion and viability of pre-osteoblasts. Such surface is bioactive, but does not allow the electrical stimuli-responsivity. Therefore, a surface treatment that makes scaffold electrically conductive is advantageous, especially for bone scaffolds because it promotes bone healing and regeneration. Ahmed El-Said et al., 2010 investigated cellular interaction on alumina substrate coated with PPy nanowire. They discovered that this surface modification exhibits better cell adhesion and proliferation of HeLa cancer cells and HMCF normal cells. In study of Jasenská et al., 2021, conductive composite films were presented. These films were prepared *via in-situ* polymerization of AH and SH or CH. From the available literature, it was found that this surface treatment approach in combination with a ceramic substrate has not yet been investigated.

To the author's best knowledge, no study has been published on the combination of alumina substrate with a defined pore size for TE prepared by PIM technology.

Another innovative feature is surface modification by composite colloidal-based coating based on PANI and biopolymers. The article "*Powder injection molded ceramic scaffolds: The role of pores size and surface functionalization on the cytocompatibility"* by **Martínková M.**, Hausnerová B., Huba J., Martínek T., Káčerová S., Kašpárková V., Humpolíček P. published in Q1 journal Materials and Design (**ARTICLE I**) is one of the first to explore this issue. Initially, the cytotoxicity of native CBS prepared by PIM was investigated on cell line NIH/3T3. Native alumina substrates did not induce cytotoxicity. Further the porosity of CBS was visualized by SEM microphotographs. The architecture of CBS was influenced by different sizes of space holder and four different space holder *vs* powder volume ratios. There are conflicting reports in the available literature on the optimal average pore size for bone scaffolds. In work of Murphy et al., 2010 it was discovered that the highest cell viability was in the scaffold with a pore size of 325 μm and was therefore evaluated as the best pore size for bone TE. However, higher cell numbers were also observed for scaffolds with 120 μm pores. In **ARTICLE I**, the space holder in size of 125-250 μm and then 250-500 μm were used. In this study, it was confirmed that cell growth was better on samples with pore sizes greater than 250 μm (Fig. 12).

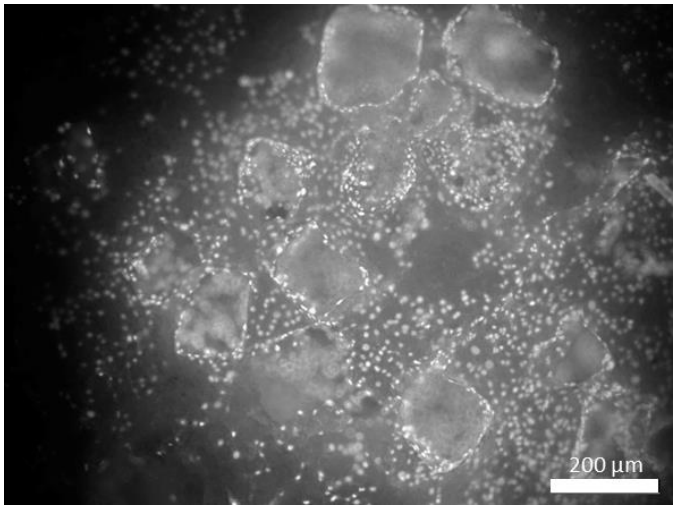

Fig. 12 Cell growth on native CBS with 30% of space holder with grain sizes greater than 250 μm

As mentioned above, the conductivity is important for bone grafts and scaffolds. The proper function of natural tissue is ensured by its ionic conductivity, therefore conductive polymers are a promising material for TE. The bioactive coatings by PANI films and PANI in combination with natural polymer stabilizers were prepared. Anyway, as reported in the study by Jasenská et al., 2021 pristine PANI does not provide an adequate cellular response. Addition of biopolymer stabilizers had a beneficial effect on cell adhesion, and proliferation. This was also confirmed in ARTICLE I, where the component of the extracellular matrix – sodium hyaluronate, or natural biopolymers such as sodium alginate and chitosan were employed, and, subsequently, the cytocompatibility of the native

and functionalized alumina scaffolds was determined. Interesting results were obtained in the determination of cytocompatibility on CBS modified with PANI films (Fig. 13). Cells on PANI film prepared on Petri dishes were unable to adhere, but PANI film on ceramic substrate allowed adhesion (Fig. 14 PANI). Overall cells could grow on the all surfaces and within the pores. However, the cell distribution on surfaces is uneven (Fig. 14).

