Composite materials based on polysaccharides

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Doctoral Thesis Summary

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Centre of Polymer Systems

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Kompozitní materiály na bázi polysacharidů

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ABSTRACT

One of the most important aspects of effective regenerative medicine is the design of biologically active materials with appropriate biological and material properties. Hydrogels have been receiving great attention as one of the most attractive form of biomaterials. The main reasons are their versatility allowing them to mimic the natural extracellular matrix composition and mechanical properties, their ability to support cell proliferation, migration, differentiation, and allowing of transport of oxygen and nutrients within their structure. Moreover, hydrogels can be engineered according to the demand of specific applications, such as scaffolds for treating various tissues or drug delivery and wound healing systems. In addition, various molecules can be incorporated into the hydrogel to provide specific bioactive properties. The conducting polymers, which could be important in treating nervous or cardiac tissues, are one of the possible bioactive agents.

Experiments described in this thesis were focused on the preparation of biomaterials based on polysaccharides due to their biocompatible, non-immunogenic, and tunable properties. Furthermore, colloidal dispersions of conducting polypyrrole were prepared in order to successfully fabricate bioactive composites for wound healing. This work also focused on the determination of the cytocompatibility of all prepared materials.

Key words: *regenerative medicine*, *biomaterials*, *composites*, *hydrogels*, *conducting polymers*

ABSTRAKT

Jedním z nejdůležitějších aspektů regenerativní medicíny je příprava biologicky aktivního biomateriálu s vhodnými biologickými, ale i materiálovými vlastnostmi. Jednou z nejaktraktivnějších forem biomateriálů jsou hydrogely, kterým se dostává velké pozornosti z důvodu jejich podobnosti s přirozeným extracelulárním matrixem, ale také pro jejich schopnost podporovat buněčnou proliferaci, migraci, diferenciaci a umožnění transportu kyslíku a živin. Hydrogely mohou být navíc navrhovány podle požadavků konkrétních aplikací, jako jsou například scaffoldy pro léčení různých tkání, nebo systémy pro cílené dodávání léčiv. Kromě toho mohou být do hydrogelu inkorporovány různé molekuly, které poskytují specifické bioaktivní vlastnosti. Jedním z bioaktivních činidel jsou vodivé polymery, které mohou být důležité při léčbě nervových nebo srdečních tkání.

Experimenty popsané v této práci byly zaměřeny na přípravu hydrogelů na bázi polysacharidů, jelikož vykazují dobrou biokompatibilitu a zároveň jsou neimunogenní. Dále byly připraveny koloidní disperse vodivého polypyrrolu za účelem vytvoření bioaktivních kompozit pro hojení ran. Tato práce se také zaměřila na stanovení cykompatibility všech připravených materiálů.

Klíčová slova: *tkáňové inženýrství, biomateriály, kompozity, hydrogely, vodivé polymery*

TABLE OF CONTENT

1. INTRODUCTION

Regenerative medicine (RM) including tissue engineering are rapidly growing multidisciplinary fields. These disciplines have great potential for better treatment of health issues. Furthermore, they have been improved by progress in bioengineering over the last few decades. RM and tissue engineering (TE) are realizing their full potential through the utilization of cells and tissue scaffolds (alone or in combination), as well as the support of the natural healing process by controlled release of bioactive compounds. Numerous methodologies for the preparation of scaffolds, drug delivery systems or wound dressings are currently used depending on the used materials and aimed application. However, the key property that distinguishes materials from each other is the ability to cohabit and interrelate with the tissues and biological systems (e.g. interstitial fluids, blood, immune cells and molecules) without causing any harmful effects. Hydrogels are excellent biomaterial candidates which can accomplish mentioned criteria. They are unique biocompatible polymeric substances that can operate as scaffolds and mimic the properties of diverse biological tissues. Moreover, hydrogels can be also used in desired drug delivery systems and wound healing applications (Chamkouri, 2021; Ebhodaghe, 2020; Guan et al., 2017; Mantha et al., 2019).

Therefore, this doctoral thesis focused on methods of fabrication of hydrogels based on polysaccharides, and the preparation of colloidal dispersions based on conducting polymers (CPs) in order to prepare smart and functional composites. The second part then focused on the determination of the biocompatibility of prepared biomaterials.

2. HYDROGELS

Hydrogels can be defined as cross-linked polymeric materials containing hydrophilic structure which makes them able to retain high amounts of water within their three-dimensional networks (Ahmed, 2015; Pina et al., 2019). The high hydrophilicity is caused by hydrophilic groups, such as hydroxyl, carboxyl, amide and amine which are distributed along the backbone of polymeric chains (El-Sherbiny and Yacoub, 2013). Hydrogels play an important role in biomedicine for more than half a century. For example, Wichterle and Lím invented the first hydrogel soft contact lenses, based on cross-linked poly(2-hydroxyethyl methacrylate), in 1960 (Wichterle and Lím, 1960).

One of the reasons why hydrogels get continuous attention is their similarity with natural tissues, e.g. flexibility and high content of water. The high-water content is one of the common properties of living organisms (considering both the cells and extracellular matrix). Hydrogels are therefore the ideal material to mimic the extracellular matrix (ECM). Water in organisms, and thus even in hydrogels, is an important medium for providing the high permeability of oxygen, nutrients, and, last but not least, water-soluble metabolites (Drury and Mooney, 2003). Another very important aspect of the successful application of hydrogels in biomedicine is biocompatibility and appropriate biodegradability (Ahmed, 2015).

3. HYDROGELS APPLICATION

Hydrogels are very desirable materials due to their previously outlined unique features (high-water content, flexibility, and biocompatibility) and are well established in the fields of RM, TE, and, last but not least, drug delivery and wound healing (Caló and Khutoryanskiy, 2015).

3.1 Tissue engineering

The term “tissue engineering” originated in 1987 at a bioengineering panel meeting at the National Science Foundation. Then, in early 1988 the first TE meeting was held at Lake Tahoe, California and TE was officially defined as “the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of the structure-function relationship in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions” (Nerem, 1991).

TE is a fast-growing interdisciplinary field including biomaterial science, cell biology, cell-material interactions, and surface characterization. This research area aims to restore, maintain, or improve tissue functions. Furthermore, TE also targets replacing diseased or damaged organs, as well as tissues that have become dysfunctional or lost due to accidents or disease. TE should meet four key elements:1) selected and isolated cells, 2) biomaterial scaffolds, 3) signaling

molecules (proteins, growth factors - GF), and 4) bioreactors that mimic living systems for cell expansion and differentiation (O'Brien, 2011). Figure 1 shows the general tissue engineering approaches currently being employed in clinical practice (El-Sherbiny and Yacoub, 2013).

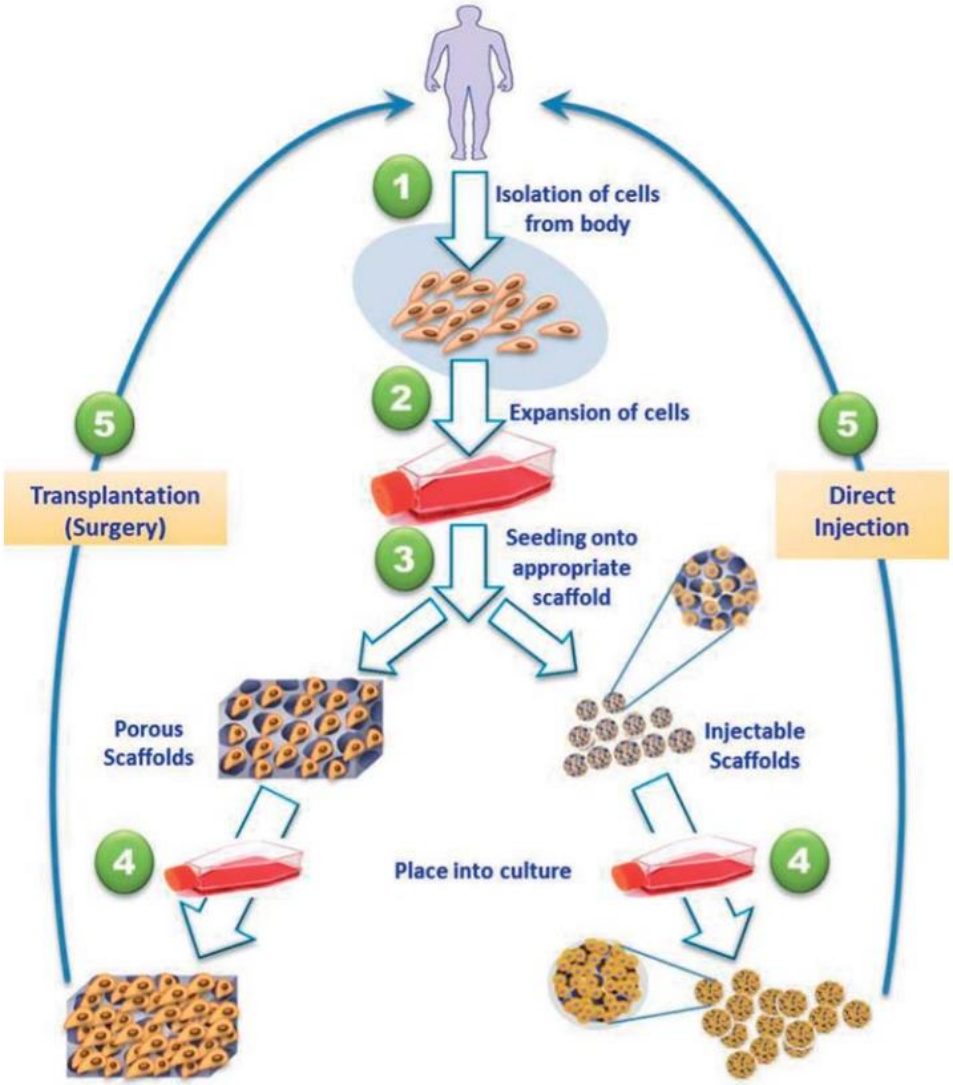

Figure 1: *A schematic illustration of TE approaches (El-Sherbiny and Yacoub, 2013)*

3.1.1 Hydrogel-based scaffolds

As it was already mentioned, hydrogels make good options for ECM mimicking. Artificial scaffolds themselves can play a significant role in regulating cell behaviour (cell-instructive properties) e.g. cell proliferation, migration, and, last but not least, ECM production. This is connected to their chemistry, architecture, mechanical properties, and surface properties. They can be also applied as delivery vehicles for bioactive substances which can further modify the cell behaviour. Scaffold material and its properties differ depending on the concrete application and specific tissue. The requirement for every scaffold is its

biocompatibility and the formation of a proper environment for cells (bulk and mechanical properties) (Courtney et al., 2006; Slaughter et al., 2009).

Cell adhesion is the first of cell-scaffold interactions occurring after the cell seeding, hence appropriate peptide motives, either on the hydrogel surface or throughout its bulk, are used. A widely used adhesion peptide sequence is the tripeptide Arg-Gly-Asp (RGD) domain, which enhances the cellular (fibroblasts, endothelial cells, smooth muscle cells, chondrocytes, and osteoblasts) proliferation, growth, migration, and organization in tissue regeneration applications (Shin et al., 2003). Other adhesives, such as fibrin glue or hydrogels composed of chitin derivatives of chitosan are used to seal small wounds and to improve the efficacy of wound dressings (Ono et al., 2000; Zhao et al., 2001). Furthermore, components of ECM, such as fibronectin, vitronectin, and laminin are also used to promote not only cell adhesion but also **cell proliferation**.

It is noticeable, that the high peptide density of materials improves cell attachment but it was shown that it may also impede cell migration and proliferation. However hydrophilic/hydrophobic nature of the material must be considered as well (Drumheller et al., 1994; Massia and Hubbell, 1991; Neff, 1999)

Moreover, scaffolds can also influence **cell differentiation**. Various factors can affect the stem cell fate, for example, hydrogel porosity, different polymer types, stiffness, or growth factors incorporation (Lee et al., 2015; Tsou et al., 2016). For instance, the impact of hydrogels with adjustable stiffness by changing the molecular weight of HA on stem cells was researched. The lowest-strength hydrogel maintained the ability of bone marrow mesenchymal stem cells (BMSCs) to self-renew in a short time. The process also activated the canonical Wnt pathway. BMSCs revealed the potential for chondrogenic differentiation as mechanical strength increased, which required calcium influx mediated by transient receptor potential vanilloid 4 (TRPV4) through the plasma membrane of the BMSCs (Ren et al., 2021).

