Study of conducting biocompatible systems based on biopolymers

Ing. Daniela Jasenská, Ph.D.

Doctoral Thesis Summary

Tomas Bata University in Zlín

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Centre of Polymer Systems

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Studium vodivých biokompatibilních systémů na bázi biopolymerů

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CONTENT

ABSTRACT

Conducting polymers (CPs) are a class of conjugated polymers currently used in a broad range of applications, including electronics and medical devices. The interest in these materials is motivated by their attractive properties, comprising simple straightforward synthesis, mixed electron and ionic conductivity, and environmental stability. However, the application of CP is limited by their difficult processability. This limitation can be removed *via* the preparation of conducting colloidal dispersions containing suitable steric stabilizers, such as polymers and biopolymers. This attractive stable form of conducting polymer can also serve for the preparation of films and scaffolds. The thesis focuses first on polyaniline colloidal dispersions stabilized by the biocompatible stabilizers sodium hyaluronate and chitosan, their preparation, the investigation of their properties, and possibilities with respect to their application. With regard to the future utilization of these systems, a precise method of preparation was established, this involving the choice of a suitable ratio of reactant and stabilizer. The colloids were characterized by UV–vis spectra, particle-size distributions, and morphology, as well as by their biological properties in terms of cytotoxicity and antibacterial activities. In the second part of the thesis, successfully prepared colloids served as precursors for the formation of conducting composite films combining polyaniline with the abovementioned polysaccharides. In addition to these films' physico-chemical characteristics, their antibacterial activity and mainly cytocompatibility with human-induced pluripotent stem cells were described. The final part of the thesis is devoted to novel, green synthesis routes for conducting polymers utilizing peroxidase enzymes. Enzymatically synthetized polyaniline colloids were described *via* their physicochemical and biological properties, including their cytotoxicity to fibroblasts and macrophages, as well as their immunomodulatory effect on macrophages and neutrophils.

ABSTRAKT

Vodivé polymery jsou konjugované polymery, které jsou v současnosti používány v široké řadě aplikací, včetně elektronických a medicínských zařízení. Důvodem zájmu o tyto materiály jsou jejich atraktivní vlastnosti, zahrnující jednoduchou přípravu, kombinaci elektronové a iontové vodivosti i dobrou stabilitu. Jejich aplikace je však omezena obtížnou zpracovatelností. Tento nedostatek lze vyřešit přípravou koloidních disperzí vodivých polymerů, která se provádí reakcí v přítomnosti vhodného sterického stabilizátoru. Koloidní systémy jsou atraktivní stabilní formy vodivých polymerů a mohou rovněž sloužit jako základ pro přípravu filmů a scaffoldů. Dizertační práce se v první části zabývá přípravou koloidních disperzí polyanilinu stabilizovaných biokompatibilními stabilizátory chitosanem a hyaluronátem sodným a zkoumá jejich vlastnosti a možnosti aplikace. Koloidy byly charakterizovány pomocí UV-vis spekter, distribuce velikosti částic a morfologie. Současně byly stanoveny i jejich biologické vlastnosti, a to cytotoxicita a antibakteriální aktivita. Úspěšně připravené koloidy byly v další části práce využity jako prekurzory pro přípravu kompozitních filmů kombinujících vodivý polymer polyanilin s biokompatibilními polysacharidy. Kromě fyzikálně-chemických vlastností filmů byla stanovena jejich antibakteriální aktivita a především cyto-kompatibilita s lidskými indukovanými pluripotentními kmenovými buňkami. Závěrečná část práce je věnována novému způsobu přípravy vodivých polymerů, takzvané zelené syntéze, která využívá enzymů z třídy peroxidáz. Enzymaticky syntetizované polyanilinové koloidy Enzymaticky syntetizované polyanilinové koloidy byly popsány pomocí fyzikálně-chemických a biologických vlastností, včetně cytotoxicity na fibroblastech a makrofázích i jejich imunomodulačního účinku na makrofágy a neutrofily.

1. INTRODUCTION

The discovery of conducting polymers initiated a novel attractive research of interesting properties and numerous application possibilities of these unique materials [1, 2]. The most promising representatives of CPs include polyaniline (PANI) and polypyrrole (PPy), especially due to their simple synthesis, low cost monomers, tunable properties, and good stability. Both polymers are commonly prepared either by chemical preparation or electrochemical. However, the potential applications of PANI and PPy are limited due to their infusibility, very low solubility in most of available solvents and lower conductivity compared to that of metals [3]. Similarly, PPy cannot be further processed once synthesized, as its molecular structure makes it non-thermoplastic, mechanically rigid, brittle and insoluble after the synthesis [4]. These shortcomings may be avoided by the preparation of conducting colloidal dispersions. Colloidal dispersions of CPs, are usually prepared by dispersion polymerization. Dispersion polymerization is a time-saving method that provides colloidal systems with long-term stability. In spite of that, environmentally sensitive polymers have received a great deal of attention in recent years, and their synthesis and applications have become a major field in polymer science. In the preparation of CPs, the use of so-called green syntheses, such as enzymatic polymerization, is therefore on the rise. Compared to the commonly used chemical synthesis employing monomers and strong oxidation agents, application of enzymes offers more exciting advantages, including a high selectivity, substrate specificity and reaction proceeding in aqueous media at a mild temperature [5]. However, the resulting properties of colloidal conducting particles prepared by either standard dispersion or enzymatic polymerization depend in particular on the reaction conditions and composition of the reaction mixture, as well as on the choice of a suitable stabilizer. Commonly, water-soluble synthetic polymers or surfactants can be used as stabilizers for synthesis of CPs colloids; nevertheless, only a few biomacromolecules, such as cellulose derivatives proteins or gelatine [6], have been employed for this purpose. In connection with biological applications, polysaccharides, including sodium hyaluronate, sodium alginate or chitosan, appear to be suitable candidates for CPs stabilization because of their good environmental and biological properties [7]. Alternative processing strategies to increase the application potential of CPs consist in surface coating with conducting thin films. Such conducting thin films are the forms of CPs providing attractive biomedical applications as biosensors, detectors, indicators, or biomedical devices due to the fact they are able to create a bioactive interface between the device and living tissue. Moreover, there are various methods of their syntheses, including polymerization in colloidal dispersion mode.

2. CONDUCTING POLYMERS

Polymers are macromolecules composed of long chains of repeating constitutional subunits called monomers. They share several macro and micro characteristics, optical properties and have been generally considered as insulators. In the mid-1970s novel organic polymer materials were produced that merge positive properties of conventional polymers and metals. Likewise most commercially available polymers, they are easy to synthesize and reasonably flexible in the processing. Furthermore, they are able to conduct a charge and provide an electrical conductivity as metals and inorganic semiconductors [1, 8]. The first discovered conducting polymer was halogen doped *trans*-polyacetylene (PA) which exhibited a 106-fold increase in the conductivity compared to unmodified *trans*-PA [9]. Although PA, a non-cyclic polyene, is still one of the most studied polymers in this field, it is unstable in the air and difficult to process. Unlike PA, polyphenylenes, which are cyclic polyenes, are known to be thermally stable [10]. Consequently, the development of aromatic CPs with a better stability and processability received more attention. Polyheterocycles, such as polypyrrole (PPy), polythiophene (PT), polyaniline (PANI), and poly(3,4-ethylenedioxythiophene) (PEDOT) have emerged as another class of aromatic CPs that exhibit good stabilities, conductivities, and are easily synthetized [11, 12]. This combination of the availability with unique properties has given these polymers a wide range of applications, such as capacitors, solar cells, sensors [13], transistors and data storage and corrosions inhibitors [8]. Recently, biological and medical applications of CPs have been on the rise [1, 14].

2.1 Polyaniline

Among electrically CPs, probably the oldest synthetic polymer that has ever been prepared is polyaniline [3]. Polyaniline consists of chains composed of aniline monomers as repeating units. It exists in a variety of forms differing in the degree of oxidation or protonation (Fig. 1). The stable form of PANI is a green-coloured emeraldine salt with the conductivity in the range of $10^{-1} – 10^{1}$ S cm^{-1} [15], produced directly by the oxidative aniline polymerization. During the direct treatment with alkali, protonated emeraldine salt converts to the blue non-conducting emeraldine base and its conductivity is reduced to $10^{-10} – 10^{-8}$ S cm^{-1} [15]. The completely oxidized form, blue pernigraniline salt, is observed as an intermediate during the oxidation occurring in the presence of the excess oxidant. Deprotonation of pernigraniline salt forms a violet non-conducting pernigraniline base. Alternatively, emeraldine can be reduced to colorless non-conducting leucoemeraldine [15-17].

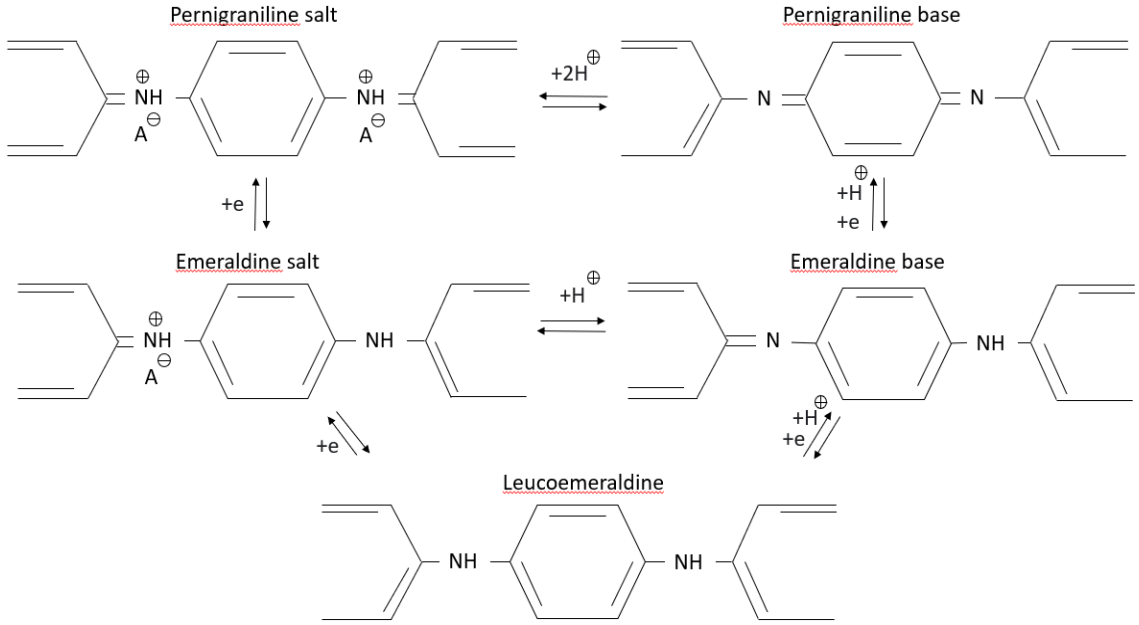


Fig. 1 Polyaniline forms and their interconversions.

In order to understand the chemical nature of PANI, it is necessary to correlate both the colour and conductivity with the structure compatibly with the conditions of the polymer preparation. Synthesis of PANI is performed by chemical or electrochemical oxidations. The chemical synthesis of PANI requires three reactants: aniline as a monomer, an oxidant and acidic medium. Typically, it is prepared by the oxidation of aniline with ammonium peroxydisulfate (APS) in the aqueous solution of HCl or H_2SO_4 as a dopant (Fig. 2) [18]. The reaction is exothermic, proceeds at room temperature and in a relatively short time leads to approximately 100% yield [17, 19]. Electrochemical synthesis of PANI involves the oxidation of aniline in electrolyte solutions by applied electric potential [20, 21]. Electrochemical process has several advantages including simple and cost-effective technique preparation. Nonetheless, compared to the chemical method, the electrochemical method provides a lower yield [17].