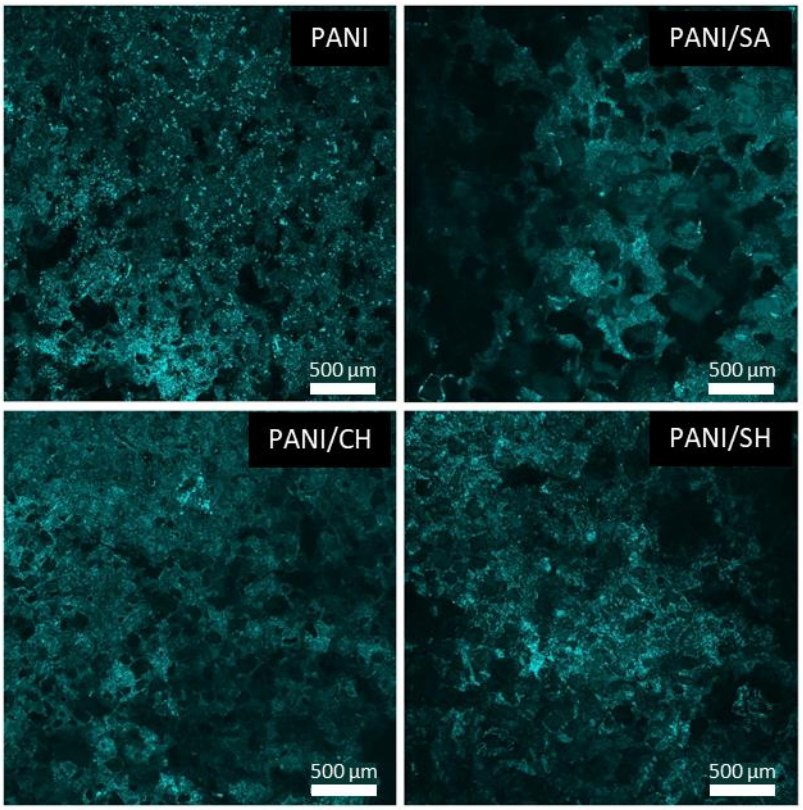

Fig. 13 Surfaces of modified CBS with 50% of space holder with grain sizes greater than 250 µm

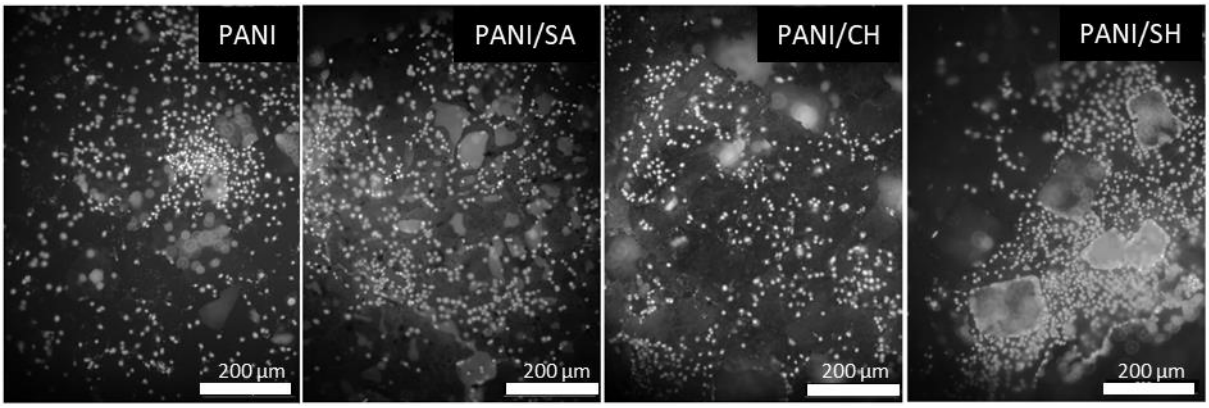

*Fig. 14 Cell grow on a modified ceramic scaffolds (CBS with 30% of space holder with grain sizes greater than 250 µm) under static cultivation (supplementary material of **ARTICLE I** (Martínková et al., 2022))*