3.2 Wound healing dressings

Another interesting usage of hydrogels is hydrogel dressings for wound healing. Although the skin is the largest organ in the human body serving as a barrier against the outside environment, it must be considered that even the smallest wound could represent a high life risk. Wound healing is a coordinated sequence of controlled stages each of which has a proper duration. The stages are **hemostasis**, **inflammation**, **proliferation,** and **remodeling** (Figure 2) (Firlar et al., 2022; Velnar et al., 2009).

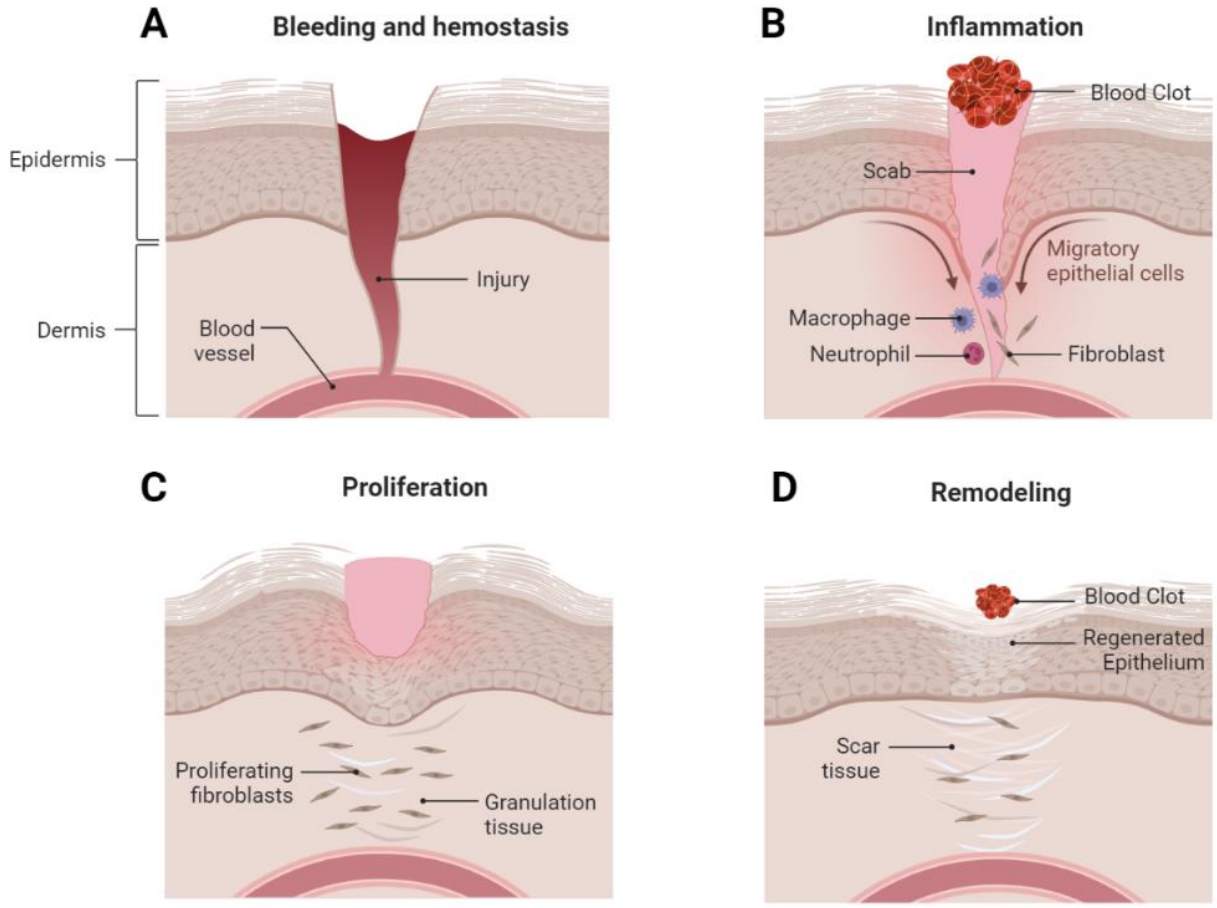

Figure 2: *Wound healing stages: A) – Bleeding and hemostasis; B) Inflammation; C) Proliferation; D) Remodeling (Created with BioRender.com, 2023)*

The first response to the damage is **homeostasis**. The blood clot is created to seal the damaged blood arteries (Han and Ceilley, 2017). Then, the **inflammatory** phase begins, while inflammatory cells (monocytes, lymphocytes, and neutrophils) are activated and respond to chemokines by moving to the wound. Neutrophils are producing reactive oxygen species (ROS) that cause the killing of invading microorganisms. Nevertheless, ROS in high concentrations can cause lipid peroxidation and cell damage and induce the development of chronic wounds. On the other hand, macrophages play an important role in the regeneration of skin tissue. They also clear the apoptotic cells, including neutrophils (Wang et al., 2018). The third phase, **proliferation**, is responsible for granulated tissue with ECM formation. And last but not least, the **remodeling** of tissue to be similar to a healthy one is taking place (Su et al., 2021).

In contrast to gauzes and bandages, hydrogel dressings excel in the similarity to biological tissues including the high-water content which can keep the wound moist and can also absorb the exudate. In addition, hydrogel's mechanical and biochemical properties can be enhanced with various bioactive molecules (Huang et al., 2022).

For illustration, the antibacterial characteristics of hydrogels can be adjusted using, for example, antibiotics and inorganic metals (M. H. Kim et al., 2018; Liang et al., 2019). Adhesion can be improved by polymers containing carboxyl groups (Shin et al., 2019). Moreover, antioxidant and anti-inflammatory properties can be enhanced for example with honey, acacia gum, and polyaniline (Mohd Zohdi et al., 2012; Singh et al., 2017; Zhao et al., 2017). However, polyaniline, as well as polypyrrole, are also used for their conducting properties which improve healing (Lu et al., 2019; Zhao et al., 2017)

Nevertheless, scientists are still developing new and improved hydrogel-based wound dressings, and some of the hydrogel materials are already commercially available. Hydrogel film Suprasorb®G based on acrylic polymers, poly-ethylene, and phenoxyethanol is being used for moisturizing and preventing the formation of necrotic tissue in chronic dry wounds ("Suprasorb®G," 2023). On the other hand, L-Mesitran® is a hydrogel dressing based on acrylic polymer gel with polyurethane film enhanced with a medical grade honey for its antibacterial and anti-inflammatory properties ("L-Mesitran," 2023). Many more hydrogel dressings are already commercially available (Firlar et al., 2022).

4. HYDROGEL PREPARATION

Many 1) **chemical** and 2) **physical** cross-linking methods have been utilized for hydrogel fabrication:

1) **Chemical** cross-links are carried out *via* covalent bonding between separate polymer chains. Hydrogels formed in this manner are forming with elastic behaviour and better resist mechanical stress (Figure 3a). Chemically crosslinked hydrogels can be prepared by, for example, chain-growth polymerization, polyaddition, and polycondensation, or gamma and electron beam polymerization accompanied by crosslinking but crosslinking copolymerization of monomer and crosslinker is the most used technique (Maitra and Shukla, 2014; Oyama, 2014).

2) On the other hand, **physically** cross-linked hydrogels rely on molecular entanglement and non-covalent (hydrogen bonding, ionic and van der Waals interactions) and hydrophobic interactions to provide coherence (Figure 3b). However, although physical cross-links may not be permanent in nature, they are able to build hydrogels that are permanent in aqueous media. Furthermore, physically cross-linked hydrogels are reversible (Maitra and Shukla, 2014). This reversibility makes the soluble polymer possible to form an insoluble gel in contact with living tissues. This property is widely used for injectable applications, mainly for drug-delivery systems (Hu et al., 2019).

Both the types of gels and degree of cross-linking provide different properties at the nano- and macro-scale (swelling, elastic modulus, and transport of molecules) (Kuo and Ma, 2001).

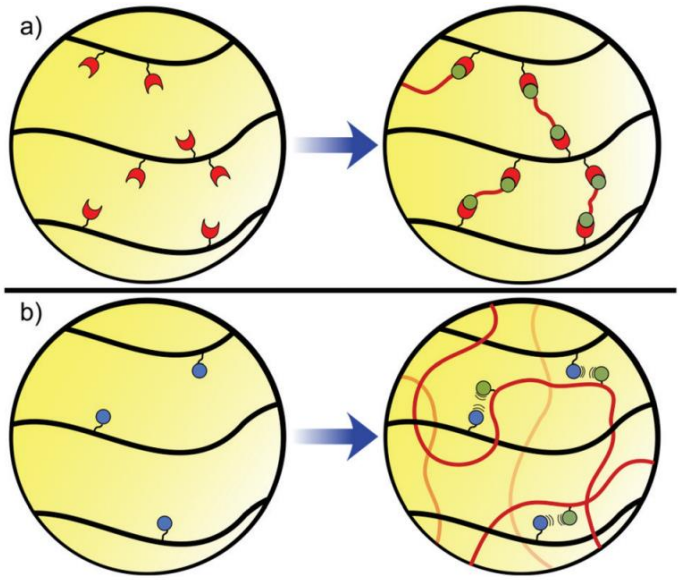

Figure 3: *a) Chemical crosslinking; b) Physical cross-linking (Spicer, 2020)*

5. DESIGN OF HYDROGEL-BASED BIOMATERIALS

Hydrogels are widely used in RM due to their ability to mimic natural tissues. However, as with every biomaterial, hydrogels must fulfil various criteria to function appropriately, such as physical parameters (e.g., mechanical properties, degradation) and also biological parameters (e.g. appropriate cell adhesion and proliferation) (Gomez-Florit et al., 2020). The most important condition of hydrogels is biocompatibility. Naturally derived polymers usually feature good biocompatibility, on the other hand, synthetic polymers may induce a relevant negative response from the body (Lee and Mooney, 2001).

5.1 Biocompatibility

The key to every biomaterial preparation is biocompatibility. It is “the ability of the material to perform with an appropriate host response in a specific application” (Williams, 1999). However, the application of the hydrogel must be considered, which means that biocompatibility is the characteristic not only of the material but of a biomaterial-host system (Williams, 2014). As the material is supposed to exist within the body, it cannot damage cells or lead to the inflammatory response of the body to the hydrogel. Furthermore, the material must be biocompatible even during degradation (Naahidi et al., 2017).

5.2 Biodegradation

Biodegradability is an important feature in the field of drug delivery and implants for TE applications. There are some ways in which degradation can occur, for instance, thanks to the a) physical or b) chemical processes and also, c) biological processes that rely on biological agents, like enzymes. It is resulting in

scaffold disassembling and dissolution of the scaffold bulk or surface degradation. It can occur in a polymer backbone, side chains, or crosslinks. Besides, the degradation rate of a polymer depends on various characteristics of the polymer, such as chemical structure, the presence of hydrolytically unstable bonds, the level of hydrophilicity and hydrophobicity, morphology, the copolymer ratio, and the molecular weight (Jeong et al., 2004; Middleton and Tipton, 2000). Furthermore, degradation can be adjusted by incorporating naturally biodegradable ECM components (hyaluronan, laminin, fibronectin etc.) (Dhandayuthapani et al., 2011; Lutolf and Hubbell, 2005). However, in some RM applications, biodegradability is not required, such as articular cartilage (Ratner, 2013).

5.3 Bulk properties

Among the bulk properties, the architecture and mechanical properties can be considered.

5.3.1 Architecture

Hydrogels should create a 3D architecture for cell growth. Such architecture more closely resembles natural tissues and enables morphology and gene expression that are impossible to achieve in 2D structures.

A very important factor in scaffold designing and preparing is their porosity. Best hydrogels are those with high **porosities** and an open **interconnected geometry** (Ahumada et al., 2019). The high interconnected porosity is critical for cell ingrowth and cell distribution. Additionally, other characteristics are significant in scaffold designing as well, such as **pore size, volume, size distribution, shape, and wall roughness** (Yang et al., 2001). For instance, small pore sizes may obstruct cellular penetration and ECM production. On the other hand, pore interconnectivity is crucial for cell migration, neovascularization, nutrients, and metabolite transfer (Griffon et al., 2006). Many researches have been studying the optimum of pore sizes. For example, for ingrowth of fibroblasts, it is 5 – 15 μm (Klawitter and Hulbert, 1971), for neovascularization, it is 5 μm (Brauker et al., 1995) and for adult mammalian skin cells 20 – 125 μm (El-Sherbiny and Yacoub, 2013; Yannas et al., 1989).