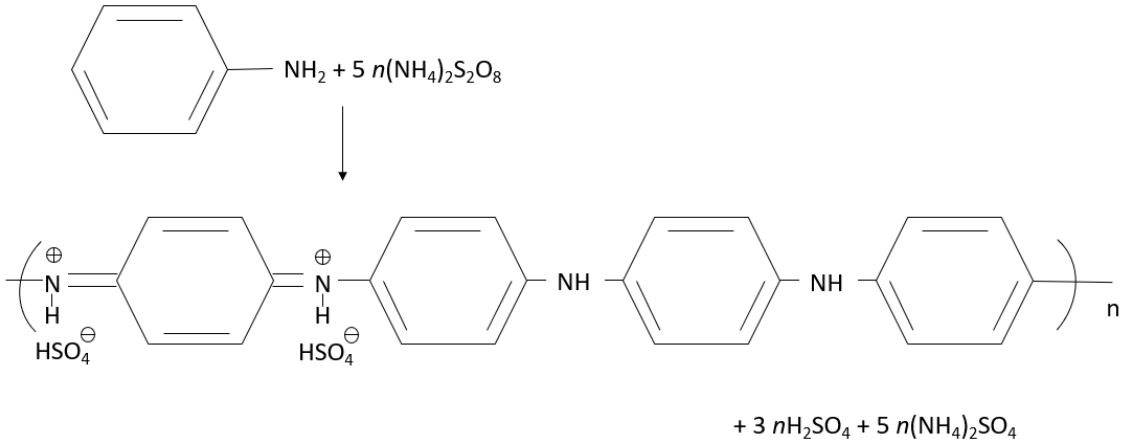


Fig. 2 Oxidation of aniline hydrochloride by peroxydisulfate.

2.2 Polypyrrole

The second and the most studied conducting polymer is polypyrrole. As for PANI, PPy can be synthetized using two major methods, either chemical or electrochemical. Even though the electrochemical oxidation has been extensively studied [22-24], its mechanism has not been fully understood yet. The polymerisation provides an electroactive, high-conducting film attached to the electrode surface. Thickness and morphology of a deposited layer may be controlled by the application of well-defined potential and known current passing through the electrochemical cell. Electrochemical deposition is irreversible and can be performed from various solvents (e.g. acetonitrile, water, etc.) The resulting properties of the films, their morphology and electrochemical behaviour depend on the conditions of preparation, including the nature of solvent, pH of the electrolyte, purity and concentration of the initial monomer, and nature and concentration of electrolytic salt [25, 26]. Where a high reaction yield is required, it is preferable to use chemical polymerization method. The principle of chemical polymerization of PPy is the same as for other conducting polymers. Polypyrrole prepared by oxidation of the monomer with chemical oxidants has a form of black powder (Fig. 3).

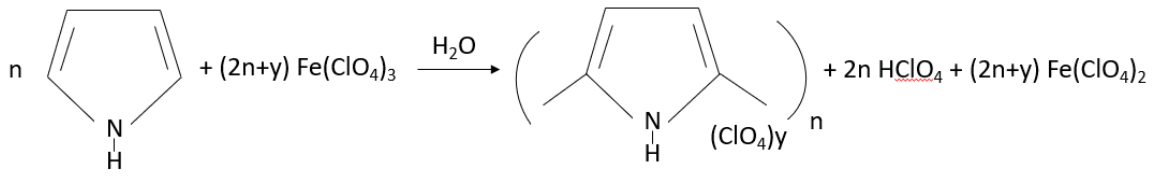


Fig. 3 Pyrrole oxidation by ferric perchlorate.

3. CONDUCTING COLLOIDAL DISPERSIONS

The combination of intrinsic electrical conductivity with simple preparation and modification procedures of conducting polymers indicates their promising utilization. Unfortunately, it is difficult to process them further once they are synthesized since their molecular structure defines them as non-thermoplastic [27, 28], mechanically rigid, brittle [28, 29] and insoluble after the synthesis. One possibility to overcome these processability issues is to prepare conducting polymers in a colloidal form [4, 15]. The efforts towards enhancing the CPs processability using the colloidal approach commenced with the preparation of polyacetylene colloids. Since 1986 several groups have described the preparation of spherical sub-micron polypyrrole colloidal particles in the aqueous media. Armes et al. stabilized the conducting PANI particles sterically by an outer layer of physically adsorbed polymeric surfactants, such as methylcellulose, poly(viny1 alcohol-co-vinyl acetate), poly(*N*-vinyl pyrrolidone) (PVP) and poly(viny1 pyridine-co-butyl methacrylate). Although most initial attempts to produce colloidal PANI by analogous methods have resulted in macroscopic precipitation of the polymer due to the inefficient adsorption of a stabilizer, in certain cases a low yield of colloidal PANI was reported. To achieve colloidal stability of PANI particles and avoid the problems associated with physical adsorption/desorption of a stabilizer, another synthetic approach consisting of graft copolymerization of aniline onto the suitable polymeric surfactant was used [30, 31]. Finally, following the paper by DeArmitt and Armes published in 1993, anionic low-molecular-weight surfactants have been also applied to prepare colloidal forms of conducting polymers. The activities in the field of conducting colloids have been reviewed by Armes and Stejskal [15].

Conducting colloidal dispersions have a wide application potential. The dispersions prepared in the presence of conventional synthetic stabilizers can be used, for example, for the preparation of anti-corrosion coatings, electronic components and circuits, batteries, light emitting diodes or sensors [15]. Nonetheless, colloids prepared in the presence of suitable biocompatible components can be used for the preparation of composite films [7], hydrogels, scaffolds for biomedicine or tissue engineering [4].

3.1 Dispersion polymerization

Colloidal dispersions of conductive polymers, such as polyaniline and polypyrrole, are preferably synthesized by dispersion polymerization. The reaction mixture for the preparation of CPs dispersions contains: 1) a monomer; 2) an oxidant; 3) a steric stabilizer; and 4) the reaction medium. The course of the reaction proceeds in three phases, namely adsorption, nucleation and particle growth. In the first phase, the oligomer is adsorbed on the stabilizer chain. In the next phase, the adsorbed oligomers stimulate the growth of a polymer chain and the nucleus is produced. Finally, due to the mechanism of automatic acceleration,

new oligomers and polymer chains close to the nucleus form and the particle grows (Fig. 4). The reaction results in the formation of colloidally stable dispersions with the particles protected from further aggregation by the surface layer of a steric stabilizer [3, 15]. Considering conducting colloidal PANI, its standard preparation according to IUPAC Technical Report should be noted [32]. Using this procedure, colloidal PANI is prepared by oxidation of 0.2 mol L^{-1} aniline hydrochloride (AH) with 0.25 mol L^{-1} APS in the aqueous medium containing the stabilizer. The AH (259 mg) is dissolved in 5 mL of 4% stabilizer solution and APS (571 mg) is separately dissolved in 5 mL of water. The polymerization starts by mixing both solutions and is completed within 10 min. Similarly, PPy is usually prepared by oxidation of pyrrole by APS or ferric(III) chloride in a solution containing a suitable stabilizer. The presence of a stabilizer plays an important role, as without its addition powder PANI is formed.

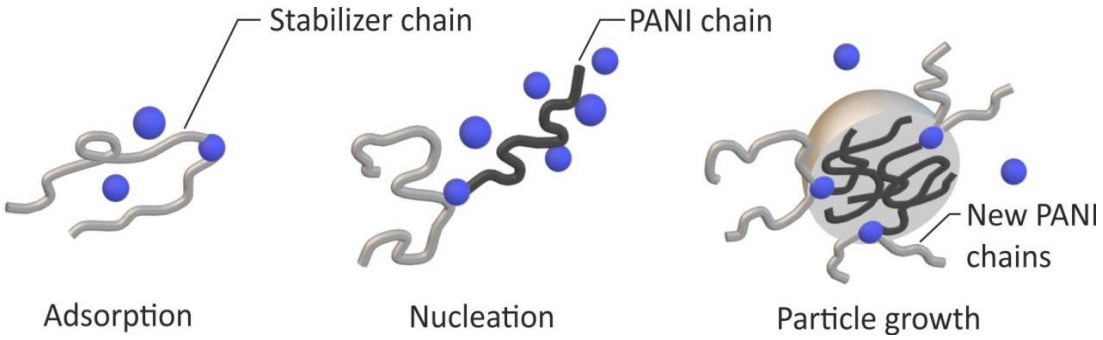


Fig. 4 The course of formation of colloidal conducting particles in the presence of a stabilizer, polymer chain.

3.1.1 Enzymatic polymerization

Enzymatic syntheses is carried out in milder conditions and has been mainly used to obtain water soluble complexes of CPs with polyanionic templates [33]. Enzymatic polymerization has been conducted with the aid of enzymes such as glucose oxidase [34], horseradish peroxidase [35] or laccase [35], which catalyse formation of respective free radicals [36]. However, the most investigated enzymes for the polymerization of conducting polymers are peroxidases. In particular, soy peroxidase and horseradish peroxidase (HRP), which share many similarities, such as presence of iron at the active centre [37]. The catalytic cycle of these peroxidases is initiated with their oxidation by hydrogen peroxide (oxidizer). The active forms of the enzyme oxidize two aromatic molecules to produce free radicals. The generated radicals diffuse into the reaction medium and combine to form dimers, trimers, further oligomers and eventually polymers through a nonenzymatic reaction, implying step growth by addition polymerization followed by propagation and termination sequences, similar to that occurring in classical chemical oxidation polymerization [36, 38]. In enzymatic synthesis, the oxidation rate is mainly dependent of the amount and

activity of the enzyme, and in contrast with chemical oxidation, it is a non-autocatalytic reaction [33].

4. BIOPOLYMERS

The growing ecological and environmental consciousness has driven efforts to develop new innovative materials. Therefore, an increased attention has been placed not only on the materials from renewable natural sources, but also on materials exhibiting a biocompatibility ensuring the contact with living systems without initiating a damaging effect [39]. In this respect, biopolymers may be suitable candidates. Strictly speaking, biopolymers are macromolecular substances produced by biochemical processes in the body of living organisms. They are formed by polycondensation of basic structural units (monomers) linked by a covalent bond. The combination of the same or different types of monomers results in the formation of a linear chain which can occupy a number of conformations in space. Due to their chemical structure, they are biodegradable, non-toxic and environmentally beneficial [40, 41].

4.1 Sodium hyaluronate

Sodium hyaluronate (SH) is the sodium salt of hyaluronic acid (HA) which is a naturally occurring polysaccharide [42, 43]. The linear macromolecule of HA consists of a disaccharide unit composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, interconnected alternately by β (1→ 4) and β (1→ 3) glycosidic bonds. From the HA structure is clear that this polymer readily binds water due to a large number of hydroxyl groups and is soluble in water due to the charge repulsion induced by carboxyl groups [42, 44]. In the solution, SH is organized in a double-twisted helix (tertiary structure). This arrangement is stabilized by hydrogen bonds between amide and carboxyl groups, van der Waals forces, mutual repulsion of carboxyl groups, and electrostatic interactions. The combination of these interactions allows the packing of a large number of SH molecules side by side and thus forming the stable polymer network [45]. The viscoelastic nature of HA, together with its biocompatibility and non-toxicity, predetermines it to many clinical applications. It is used in orthopaedics, ophthalmology [46, 47], dermatology [48, 49], in the pharmaceutical products designed for topical applications, injections or as functional components of nebuliser solutions, in the biomedicine [50, 51] or tissue engineering [52-54].