Since the static cultivation has its limits, testing continued with dynamic cultivation in a bioreactor. Dynamic cultivation allows simulating the cell's surrounding microenvironment. It enhances mass transfer and mechanotransductive effects. The flow rate of the culture medium in the bioreactor is essential for bone scaffolds as it can promote bone regeneration. Furthermore, electrical stimulation also plays a major role in bone regeneration. A report by Kumar et al., 2016 investigated electric field-mediated cell growth to accelerate wound healing. In this research the direct current pulses with electric field intensity of 0.5-1 $V \cdot cm^{-1}$ were applied for 10 minutes (square waveform, 100 Hz frequency and 50% duty cycle). This study showed that cell growth under dynamic conditions with electrical field stimulation is higher than under static conditions with stimulation. Mobini et al., 2017 in their publication invastegated the effect of direct current electrical stimulation on rat mesenchymal stem cells. The cells were exposed to 0.01 $V \cdot cm^{-1}$ for 1 hour per day for 3, 7, and 14 days. The findings revealed that the ES changed expression patterns of certain osteogenic genes. The settings for electrical stimulation of cells vary in the literature, so this part of the study required a lot of time. For example, in article by Wen et al., 2013 MSC and cardiac myocytes coculture monolayer was stimulated by electric pulses current of power 40 μA, rectangular waveform 2 ms, frequency 2 Hz. These pulses were applied 3, 6 hours per day for 5days). Electric stimulation in neurogenesis were discussed in the research of Chang et al., 2011 used electrical stimulation with magnitude in range from 4 to 32 $\mu A/cm^2$ for 50 and 200 μs at 100 Hz. Study revealed that current density 8 $\mu A/cm^2$ for 200 μs at 100 Hz increased fetal NSC proliferation.

In the first few experiments, the bioreactor settings were too extreme and the cells did not proliferate under dynamic conditions. The morphology of the cells under simulated *in vivo* conditions with electric stimulation was not typically triangular (Fig. 15).

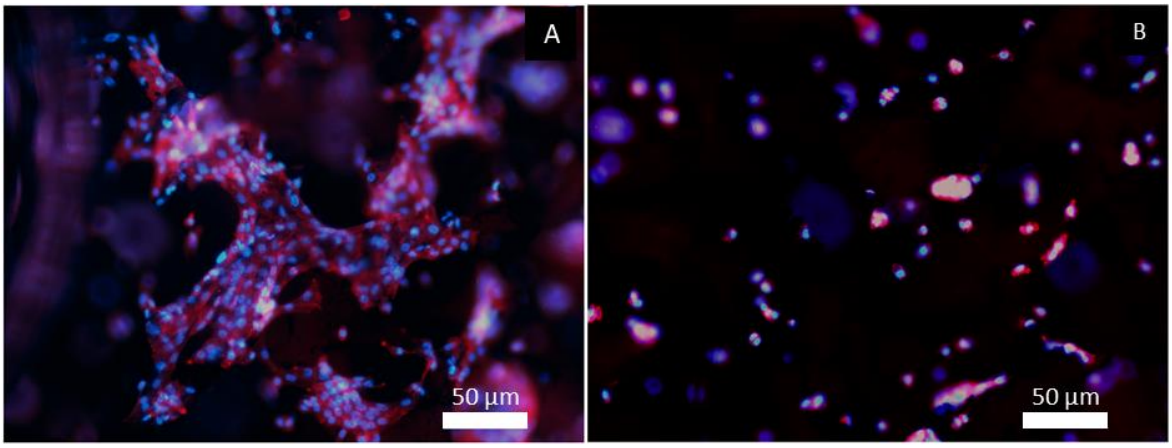

Fig. 15 Cell grow on CBS PANI-SH (CBS with 30% of space holder with grain sizes greater than 250 μm); (A) static cultivation; (B) dynamic cultivation

After a number of unsuccessful attempts, the settings of the medium flow and electrical pulses were optimized. The results were published in **ARTICLE I** and *"the electrical stimulation parameters were as follows: voltage 0.1V, pulse width 3000 ms, arrangement on square-wave "* (Martínková et al., 2022) with media flow 54 RPMI. Application of shear stress and external electrical stimulation resulted in a homogeneous cellular distribution. Both the absence of cytotoxicity and the cytocompatibility that were revealed demonstrate the application potential of these scaffolds.