5.3.2 Swelling

Moreover, hydrogels can show a detectable change in volume in response to external stimuli – swelling. The capability of the hydrogel network to diffuse water is influenced by the degree of crosslinking. Also, the capacity of hydrogels to take up water needs to be considered (Peppas, 2000). The water capacity is the ratio of the mass of a fully swollen hydrogel (in a state of equilibrium with an aqueous medium) to the mass of a dehydrated hydrogel, where M is hydrogel mass (Brannon-Peppas and Peppas, 1991).

$$\text{Swelling ratio} = \frac{M_{hydrated} - M_{dehydrated}}{M_{dehydrated}}$$

Hydrogels, which are made up of networks of crosslinked hydrophilic polymers, swell, rather than dissolve in water. On the other hand, polyelectrolyte hydrogels swell more because of the charge repulsion across polymer chains. This swelling property is advantageous in environment-sensitive hydrogel swelling for controlled drug release (Holback et al., 2011; Peppas, 2000; Roy and Gupta, 2003).

5.3.3 Mechanical properties

The mechanical properties of scaffolds must be similar to those of native tissues to be replaced. They have a crucial impact on attached cells and encapsulated cells. There is a specific level of isometric tension in the ECM between the cells in a certain tissue (Ingber, 2006). This fact must be considered in the hydrogel designing, as each tissue has specific mechanical characteristics. For example, it has been researched that the differentiation of mesenchymal stem cells (MSCs) can be controlled by the hydrogel stiffness (Engler et al., 2006).

The mechanical properties can be adjusted not only by cross-linker type, cross-linker density, and monomer type but also by incorporating various materials, such as inorganic nanoparticles. For instance, calcium phosphates and silicates have been used for modulating the mechanical strength and osteogenic properties of hydrogels for bone tissue engineering (Drury and Mooney, 2003; Xavier et al., 2015; Zhao et al., 2010).

6. TYPES OF POLYMERS USED IN BIOMEDICAL APPLICATIONS

Based on the source of material, hydrogels can be divided into natural, synthetic, and hybrid polymer-based hydrogels, which is a combination of both materials (El-Sherbiny and Yacoub, 2013). Here, I focused on naturally derived polymers – chitosan and hyaluronan (HA), thus they are described below.

6.1 Natural polymers

Naturally derived polymers originate from living organisms, which is the main reason why they generally possess adequate biocompatibility with low cytotoxicity. For instance, the main proteins of mammalian tissue ECM are collagens and composing about 25 % of the overall protein mass of most mammals (Lee et al., 2001; Vella, 1994). Comparably, HA is found in all tissues of adult animals (Lee et al., 2001). Other natural polymers occurring in living organisms are for example chitosan, alginates, and fibrin. Furthermore, these materials do not induce a chronic inflammatory response (Singh et al., 2016).

6.1.1 Chitin and chitosan

Chitin is the second most plentiful polysaccharide found in the exoskeletons of crustaceans, insects, and fungi. However, chitin is mostly used in its deacetylated form, chitosan, built of N-glucosamine and N-acetyl glucosamine units linked by a β (1→4) glycoside bond. Its molecular weight may scope from 300 to 1000 kDa depending on the source and production process used. Chitosan (Figure 4) is water-soluble only in diluted acid with a pH of less than 6. This fact is caused by the protonated free amino group on glucosamine, which eases the solubility of the molecule, while in water and aqueous solutions above pH 7 chitosan is usually insoluble (Madihally and Matthew, 1999). The positive charge of the molecule can be used to complex with negatively charged substances, such as GF, nucleic acids, or cytokines (Aibani et al., 2021).

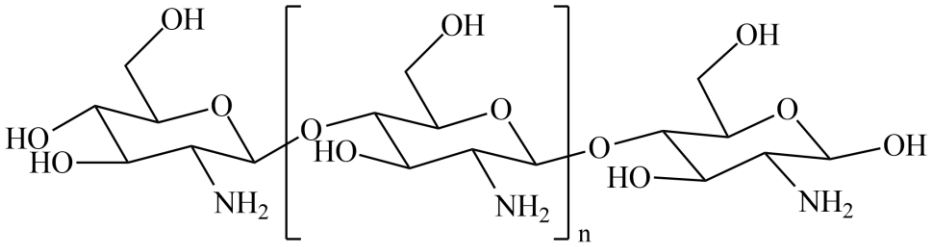

Figure 4: *Structure of chitosan*

Chitosan-based materials are well-established in the biomedical field. These substances are biocompatible and show wound-healing and antibacterial properties (Kim et al., 2023). Furthermore, chitosan can be degraded by human enzymes. Lysozymes are primarily responsible for its biodegradation because they can break the linkage between acetylated units and reduce chitosan to monosaccharides (S. Kim et al., 2018). The highly deacetylated form exhibits the lowest degradation rates resulting in a longer-lasting material (up to several months *in vivo*). For this reason, chemical modification of chitosan is desired, as it can remarkably affect its solubility and degradation rate (Shi et al., 2006). One of the possible modifications is the partial re-acetylation of chitosan resulting in the preparation of water-soluble half N-acetylated chitosan. No artificial functional groups are added. Thus, the modification is natural and resulting in low toxicity and good biocompatibility (Kubota et al., 2000; Qin et al., 2006).

Chitosan is also a promising material for bone TE because it supports the adhesion and proliferation of osteoblasts (Khor and Lim, 2003; Przekora and Ginalska, 2014).

7. HYALURONAN

HA is a linear glycosaminoglycan (GAG) with disaccharide units consisting of α-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine. It exists in molecular weights from 100 000 Da in serum to 8 000 000 Da in the vitreous

(Burdick and Prestwich, 2011). HA is a hydrophilic molecule, so it forms hydrogen bonds between water molecules and carboxyl- as well as acetyl-groups. HA is highly viscous in an aqueous solution because of its high molecular weight and strong binding with water (Day and Sheehan, 2001). HA has a negative charge, because of the presence of glucuronic acid, resulting in the binding of cations, such as Ca^{2+}, K^{+}, Na^{+} and H^{+} (Maleki et al., 2008).

HA plays a major role in the structure and composition of the ECM. It is found throughout the body, mainly in synovial fluid and cartilage tissues. Besides giving the tissues their structure, it also provides hydration (Fraser et al., 1997). HA is a biodegradable polymer with a half-life of a maximal 24 h. It is degraded either by the organism's hyaluronidase enzymes or *via* HA cell internalization by CD44 cell surface receptors (Brown et al., 1991). Furthermore, HA can be also cleaved by acidic or alkaline hydrolysis, thermal degradation, and degradation by oxidants (Stern et al., 2007).

7.1 Scaffolds based on HA

In terms of preparing a scaffold for TE, HA without chemical modification and crosslinking could represent a problem. Modification of HA improves not only degradation time but also mechanical stability *in vivo* (Kafedjiiski et al., 2007). The chemical modifications of HA target three functional groups: carboxylic acid of the glucuronic acid, the primary and secondary hydroxyl groups, and the *N*-acetyl group (following deamidation). Mostly **carboxylates** have been modified by carbodiimide-mediated reactions, esterification, and amidation. On the other hand, **hydroxyls** have been altered by etherification, divinylsulfone crosslinking, esterification, and bis-epoxide crosslinking (Burdick and Prestwich, 2011).

7.2 Hydrogels from functionalized HA

HA can be functionalized using for example a) **adipic acid dihydrazide (ADH)**. HA-ADH provides binding sites for ketones, aldehydes, and acylhydrazides with acylating agents, which allows cross-linking, the addition of the hydrophobic moieties, and attachment of drugs, GF, and cytokines (Burdick and Prestwich, 2011; Vercruysse et al., 1997). In addition, hydrogels prepared from HA-ADH and poly (ethylene glycol) bis(succinimidyl propionate) with incorporated bone morphogenetic protein-2 (BMP-2) exhibited good cell infiltration and chondrogenic differentiation (Bulpitt and Aeschlimann, 1999). Moreover, hydrazide- and aldehyde-functionalized HA resulted in the formation of a prostate cancer model that was used for testing the effectiveness of anticancer drugs (Gurski et al., 2009).

Another possibility of modification of HA is *via* b) **polymerizable methacrylate residues to the hydroxyl group**. The benefit is that photopolymerization allows controlling gel geometry (Walimbe et al., 2017). Hydrogels with good tunability and biocompatibility were prepared by the

reaction of photo crosslinkable methacrylate with oxidized HA or oxidized HA with a functional acrylamide (Jia et al., 2004). Such hydrogels promote cell adhesion and proliferation (Hansen et al., 2005).

One of the most promising methods is **c) thiolation**. It involves the modification of the carboxylic group on the HA backbone resulting in thiols as sites for cross-linking. Many researchers are being attracted by disulfide bond hydrogels for the simple way of fabrication, non-toxicity, and *in-situ* gelation properties. Furthermore, these hydrogels can be prepared from a single component. The principle of biodegradation of the disulfide bond of HA relies on dissociation by reductant glutathione (GSH), which can be synthesized in cells (Bian et al., 2016). Biodegradation and mechanical characteristics can be improved, for example, by changing the molecular weight of HA or the degree of thiolation (Burdick and Prestwich, 2011). Moreover, the combination of thiolated-HA with thiolated gelatin or methacrylated collagen to provide cell adhesion motifs resulted in the development of complex patient-specific tumour-derived organoids from a lot of types of tumour biopsies (melanoma, colorectal, etc.) (Devarasetty et al., 2018).

Another potential technique for functionalization of HA is using **d) CPs**, such as polyaniline (PANI) (Figure 5b), poly(3,4-ethylenedioxythiophene) (PEDOT) and polypyrrole (PPy) (Figure 5a), that are popular constituents for biomaterials and their applications where electrical signalling is desirable (Balint et al., 2014).

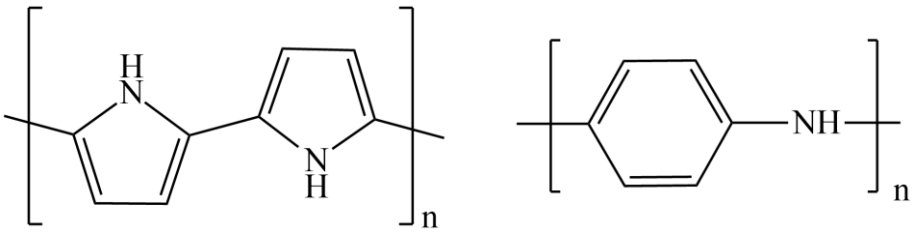

Figure 5: *a) polypyrrole and b) polyaniline*

CPs are simple to work with and to modify (Guimard et al., 2007). Moreover, at the interfaces between cells and conductive surfaces, electrical impulses can be efficiently conveyed, resulting in electrical communication between cells and stimulation of cellular activities, such as differentiation (Thrivikraman et al., 2014). Conductive hydrogels were prepared by the combination of pyrrole-incorporating hyaluronic acid and PPy *via* covalent bond formation and PPy polymerization. These hydrogels showed good cell adhesion and proliferation (Yang et al., 2016). Furthermore, hydrogels based on HA with incorporating single-walled carbon nanotubes (CNTs) and/or PPy were fabricated to support the differentiation of human neural stem/progenitor cells (Shin et al., 2017).

8. AIMS OF DOCTORAL THESIS

The main aims of my experimental work during the Ph.D. study were the preparation of hydrogels based on polysaccharides, the preparation of colloidal dispersions formed from CPs for subsequent incorporation into the hydrogels, and last but not least, the study of interactions between materials and cells, concretely:

- The preparation and characterization of hydrogels based on hyaluronic acid
- The fabrication and characterization of polypyrrole colloids
- The preparation and characterization of smart functional wound dressing composites based on chitosan and polypyrrole colloidal dispersions
- Determination of biocompatibility of above-mentioned materials

9. EXPERIMENTAL PART

The experimental part of this work focused on the preparation of hydrogel composites based on polysaccharides and the testing of interactions between materials and cells. The fabrication of hydrogels based on hyaluronan and/or chitosan was optimized. Furthermore, conducting polymer – polypyrrole –was used for the preparation of colloidal dispersions. CPs are widely used for their unique characteristics, mainly for application where bioelectricity play a role. Here, the PPy colloidal dispersion was used for the preparation of bioactive wound dressing composites based on chitosan. Then, materials were tested using the NIH/3T3 mouse fibroblast cell line to evaluate the cytotoxicity, cell proliferation, and migration. Furthermore, the skin irritation potential of prepared wound dressings was also tested using a 3D reconstructed human epidermal tissue model EpiDerm^{TM}. The following chapters describe the methodology required to assess the experiments during the doctoral study.