4.2 Chitosan

Chitosan is the only naturally occurring cationic polysaccharide. It is essentially a copolymer composed of two basic subunits, 2-amino-2-deoxy-β-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose linked by a β (1→ 4) bond. Chitosan is characterized by its poor solubility, ionic character in the

solution, and the presence of three-dimensional networks formed by hydrogen bonds [40]. Chitosan is insoluble in organic solvents including water. It is, however, soluble in the acidic aqueous media with a pH lower than 6.5. Its solubility in the acidic environment is facilitated by the presence of free amino groups in the polymer chain. At low pH, these amino groups protonate and chitosan behaves as a polycation [55]. In the solution, chitosan is able to form a dense elastic network due to the covalent bonds between the chitosan chains. Therefore, chitosan contributes to the stabilization of dispersion systems both by its gelling capabilities and by electro-steric stabilization mechanisms [56]. Previously, Cruz-Silva et al. [57] reported on the application of chitosan as a steric stabilizer in the enzymatic synthesis of PANI colloids. Such prepared colloidal particles had a strong pH-dependent colloidal stability and performed a rapid flocculation in the near-neutral or alkaline media. In addition to its beneficial stabilization properties, chitosan owns an intrinsic antibacterial nature and the ability to be formed in various geometries and shapes, including porous structures. Behzad Mohammadi et al. [58] utilized the unique antimicrobial properties of chitosan in conjunction with conducting polymers and examined the physical, electrical, and mechanical properties of chitosan–PANI nanocomposite films. The most important fields where the advantageous properties of chitosan are employed include the pharmaceutical, biomedical applications and tissue engineering [59]. As an example, the study of Di Martino et al. [60] described the use of chitosan for the orthopaedic tissue-engineering where it is employed as a part of supportive scaffolds for the cell ingrowth and osteoconduction.

5. CONDUCTING THIN FILMS

As already mentioned, conducting polymers are flexible organic electronic materials extensively utilized in the multidisciplinary fields. They can be prepared in a variety of different forms, powder particles of different sizes and shapes, colloidal dispersions, or thin films. Nevertheless, the most attractive forms of conducting polymers suitable for commercial applications are the films. In the context of biomedicine, films are for example used in the sensors, detectors, or indicators. One of the most common uses of conducting films is the coating of various surfaces to form efficient bioactive interfaces between living tissues and electronic devices. Chen et al. successfully developed flexible sub-ppm ammonia gas-sensing films based on PANI nanoparticles on the porous poly(vinylidene fluoride) substrate prepared by in situ chemical oxidative polymerization [61]. David et al. [62] developed a new glucose label-free biosensor. Using electropolymerization, they incorporated glucose oxidase in a multilayer film on a gold electrode, previously modified with a film of the conducting polymer PEDOT. The analytical properties of the glucose biosensor were determined and showed a substantial enhancement of the biosensing sensitivity in the presence of PEDOT. A few reviews dealing with the synthesis and applications of CPs for

sensing of various substances have been published [63, 64]. Most of the reviews are based on the sensory principles, such as gas sensors [65], potentiometric ion sensors [66], and electrochemical biosensors [67].

5.1 Dispersion polymerization

As with the dispersion polymerization resulting to formation of colloidal particles, the film-forming mixture contains a monomer, oxidant and steric stabilizer [15]. The process of PANI film formation comprises three phases (Fig. 5). It starts with the oxidation of aniline when a cationic radical is formed. Electrophilic substitution of the aniline molecule for hydrogen then produces a dimer. Dimers are transformed to higher, more hydrophobic oligomers that are readily adsorbed on various types of surfaces. The stabilizer increases the surface area of the available interfaces in the solution and the PANI chain grows on the dissolved polymer as well as on the surface. The first PANI chain anchored at the surface produces a nucleus of the future film. New oligomers are adsorbed close to the nucleus and stimulate the growth of new PANI chains. These PANI chains forming a film thus proliferate along the surface. Due to the change in the ratio of available surface area to the amount of monomer, the films formed by dispersion polymerization are thinner in comparison to PANI films grown without a stabilizer. The structure of the films prepared from colloidal dispersions is not formed by brush-like chains, as it is the case with standard PANI films (Fig. 6 a), but the chains lie loosely twisted on a support and they prevent the other reaction centre from obtaining the contact with the bulk reaction medium (Fig. 6 b). As a result, dispersion films are much more isotropic than the standard PANI films [32, 68]. Similarly to PANI, PPy is transformed into its radical cation after the oxidation and then two such radicals form a pyrrole dimer. Finally, a large number of pyrrole dimer cations interact with radical cations to form the polymer chain of PPy [69]. Nevertheless, PPy films produced by dispersion polymerization in conjunction with the preparation of colloidal dispersions are rarely described [70, 71].

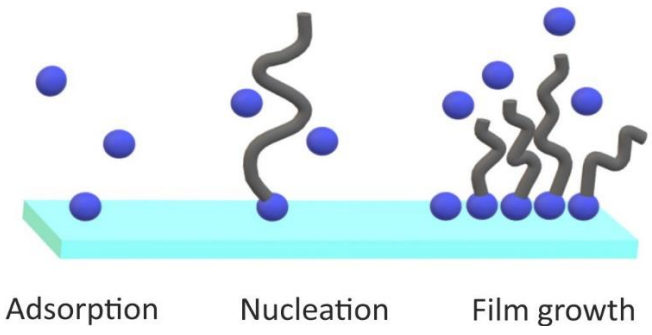


Fig. 5 The model of in situ formation of conducting films.

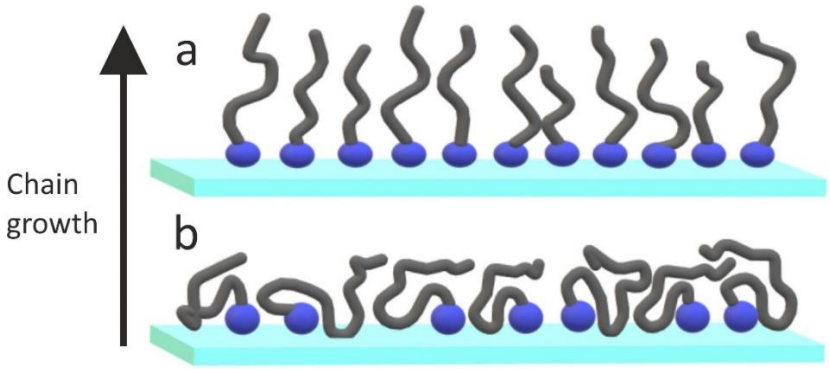


Fig. 6 The model of the structure of: a) brush-like film of standard polyaniline and b) film prepared of colloidal dispersion.

6. AIMS OF DOCTORAL THESIS

With respect to the preparation of biocompatible stimuli-responsive materials, colloidal forms of conducting polymers exhibit attractive properties. However, the synthesis and properties of these materials are yet to be sufficiently investigated. Therefore, the general aim of the thesis was to increase our understanding of conducting colloids, especially polyaniline (PANI) and polypyrrole (PPy), and explore their preparation, stabilization, and physico-chemical and biological properties. This general aim further developed towards applying colloidal particles in the preparation of conducting composite films and finally towards developing enzymatic synthesis as a novel approach to the preparation of conducting colloids. The fulfilment of these aims was achieved by accomplishing the following objectives:

- To investigate the optimum composition of PANI in colloidal form and to establish a procedure for its chemical synthesis with the support of polysaccharide-based biocompatible stabilizers, namely chitosan and sodium hyaluronate.

- To investigate the properties of PANI colloidal dispersions as suitable precursors for the preparation of conducting layers/coatings/films and to use these dispersions practically to prepare thin composite films. More specifically, the goal of the thesis was to conduct film synthesis *via* the *in-situ* polymerization of aniline hydrochloride with ammonium peroxydisulfate in the presence of the above-named polysaccharides.

- To develop and verify a procedure for the enzymatically-catalysed polymerization of conducting polymers. Specifically, to prepare PANI colloidal particles stabilized with poly(vinylalcohol) and chitosan with the aid of peroxidase enzymes, and to compare enzymatically and chemically synthetized colloids with respect to their physico-chemical properties.

7. EXPERIMENTAL

For clarity, sections Materials, Methods, Result and Discussion together with Summary of individual aims of the thesis are divided into:

a) *Study on CP colloidal particles:* the initial study dealt with the synthesis, characterization, and biocompatibility of PANI colloidal particles prepared by the oxidative polymerization of aniline hydrochloride in the presence of the bioactive polysaccharides chitosan and sodium hyaluronate. The main aim of the study was to find a suitable composition for the reaction mixture, one which could provide stable conducting colloids, and to characterize the colloids with respect to their physicochemical and biological properties in terms of cytotoxicity and antimicrobial efficacy. This study was conducted to look more closely into colloids stabilized with polysaccharides.

b) *Study on conducting dispersion films:* the follow-up study was aimed at using colloidal dispersions for the preparation of novel composite films combining conducting PANI with biocompatible polysaccharides. The films were synthetized *via* the *in-situ* polymerization of precursors in the presence of sodium hyaluronate or chitosan. In addition to the physicochemical characteristics of these materials, the study focused on investigating their antibacterial activity and mainly their cytocompatibility with human induced pluripotent stem cells (hiPSC).

c) *Study on enzymatic polymerization of PANI:* the aim of the study was to prepare PANI colloidal particles with the aid of the peroxidase-mediated polymerization of aniline. The study represents the currently preferred approach to the preparation of such materials – green synthesis. The successfully prepared PANI colloids stabilized with chitosan or PVA were described by means of their physicochemical and biological properties. In addition to the antibacterial properties of the resulting colloidal dispersions, their cytotoxicity was determined on fibroblasts and macrophages, together with their immunomodulatory effect on macrophages and neutrophils.

7.1 Materials and Methods

Reagent-grade aniline hydrochloride (AH), ammonium persulfate (APS), chitosan (CH), poly(vinyl alcohol) (PVA), *p*-toluenesulfonic acid (TSA), and horseradish peroxidase (HRP) were purchased from Sigma Aldrich (Germany). Sodium hyaluronate (SH) was acquired from Contipro a.s. (Czech Republic). Reagent-grade Aniline (≥ 98 %) was obtained from PENTA (Czech Republic). Hydrogen peroxide (30 %) was purchased from Kittford (Czech Republic). In all studies, Milli-Q water was employed.

CP colloidal particles were prepared *via* the oxidation of AH with APS in the presence of each of the sodium hyaluronate or chitosan as polymer stabilizers. The SH solution was prepared by the dissolution of SH in demineralized water; correspondingly CH was dissolved in 1M aqueous hydrochloric acid. In both cases, the dissolution was carried out under stirring at 55 °C overnight. Chitosan solutions were then filtered to remove any non-dissolved polymer residues. Aniline hydrochloride was dissolved in 5 mL of the respective polymer solution. Polymerization was started by adding 5 mL of aqueous solution APS to the reaction mixture. Concentrations of AH and APS are given in Tab. 1. The mixture was stirred for 10 min and then left at rest to polymerize. Polymerization was completed within 24 h. Colloidal dispersions were purified through exhaustive dialysis using Spectra Por 2 dialysis tubing (molecular weight cut-off 12 000–14 000; Spectrum Laboratories Inc., USA) again 0.2 mol L^{-1} hydrochloric acid solution.