2) *PEEK has high potential for use in bone TE* and connective tissue replacement. In particular, it is biocompatible, chemically stable and has an elastic modulus similar to that of natural bone (Gu et al., 2021). This material is widely studied for another numerous application such as dental implants (Sarot et al., 2010), orthopaedic (Ma et al., 2021), and spine implants (Mavrogenis et al., 2014). However, this material is bioinert and exhibits poor osteoconduction, which can lead to clinical failure. Another disadvantage is the poor antibacterial activity. These properties can be modified by additives or surface treatment. Since calcium phosphate occurs naturally in bone, many studies have focused on these materials for bone TE. For example, in research by Manzoor et al., 2021, hydroxyapatite was added to PEEK. The bioactivity was investigated and the formation of apatite was observed on the surfaces of samples. Other publications of Abdulkareem et al., 2019 focused on the antibacterial activity of chitosan-enriched PEEK-hydroxyapatite coatings. Based on this study, it was summarized that PEEK-chitosan-hydroxyapatite coatings have broad-spectrum antibacterial activity with potential for biomedical applications. However, the type of mineral additive can affect the determining the properties of the composites. As summarized in the study by Sikder et al., 2020, the addition of amorphous magnesium phosphate to PEEK enhanced biological activity and helped to significantly increase pre-osteoblast cellular response. This indicates that phosphate-based minerals such as farringtonite $Mg_3(PO_4)_2$ will positively affect the final properties of the biomaterial.

Most publications deal with the production of PEEK materials using 3D printing (Manzoor et al., 2021), extrusion (Tseng et al., 2018), or injection molding (Sagomonyants et al., 2008). Nevertheles, another option of manufacturing seems to be the manually hot-pressed technique. This method of preparation PEEK with farringtonite was used in our submitted paper by **Martínková M.**, Zárybnická L., Viani A., Killinger M., Mácová P., Sedláček T., Oralová V., Klepárník K. and Humpolíček P. entitled *"Polyetheretherketone Bioactivity Induced by Farringtonite: The Effect on Mineralization and Differentiation of Osteoblasts"* (**APPENDIX I**). Initially, the farringtonite material, which was synthesized from an analytical grade powder mixture, was characterized by XRPD. Then the particle mean diameter and specific surface area

of the powder was measured. The results were consistent with those from the farringtonite powder proposed for bioapplication. Main part of this work focused on characterization of physical-chemical and mechanical properties of grafts for bone TE. Fourier transform infrared (FTIR) maps were obtained to determine the distribution of farringtonite on the PEEK graft surface. The maps showed relatively homogeneous distribution of farringtonite in the PEEK matrix. Since this material is intended for use in bone TE, mechanical properties are important. Trabecular bone is estimated to have a modulus of elasticity around 5.4 GPa (Choi et al., 1990) to 14.8 GPa (Rho et al., 1993). There are studies that have measured a lower modulus of elasticity, approximately 1.3 GPa (Williams and Lewis, 1982). PEEK matrix had Young's modulus 5.8 GPa and as the concentration of the mineral additive increased, Young's modulus increased to 7.9 GPa (for more details see attached **APPENDIX I**). In order to determine whether osteoblasts affect grafts, the surface properties of grafts were tested before and after bioassays. As mentioned in the theoretical part of the thesis, the biomaterial surface influences cell adhesion and proliferation. The contact angle of the PEEK grafts was measured, which was approximately 75°. The results are consistent with the publication by Ren et al., 2018. Measurements after bioassays showed lower contact angle values, this could be due to protein binding to the surface.

One of the studies by Sikder et al., 2020 dealt with the preparation of PEEK with the additive amorphous magnesium phosphate. In this research, the material was processed using 3D printing. The research also confirmed that the incorporation of amorphous magnesium phosphate increased the bioactivity of PEEK and promoted a significant increase in the adhesion and proliferation of preosteoblasts. In our research (**APPENDIX I**) cytotoxicity was determined on a mouse fibroblast line. There was a slight decrease in cell viability below the cytotoxicity threshold. On the other hand, the results from cell adhesion and proliferation of osteoblast were comparable to the reference surfaces and even higher in the case of PEEK with 1% farringtonite. It was also found that the addition of farringtonite led to the mineralization process. Overall, the bone grafts made of PEEK and farringtonite was biocompatible, bioactive and could be used for treatement of bone defects and disiases. This material could also be further modifies using a surface coating to make it stimuli responsive.