9.1 Materials

HA (sodium salt, *Mw* = 1.5 MDa, Contipro), 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDCl, Sigma Aldrich Co.), N-hydroxysuccinimide (NHS, Sigma Aldrich, Co.) and cysteamine hydrochloride (CSA·HCl, Sigma Aldrich Co.) were used in the HA-SH preparation.

Polypyrrole colloids stabilized by poly(vinylpyrrolidone) (PPy/PVP) were prepared using pyrrole (Sigma-Aldrich Co.), iron (III) chloride (Sigma-Aldrich) and poly(vinylpyrrolidone) (PVP; Fluka, K 90, molecular weight M_w = 360,000).

Cellulose SigmaCell type 20 (Sigma Aldrich Co.), sodium periodate ($NaIO_4$; Penta, Czech Republic), and ethylene glycol (Penta, Czech Republic) were used in the preparation of dialdehyde cellulose (DAC). Low-molecular-weight chitosan (Sigma Aldrich Co.), glacial acetic acid (CH_3COOH; Sigma Aldrich Co.), absolute ethanol (VWR, Czech Republic), acetic anhydride (Ac_2O; Sigma Aldrich Co.), hydrochloric acid (HCl; Penta, Czech Republic) and sodium hydroxide (NaOH; Penta, Czech Republic) were used in the preparation of soluble chitosan.

Sample preparation

Most of the samples were prepared and tested by myself, however, in some cases, the optimization of sample fabrication and testing was done in cooperation with other colleagues and institutes (Institute of Biophysics, Czech Academy of Science).

9.1.1 The fabrication of thiolated hyaluronan

A modified procedure based on research by Bian et al. was used for the preparation of HA-SH (Bian et al., 2016). In the first step, HA was dissolved in

UPW for 24 h. Next, pH was adjusted to 5.5 and NHS and EDCl were transferred into the mixture and let to react for 2 h to activate the carboxylic groups of HA. In the next step, pH was adjusted to 4.75 and CSA·HCl dissolved in UPW was added to the solution (molecular ratio – HA:NHS:EDCl:CSA·HCl = 1:2:2:2). The mixture was stirred for 24 h and then dialyzed against dilute HCl solution (pH 3.5) containing NaCl for 48 h. In the last step, the solution was lyophilized to get the solid HA-SH sample. The synthesis was prepared under the nitrogen atmosphere. The degree of thiol substitution was analysed by Ellman's test (Ellman, 1959).

Preparation of HA-SH hydrogel

The solution of HA-SH was able to crosslink itself and form the hydrogel thanks to the oxidation reaction of free thiol groups resulting in disulfide bonds. To achieve this, solid HA-SH polymer was dissolved in UPW (pH 3.5). In the next step, the pH value of the solution was adjusted to 7.5 – 8 with the addition of 1.0 M NaOH. Then the solution was immediately injected into a vessel and exposed to the air at room temperature for 1 h. To get stiffer hydrogel, hydrogen peroxide was added (w/v = 0.35%) to oxidize residual thiol groups. Finally, prepared hydrogels were put into reaction flasks with PBS (Phosphate Buffered Saline) and shaken for 24 h.

The gelation time of HA-SH hydrogel

Freshly prepared HA-SH solution in the vessel was determined by inverse tube test, i.e. test tubes inverted after a given time to confirm gel formation. If there was not any observed fluidity after 1 min since the test tube was inverted, it could be considered that the hydrogel was formed. More polymer concentrations were prepared to see the limit concentration when the hydrogel is still formed (w/v = 2%, 3%, and 5%).

9.1.2 The preparation of PPy colloidal dispersions

Solutions of different concentrations of PVP (2 and 4 wt.%) were prepared by dissolving a specific amount of PVP powder in UPW. Then, to each of the PVP solutions, pyrrole was added, and the volume was adjusted to 50 mL. The mixture was put under ultrasonic treatment for 30 min to get a solution. Each solution was mixed with iron(III) chloride to initiate the polymerization of PPy. The mixture was left to polymerize at room temperature for 24 h. Subsequently, the colloidal dispersions were dialyzed against aqueous HCl to remove the unreacted monomer and oxidant ions (Li et al., 2016).

Zetasizer Nano ZS instrument (Malvern Instrument, UK) was used for the determination of the particle sizes by dynamic scattering (DLS).

Details are given in the ready-to-submit manuscript ***Káčerová S.**, Valášková K., Humpolíček P., Vícha J., Vašíček O., Kašpárková V., Víchová Z. Biocompatibility of polypyrrole based colloidal dispersions*.

9.1.3 Synthesis of water-soluble chitosan

A modified synthesis process by Qin et al. was used for the water-soluble half N-acetylated (50%) chitosan (SCN, Soluble ChitosaN) fabrication (Qin et al., 2006). Briefly, low-molecular-weight chitosan was dissolved in 10% acetic acid for 6h. In the next step, a mixture of ethanol and Ac_2O was added dropwise and stirred for 15 h at 40 °C. The pH was set to 8.5 to initiate the formation of gel particles in the solution. The mixture was dialyzed against water for 72 h. Subsequently, the pH was decreased to 6.5 which led to the dissolution of gel particles. The sample was centrifuged, filtered, and lyophilized.

9.1.4 Synthesis of crosslinker – dialdehyde cellulose

$NaIO_4$ was used for cellulose oxidation (molar ratio of anhydroglucose unit: $NaIO_4$ was 1:1:25) (Münster et al., 2018, 2017). Next, ethylene glycol was added to terminate the reaction. Dialdehyde cellulose (DAC) was purified by repeated centrifugation and mechanical homogenization. Subsequently, the DAC was solubilized. In the next step, centrifugation and filtration were used for the removing of insoluble residues. Finally, the sample was dialyzed and the solubilized product was lyophilized.

9.1.5 Wound dressings preparation

The SCN-DAC films were prepared by adding either 2 or 5 mol.% of DAC relative to SCN (SCN-DAC-2% and SCN-DAC-5%).

PPy-containing wound dressings were prepared by adding the 5 or 10 wt.% PPy (relative to SCN) to the SCN solution and subsequently crosslinked with 2 mol.% of DAC (SCN-DAC-PPy5%, SCN-DAC-PPy10%).

Details are given in the ready-to-submit manuscript ***Káčerová S.**, Muchová M., Doudová H., Münster L., Hanulíková B., Víchová Z., Valášková K., Kašpárková V., Kuřitka I., Humpolíček P., Vašíček O., Vícha J. Antibacterial, anti-oxidant, conductive, and anti-inflammatory polypyrrole/chitosan/dialdehyde cellulose hydrogel wound dressings*.

9.2 Biological properties

The biological properties of materials were characterized in accordance with ISO standard 10993 Biological evaluation of medical devices.

9.2.1 Cell lines

In this chapter, cell lines used for the biological testing of the materials are described.

Mouse embryonic fibroblast cell line NIH/3T3

The biological testing was exhibited using a mouse embryonic fibroblast cell line NIH/3T3 (EACC 93061524, England). Dulbecco's Modified Eagle's Medium (Biosera, France) with the content of 10% of calf serum (Biosera, France) and 100 U/mL of Penicillin/Streptomycin (Biosera, France) was used for the cultivation of NIH/3T3 cells. Cells were incubated at 37 °C under the 5% CO_2 atmosphere in the humidified air (incubator HERAcell 150i, Thermo Scientific, USA).

Embryonic stem cell line ESCs

The embryonic stem cell ES R1 line was cultured in an undifferentiated state using gelatinized tussie culture dishes (0.1% gelatine in UPW) in DMEM containing 15% fetal calf serum, 100 U/mL Penicillin/Streptomycin, 100 mM non-essential amino acids solution (Thermo Fisher, USA), 0.05 mM 2-mercaptoethanol (Sigma, USA) and 1000 U/mL of LIF (Gibco, USA).

9.2.2 Biological testing

Material **cytotoxicity** was examined using the MTT assay which evaluates the cell viability (Freimoser et al., 1999; Riss et al., 2004). Extracts from samples were prepared according to ISO standard 10993-12. Cytotoxicity itself was done according to ISO 10993-5 protocol. Furthermore, cell **proliferation** on the samples was researched (Adan et al., 2016). **Skin irritation** of topically applied mateials was studied according to OECD 439 (OECD, 2021) using a 3D reconstructed human epidermal (RhE) tissue model EpiDerm™ (EPI-200, MatTek, Ashland, USA and MatTek In Vitro Science Laboratories, Bratislava, Slovakia). And last but not least, the **migration assay** was used for the evaluation of wound healing activity (Liang et al., 2007).

10. SUMMARY OF RESULTS

The presented doctoral thesis is mainly focused on the preparation, optimization, and biological testing of biomaterials. As was previously discussed in the theoretical part, they must meet many aspects in terms of biochemical, mechanical and physical properties (Pina et al., 2019). Their applicability can be documented by the fact, that they are employed since the 1960s for contact lenses and intraocular lens applications (Singh, 2009). Moreover, hydrogels are used as, for example, 3D scaffolds for the biomimetic 3D microenvironment for cell growth, but also drug delivery and wound healing (Bordbar-Khiabani and Gasik, 2022; Cui et al., 2023; Xu et al., 2020). Though they can be prepared from many different materials, whether natural, synthetic, or their composites, natural ones were chosen in this thesis. The main reasons for this decision were good biocompatibility caused by the natural origin processed from living organisms. However, the naturally derived materials also exhibit some limitations, such as weak mechanical properties. Thus they must be very often chemically modified (Naahidi et al., 2017). Their potential can be also enhanced by the incorporation of various bioactive molecules (Fiorati et al., 2021). Nevertheless, it should be noted that all of the modifications of the materials must depend on the intended field of application while considering biocompatibility.

In my experimental work, I focused on 1) the fabrication and optimization of HA-SH hydrogels for future preparation of scaffolds for reconstituted intestinal tissue; 2) the preparation and biological testing of conducting PPy/PVP colloidal dispersions; and 3) the preparation and biological testing of active hydrogel wound dressing composites based on chitosan and PPy/PVP colloidal dispersions

1) *The fabrication and optimization of HA-SH hydrogels for future preparation of reconstituted intestinal tissue*. Hyaluronic acid is the only non-sulfated glucosaminoglycan found in embryos and the niches of progenitor/neural cells. HA naturally exhibits low stiffness and high degradability, however, researchers all over the world have been studying multiple strategies to improve the control over these properties. Among others, modifications of HA involve the functionalization of hydrazide or thiol groups, or methacrylation (for more information see chapter 7) (Xu et al., 2012). When I began my postgraduate studies, I started researching the modification of HA by thiol groups. This method relies on methods of green chemistry involving non-toxic urea and succinic acid derivatives as by-products. Furthermore, cysteamine is used as a non-toxic crosslinker. The gelation itself is initiated by the controllable formation of –S–S– groups when the precursor solution is set close to neutral pH, –SH groups start to create mentioned disulfide bridges. The gelation can be controlled by the concentration of the polymer as well as the amount of –SH groups (Bian et al., 2016). However, as is common in science, many challenges arose during the modification process that needed to be solved. Hence, I present below the specific

situations that occurred and describe the approaches I employed to overcome them.

The HA-modification in this thesis was inspired by the work of Bian et al. (Figure 6) (Bian et al., 2016). In the first step, HA was dissolved in water, then EDC and NHS were added into the solution to activate the –COOH groups. Subsequently, cysteamine was added as a bearer of thiol and amine groups.