Tab. 1 Compositions of the reaction mixtures of PANI colloids stabilized with sodium hyaluronate (SH) of two different molecular weights and chitosan (CH).

Sample	Stabilizer (wt.%)	AH (mol L^{-1})	APS (mol L^{-1})
SH-A1	1.0	0.2	0.1
SH-A2	1.0	0.2	0.05
SH-B1	2.0	0.2	0.05
CH-A1	2.0	0.2	0.01
CH-A2	2.0	0.1	0.01

The samples from were analysed by several analytical methods, such as by UV-vis spectroscopy, dynamic light scattering (DLS) and transmission electron microscope (TEM). Biological properties testing included MTT assay and determination of minimum inhibitory concentration (MIC).

Conducting dispersion films were formed in situ from colloidal PANI dispersions directly on the substrates. In the production of film stabilized by sodium hyaluronate, APS (0.1 mol L^{-1}) was dissolved in water and AH (0.2 mol L^{-1}) was similarly dissolved in aqueous solution of sodium hyaluronate (SH). SH solution (1 wt.%) was prepared under the corresponding conditions as in the study on CP colloidal particles. The polymerization of AH was initiated by mixing the solutions of AH/SH and APS. Polymerization was completed within 4 h (PANI-SH). Similar films were prepared with CH as a stabilizer. Chitosan (2 wt.%) was prepared in the same way as in the previous study. An APS solution (0.25 mol L^{-1}) was added to the solution containing AH (0.2 mol L^{-1}) and CH. The oxidation of AH was allowed to proceed for 12 h (PANI-CH). The procedure was repeated and a second layer of the film was deposited on this first layer. The supports with deposited both types of films (PANI-SH and PANI-CH) were rinsed with 0.2 mol L^{-1} hydrochloric acid, followed by methanol, and allowed to air dry. Standard PANI films, used as a reference surfaces, were prepared in a similar manner without any stabilizer [32] and designated as PANI.

Physico-chemical properties of PANI films was analysed by infrared spectroscopy (FT-IR), tunnelling atomic force microscopy (AFM) and determination of conductivity and surface energy. Biological properties were determined by testing of protein adsorption, cytocompatibility with hiPSC and antibacterial efficacy.

In the last **study on enzymatic polymerization** of PANI was colloidal PANI synthesized using both PVA or CH as the steric stabilizers. The dispersion polymerization was based on the procedure published by to Cruz-Silva et al. [57] and was carried out as follows: 1.2 g of PVA 215 μL of aniline, and 456 mg of TSA were dissolved in 20 mL water. The reaction mixture containing the steric stabilizer, the aniline, and TSA was kept under vigorous stirring in a water/ice bath for 6 h. Afterward 2,0 mL of a freshly prepared enzyme solution in Milli-Q water (1.2 mg mL^{-1}) was added to the reaction mixture under stirring. The reaction was initiated by adding 2 mL of a hydrogen peroxide (3.75 wt.%). The hydrogen peroxide solution was added dropwise to stirred reaction mixture over a time of 2 h. The resulting dispersion of PANI composite particles (PANI-PVA) was dialyzed against 0.2 mol L^{-1} hydrochloric acid (molecular weight cut-off 12 000–14 000; Spectrum Laboratories Inc., USA). For the production of colloidal particles stabilized with chitosan (PANI-CH), 0.040 g of CH and 0.215 mL of aniline were added to 20 mL of Milli-Q water, after which TSA was added slowly until a pH of 3.0 was achieved. The procedure for particle production was the

same as that described above for PANI-PVA. A reference PANI sample prepared (designated PANI-N) was produced in a similar manner as PANI-PVA, however, with absence of PVA stabilizer.

Enzymatically prepared colloids were investigated by UV-vis spectroscopy, DLS, TEM and by AFM. Biological properties including cytotoxicity, immunomodulatory effect together with the MIC were determined.

7.2 Results, discussions

The presented doctoral thesis is focused on the preparation of biocompatible colloidal systems and conducting films systems stabilized by biopolymers, especially sodium hyaluronate (SH) or chitosan (CH). Investigation of physico-chemical and biological properties of the system with regard to their use in biomedical or tissue engineering.

Study on CP colloidal particles

The objective of this study was to contribute to a more detailed investigation of these promising systems and to formulate colloidal PANI dispersions with enhanced bioavailability based on each of the two above-mentioned polysaccharides. In present study, the colloids were prepared by means of the oxidative polymerization of AH with APS in the presence of each stabilizer, with various ratios of reactants in the reaction mixture. The most important findings are discussed below briefly.

The preparation of polysaccharide-based PANI colloids was carried out using a modified protocol for the synthesis of colloidal PANI stabilized with PVP [32]. In the case of SH with a molecular weight of between 1.8 and 2.1×10^6, only a 1% solution was employed, as SH is known to form highly viscous gel-like solutions at concentrations above 1 wt.%. Concentrations of chitosan in the reaction mixture were kept at the usual level of 2 wt.% during synthesis. DLS analyses as well as visual inspection revealed that SH-based samples synthetized with 0.1 and 0.05 mol L^{-1} APS were of colloidal character, irrespective of the molecular weight of polymer used. The colloidal particles, however, had different sizes (Tab. 2) and, not surprisingly, the lower-molecular-weight SH afforded colloidal particles with smaller diameters. The samples stabilized with lower-molecular weight polymer (SH-B1) polymerized with 0.05M APS contained the smallest particles (574 ± 5 nm). In contrast, the largest particles were observed in sample SH-A1 prepared in the presence of 0.1 mol L^{-1} APS and higher-molecular weight SH. Depending on the composition of the reaction mixture, PANI colloids prepared in the presence of chitosan of "average" molecular weight (CH-A) contained particles with sizes ranging from 376 to 512 nm and PDI ranging from 0.26 to 0.39 (Tab. 2). Colloids with the smallest particles were prepared with 0.01 mol L^{-1} APS with both 0.2 and 0.1 mol L^{-1} AH. Under these reaction conditions, the

particles were of 376 ± 1 nm in diameter for samples CH-A2. Particle sizing analyses therefore lead to the conclusion that the size of colloidal particles is directly influenced by the ratio of AH to APS in the reaction mixture. In the comparison with PVP-stabilized PANI colloid (average particle size 240 ± 50 nm, PDI 0.264 ± 0.116), which is the most studied and described conducting colloid [32], particles stabilized with both polysaccharides were bigger and also their size distributions were broader.

The long-term stability of the prepared colloids was determined after 6 months storage at room temperature via the measurement of their particle sizes (Tab. 2) and the obtained data evidenced that changes in particle sizes clearly depended on the composition of the reaction mixture and the used stabilizing polymer. However, only a slight variation in the colloidal behaviour of samples was observed. While the samples (SH-A1, SH-A2) stabilized with higher-molecular weight SH showed an expected increase in particle size, the size of particles in sample (SH-B1) stabilized with lower-molecular weight SH decreased. The chitosan-containing sample showed only slight changes in particle size during the storage.

Tab. 2 Z-average particle diameter (z-average ± SD) and polydispersity index (PDI ± SD) of colloidal particles prepared by the oxidation of aniline hydrochloride (AH) with ammonium persulfate (APS) in the presence of sodium hyaluronate (SH) or chitosan (CH).

Sample	After preparation		After six months	
	z-average (nm)	PDI	*z*-average (nm)	PDI
SH-A1	1.100 ± 15	0.450 ± 0.011	1.393 ± 14	0.470 ± 0.008
SH-A2	655 ± 2	0.350 ± 0.014	741 ± 5	0.300 ± 0.007
SH-B1	574 ± 5	0.280 ± 0.001	489 ± 2	0.160 ± 0.006
CH-A1	512 ± 2	0.390 ± 0.001	356 ± 2	0.220 ± 0.004
CH-A2	376 ± 0	0.260 ± 0.004	481 ± 2	0.360 ± 0.013

Investigation of morphology of colloidal particles by transmission electron microscopy revealed their differences (Fig. 7), and undoubtedly confirmed the influence of the polysaccharides on the shape and size of colloidal particles. A typical image of colloids stabilized with low-molecular-weight SH shows particles with average sizes ranging from 100–400 nm in diameter, which roughly correspond to the results obtained by DLS. The fact that sizes of particles determined by DLS are expressed as intensity weighted z-average diameters and

that TEM provides number-averaged particle sizes has to be considered in this respect. The morphology of samples stabilized with high-molecular-weight SH is more complicated and the figure depicts round particles (darker) connected by oblong rods/tubes (brighter part), which are distributed around them. The appearance of chitosan stabilized colloids is even more different, showing rod-like structures with a higher aspect ratio (Fig. 7) The morphology of colloids stabilized with two the most used synthetic polymers, PVA [32] and poly(N-vinylpyrrolidone) [16] was significantly different. The samples formed irregular rice-grain particles and were noticeably polydisperse in their sizes. On the other side, the PANI colloids prepared in the presence of biodegradable and biocompatible gelatine yielded particles with spindle-like morphology and the authors reported on their non-uniformity in size, which decreased with increasing concentration of gelatine [6]. Influence of the type and molecular weight of stabilizing polymer on the shape, size and size distribution of colloidal particles is, therefore, obvious.

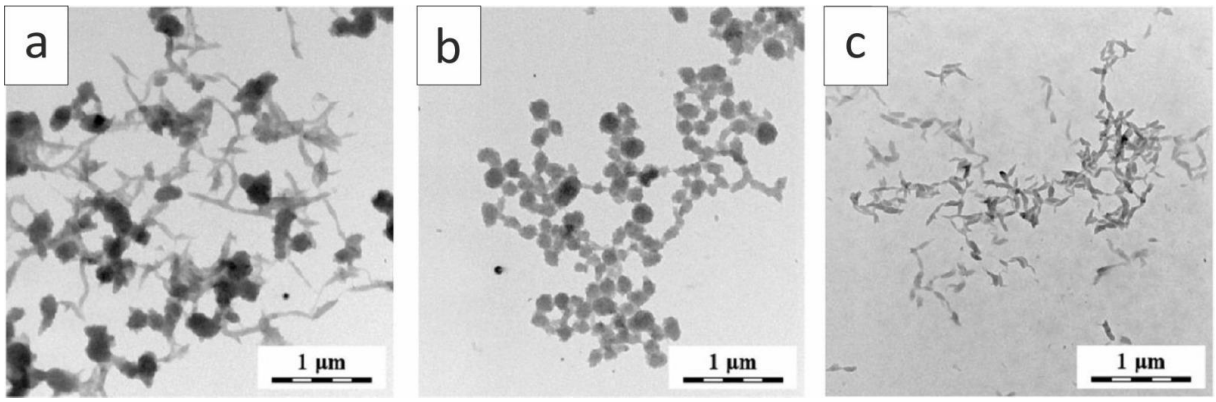


Fig. 7 Transmission electron micrographs of PANI colloids stabilized with sodium hyaluronate of a) lower molecular weight (SH-B), b) higher molecular weight (SH-A), and c) chitosan (CH-A) [7].