8.1 Scaffolds for soft TE

Soft tissues play a crucial role in connecting, supporting, and enveloping various structures and organs within the body encompassing skeletal muscles, tendon vessels, and the nerves that supply them. Additionally, vital organs like the heart, brain, liver, and kidney are classified as soft tissues. Unfortunately, acute or chronic injuries can result in temporary or permanent damage to these organs and soft tissues. In cases of severe damage, the body's natural physiological repair

and restoration mechanisms may be insufficient. To address this, TE scaffolds have emerged as a promising clinical solution for repair or regeneration.

3) *Cellulose combined with conductive polypyrrole* has potential applications in biomedicine due to its unique mechanical, biochemical and physical properties. Cellulose is studied for its biocompatibility and possibility of tunable mechanical properties. Cellulose could be prepared in different forms such as nanocrystals (Abraham et al., 2017), nanofibrils (Olsson et al., 2010), hydrogels (Kundu et al., 2014) etc. When neural TE is considered, the addition of CPs is often studied. Publication by Zha et al., 2020 concerned with electrospun cellulose in combination of CPs (such as poly N-vinylpyrrole and Poly(3-hexylthiophene)) incorporated through *in situ* polymerization. *In vivo* cytocompatibility testing revealed that the scaffolds with CPs exhibited cell adhesion and proliferation. In research paper by Thunberg et al., 2015 *in situ* polymerization of PPy on electrospun cellulose nanofibers was described. During the investigation, it was found that no tested concentration of PPy does not cause cytotoxicity. Also, the results suggested that the addition of PPy helped cell adhesion and affected the differentiation of human neuroblastoma cell line. Nevertheless, this type of preparation often led to the inhomogeneous distribution of PPy in the matrix which caused inhomogeneous properties. In addition, PPy is often flaking off the surface. A stronger bond between the polypyrrole and the matrix could solve this problem.

However, none of the studies suggested the possibility of covalently binded PPy. This is possible due to condensation reaction between pyrrole cycles and aldehyde groups of dialdehydes of polysaccharides. For this reason, cellulose in our investigation was partly oxidized to create cellulose dialdehydes (SEM photographes of cellulose nanofibers see in Fig. 16). This idea is described in our patent that is currently under review.

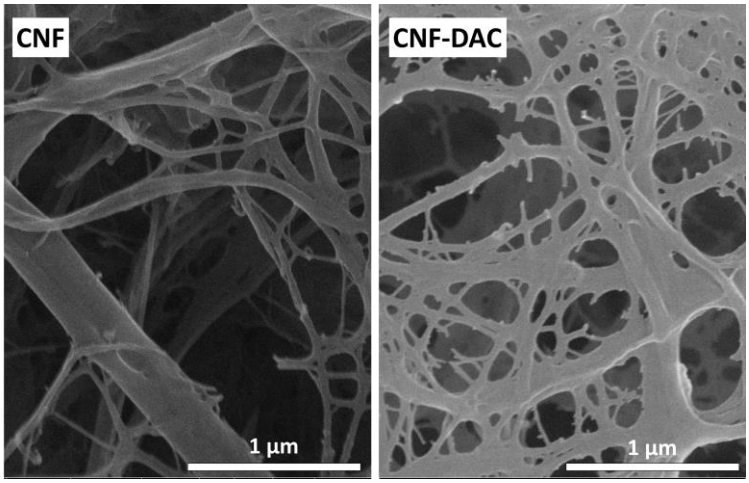

Fig. 16 SEM photographs of CNF and CNF-DAC suspensions

Subsequently, composites with covalently bound PPy were prepared. PPy was deposited on the CNF-DAC using template-controlled polymerization. SEM analysis revealed the presence of PPy grains on composites CNF-DAC-PPy in all samples, with the amount of deposited PPy increasing with increasing ratio of pyrrole to aldehyde groups during synthesis (Fig. 17).