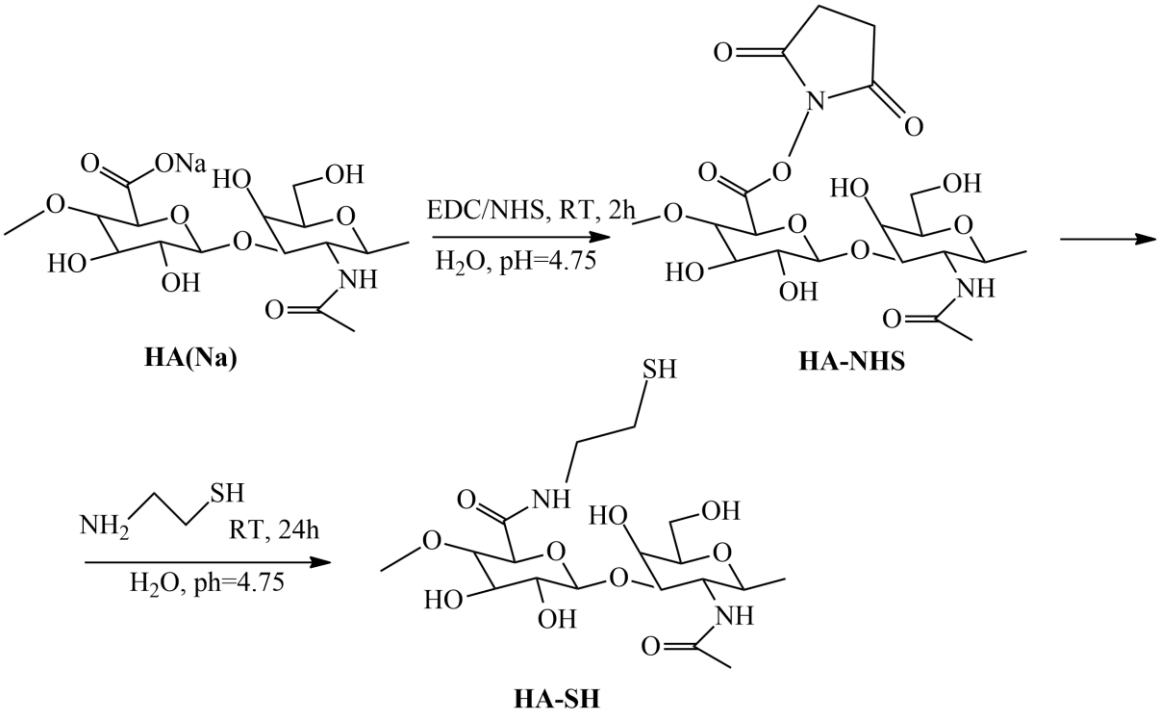

Figure 6: *Scheme of HA-SH synthesis (Bian et al., 2016)*

To obtain the pure product, HA-SH was dialyzed against an aqueous HCl solution (pH = 3.5) and lyophilized. The acidic environment prevented the thiol groups to react with each other. However, it was important to choose the appropriate molar ratio of reactants to prepare the demanded degree of thiolation. The degree of thiol substitution was analyzed according to Ellman's test (Ellman, 1959). The results showed that if the coupling rate was too high (the molar ratio of HA:NHS:EDCl:CSA·HCl = 1:4:4:4), the degree of thiol groups was about 20%, but the sample was not soluble. Carboxyl groups that are providing a negative charge of HA, thus making the molecule hydrophilic, are in this modification substituted with thiol groups. This makes the HA more hydrophobic. Consequently, the molar ratio was adjusted to HA:NHS:EDCl:CSA·HCl = 1:2:2:2 but the degree of thiol groups was only about 4 – 5%. Nevertheless, it was not clear why it was not possible to get higher degrees of thiolation as well as the problems with dissolving the samples occurred again. Thus, the samples were synthesized under the nitrogen atmosphere which prevented oxidation during the fabrication and also during the storage and resulted in higher concentrations of thiol groups (10 – 11%). Furthermore, according to the research by Cao et al., the

dialysis solution was enriched with NaCl solution which decreased the viscosity of the solution and helped with the dissolution of the final product (Cao et al., 2019). After the modification of the HA-SH, the polymer with the exact concentration was dissolved and disulfide-crosslinked in the neutral environment to get hydrogel (Figure 7). Furthermore, to get a firmer hydrogel, hydrogen peroxide was added as it immediately oxidizes all of the residual thiol groups to disulfide.

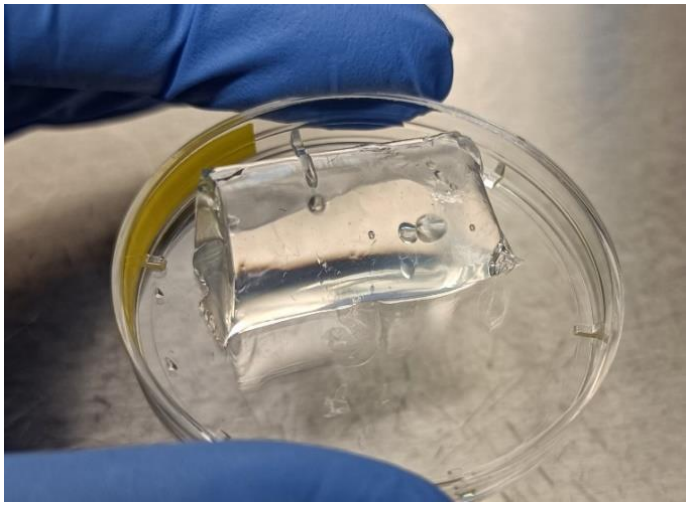

Figure 7: *HA-SH_2% hydrogel sample*

The viscoelastic properties of the samples were studied (the molar ratio of HA:NHS:EDCl:CSA·HCl = 1:2:2:2; degree of thiol groups – 10.44%). The dependence of storage modulus, G', on angular frequency is given in Figure 8. Four types of HA-SH hydrogels were tested; 2%, 3%, 5% and 3% + H_2O_2. It can be observed that the higher the concentration of HA-SH is, the bigger the storage modulus and the elasticity of HA-SH gel. It is because of the denser network of the hydrogel, which can more easily store deformation the energy. Moreover, Bian et. al. have also reported, that the higher the degree of thiol substation, the bigger the G' (Bian et al., 2016).

Nevertheless, it is noticeable that 3% HA-SH hydrogel with the addition of H_2O_2 has a 15 times smaller storage modulus, which is probably caused by side reactions of HA-SH and H_2O_2, causing over-oxidation of SH groups. Also, the decrease of the curve with higher frequency indicates a shearing response and thus more viscous behaviour. This fact rejects our hypothesis about getting a firmer hydrogel after adding the hydrogen peroxide. However, less concentrated H_2O_2 solution could help to overcome this issue, thus further research is needed.

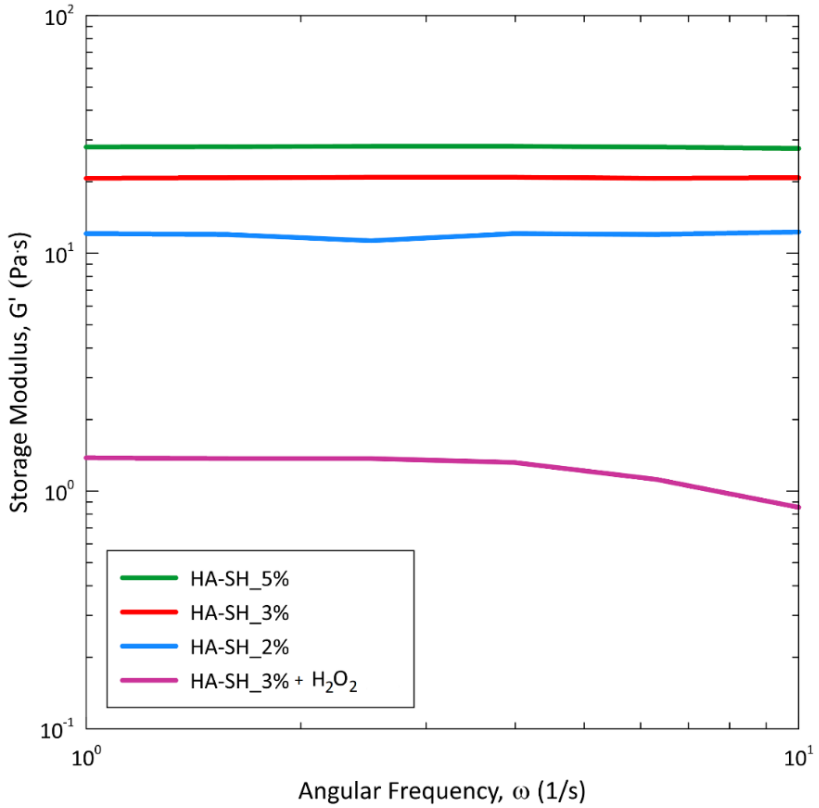

Figure 8: *Dependence of storage modulus (G') of HA-SH hydrogels on the angular frequency*

The cytotoxicity of prepared hydrogels was determined according to ISO 10993-5 using a mouse embryonic fibroblast cell line (NIH/3T3). As expected, the absence of any cytotoxicity (viability of cells higher than 70%) was observed in the case of all samples. This is a confirmation of the successful use of green chemistry methodes in this work. Also, cell proliferation was studied. However, the cells were not able to proliferate on the surface of the hydrogels as a result of a highly hydrophilic and polyanionic surface of HA. This could be overcome by the combination of HA, for example, with growth factors or collagen. For illustration, Gao et al. prepared HA/collagen composite hydrogel with tunable properties resulting in good cell proliferation (Gao et al., 2020).

In conclusion, this pilot study performed by me has led to the idea of the preparation of man-made scaffolds mimicking the mechanical and biochemical composition of small intestine tissue (SIT). The material should be formed by HA-SH and collagen as the main constituents and combined also with appropriate growth factors as well as intestinal stem cells. Currently, this research is being finished and the results will be published in a research article.

Furthermore, after a not-so-simple start to my studies, I moved from hydrogels for a while and focused on the preparation of colloidal PPy.

2) *The preparation and biological testing of conducting PPy/PVP colloidal dispersions*. It is known, that CPs are having unique properties, which are widely

used for the preparation of neural implants, biosensors, tissue engineering scaffolds, and drug delivery systems. For instance, they have not only good electrical properties, but they are also biocompatible, easy to synthesize and work with (Guimard et al., 2007; Jasenská et al., 2021). One of the most popular CPs is PPy. Due to its p-type conductivity, PPy exhibits good electrical conductivity under physiological circumstances. Furthermore, the synthesis of PPy can be done through chemical and electrochemical polymerization (Wang et al., 2004). Even though PPy can be prepared in the forms of colloidal dispersions, insoluble powder, or film, colloidal suspensions are a best choice for further usages, such as scaffold preparation. Colloidal dispersions are prepared using a proper stabilizer. The main reason for stabilizer useage is its ability to create a shell over the particle surface preventing the PPy from precipitation. For example, nanocrystalline cellulose or PVA can be used (Al-Dulaimi and Wanrosli, 2016; Gangopadhyay and Molla, 2011). The reaction medium should not be forgotten either, as water is used the most in the biomedical sciences. Here, PVP was employed as a stabilizer. PVP is a non-toxic biocompatible polymer and is thus widely used as a carrier in biomedical or pharmaceutical fields. Furthermore, this substance was also accepted by the FDA for biomedical applications (Bothiraja et al., 2010; Franco and De Marco, 2020).

The main goal of this study was **to evaluate the biological characteristics of pristine PPy colloidal dispersions** for the first time as only the PPy salts were already studied previously (Humpolíček et al., 2018).

Firstly, in this thesis, the PPy colloidal dispersions were prepared using 2 and 4 wt.% of the PVP stabilizer. According to the literature, the characteristic peaks around 450 nm, corresponding to the π–π* transition from the valence to the antibonding band because of the polaron presence emerged in the **UV-vis spectra**, are confirming the PPy development (Figure 9) (Li et al., 2016; Weng et al., 2011).

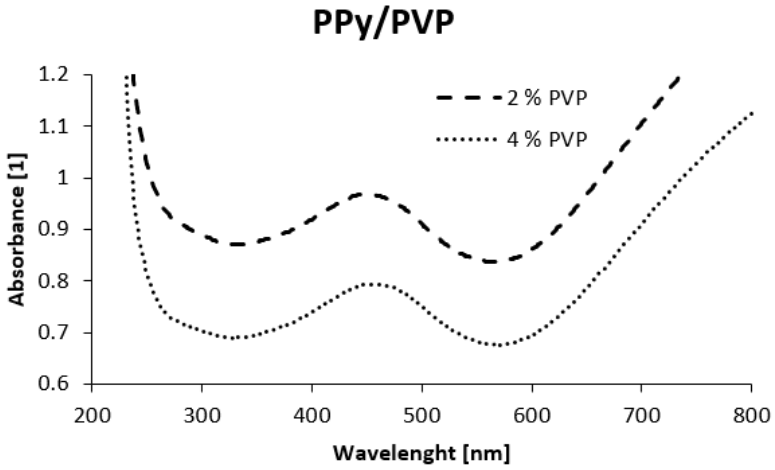

Figure 9: *UV-vis spectra of PPy/PVP colloidal dispersions*

Sizes of the prepared **particles** were also characterized. As illustrated in Table 1, the results showed that the concentration of stabilizer did not significantly affect the z-average diameter of particles. A difference of 50 nm was found between PPy/PVP-2%, where the z-average was 131±7 nm, and PPy/PVP-4% with a value of 179± nm. The polydispersity index (PDI) for both colloids was below 0.3 which is generally considered as polydisperse system with **good homogenity** (Raval et al., 2019).