The course of colloid formation was monitored by UV–vis spectroscopy and corresponding spectra are compared in Fig 8. From the spectra, it is obvious that the course of the reaction and the formation of the final product are controlled both by the type of stabilizing polymer and by the composition of the reaction mixture. The formation of a colloid stabilized with chitosan in the reaction mixture containing the higher concentration of aniline hydrochloride (0.2 mol L^{-1}) proceeded uniformly with a gradual increase in absorbance over time. The lower concentration of AH in the reaction mixture (0.1 mol L^{-1}) led to lower absorbance at the reaction end when compared with the previously described sample; however, almost identical spectra were recorded for reaction times of 240 min, 480 min, and 24 h. This may indicate the termination of the reaction occurring after 240 min, due to the depletion of reactants in the reaction mixture as lower than stochiometric amount of APS was used. In the case of SH-stabilized colloids,

in addition to the concentrations of AH and APS, the reaction course could also be influenced by the molecular weight of the polymer; however, the resulting colloids were, as for UV–vis spectra, identical. A comparison with the most studied colloids prepared in a similar way but stabilized synthetic polymers may be useful. Stejskal and Sapurina [32] reported UV–vis spectra of PANI films prepared by dispersion polymerization in the presence of water-soluble polymer, poly(*N*-vinylpyrrolidone). The spectra of PVP-based colloid are characterized by two maxima located at $\lambda = 356$ and 852 nm, when determined in 1 mol L^{-1} HCl. Whilst the first absorption band arises from the $\pi - \pi^*$ electron transition within the aromatic ring, the second is typical for presence of polaron states (charged cation radicals) and is assigned to $\pi -$ polaron and polaron $- \pi^*$ transitions. Stejskal et al. [72] also recorded the absorption spectra of colloidal PANI stabilized with PVA. They reported on an absorption band at 430 nm which is often merged with the above-mentioned peak located at around 356 nm into a flat or distorted single peak and represents $\pi - \pi^*$ transition. In comparison with these data, the recorded spectra from SH colloids (Fig. 8) are more similar to those recorded for the mentioned PVA-based colloid, exhibiting maxima at 430 and 800 nm. The maximum at about 356 nm is absent and a plateau is observed in the wavelength region from about 300–400 nm. Also, the maximum in the red region is less notable in comparison with PVP based samples. On the other hand, the spectrum recorded for CH colloids includes distinct maxima at 390 nm and 800 nm and is, therefore, more similar to spectra from samples containing PVP.

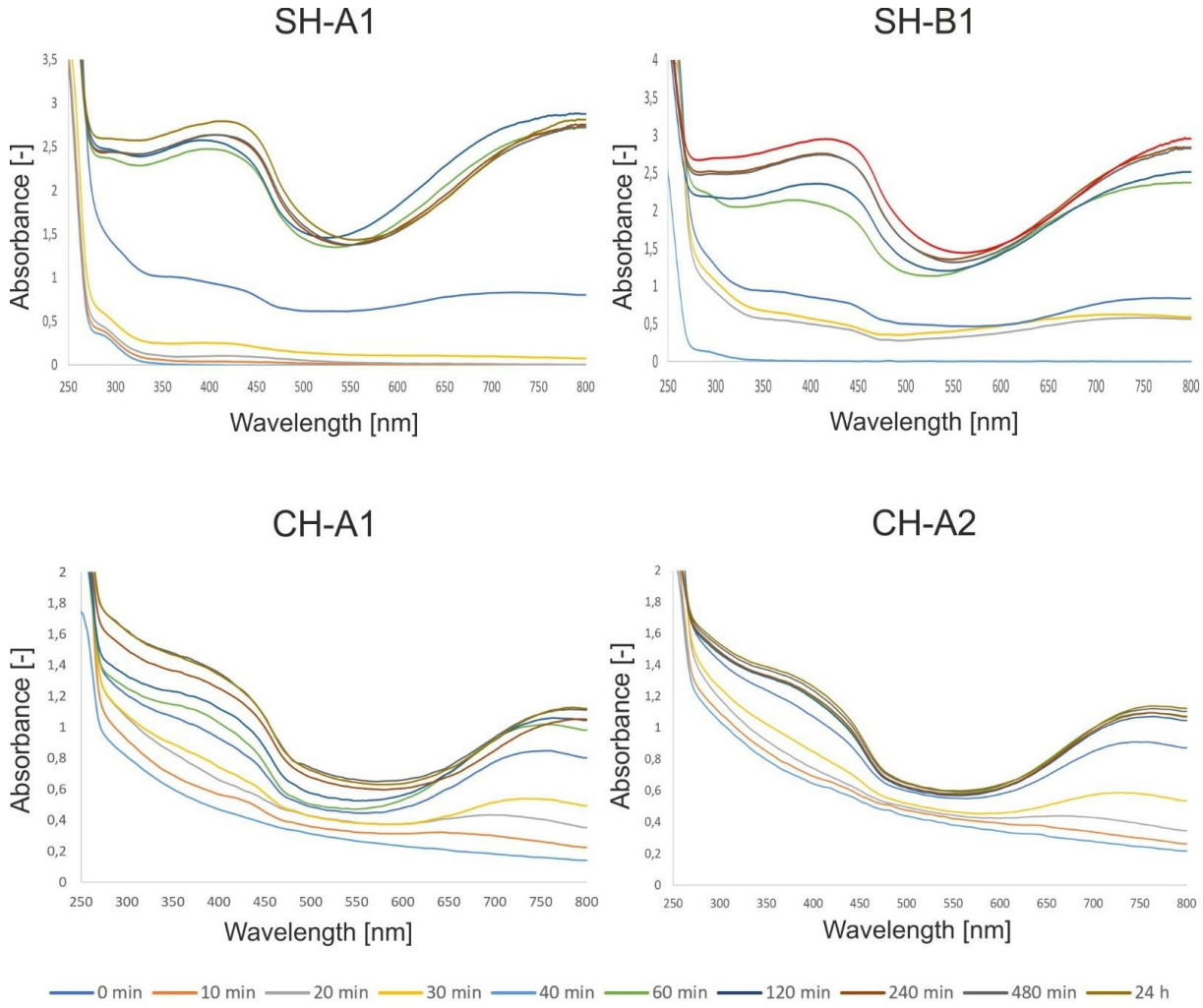


Fig. 8 The course of formation of colloidal PANI prepared with SH and CH as stabilizers followed by the changes in UV–vis spectra.

In conclusion, the physico-chemical characteristics of prepared colloids proved that all their properties depend upon composition of reaction mixture in terms of concentrations of reactants, molecular weight and type of the stabilizer.

The following findings resulted from the examination of biological properties conducting colloids. The tests on cytotoxicity performed on mouse embryonic fibroblasts (NIH/3T3) demonstrated that both hyaluronate and chitosan stabilized colloids have low toxicity, which mainly depends on the concentration of polyaniline in the respective sample. The threshold for the absence of cytotoxicity (cell viability higher than 80 %) appeared at PANI concentration of 465 μg mL^{-1}. The antibacterial testing evidenced activity of colloids both against gram positive *S. aureus* and gram negative *E. coli* strains. The best-performing sample was the polyaniline stabilized with sodium hyaluronate (molecular weight 1.8–2.1×10^6 g mol^{-1}), which had minimum inhibitory concentration of 1550 μg mL^{-1}. Colloidal polyaniline dispersions can be considered as promising materials

applicable in their original colloidal form as well as precursors for preparation of conducting layers mediating contact with cells and tissues capable to respond to electrical stimuli (e.g., cardiac or nervous cells).

The results of the study were published in *"Kašpárková. V., et al. Polyaniline colloids stabilized with bioactive polysaccharides: Non-cytotoxic antibacterial materials by: Carbohydrate polymers. 219 (2019) 423–430*" and served as the background for the following study aimed at the development and characterization of advanced composite films of conducting PANI.

Study on conducting dispersion films

Considering the vast amount of new possibilities conducting polymers offer, the next study included in the thesis focuses on the *in situ* preparation and more detailed investigation of novel conducting composite films produced on immersed supports during the preparation of colloidal PANI dispersions containing SH or chitosan as steric stabilizers. The novelty of the research presented here relies not only on the materials prepared but also on the characterization of the biological properties of the films when in contact with hiPSC. The physico-chemical characteristics of the prepared composite films determined here serve as a natural basis for understanding the cell behaviour on these surfaces.

Observations of film morphology demonstrated significant differences between PANI-CH and PANI-SH samples. While PANI-CH film is smooth with some adhering spherical colloidal particles (Fig. 9 b), in PANI-SH films fibre-like structures with green elements of protonated PANI within the shell of stabilizing polymer are observed (Fig. 9 a). This morphology can be ascribed to the presence of stabilizing high-molecular-weight SH polymer which displays a similar fibrillar morphology in the absence of PANI as well.

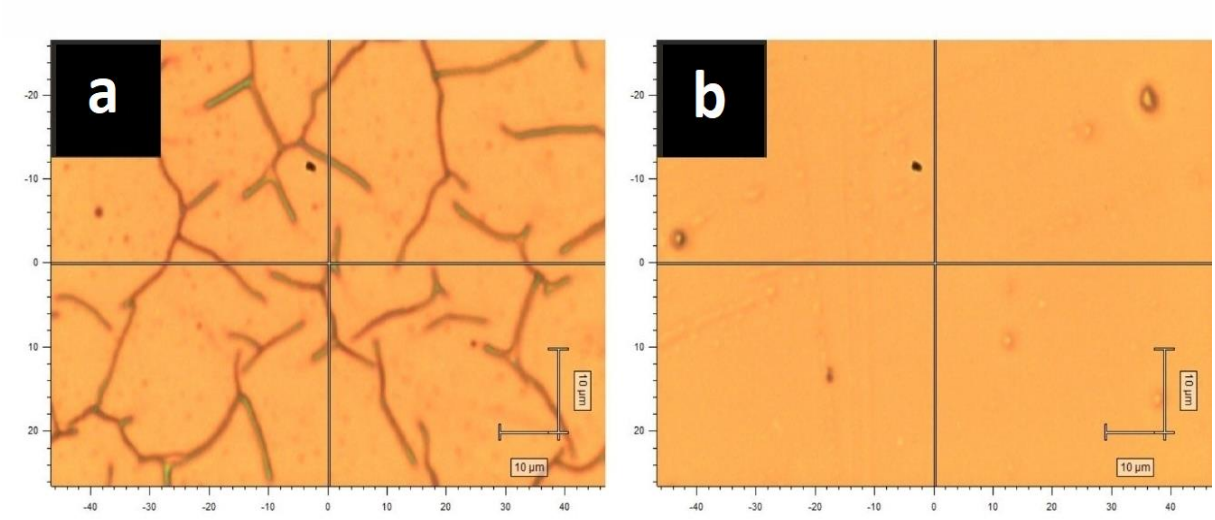


Fig. 9 Optical micrographs of the films of PANI-SH (a) and PANI-CH (b) on silicon window, bar is 10 µm [73].

Cell attachment and proliferation on solid surfaces depend on a number of variables including surface chemistry and topography. Roughness together with the surface electrical properties were determined using AFM. The roughness parameters *Sz* and *Sa* (Fig. 10) were significantly different between native PANI (28 nm) and both biopolymer-modified films with notably smoother surfaces. *Sa* values for PANI-SH and PANI-CH were 4 and 14 nm, respectively. Surprisingly, the SH-modified film containing a very high molecular weight polymer exhibited a rather uniform surface which might be due to the special morphology of the sample revealed by optical microscopy (Fig. 9 a, b).