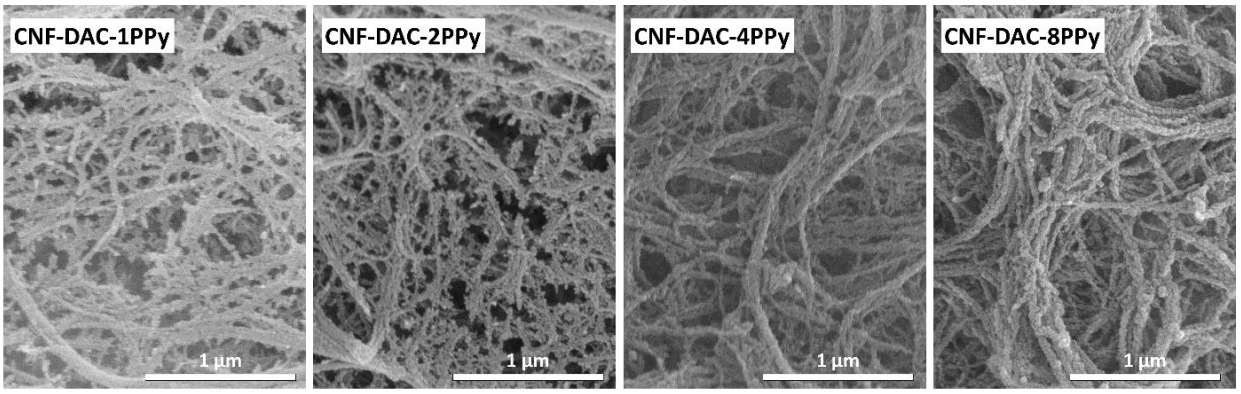

Fig. 17 SEM photographs of CNF-DAC with four different concentraion of PPy

CNF-DAC-2PPy and CNF-DAC-8PPy samples (n_{py} : n_{CHO} 2:1 and 8:1) were selected for further analysis. The conductivity was measured using the Van der Pauw four-electrode method (Keithley 6517B digital electrometer; Keithley 2410 voltage source; Keithley 7002 scanner). The specific conductivity of the CNF-DAC-2PPy sample was 0.708 mS/cm, while that of the 8PPy sample was 0.91 S/cm, which is due to the larger amount of deposited PPy.

To show the advantage of a stronger covalent bond, composites of unoxidized CNFs were also prepared in an analogous manner to the preparation of CNF-DAC-2PPy-8PPy. These samples are hereinafter referred to as CNF-2PPy-8PPy. These composites without cellulose dialdehydes had PPy attached by weak bonding interactions and thus were more susceptible to mechanical damage. CNF-DAC-2PPy-8PPy and CNF-2PPy-8PPy subjected to mechanical degradation in 30 min sonication using an MS 73 micro tip ultrasonicator with a Bandelin Sonopuls HD 2070.he composites prepared using unoxidized CNF fibers have lost a significant part of the deposited PPy layer. Especially in the CNF-2PPy samples where the PPy layer is almost completely absent, the PPy layer is still present in the CNF-DAC-2PPy and CNF-DAC-8PPy samples. The differences between the CNF and CNF-DAC samples are also apparent on a macroscopic scale, with much less fragmentation due to ultrasound in the CNF-DAC samples than in the CNF series. See details of the petri dishes in Fig. 18.

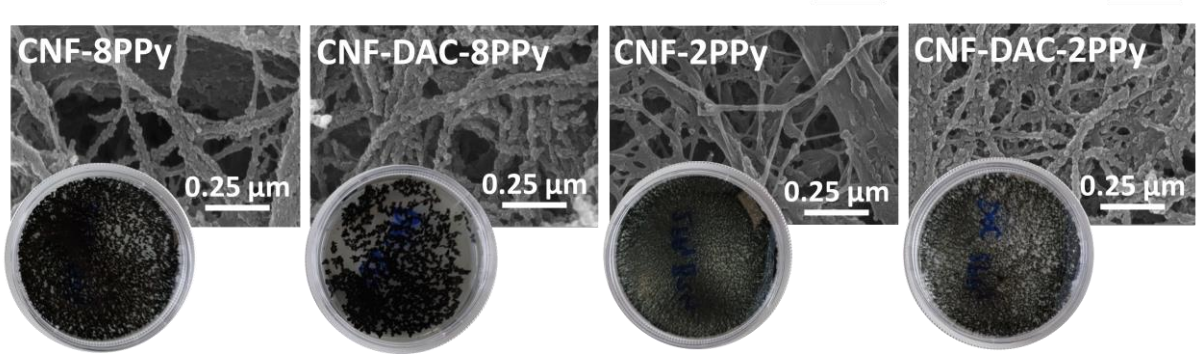

Fig. 18 SEM analysis of CNF-2PPy, CNF-DAC-2PPy, CNF-8PPy, CNF-DAC-8PPy after 30 min of sonication, demonstrating PPy layer damage in samples of the CNF-PPy series; Petri dishes with samples after sonication to compare fragmentation of samples after sonication

Thus, covalent anchoring leads to an increase in the coverage efficiency of the dialdehyde polysaccharide and an increase in the flaking resistance of the deposited polypyrrole layer from the matrix while maintaining the electrical conductivity of the as-prepared composites.