Table 1: *Size (z-Average Diameter ± SD) and Polydispersity Index (PDI ± SD) of PPy colloids*

	Fresh		**24 months of storage**		**pH 7.4**	
	Z-Ave ± SD **[d.nm]**	**PDI**	**Z-Ave** ± SD **[d.nm]**	**PDI**	**Z-Ave** ± SD **[d.nm]**	**PDI**
PPy/PVP-2%	131±7	0.25±0.03	179±2	0.24±0.03	152±3	0.23±0.01
PPy/PVP-4%	179±7	0.29±0.01	73±1	0.20±0.03	211±4	0.42±0.02

Furthermore, the long-term stability of the colloids was assessed after 24 months of storage at 4°C. Both colloids (PPy/PVP-2% and PPy/PVP-4%) were visually homogenous (Figure 10 a, b). The z-average of PPy/PVP-4% significantly decreased from 179±7 nm to 73±1 nm, as well as the PDI. This suggests that the particles are maturing and creating a new thermodynamic equilibrium state, which was probably made possible by an excess of PVP that could stabilize much smaller particles. Nevertheless, no traces of aggregation or particle sedimentation were found.

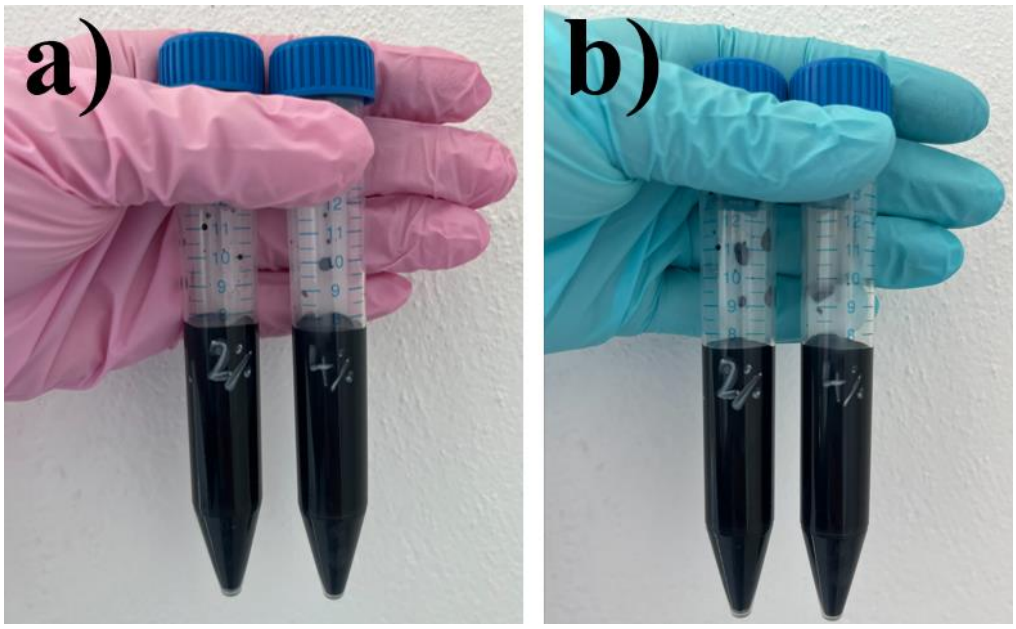

Figure 10: *The long-term stability of the PPy/PVP-2% (left) and PPy/PVP-4% (right): a) freshly prepared samples; b) samples after 24 months storage at 4°C*

The pH stability of materials is another crucial property as it is meant to come in touch with biological systems. For this reason, the z-average diameter and PDI were studied at pH 7.4 in PBS. The results showed that the z-average and PDI of PPy/PVP-2% did not remarkably change. However, good stability cannot be claimed in the case of PPy/PVP-4%. Finally, it is evident from the obtained results that the colloidal particles in PPy/PVP-2% are stable in the physiological setting.

Nevertheless, DLS expresses particle sizes as their hydrodynamic diameter, which includes also the ionic corona etc. Due to this fact, TEM analysis was also used. The results showed that the morphology of colloidal particles was homogenous and the sizes were approximately 35 nm for both colloids.

Finally, the **biological properties** of colloidal PPy particles were evaluated in cooperation with the Institute of Biophysics (Czech Academy of Sciences). As it was previously said, cytotoxicity, antioxidant activity, and antibacterial tests were established and reported for the first time.

The initiative step of biological testing was the **cytotoxicity** evaluation followed by the ISO standard 10993 part 5 using a mouse fibroblast cell line (NIH/3T3). Figure 11 shows the reported data. As can be seen, both of the samples having a concentration of PPy 200 $\mu g.mL^{-1}$ or less did not show any cytotoxicity.

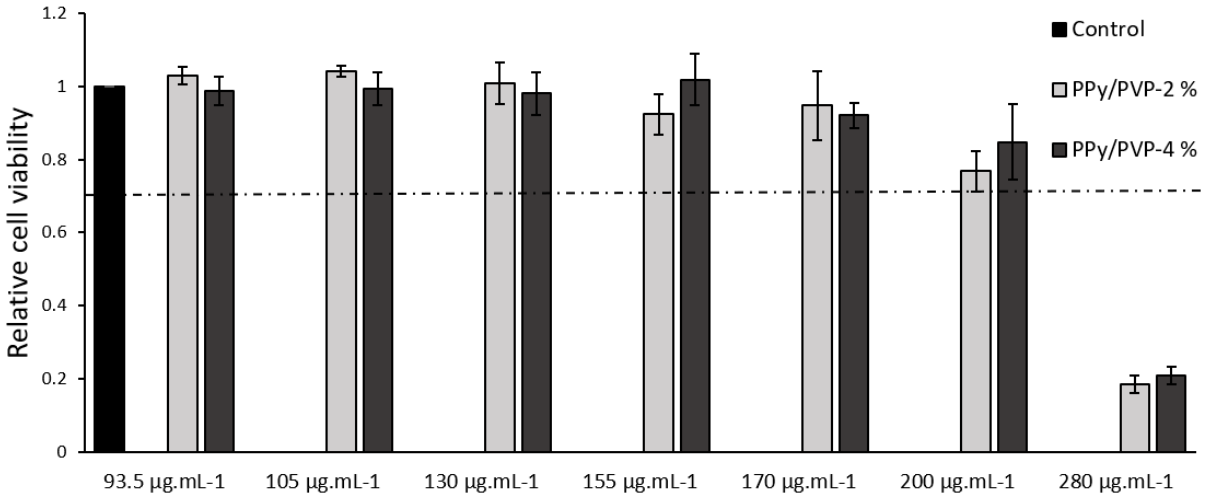

Figure 11: *Cytotoxicity of PPy colloids with different concentrations of PVP. Relative values to the reference (set as 1) are used to express data.*

For illustration, Vaitkuviene et al. studied the PPy nanoparticles prepared by the oxidative polymerization route. The higher concentrations were toxic to mouse hepatoma cells (MH-22A), primary mouse embryonic fibroblast cells (MEF) and human T lymphocyte Jurkat cells, while concentrations lower than 9.7 $\mu g.mL^{-1}$ did not affect cell viability. This value is significantly lower than the concentration of PPy in PPy/PVP colloids thus it can be concluded that PVP improves the biocompatibility (Vaitkuviene et al., 2013). Furthermore, Guo et al. researched the cytotoxicity of PPy nanoparticles mediated by PVP. They found

that the cell lines mouse fibroblasts (L929), pancreatic acinar (266-6) and colorectal adenocarcinoma (HT29) were viable at the tested nanoparticles concentration of 100 $\mu g.mL^{-1}$ (Guo et al., 2019).

As was previously discussed in the theoretical part, biomaterials can be immunomodulatory active and induce a variety of adverse reactions, such as inflammation. This reaction can be activated immediately once the material comes in contact with living tissues. Immune cells start to produce ROS, aiding in the healing process but also having the potential to cause oxidative stress (Tu et al., 2022). The existing studies on the antioxidant activity of PPy are limited. For instance, research paper by Upadhyay et al. examined the antioxidant activity of PPy nanotubes with different diameters. They explored that not only do higher concentrations of PPy have an impact on antioxidant activity, but also that nanotubes with smaller diameter showed enhanced activity (Upadhyay et al., 2014). In our study, we investigated **the antioxidant activity** of colloidal PPy as potential immunomodulatory markers. Based on the obtained data, it was researched that colloidal PPy itself revealed a **significant antioxidant effect** even in low concentrations. Tested concentrations of PPy ranged from 1.87 – 93.5 $\mu g.mL^{-1}$. The concetrations 9.35 – 93.5 $\mu g.mL^{-1}$ showed a nearly complete loss of the signal and the lowest concentration 1.87 $\mu g.mL^{-1}$ reducted the signal below 50 % of the control. Additionally, the production of ROS both before and after the neutrophil activation by opsonized zymosan particles (OZP) was examined. Even the lowest tested concentration of PPy 1.87 $\mu g.mL^{-1}$ significantly reduced both the OZP-activated and spontaneously produced ROS. Furthermore, concentrations 9.35 $\mu g.mL^{-1}$ and above resulted in remarkable reduction of over 90% in spontaneous ROS generation which is a potential counterproductive outcome. As previously researched, basal levels of ROS are essential for maintaining normal cellular functioning (Dunnill et al., 2017). However, in the case of OZP-activated ROS generation, only the highest concentration of PPy (93.5 $\mu g.mL^{-1}$) achieved complete ROS production, indicating promising results. These consequences lead to the conclusion that **PPy colloids make excellent free radical scavengers**.

Biological testing should undoubtedly include **antimicrobial properties**. It is known that bacteria have a negatively charged wall cell thus the positive net charge of PPy chains electrostatically interacts with it (Varesano et al., 2013). Here, in this thesis, the antibacterial activity expressed as a minimal inhibitory concentration (MIC) against both gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria was investigated. The best results were obtained for PPy/PVP-2% with the MIC value 234 $\mu g.mL^{-1}$ for both gram-positive and gram-negative bacteria. The same value was observed for the PPy/PVP-4% against *S. aureus*, in the case of *E. coli*, the MIC value was higher – 468 $\mu g.mL^{-1}$. For comparison, the research group Sayyah et al. studied the MIC against *E. faecalis* and *S. aureus* and the values ranged from 62,5 to

125 mg mL^{-1}. However, they prepared PPy by a cyclic voltammetry without the usage of stabilizing agent with antibacterial properties (Sayyah et al., 2014).

Based on these results it can be conluded that **PPy in colloidal form** is not only **easy to process** but especically samples PPy/PVP-2% exhibited **good stability** under the physiological conditions and in long-term storage. Furthermore, good results were obtained also in biological testing. **No cytotoxic effect** was found under the concentration of 200 $\mu g.mL^{-1}$ colloidal PPy. As measured by ROS scavenging, samples also demonstrated **high antioxidant effects** and related immunomodulatory activities. The sample PPy/PVP-2% exhibited good **antibacterial activity** against both *E. coli* and *S. aureus*.

Details are given in the ready-to-submit manuscript ***Káčerová S.**, Valášková K., Humpolíček P., Vícha J., Vašíček O., Kašpárková V., Víchová Z. Biocompatibility of polypyrrole based colloidal dispersions*.

As this material is suitable for biomedical applications, the second part of this thesis studied the incorporation of PPy/PVP-2% into the hydrogel wound dressings based on chitosan to prepare bioactive composites (see the next part 3).

3) *The preparation and biological testing of active hydrogel wound dressing composites based on chitosan and PPy/PVP colloidal dispersions.* As was previously discussed in chapter 3.2, even though the skin is the body's biggest organ and acts as a protective layer against the external world, it's crucial to consider that even a minor injury can pose a significant risk to life. Traditional wound dressings, such as gauzes and bandages are typically utilized primarily to bleeding and shielding the wound from external factors. However, they provide minimal benefits in terms of enhancing the healing process, preventing infection, or reducing overactive inflammation. For these reasons, bioactive wound dressings intended to promote various aspects of the wound healing process have been created (Das and Baker, 2016). Hydrogels are excellent choices for numerous reasons, such as high-water content, similarity to ECM, mechanical properties, they can keep a moist wound environment and absorb excessive exudate and last but not least, they can be enhanced by a variety of bioactive molecules to generate further therapeutic effects (Bano et al., 2017).