As already mentioned by Wang et al. [74] the nano-scale morphology of PANI can be a factor influencing cell adhesion. Correspondingly to roughness, differences between the samples were also observed with respect to their surface electrical properties. Specifically, the average TUNA current decreased in accordance with a decreasing surface roughness in the following order: PANI> PANI-CH> PANI-SH with all values ranging from 24 to 5 pA.

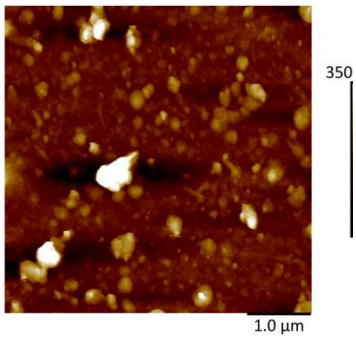
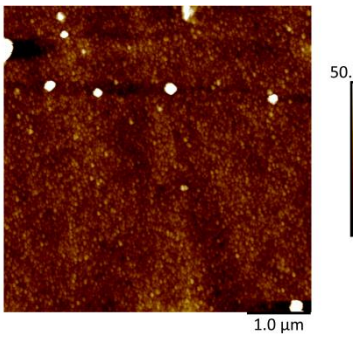
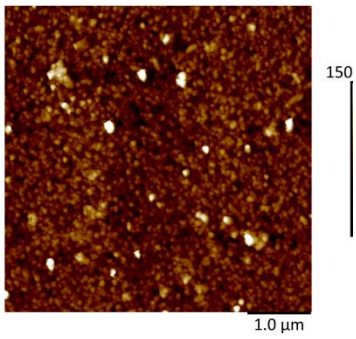
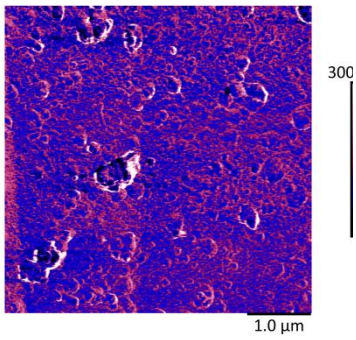
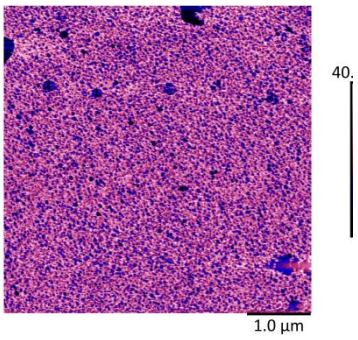
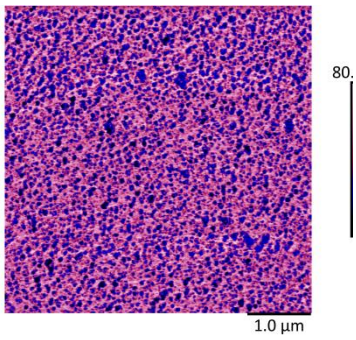
PANI	PANI-SH	PANI-CH
		
***Sz* = 577 nm** ***Sa* = 28 nm**	***Sz* = 120 nm** ***Sa* = 4 nm**	***Sz* = 236 nm** ***Sa* = 14 nm**
		
Max. TUNA current = 860 pA **Average TUNA current = 24 pA**	**Max. TUNA current = 53 pA** **Average TUNA current = 5 pA**	**Max. TUNA current = 103 pA** **Average TUNA current = 12 pA**

Fig. 10 AFM images of PANI, PANI-SH, PANI-CH films. Top images show height changes, below TUNA current maps. The scan area was 5x5 mm [73].

The value of surface energy is also important parameter to evaluate the surface properties of the material. As already mentioned, surface properties, including surface energy, can affect biological properties, such as for example cell adhesion. The measurements from the current study show that the surface energies of the films prepared in the presence of both biopolymers were different in comparison with pure PANI films (Tab. 3). The surface energies of pristine PANI films prepared in the absence of stabilizing polymers were determined earlier. [75] They differed in their values of γ_{TOT}, which were both lower for PANI-SH and PANI-CH than for PANI film without polysaccharides. Moreover, the components representing the dispersive (γ_{LW}) and polar (γ_{AB}) parts of surface energy were different for PANI-SH and PANI-CH. A significant difference was observed for PANI-CH which showed the lowest γ_{LW} value and correspondingly

the highest γ_{AB} value among the studied samples. Meanwhile, the lowest polar component was observed for PANI-SH film.

Tab. 3 Surface energy of polyaniline films prepared in situ in the absence (PANI) and in the presence of polysaccharides (PANI-SH, PANI-CH).

	Surface energy components (mN m^{-1})		
Sample	γ_{TOT}	γ_{LW}	γ_{AB}
PANI	52.5	46.1	6.5
PANI-SH	48.0	45.9	2.1
PANI-CH	46.6	35.4	11.2

Surface energy values were obtained from measurements of contact angles between the film surface and the test liquids. Three different liquids with different polarity were used to measure the contact angles, namely Milli-Q water, ethylene glycol, and diiodomethane. In general, the larger the contact angle, the higher the surface energy. This can be observed in Fig. 11 where the contact angles of PANI-CH and PANI-SH films in the presence Milli-Q water as a test liquid are shown. The average values of the contact angles of the polysaccharide films for the individual liquids are given in Tab. 4.

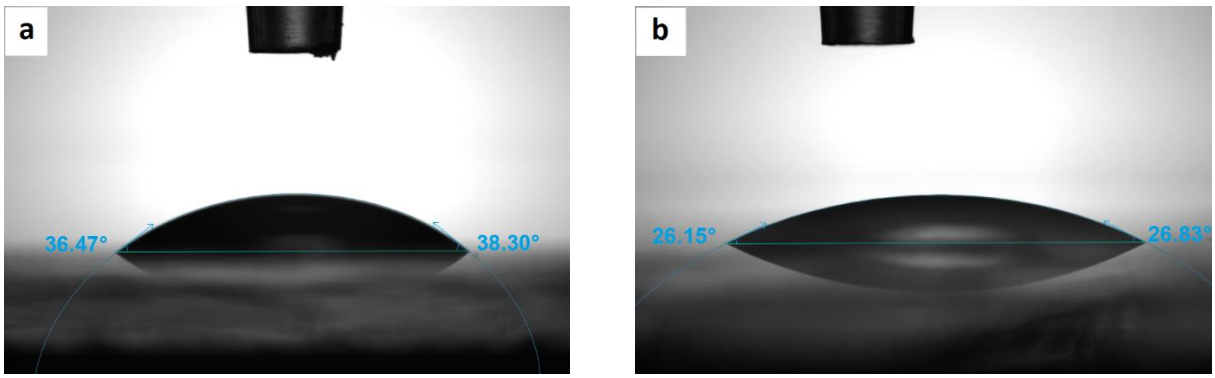


Fig. 11 Images recorded during measurements of contact angles of (a) PANI-SH and (b) PANI-CH in water.

Tab. 4 Contact angle value of polyaniline films prepared in situ in the presence of polysaccharides (PANI-SH, PANI-CH).

Test liquid	Contact angle PANI-SH (°)			Contact angle PANI-CH (°)		
	left	right	**mean**	left	right	**mean**
Milli-Q water	34.2	34.1	**34.1**	26.7	27.5	**27.1**
Ethylene glycol	15.9	17.4	**16.7**	13.1	12.5	**12.8**

Diiodomenthane	24.8	26.5	**25.7**	47.6	46.8	**47.2**

Another key surface parameter of materials prepared in this way is conductivity. Slightly higher conductivity was achieved for standard PANI films in comparison with the samples prepared from the stabilizer-containing reaction mixtures. The conductivity of pure PANI film was 5.5 ± 0.7 S cm^{-1} which is of the same order of magnitude as the typical values reported by Stejskal and Sapurina [32] for thin PANI films stabilized with PVP (2.6 ± 0.7 S cm^{-1}). Also, compressed powder of polyaniline hydrochloride exhibits the conductivity values (4.4 ± 1.7 S cm^{-1}) close to those determined in the present work. The conductivities of films containing SH or CH ranged from 1.1 to 2.3 S cm^{-1} with only insignificant differences. The results obviously show that the presence of polysaccharides in composite films did not significantly decrease the electrical properties of the products.

In summary, the physicochemical testing revealed that the presence of polysaccharides in PANI conducting films did not decrease their conductivities; however, it modified their surface energies and topography which could influence the biological properties.

The main novelty of the study rests in the comprehensive investigation of biological properties of composite films. The tests have demonstrated the ability of proteins to adsorb onto their surfaces. Here, the amounts of bovine serum albumin (BSA) adsorbed on PANI-SH films were twice those absorbed on the reference surface, while PANI-CH displayed adsorption comparable to the reference. The PANI-SH films also exhibited excellent antibacterial activity towards the common gram negative spoilage specie *E. coli*. The effect of PANI-CH was lower, and the activity of both films against gram positive *S. aureus* was only minor. The present study provides clear evidence of the unique properties of PANI/polysaccharide composite films in terms of their cytocompatibility with hiPSCs. Both used cell lines (Neod1 and M67) were able to adhere and proliferate on the tested surfaces, at least comparable to the reference material, tissue-culture polystyrene. The differentiation protocol led to cardiomyogenesis accompanied by the formation on these films of spontaneously beating clusters of cardiomyocytes. The presence of cardiomyocytes was also confirmed by immunohistochemistry through the detection of cells positive to cardiomyocyte-specific sarcomeric α-actinin within cells growing on the films. As the presence of beating clusters is one of the markers of the absence of embryotoxicity, it can also be concluded that the films do not induce embryotoxic effects.

Results of the study were summarized and published in *"Jasenská. D., et al. Conducting composite films based on chitosan or sodium hyaluronate. Properties and cytocompatibility with human induced pluripotent stem cells by: Carbohydrate polymers. 253 (2021), 117244"*.

Study on enzymatic polymerization of PANI

The aim of last study was to verify possibility to synthetize PANI colloidal particles using enzymatic polymerization of aniline in aqueous media using either poly(vinyl alcohol) or chitosan as steric stabilizers, and compare properties of enzymatically prepared polymers with those synthetized with the aid of chemical oxidation. The intention was to examine not only the physicochemical properties of the prepared colloids, but also the biological properties, especially those connected with their antioxidant activity.

The process of formation of polymer-stabilized PANI colloids and non-stabilized PANI was monitored by UV-vis spectroscopy. During the synthesis, the reaction mixtures became blue, indicating the rapid formation of PANI oligomers after starting the addition of hydrogen peroxide. This reaction step (20 s) was followed by a rapid change in colour of the reaction mixture to a dark green, indicating thus formation of PANI emeraldine salt. An exception from this course was PANI-N sample prepared in absence of stabilizer, in which the reaction mixture did not turn dark green but was brown. The UV-vis spectra of all samples (PANI-PVA, PANI-CH, PANI-N) recorded in two different media (1 mol L^{-1} HCl and water) within the wavelength range from 300 to 800 nm are displayed in Fig. 12. The UV–vis spectra of PANI-PVA colloid were similar in both media and showed presence of a broad peak at 400 nm and a peak at with maximum at 800 nm. The broad peak around 400 nm arises from the π–π* electron transition within the benzoid ring and a peak around 750 nm evidences the formation of a polaron structure. The λ_{max} of the latter peak appears shifted to 740 nm for PANI-PVA measured in HCl in comparison with spectra in water, indicating differences in the polaron delocalization. When measured at fixed acidity of the medium, in 1 mol L^{-1} HCl, the maximum absorbance in the λ range 350 and 430 nm may be taken as an approximate measure of the concentration of PANI in dispersion. The spectrum recorded for PANI-CH colloids includes distinct maxima at λ ~330 and 770 nm in water, ~300 and 780 nm in HCl. The study on CP colloidal particles, where PANI colloids were stabilized with chitosan and prepared by standard chemical oxidation with APS, showed similar spectra with distinct maxima at 390 and 800 nm. The above mentioned difference in colour of non-stabilized PANI-N (brown) in comparison with PANI-PVA and PANI-CH (green) is also evident from the spectra obtained on the PANI-N sample dispersed in water, which does not show course typical for green PANI salt [18]. After the dispersion medium was changed to 1 mol L^{-1} hydrochloric acid, the spectra have converted to their common course (Fig. 12). The reason is obvious as the experimental data published in [76] refer that oxidation products of PANI formed in basic, neutral and weakly acidic media gives rise to brown product with low conductivity.