Furthermore, during my studies I was involved in testing the biological properties of conducting polymers. First co-authorship is in research by Skopalová K., Radaszkiewicz K.A., Kašpárková V., Stejskal J., Bober P., Junkar I., Mozetič M., Capáková Z., Lehocký M., **Kašparová M.**, Pacherník J., Humpolíček P., 2021. *Modulation of Differentiation of Embryonic Stem Cells by Polypyrrole: The Impact on Neurogenesis*. International Journal of Molecular Sciences 22, 501. Second publication by Gupta S., Acharya U., Pištěková H., Taboubi O., Morávková Z, **Kašparová M.**, Humpolíček P., Bober P., 2021. *Tuning the Conductivity, Morphology, and Capacitance with Enhanced Antibacterial Properties of Polypyrrole by Acriflavine Hydrochloride.* ACS Appl. Polym. Mater. 3, 6063–6069. In the third article the cytotoxicity of cryogels was tested together with the proliferation of the NIH 3T3 cell line. Milakin K.A., Morávková Z., Acharya U., **Kašparová M.**, Breitenbach S., Taboubi O., Hodan J., Hromádková J., Unterweger C., Humpolíček P., Bober P., 2021. *Enhancement of conductivity, mechanical and biological properties of polyaniline-poly(N-vinylpyrrolidone) cryogels by phytic acid*. Polymer 217, 123450.

Another paper where I tested cytotoxicity was by Musilová L., Achbergerová E., Vítková L., Kolařík R., **Martínková M.**, Minařík A., Mráček A., Humpolíček P., Pecha J., 2022. *Cross-Linked Gelatine by Modified Dextran as a Potential Bioink Prepared by a Simple and Non-Toxic Process*. Polymers 14, 391. In this research, the distribution of fixed and contrast-stained cells in a 3D printed structure was investigated.

9. CONTRIBUTION TO SCIENCE

Stimuli-responsive materials for tissue engineering are frequent subjects of research. Researchers are exploring a range of stimuli such as temperature, light, pH, magnetic fields, and electric fields with the aim of harnessing their potential to alter the properties, interactions, structure, and dimensions of materials. These stimuli-responsive materials hold great promise in the biomedical sector, field of TE, but also in drug delivery systems for diagnostics and treatment purposes. Despite a notable increase in the number of publications concerning stimuli-responsive biomaterials in recent years, there are still encountering challenges related to the fabrication methods and the composition of the material that would provide the cell-instructive potential.

Part of this work is focused on bones TE. The specificity of response to stimuli and the ability to respond to stimuli is essential for bone TE. When bone is mechanically deformed it generates a small electrical current that aids bone regeneration. Therefore, the possibility of electrical field stimulation is mainly investigated in this work, so this work also focuses on conducting polymers. However, mechanical fragility and poor processability of CPs limit their use. Therefore, here we come up with a solution based on a combination of materials that have suitable bulk properties (ceramic – ARTICLE I, PEEK - APPENDIX I) for bone TE with a thin conducting polymer film (pristine or prepared in colloidal dispersion mode ARTICLE I) on the surface of the material. The main contributions of this research to science include an innovative approach of surface modification by films of conductive polymers such as or prepared in colloidal dispersion mode. These modifications lead to different surface characteristics but also significantly change the cytocompatibility of materials. Coatings based on conductive polymers and biopolymers are electrically-conductive and cytocompatible.

Another achievement is in the new way of biomaterials preparation. This work introduces two manufacturing options that are not commonly used, yet enable the production of personalized medical devices. In our work (ARTICLE I) was declared that not only the architecture but also the porosity can be controlled using Powder injection molding technology moreover using an environmentally friendly approach. The second used fabrication method (APPENDIX I) was used for the bone graft of PEEK prepared by manually hot-pressed technique. This material is not stimuli-responsive but has suitable properties for bone TE. Due to the lack of time, it was not possible to finalize the surface modification or introduce the CP into the structure of PEEK grafts to be stimuli-responsive.