In this thesis, chitosan was chosen as it is considered one of the most potent options for treating wounds due to its favourable qualities, including biocompatibility, antimicrobial, and antioxidant properties. Chitosan-based materials are already well-established in the biomedical fields (Ali Khan et al., 2020). Chitosan, for example, was used in the preparation of sponges, nanoparticles, hydrogels, membranes, and wound dressings (Dev et al., 2010; Portero et al., 2007; Tamura et al., 2011). However, chitosan is soluble in water only if its NH_2 groups are protonated to $–NH_3+$ groups, i.e. while the pH is lower than its pK_a value of ~6.5. At physiological pH, chitosan loses its charge, becomes

insoluble and also its antibacterial function associated with the positive charge vanishes (Wang et al., 2006). To overcome these issues, water-soluble chitosan derivatives were studied. In this thesis, the fabrication of water-soluble half N-acetylated chitosan (SCN) was researched (Qin et al., 2006). SCN, with a degree of deacetylation (DD) adjusted to ~50% is soluble at physiological pH. Moreover, SCN preparation is completely natural without a need to use artificial functional groups and it also exhibits high antioxidant activity (Feng et al., 2007). Properties of chitosan-based materials are also often enhanced with various bioactive substances to improve, for example, antibacterial activity or antioxidant and anti-inflammatory properties (Singh et al., 2017; Zhao et al., 2017). Here, the colloidal conductive PPy stabilized by 2% of PVP was incorporated into the SCN hydrogels resulting in better biological properties, such as good biocompatibility, antioxidant activity, and improved antimicrobial activity. The increased conductivity of the hydrogels further improved their potential for promoting wound healing by stimulating cell migration and proliferation (Talikowska et al., 2019). Furthermore, for the first time, dialdehyde cellulose (DAC) was used not only as a crosslinker of the SCN, but also to covalently anchor the prepared PPy particles (Figure 12). DAC is a crosslinking agent with very low toxicity compared to organic crosslinkers, such as glutaraldehyde (Muchová et al., 2020; Münster et al., 2018).

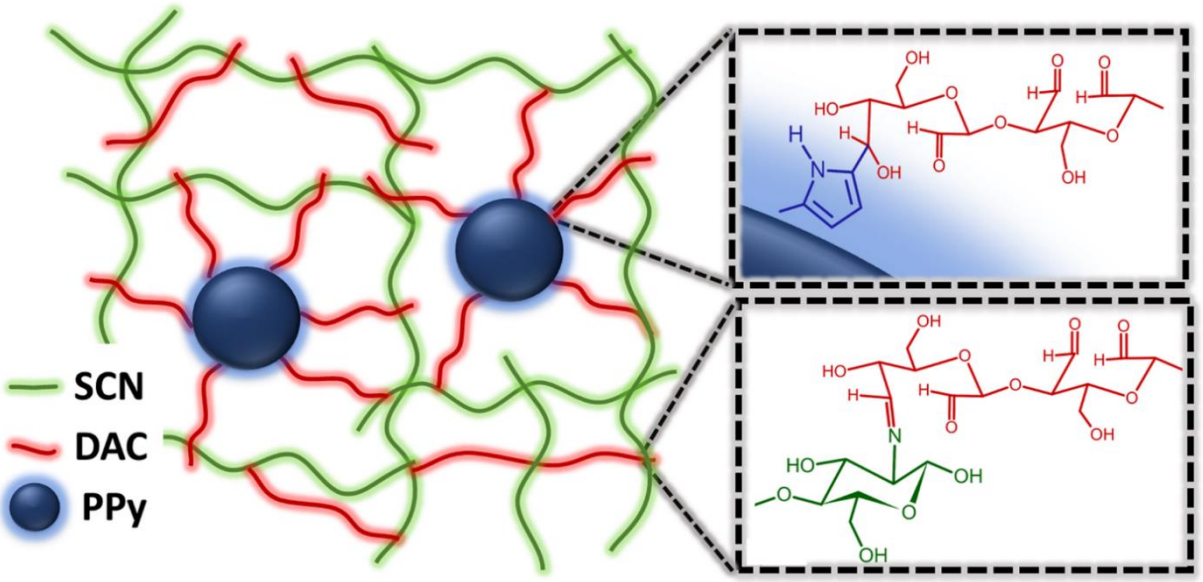

***Figure 12**: Scheme of SCN-DAC-PPy wound dressing hydrogel with details of bonding between DAC+PPy (top) and DAC+SCN (bottom)*

SCN was prepared using a modified method by Qin et al. (Qin et al., 2006), DAC fabrication was based on the works by Münster et al. (Münster et al., 2018, 2017), and finally, colloidal PPy was prepared as described above. The method for determining the degree of deacetylation (DD) of the SCN involved comparing the intensity of H2 signals in ^{1}H NMR spectra (Kasaai, 2010; Kubota et al., 2000),

while PPy/PVP-2% characteristics were described previously. The **IR** and **Raman** spcetra DAC, PPy, and their aqueous mixtures (Raman) and dried-out wound dressings (FT-IR) in Figure 13 a, b proves the spontaneous DAC+PPy reaction by the selective disappearance bands associated with aldehyde groups of DAC. The Raman and FT-IR spectra of DAC showed that the band corresponding to the free carbonyl groups of DAC, which was initially present at 1637 cm^{-1} in Raman and 1732 cm^{-1} in FT-IR spectra, disappeared when DAC was mixed with PPy. Also, the Raman and FT-IR DAC+PPy spectra still clearly show additional DAC bands between 800 and 1200 cm^{-1}.

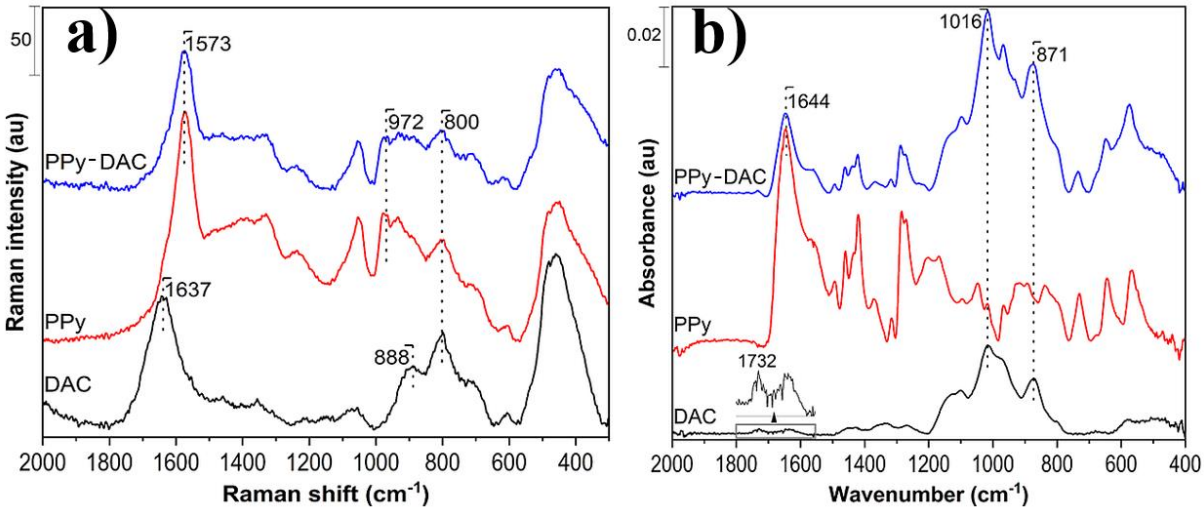

Figure 13: *a) Raman spectra of DAC, PPy, and their mixture, b) FT-IR spectra of DAC, PPy, and wound dressing prepared by drying the PPy-DAC mixture*

Firstly, the right amount of DAC (2 and 5 mol.%) as a crosslinker for the wound dressing preparation was studied. Based on the obtained results, the sample SCN-DAC-2% was chosen for the incorporation of PPy colloid. Mainly for the lower elasticity which allows better adhesion to the skin and also for the higher swelling capacity which allows better exudate absorption and lower cell attachment and growth on the surface.

Subsequently, two different SCN-DAC-2% samples with 5 wt.% of PPy and 10 wt.% of PPy relative to SCN (SCN-DAC-PPy5% and SCN-DAC-PPy10%) were prepared and studied (Figure 14). The network parameters showed that SCN-DAC-PPy5% swelled almost the same as SCN-DAC-2% (31±9 times), with the EWC of 97±1% and gel fraction of 59±2%. On the other hand, SCN-DAC-PPy10% swelled significantly more - 77±9 times, the EWC was 99±0.2% and the gel fraction 62±92%. If compared to SCN-DAC-2%, the storage modulus, loss modulus, and complex modulus of both SCN-DAC-PPy5% and SCN-DAC-PPy10% were higher, which might be explained by the network's integration of stiffer PPy nanoparticles into the network. However, the sample containing 10% of PPy had, for example, a significantly lower storage modulus than the sample

with 5% of PPy. The higher SCN-DAC-PPy10% swelling and lower storage modulus may be explained by an increased PPy:DAC ratio. Because as more pyrrole groups are available for aldehyde condensation, more DAC might attach to the PPy particles and less is available for crosslinking of farther-reaching SCN chains.

Figure 14: *SCN-DAC-PPy5% wound dressing hydrogel samples in UPW*

Moreover, PPy colloids exhibit an ionic/electronic conductivity mechanism, thus the specific conductivity (K) was measured. It is not surprising that no K was observed for dry wound dressings as the ion-conducting environment was missing. However, all swelled samples in UPW showed specific conductivity, between 2.0 $mS.cm^{-1}$ for SCN-DAC-2% and 5.1 $mS.cm^{-1}$ measured for SCN-DAC-PPy10% with an increased quantity of PPy. The hydrogel wound dressings thus possess a conductivity level similar to various human tissues, such as the human epidermis (0.26 $mS.cm^{-1}$), human dermis (2.2 $mS.cm^{-1}$), and muscle (4.1 $mS.cm^{-1}$), as reported in some studies (Duck, 1990; J. Peters, G. Stinstra, M. Hendriks, 2001). Such conductive hydrogels are capable of transmitting bioelectrical signals and potentially aiding in the process of wound healing (Zhao et al., 2017). For example, He et al. prepared hydrogels based on N-carboxyethyl chitosan and benzaldehyde-terminated Pluronic F127/carbon nanotubes with conductivity (1.37 $mS.\ cm^{-1}$ – 8.45 $mS.\ cm^{-1}$) also similar to skin tissue (He et al., 2020).

As illustrated in Figure 15, SEM micrographs of dried samples prepared in this thesis show the amount of PPy particles, represented as the quantity of bright dots, increasing with the initial dose of colloidal PPy. Additionally, the

distribution of PPy particles is homogenous because no aggregation of particles was observed.

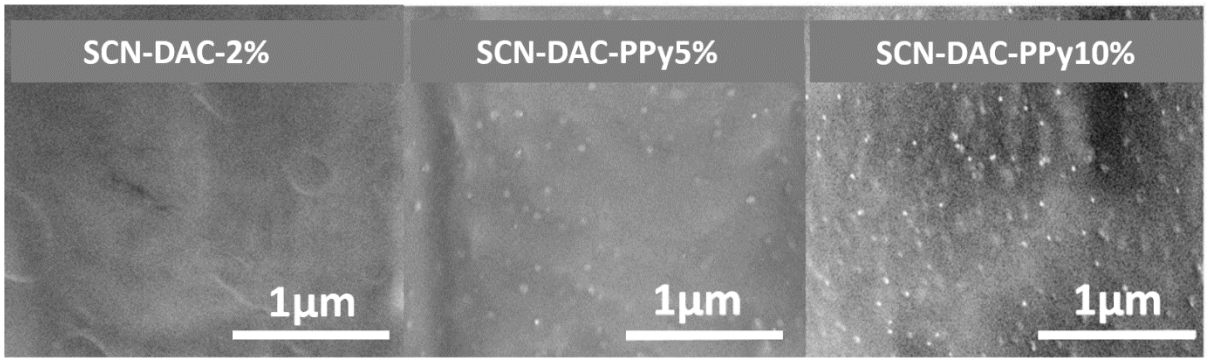

Figure 15: *SEM images of dried samples*

As the prepared wound healing dressings are supposed to interact with living tissues, appropriate **biological tests** were performed in cooperation with the Institute of Biophysics (Czech Academy of Sciences).