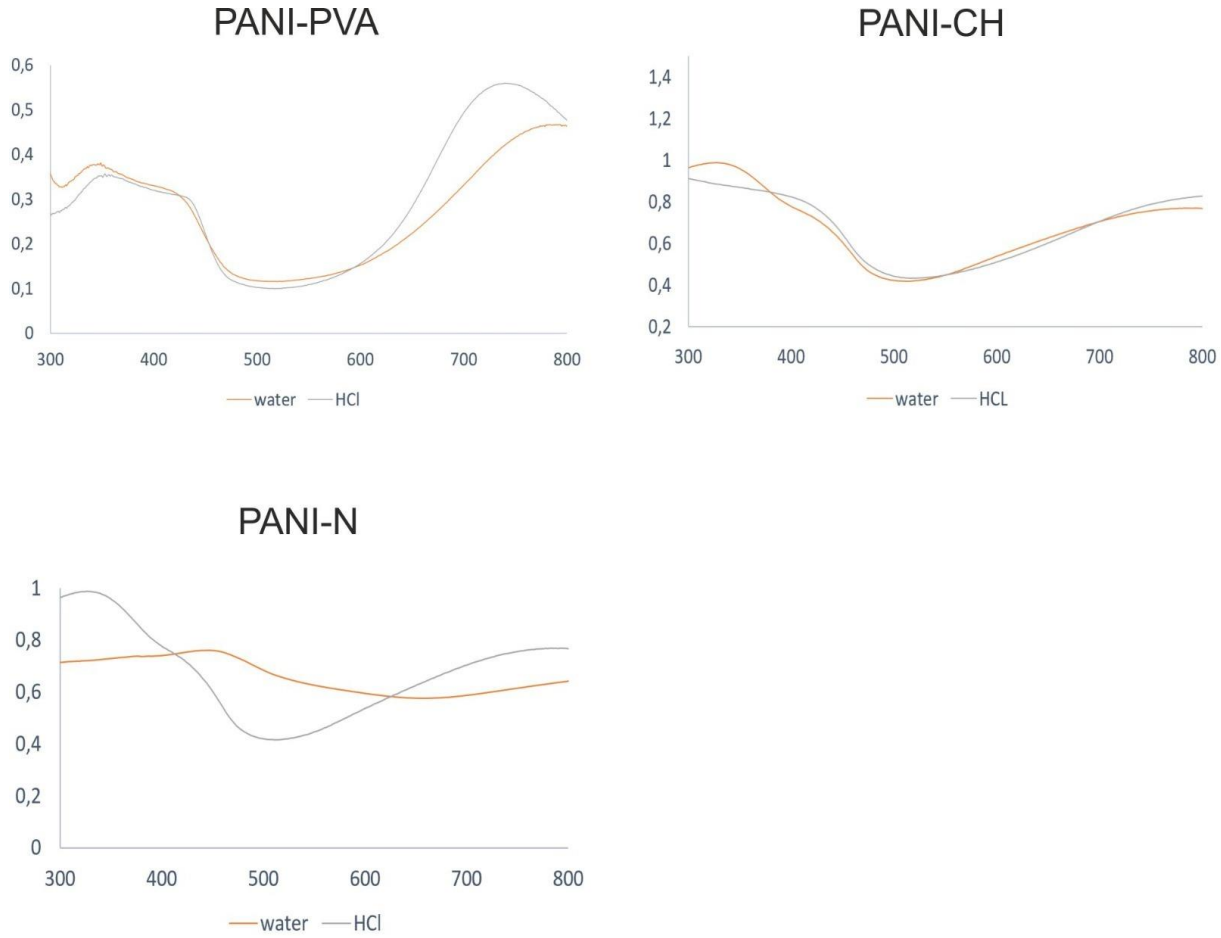


Fig. 12 UV–vis spectra of enzymatically synthesized PANI stabilized with poly(vinyl)alcohol PANI-PVA, chitosan PANI-CH and without stabilizer (PANI-N).

Particle size analyses of prepared samples revealed that samples synthetized in the presence of steric stabilizers (PANI-PVA, PANI-CH) were of colloidal character. The colloidal particles exhibited different sizes depending on the type of polymer used for their stabilization (Tab. 5), and PANI-PVA colloids were smaller in size (206 ± 2 nm) than samples prepared in presence of chitosan showing z-average diameter of 585 ± 10 nm. Widths of distributions expressed as PDI were 0.55 and 0.45 for PANI-PVA and PANI-CH, respectively. After dialysis conducted with the aim to remove residual impurities, which might compromise the biological tests, the average particle size in the samples increased. In the contrast to the PANI-PVA or PANI-CH, freshly prepared sample without stabilizer, PANI-N, was not of true colloidal character. The enzymatic reactions resulted in the formation of unstable coarse dispersions with particle sizes greater than 3 μm and sedimentation occurring soon after dialysis. Nevertheless, after homogenization, a uniform dispersion formed again, which was stable for several hours. If we compare enzymatically prepared PANI-N with PANI prepared by polymerization with APS, both without stabilizing polymer, nature of enzymatically prepared sample was different and its colloidal stability was

significantly higher. The explanation may lie in the stabilizing effect of peroxidase [77].

Tab. 5 Size (z-average diameter ± SD) and polydispersity index (PDI ± SD) of colloidal particles stabilized with poly(vinyl alcohol) or chitosan as the steric stabilizer. Comparison with PANI prepared without stabilizer.

Sample	Before dialysis		After dialysis	
	z-average (nm)	PDI	*z*-average (nm)	PDI
PANI-PVA	206 ± 2	0.50 ± 0.04	240 ± 2	0.55 ± 0.01
PANI-CH	585 ± 10	0.41 ± 0.06	655 ± 7	0.45 ± 0.03
PANI-N	2800 ± 81	0.33 ± 0.05	3500 ± 74	0.33 ± 0.13

Morphology, size and shape of prepared PANI samples were depicted using TEM or AFM. The microscopy observations by TEM proved regular, spherical nanoparticles of similar sizes with relatively narrow distributions for both PVA and CH stabilized samples (Fig. 13). The average particle diameters estimated by TEM were similar being of 126 ± 9 nm and 133 ± 13 nm for PANI-CH and PANI-PVA, respectively. In comparison with polymer-stabilized samples, individual PANI-N particles synthetized without stabilizing agent were connected to aggregates/clusters and were obviously bigger than those present in samples where polymers were used. However, as it was mentioned above the agglomerated PANI-N could be reconstituted by homogenisation to original dispersion. The TEM analyses showed, as regards particle sizes, similar trend as DLS. However, thanks to different measuring principle of both techniques, differences between the sizes of PVA and CH stabilized PANI colloids measured by DLS were bigger in comparison with TEM. Compared to PANI-based colloids prepared *via* chemical oxidation, enzymatically polymerized colloids stabilized with a CH and PVA are spherically symmetrical. After deposition on a solid substrate for TEM imaging, they do not disintegrate into randomly arranged elliptical or fibrillar structures discussed in colloidal study mentioned above.

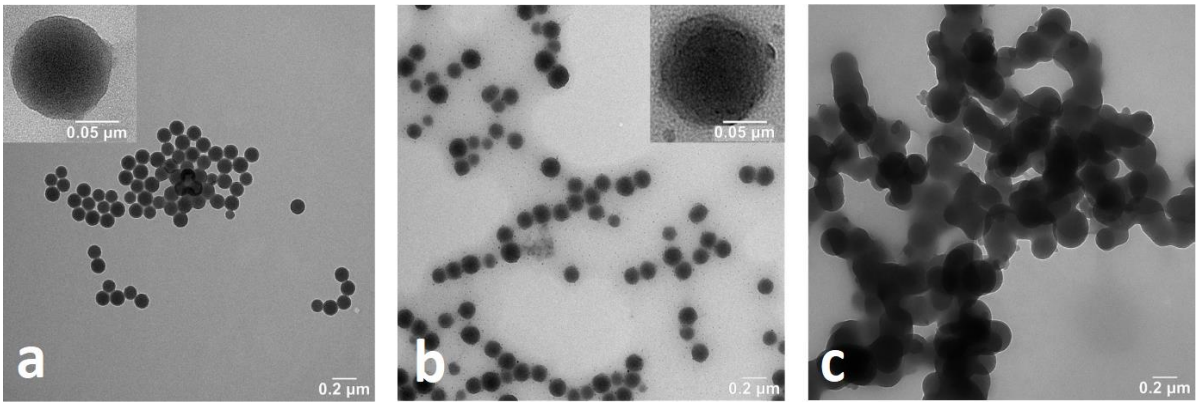


Fig. 13 Transmission electron micrographs of PANI-CH, PANI-PVA and PANI-N colloids.

Atomic force microscopy confirmed spherical shapes of the PANI-PVA and PANI-CH samples on the mica surface. In contrast, the AFM analysis of PANI-N was not feasible, which was conditioned by the size of the particles/agglomerates unsuitable for AFM analysis The particles stabilized by chitosan are characterized by average diameter ranging from 50 to 110 nm and height from 60 to 120 nm. Colloidal particle stabilised by PVA show diameter from 90 to 150 nm and height from 100 to 130 nm. The non-stabilized PANI-N forms aggregate structures with an average width and height in the order of units of micrometres on the mica surface (Fig. 14). The resulting sizes from AFM differ significantly from those measured by DLS analysis. However, they fully comply with TEM.