The last present study introduces a novel method for the preparation of conductive composites based on PPy and polysaccharide dialdehydes. Covalent bonding

of PPy on polysaccharide dialdehydes enhances the homogeneity of decoration efficiency, improves the resistance of the deposited polypyrrole layer against flaking from the matrix, and provides the electrical conductivity of the composites. To summarize the main contribution of this disertation to science is the extension of knowledge about the preparation of CPs based stimuli-responsive biomaterials.

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LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Alphabetically ordered

AH	Aniline hydrochloride
APS	Amonium persulfate
CBS	Ceramic-based substrate
CH	Chitosan
CPs	Conductive polymers
CNF	Cellulose nanofibrils
CNF-DAC	Cellulose nanofibrils dialdehyde
CNF-DAC PPy	Cellulose nanofibrils dialdehyde with polypyrrole
CNF/PPy	Cellulose nanofibrils with polypyrrole
DAAL	Dialdehyde alginate
DAC	Dialdehyde cellulose
DAH	Dialdehyde hyaluronate
ECM	Extracellular matrix
HA	Hyaluronic acid
PANI	Polyaniline
PEDOT	Poly(3,4-ethylenedioxythiophene)
PEEK	Polyetheretherketone
PHS	Powder space holdes
PIM	Powder Incjecton Molding
PNIPAAm	Poly(N-isopropyl acrylamide
PPy	Polypyrrole
SA	Sodium alginate
SH	Sodium hyaluronate
TE	Tissue engineering
UPW	Ultra-pure water

CURRICULUM VITAE

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Research projects

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2020-2022	Project OP RDE Junior Grants of TBU in Zlín, JUNG-2020-001, Smart biomaterials based on conducting polymers Member of project team
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2020 – 2021	Project GAČR 20-28732S, Colloidal systems for topical formulations. Pickering emulsions and polymer based colloids. Member of project team

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2019	Project IGA/CPS/2019/004, Biological properties of materials Member of project team
2019	Project GAČR 19-16861S, Interactions between materials and stem cells in simulated *in vivo* conditions Member of project team

Conferences and seminars

04/09/2022 – 09/09/2022	*36th European Colloid & Interface Society Conference* Chania, Crete, Greece Poster title "Conducting film prepared in colloidal dispersion mode, the efficient way of surface functionalization"
28/06/2022 – 01/07/2022	*Tissue Engineering and Regenerative Medicine International Society (TERMIS) European Chapter Conference 2022* Krakow, Poland Poster title "Conducting polyaniline films prepared in colloidal dispersion mode in presence of bioactive polysaccharides"
08/11/2021 – 12/11/2021	*Fellowship on Institute of Biophysics of the CAS* Institute of Biophysics of the CAS, Brno Practical training of isolation and manipulation of mouse Caco-2 cell line

Pedagogic activities

Student training in laboratories

Mentoring the bachelor thesis:
Exosomes

LIST OF PUBLICATIONS

Articles published in journals indexed on Web of Science:

Martínková M., Hausnerová B., Huba J., Martínek T., Káčerová S., Kašpárková V., Humpolíček P., 2022. Powder injection molded ceramic scaffolds: The role of pores size and surface functionalization on the cytocompatibility. Materias and Design 224. ISSN 02641275. DOI 10.1016/j.matdes.2022.111274

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Skopalová K., Radaszkiewicz K.A., Kašpárková V., Stejskal J., Bober P., Junkar I., Mozetič M., Capáková Z., Lehocký M., **Kašparová M.**, Pacherník J., Humpolíček P., 2021. Modulation of Differentiation of Embryonic Stem Cells by Polypyrrole: The Impact on Neurogenesis. International Journal of Molecular Sciences 22, 501. DOI 10.3390/ijms22020501

Submitted article to the editors of international journals with an impact factor:

Martínková M., Zárybnická L., Viani A., Killinger M., Mácová P., Sedláček T., Oralová V., Klepárník K. and Humpolíček P., Polyetheretherketone Bioactivity Induced by Farringtonite: The Effect on Mineralization and Differentiation of Osteoblasts

Ing. Martina Martínková, Ph.D.

Stimuli-responsive scaffolds

Stimuli-responsivní scaffoldy

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