The **cytotoxicity testing** using a mouse fibroblast cell line (NIH/3T3) in direct contact with the samples did not show any cytotoxicity. Based on these results, it can be confirmed that PPy in hydrogels does not have a negative impact on cytotoxicity similarly to the previous study. Furthermore, cell **proliferation** was assessed. The morphology of cells on the tissue plastic in the presence of the hydrogels was physiological when compared to the reference (cell proliferation on the tissue plastic). The adhesion and proliferation were not observed on the surface of the hydrogels, especially in the case of SCN-DAC-PPy5%. However, this fact is advantageous in wound healing patches as cell ingrowth is not desirable.

In the next step, **skin irritation** was evaluated. Skin irritation test is crucial as it helps to assess the safety of the materials that come in contact with the skin. Such tests can help identify potential irritants or allergens that could cause undesirable reactions, such as redness, itching, or swelling (Kandarova et al., 2018). Here, *in vitro* skin irritation test was evaluated using a 3D reconstructed human epidermal tissue model EpiDerm^{TM} according to OECD 439 (OECD, 2021). The testing was performed using the neat SCN-DAC hydrogel and the sample with 5% of PPy, which was chosen as the best-performing wound dressing considering its excellent biological and mechanical characteristics. The results showed that both materials have non-irritating properties.

The **wound healing** was studied by a common *in vitro* technique using a **scratch assay** on NIH/3T3 cell line (Liang et al., 2007). Based on the acquired data, it was clear that the presence of all hydrogel wound dressings, even the neat one, significantly enhanced wound healing *in vitro*. It may be expected that the higher the value of PPy, the better results will be observed. However, the best results were observed in the case of SCN-DAC-PPy5% as the percentage of open

wound area remaining after 10h of incubation was the lowest (37±2%). In the case of SCN-DAC-PPy10%, the value was comparable to the neat hydrogel. While the cause behind this behaviour is unclear, the wound healing rate does not increase proportionally with the quantity of PPy.

The development of biomaterials with antioxidant properties has become another important goal in wound dressing preparation. As already discussed in chapter 3.2, excessive ROS production can lead to, for example, deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) damage or cell apoptosis. Antioxidants are a good solution to overcome these limitations as they repair the normal level of ROS (Fadilah et al., 2023). For example, Bektas et al. researched the effects of incorporating vitexin into a gel based on chitosan to enhance wound healing. They observed good results as the material was able to improve the healing process in both *in vitro* and *in vivo* tests (Bektas et al., 2020). In our study, we first focused on the testing of the **antioxidant activity** of the SCN-DAC samples with 0, 5, and 10% of PPy using a luminol-horseradish peroxidase-H_2O_2-free system. The neat SCN-DAC sample showed the signal reduced by a third of the control, thus, the obtained data confirmed the previous research about the antioxidative characteristics of SCN (Feng et al., 2007). However, the samples containing PPy reduced the signal more than twice of the control and the values for both amounts of PPy were nearly the same. Next, the spontaneous and OZP-activated ROS production by neutrophils was researched and similar trends were observed – the antioxidant effect was recorded for all of the samples, including neat SCN-DAC. However, the greater the amount of PPy, the better antioxidant activity. Also, no cytotoxic effect of the samples on macrophages was observed. Moreover, as was observed in nitric oxide (NO) and interleukin 6 (IL-6) production, the tested wound dressings activated and positively modulated macrophage response.

The utilization of antibacterial wound dressings aids can reduce the entry of pathogens into the wound which is resulting in the reduction of inflammatory response. Thus, the **antimicrobial activity** of the SCN-DAC and PPy-containing hydrogels was studied according to ISO standard 22196 using *S. aureus* and *E. coli* bacteria. The results revealed, that neat SCN-DAC hydrogel was not effective against either of the bacterial strains as expected. The reason was already explained previously – materials based on chitosan do not show an antibacterial effect in neutral pH (Qin et al., 2006). On the other hand, the wound dressings containing PPy showed improved antimicrobial activity, for example, both samples exhibited significant activity against *S. aureus* (R ~2) and against *E. coli* the value was 0.8 – 1.0.

Based on obtained results it can be concluded that **active hydrogel dressings** with **non-cytotoxic**, **antioxidant**, **non-irritating**, **antibacterial**, and **conducting properties** were prepared. Furthermore, the samples also exhibited **enhancing *in vitro* wound healing**. Also, it must be noted that in the future the application of

the reaction of DAC+PPy could have wide-ranging uses in the development of conductive materials for biomedical applications.

Details are given in the ready-to-submit manuscript ***Káčerová S.**, Muchová M., Doudová H., Münster L., Hanulíková B., Víchová Z., Valášková K., Kašpárková V., Kuřitka I., Humpolíček P., Vašíček O., Vícha J. Antibacterial, anti-oxidant, conductive, and anti-inflammatory polypyrrole/chitosan/dialdehyde cellulose hydrogel wound dressings*.

During my doctoral studies, I also participated in several cooperations with other researchers to evaluate the biological testing of different biomaterials. Specifically, in a research paper by *Münster L., Fojtů M., Muchová M, Latečka F., **Káčerová S.**, Capáková Z., Juriňáková T., Kuřitka I., Masařík M., Vícha J. Enhancing cisplatin anticancer effectivity and migrastatic potential by modulation of molecular weight of oxidized dextran carrier. Carbohydrate Polymers. 2021, 272, 118461,* I tested the scratch assay performed on ovarian cancer cell line A2780, which was treated with nanogels based on dextran loaded with cisplatin.

Furthermore, in the work by *Štěpánková K., Ozaltin K., Pelková J., Pištěková H., Karakurt I., **Káčerová S**., Lehocký M., Humpolíček P., Vesel A., Mozetic M. Furcellaran surface deposition and its potential in biomedical applications. International Journal of Molecular Sciences. 2022, 23(13),7439*, I tested cytotoxicity and cell proliferation of the embryonic stem cell ES R1 line on samples that where prepared by furcellaran deposition onto a poly(ethylene terephthalate) (PET) surface via RF air plasma discharge activation followed by grafting of a N-allylmethylamine (MAAM) monomer.

In a research paper by, *Jasenská D., Kašpárková V., Vašíček O., Münster L., Minařík A., **Káčerová S.**, Korábková E., Urbánková L., Vícha J., Capáková Z., Falleta E., Pina C. D., Lehocký M., Skopalová K., Humpolíček P. Enzyme-catalyzed polymerization process: a novel approach to the preparation of polyaniline colloidal dispersions with and immunomodulatory effect. Biomacromolecules. 2022, 23(8), 3359-3370,* I tested the effect of biocompatible polymers based on PANI colloidal particles prepared by the enzymatic polymerization of aniline stabilized by PVA or chitosan on NIH/3T3 mouse fibroblast cell viability.

Similarly, in the work by *Martínková M., Hausnerová B., Huba J., Martínek T., **Káčerová S.**, Kašpárková V., Humpolíček P. Powder injection molded ceramic scaffolds: The role of pores size and surface functionalization on the cytocompatibility. Materials and Design. 2022, 224, 111274,* I worked on the cytocompatibility tests of ceramic-based scaffolds functionalized by PANI-based films using NIH/3T3 mouse fibroblast cell line.

11. CONTRIBUTION TO SCIENCE

Although biomaterials have unquestionably revolutionized the medicine, their great potential and uncharted territories continue to attract researches towards an exciting journey of exploration and discovery. The presented Ph.D. study contributes to the current knowledge of hydrogels, conducting polymers, and their composites.

The initial phase of this study focused on the modification of carboxylic groups along the hyaluronic acid backbone, leading to the creation of thiol groups that serve as crosslinking sites. Though some problems appeared during the synthesis, appropriate adjustments led to the successful modification of HA with the demanded degree of thiolation. Such materials could be used for the preparation of the model of small intestine tissue where thiolated hyaluronic acid, as well as collagen would be the main structural materials further modified by growth factors. Together the material should promote the desired intestinal stem cells behaviour. It is based on the idea of making the gradients both biochemical and mechanical. This research is being finished and the results will be published in an international journal.

Furthermore, the colloidal dispersions based on conductive polypyrrole stabilized with poly(vinylpyrrolidone) were studied in terms of material and biological properties. While previous investigations explored the biological properties of PPy powders, nanoparticles, films, and composites, the colloidal form of PPy remained unexplored until now. Consequently, the primary objective of this research was to assess the influence of colloidal PPy on cell viability, antioxidant activity, and antibacterial efficacy, marking the first analysis in this field. The obtained data showed an exceptional antioxidative effect of colloidal PPy, positioning it as a highly promising candidate for composite preparation for biomedical applications. Thus, the next study concentrated on the incorporation of PPy into the hydrogels based on chitosan.

The investigation was focused on partially re-acetylated modification of chitosan and subsequently dialdehyde cellulose was used as a low-toxic crosslinker. Additionally, colloidal PPy was employed to enhance the hydrogel properties. The obtained results demonstrated the exceptional capabilities of these samples, as potential wound dressings, exhibiting good ROS scavenging abilities, antibacterial properties, and significant *in vitro* wound healing potential. These findings emphasize the great promise of these bioactive hydrogel dressings in advancing the field of wound care and promoting the accelerated healing process. Furthermore, the condensation reaction between DAC and PPy described here for the first time opens up the doors to the development of many more conductive biomaterials.

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LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Alphabetically ordered

2D	Two-dimensional
3D	Three-dimensional
ADH	Adipic acid dihydrazide
BMSCs	Bone marrow mesenchymal stem cells
CPs	Conductive polymers
CNTs	Carbon nanotubes
CSA·HCl	Cysteamine hydrochloride
DAC	Dialdehyde cellulose
ECM	Extracellular matrix
EDCl	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
FDA	Food and Drug Administration
GAGs	Glucosaminoglycans
GF	Growth factors
GSH	Glutathione
HA	Hyaluronic acid
HA-SH	Thiolated hyaluronic acid
IL-6	Interleukin 6
MAAM	N-allylmethylamine
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHS	N-hydroxysuccinimide
NO	Nitric oxide

OZP	Opsonized zymosan particles
PANI	Polyaniline
PDI	Polydispersity index
PET	Poly(ethylene terephthalate)
PPy	Polypyrrole
PVA	Poly(vinyl alcohol)
PVP	Polyvinylpyrrolidon
RGD	Tripeptide Arg-Gly-Asp
RM	Regenerative medicine
ROS	Reactive oxygen species
SC	Stem cells
SCN	Soluble chitosan
SIT	Small intestine tissue
TE	Tissue engineering
TRPV4	Transient receptor potential vanilloid 4
UPW	Ultrapure water

LIST OF PUBLICATIONS

Articles published in journals indexed on Web of Science:

Jasenská, D., Kašpárková, V., Vašíček, O., Münster, L., Minařík, A., **Káčerová, S.**, Korábková, E., Urbánková, L., Vícha, J., Capáková, Z., Falleta, E., Della Pina, C., Lehocký, M., Skopalová, K., Humpolíček, P., 2022. Enzyme-Catalyzed Polymerization Process: A Novel Approach to the Preparation of Polyaniline Colloidal Dispersions with an Immunomodulatory Effect. Biomacromolecules 23, 3359–3370. https://doi.org/10.1021/acs.biomac.2c00371

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Káčerová S., Valášková K., Humpolíček P., Vícha J., Vašíček O., Kašpárková V., Víchová Z. Biocompatibility of polypyrrole based colloidal dispersions

Káčerová S., Muchová M., Doudová H., Münster L., Hanulíková B., Víchová Z., Valášková K., Kašpárková V., Kuřitka I., Humpolíček P., Vašíček O., Vícha J. Antibacterial, anti-oxidant, conductive, and anti-inflammatory polypyrrole/chitosan/dialdehyde cellulose hydrogel wound dressings

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Project IGA/CPS/2020/001, Biocompatibility and antimicrobial activity of materials
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Project GAČR 20-28732S, Colloidal systems for topical formulations. Pickering emulsions and polymer-based colloids.
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2019
Project IGA/CPS/2019/004, Biological properties of materials
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	Preparation and characterization of hydrogels

Ing. Simona Káčerová, Ph.D.

Composite materials based on polysaccharides

Kompozitní materiály na bázi polysacharidů

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