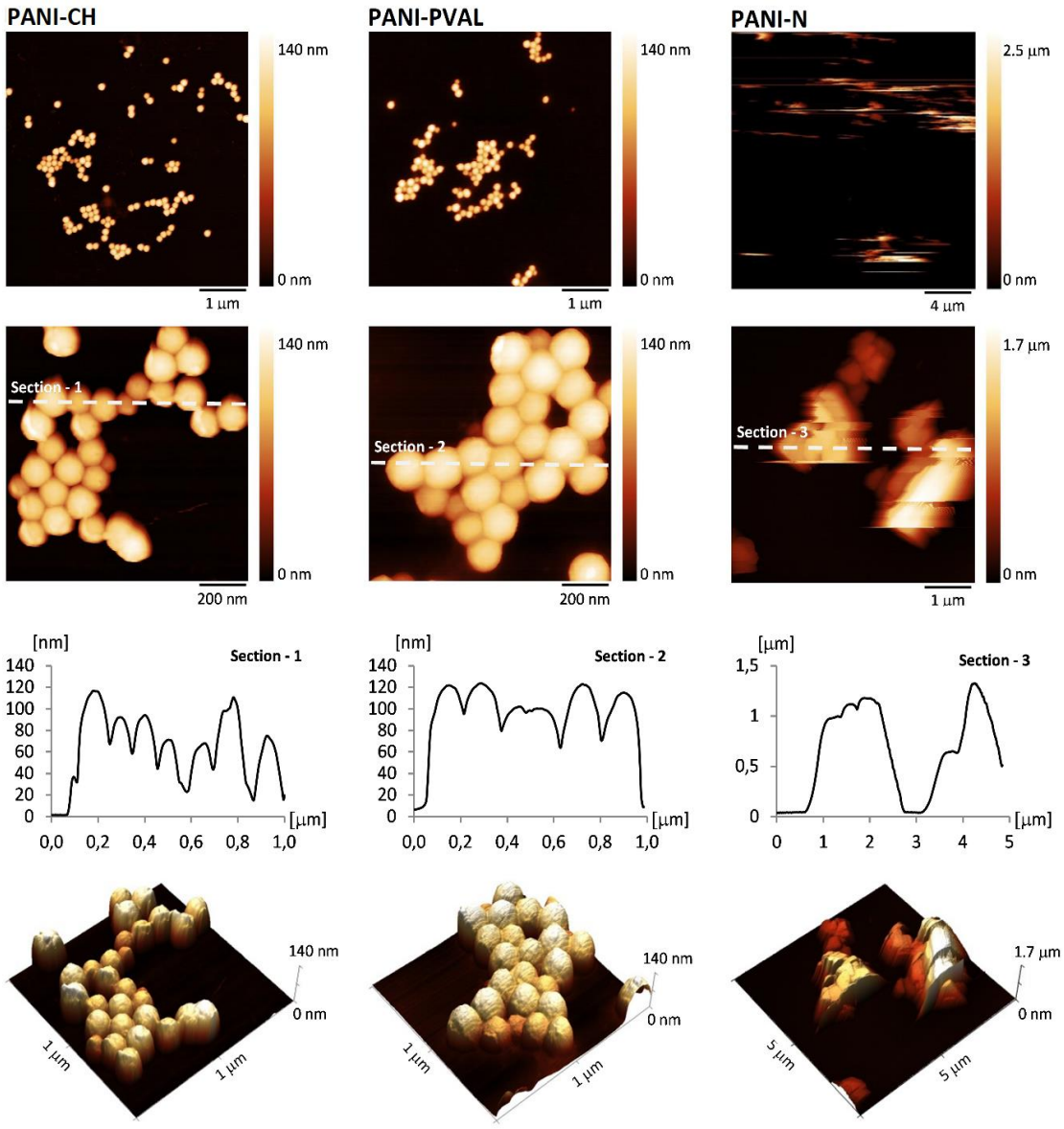


Fig. 14 Colloidal particles on mica surfaces characterized by AFM. Height images and profiles PANI-CH (left), PANI-PVA (middle), PANI-N (right) column.

In summary, this study shows the original approach to PANI synthesis employing environmentally-friendly and biocompatible horseradish peroxidase. UV–vis spectroscopy revealed the successful formation of polyaniline emeraldine salt, and testing of physico-chemical properties demonstrated the effect of the stabilizer on the morphology of colloidal particles.

Cytotoxicity testing performed on mouse embryonic fibroblasts (NIH/3T3) and macrophages (RAW264.7) demonstrated that all colloids exhibited low toxicity. Samples up to concentrations of 31 µg mL^{-1} (PANI-PVA) and 40 µg mL^{-1} (PANI-CH) did not show cytotoxicity (the cell viability was higher than 70 %). The cytotoxicity depended mainly on the polyaniline concentration and somewhat on

the type of stabilizer. All tested colloidal particles were able to reduce oxidative stress and inhibit the production of reactive oxygen species by neutrophils and inflammatory cytokines by macrophages. The anti-inflammatory effect observed was related to their antioxidant activity, especially in the case of neutrophils. The particles can be useful as active components of biomaterials modulating the early stages of inflammation.

Results of the study were summarized in *"Jasenská. D., et al. Enzyme-catalysed polymerization process: a novel approach to the preparation of polyaniline colloidal dispersions with immunomodulatory effect". The study has been submitted to Biomacromolecules.*

8. CONTRIBUTION TO SCIENCE AND PRACTICE

Although conductive polymers are a frequent subject of research due to their unique properties, such research still encounters challenges related to particular aspects of their application, such as their processability or solubility, which hinder their use in practice. The summary given below demonstrates that the outcomes of the study presented here significantly contribute to current knowledge on conductive polymers, these results achieved through research of colloidal, water-dispersible forms prepared by means of standard oxidative polymerization as well as green, environmentally friendly enzyme-assisted synthesis.

The first part of the thesis expanded knowledge concerning the preparation of conductive polymers, such as polyaniline and polypyrrole, and developed a methodology for the synthesis of biocompatible colloidal systems based on the two mentioned polymers. Specifically, colloidal, biocompatible dispersions were prepared *via* dispersion polymerization in the presence of the biodegradable polysaccharides sodium hyaluronate or chitosan by means of standard chemical oxidation. The study concludes that properties of conducting colloidal particles can be tuned both *via* the variation of precursors (aniline and oxidant) and also though the application of different types, concentrations, and molecular weights of stabilizer.

Successfully prepared colloids were used to fabricate novel thin conducting composite films. Polyaniline films based on chitosan or sodium hyaluronate exhibited interesting properties in terms of physico-chemical properties (conductivity, surface properties), biocompatibility with human pluripotent stem cells, and antibacterial activity, all of which are of practical value and applicable for the tissue engineering of stimuli-responsive tissues. The advantage of these films is that they can cover *in situ* different types of surfaces, which gives these surfaces conductivity and modifies their morphology.

The thesis also contributes to a deeper knowledge of environmentally friendly methods for the synthesis of conductive polymers. On the basis of previous experience in the field of conducting colloids, a method for the preparation of colloidal polyaniline dispersions in the presence of peroxidase enzymes instead of aggressive oxidizing agents was devised. The approach using biocompatible horseradish peroxidase illustrates a way of preparing particles with unique properties, which can be further modified by biocompatible and biodegradable stabilizers.

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LIST OF ABBREVIATIONS

Alphabetically ordered

AFM	Atomic force microscopy
AH	Aniline hydrochloride
APS	Ammonium peroxydisulfate
BSA	Bovine serum albumin
CPs	Conducting polymers
DLS	Dynamic light scattering
FT-IR	Fourier-transform infrared spectroscopy
H_2SO_4	Sulfuric Acid
HA	Hyaluronic acid
HCl	Hydrochloric Acid
hiPSC	Human induced pluripotent stem cells
HRP	Horseradish peroxidase
CH	Chitosan
IL-6	Inflammatory cytokine interleukine-6
IUPAC	International Union of Pure and Applied Chemistry
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium
NaOH	Sodium hydroxide
NIR	Near-infrared spectroscopy
PA	Polyacetylene
PANI	Polyaniline

PEDOT	poly(3,4-ethylenedioxythiophene)
PPy	Polypyrrole
PT	Polythiophene
PVA	poly(vinyl alcohol)
PVP	poly(N-vinyl pyrrolidone)
SH	Sodium Hyaluronate
TEM	Transmission electron microscopy
TSA	*p*-toluenesulfonic acid

LIST OF SYMBOLS

Alphabetically ordered

A	Absorbance
c	Concentration
l	Length of optical path
PDI	Index of polydispersity
pH	Potential of hydrogen
SD	Standard deviation
wt.%	Percentage by weight
z	Zeta
γ_{AB}	Polar parts of surface energy
γ_{LW}	Dispersive parts of surface energy
γ_{TOT}	Total surface energy
ε	Absorption coefficient
λ	Wavelength
π	Pi covalent bond

LIST OF UNITS

Alphabetically ordered

%	Percent
°	Degree
°C	Degree Celsius
g	Gram
g mol^{-1}	Gram per mole
h	Hour
mg	Milligram
mg mL^{-1}	Milligram per millilitre
min	Minutes
mL	Millilitre
mm	Millimetre
mol L^{-1}	Mole per litre
N m^{-1}	Newton per metre
nm	Nanometre
pA	Picoampere
s	Second
S cm^{-1}	Siemens per centimetre
µg mL^{-1}	Microgram per millilitre
µL	Microlitre
µm	Micrometre

LIST OF PUBLICATIONS

Publications related to the topic of the thesis

Jasenská D., Kašpárková V., Radaszkiewicz K.A., Capáková Z., Pacherník j., Trchová M., Minařík A., Vajďák J., Bárta T., Stejskal J., Lehocký M., Truong T. H., Moučka R., Humpolíček P. Conducting composite films based on chitosan or sodium hyaluronate. Properties and cytocompatibility with human induced pluripotent stem cells. Carbohydrate Polymers. 2021, 253. ISSN 01448617. doi:10.1016/j.carbpol.2020.117244

Trchová M., **Jasenská D.**, Bláha M., Prokeš J., Stejskal J. Conducting polyaniline prepared in the solutions of formic acid: Does functionalization with carboxyl groups occur? Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2020, 235. ISSN 13861425. doi:10.1016/j.saa.2020.118300

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Other publications

Korábková E., Kašpárková V., **Jasenská D**., Moricová D., Daďová E., Huong T. T., Capáková Z., Vícha J., Pelková J., Humpolíček P. Behaviour of Titanium Dioxide Particles in Artificial Body Fluids and Human Blood Plasma. International Journal of Molecular Sciences. 2021, 22, ISSN 1422-0067; doi: 10.3390/ijms221910614

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CONFERENCE CONTRIBUTIONS

Jasenská D., Maráková N., Capáková Z., Kašpárková V., Humpolíček P. Colloidal Polyaniline Dispersions Stabilized with Biopolymers. P. SAHA.18, 2018, Zlín, Czech Republic

Jasenská D., Maráková N., Capáková Z., Kašpárková V., Humpolíček P. Colloidal Polyaniline Dispersions Stabilized with Polysaccharides. 9th International Colloids Conference. 2019, Sitges, Spain

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Internship at the Institute of Macromolecular Chemistry, Czech Academy of Sciences (IMC), Standard polymerization of aniline in formic acid solutions (22/04/2019–26/04/2019; Prague)

Internship at the Institute of Macromolecular Chemistry, Czech Academy of Sciences (IMC), Standard polymerization of aniline in formic acid solutions (22/10/2018–9/11/2018; Prague)

Basics of scientific work course at the Czech Academy of Sciences (18/09/2017–22/09/2017; Brno)

	Internship at the Tomas Bata University, Faculty of Technology, Preparation and Characterization of Emulsions (01/07/2016–31/08/2016; Zlín)
Training abroad:	HR Mobility – Project funded from OP RDE, The Development of Capacity for Research and Development of TBU in Zlín, CZ.02.2.69/0.0/0.0/16_028/0006243 – Università degli Studi di Milano, Dipartimento di Chimica (28/06/2021–28/07/2021; Italy) Summer School in Bordeaux, Field of study: Biomaterials for Medical Devices and Regenerative Medicine (25/06/2018–29/06/2018; France)
Pedagogic activities:	Participation in teaching in laboratory classes of Course Chemistry and Technology of Fats I, Sensory Analysis of Cosmetics

Daniela Jasenská

Study of conducting biocompatible systems based on biopolymers

Studium vodivých biokompatibilních systémů na bázi biopolymerů

Doctoral Thesis Summary

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